DIETARY POLYPHENOLS DISPLAY ZINC IONOPHORE ACTIVITY AND MODULATE ZINC SIGNALING IN HEPATOCARCINOMA CELLS

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Dipòsit Legal: T 770-2015

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Doctoral Thesis

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UNIVERSITAT ROVIRA I VIRGILI

Tarragona 2015

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FAIG CONSTAR que aquest treball, titulat “Dietary polyphenols display zinc ionophore activity and modulate zinc signaling in hepatocarcinoma cells”, que presenta en Husam Dabbagh Bazarbachi per a l’obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament de Bioquímica i Biotecnologia d’aquesta universitat i que acompleix els requeriments per poder optar a la Menció Internacional.

Tarragona, 5 de Gener de 2015

El director de la tesi doctoral

Dr. Juan Bautista Fernández Larrea

A mi Familia,

y a Rama.

Agradecimientos

Cuatro años han pasado desde que inicié el camino hacia el doctorado. Parecía extraordinariamente largo, sin embargo sigo recordando el primer día como si fuera ayer. Ha sido un recorrido lleno de emociones y sentimientos. Estaban los días “buenos”, en los que todo parecía ir sobre ruedas, donde todo eran alegrías y sensaciones positivas, aunque también estaban los días difíciles o “malos”, donde el tiempo parecía detenerse en un bucle lleno de frustraciones y desánimo y uno se empezaba a cuestionar si ese era el camino correcto que debía seguir. En esos días es cuando realmente uno se da cuenta del apoyo que recibe de las personas que le rodean. Gracias a esas personas hoy puedo escribir estos agradecimientos de mi tesis doctoral.

Mi primer contacto con los laboratorios fue en el departamento de Enología. Fue allí donde conocí a Braulio, quien pacientemente me inició en este “mundillo” de pipetas y tubos eppendorf. Con Claudio, empezó realmente mi aventura. Con él empezaron mis primeros ensayos en el laboratorio haciendo vinagre de fresa. Fuero unos días que difícilmente podré olvidar ya que gracias a eso, junto con el apoyo de Esti y Albert Mas a quien agradezco enormemente me animé a seguir el camino.

En el otro lado, ya en Nutrigenómica, me gustaría agradecer a Niurka, Rosa y Yaiza por toda la paciencia y la ayuda que me habéis proporcionado. Sin vosotras nada de esto hubiese sido posible, así que gracias.

Con diferencia, la mayor parte del tiempo lo compartí en el despacho con los “Nutris”. Si no fuese por el “buen rollo” que hubo durante todo este tiempo, los cafés, los “minibreaks”, los “pollocatas” y demás, seguro que no hubiese tenido el ánimo y la fuerza necesaria para continuar. Muchas gracias a todos los que estuvisteis desde el principio y ya terminasteis, así como los que llegasteis mas tarde, y con quien compartí algo mas de tiempo. Víctor, Lídia, Cris Rojas, Anabel, Ester, Laura, Noe, Neus, Aleix, Zara, María M., Aída Susana, Sarah, Joan, Ángela, M. José, Adri, María I. y Cate. Ha sido una alegría poder compartir todos esos momentos con vosotros. Gracias de todo corazón!

También quiero agradecer el apoyo recibido por algunos miembros del departamento, así como agradecer a Juan el tiempo dedicado en dirigir mi tesis. Gracias. Mencionar especialmente a Ciara por acogerme en su grupo de investigación y ayudarme a mejorar en el ámbito profesional, así como a Gael, con el que compartí muchas horas haciendo los liposomas y comiendo casi a la hora de cenar.

I would also like to thank Dr. Salih Özçubukçu and Dr. Rükan Genç for accepting me in their lab of the chemical department in METU, Ankara (Turkey) and for their hospitality as well as the rest of lab team. Çok teşekkür ederim!

A mi familia, en especial a mis padres. Alhamdulillah sin ellos nada de esto hubiese sido posible. Fuisteis vosotros los que me disteis el mayor apoyo en todos los niveles, y no solo ahora, sino desde mis inicios como estudiante. También a mis hermanos Usama, Tarek y Salma. Infinitamente gracias! SHUKRAN.

Finalmente quisiera agradecer a mi mujer Rama toda su paciencia y comprensión que ha tenido, animándome en todo momento incluso desde la distancia. Muchas gracias amor. Nuestro camino no ha hecho más que empezar, pero estoy seguro que juntos lograremos todos nuestros objetivos. De nuevo gracias a todos!

اللهم ان شكرك نعمة تستحق الشكر فعلمنى كيف اشكرك ، الحمد لله كما ينبغى لجلال وجهك وعظيم سلطانك...

"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."

Marie Curie

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ABBREVIATIONS

BAPTA	1,2-bis(2-aminophenoxy) ethane N,N N',N' tetra-acetic acid
BCL2	B-cell lymphoma 2
CAF	Caffeic acid
CAT	Catechol
CAT HYD	Catechin hydrate
COX-2	Prostaglandin-endoperoxide synthase 2
CQ	Clioquinol
CRIP	Cysteine-rich Intestinal Protein
DMSO	Dimethyl sulfoxide
DPPC	1,2-dipalmitoyl-sn -glycero-3-phosphocholine
DTPA	Diethylene triamine pentaacetic acid
EDTA	Ethylenediamineteraacetic acid
EGCG	Epigallocatechin-3-gallate
EGTA	Ethylene glicol-bis(2-aminoethylesther)-N,N,N',N' tetra-acetic acid
eNOS	Endothelial nitric oxide synthase
GAL	Gallic acid
GEN	Genistein
GSK3	Glycogen synthase kinase 3
IRAP	Insulin-responsive amino peptidase
LUT	Luteolin
MAPK	Mitogen-activated protein kinases
MRE	Metal response element
MT	Metallothionein
MTF-1	Metal-responsive transcription factor-1
MTT	3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-tetrazolium bromide
NAR	Naringenin
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PBS	Phosphate buffered saline
PDTC	Pyrrolidine dithiocarbamate
PF	Polyphenol

PHLO	Phloretin
PI	Propidium iodide
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP	Phosphatidylinositol- 4-phosphate
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PKB	Protein kinase B (Akt)
PKC	Protein kinase C
PTP	Protein tyrosine phosphatase
PYR	Pyrithione
QCT	Quercetin
RSV	Resveratrol
RUT	Rutin
SLC	Solute carrier superfamily
TAN	Tannic acid
TAX	Taxifolin (Dihydroquercetin)
TMD	Transmembrane domain
TNF	Tumor necrosis factor
TPEN	N,N,N′,N′-Tetrakis (2-pyridylmethyl) ethylenediamine)
Zinquin	Ethyl (2-methyl-8-p -toluenesulfonamido-6-quinolyloxy)
ZIP	ZRT/IRT related protein (SLC39 family of zinc transporters)
Zn	Zinc
ZnT	Zinc transporter (SLC30 family of zinc transporters)

Resum

El zinc, és el metall de transició més important a l'organisme després del ferro. Fa aproximadament 75 anys que es van descobrir les primeres evidències en la importància del zinc i la seva relació amb el correcte funcionament biològic sobre certs tipus de microorganismes. Posteriorment, ara fa varies dècades, es va establir la seva importància biològica en humans fins al punt d' esdevenir un micronutrient essencial per a la vida. Hi ha una gran varietat de proteïnes directament dependents de zinc, així com moltes altres on aquest element és de gran importància pel la seva estructura i funcionalitat. La correcta regulació i el manteniment d'unes concentracions dintre d'un rang fisiològic és un dels punts clau pel bon funcionament de l'organisme. Aquesta regulació ve donada principalment mitjançant la coordinació d'elements clau en la homeòstasi del zinc com ara transportadors específics pel zinc (importadors i exportadors), la metal·lotioneïna i factors de transcripció específics. Tant l'excés com el dèficit en els nivells de zinc poden causar processos patològics, de vegades irreversibles. Així mateix, s'ha observat que aquest metall té propietats farmacològiques beneficioses en vers patologies com ara malalties immunològiques, síndrome metabòlic, diabetis o certs tipus de càncer, induint la mort cel·lular programada (apoptosis) a les cèl·lules tumorals.

La singularitat del zinc, a diferència d'altres metalls com el ferro o el coure, va més enllà del seu paper com a cofactor d'un gran nombre d'enzims i proteïnes ja que a més, actua com a senyalitzador i transmissor d'informació d'una manera similar a com ho fa el calci o altres segons missatgers. Concentracions al rang nanomolar són suficients per activar certes vies metabòliques, i per tant pot ser de gran importància conèixer i modular la concentració de zinc d'una manera controlada. És en aquesta nova etapa on més èmfasi s'està donant en les investigacions més recents, ja que la senyalització cel·lular pot ser utilitzada com una nova eina terapèutica pel tractament de diverses patologies. Gràcies als fluorocroms específics pel zinc, aquest metall pot ser fàcilment detectat i quantificat. De fet, s'han descobert i dissenyat una gran varietat de fàrmacs sintètics capaços d'interaccionar amb el zinc, formant complexos i modulant els nivells de zinc intracel·lular.

Existeixen compostos naturals d'origen vegetal capaços d'interaccionar amb el zinc, tot i que encara no se sap del cert quina importància biològica podrien tenir aquests

complexos. Es tracta dels compostos fenòlics, més comunament anomenats polifenols, metabòlits secundaris de les plantes, distribuïts de manera molt abundant al regne vegetal. Es tracta de compostos que es formen com a mecanismes de defensa de les plantes en vers factors d'estrès, i que un cop consumits a la dieta aporten beneficis sobre la salut. El consum d'aquest compostos fito químics ha estat estudiant durant molt de temps, principalment arran de la paradoxa asiàtica i francesa respectivament. La primera fou descrita per la evidència que hi havia entre el consum de te verd i la incidència de càncer pulmonar als països asiàtics, ja que es tracta d'una de les regions que més cigarrets consumeix, però sorprenentment amb més baixa incidència de càncer. Es pensa que pot ser degut a l'alt consum de te verd que conté la seva dieta, i que els aporta una gran quantitat de polifenols de manera constant. Per altra banda, la paradoxa francesa que primer es va descriure per la població de França, però que actualment engloba a la majoria de països mediterranis, destaca la baixa incidència de malalties coronàries tot i el consum d'una dieta alta en greixos, la falta d'exercici físic y el consum de tabac. L'explicació d'aquest fenomen es deu, majoritàriament, al consum regular de vi que inclou la dieta francesa, ja que aquest, conté altes quantitats de polifenols, juntament amb els antioxidants continguts a l'oli d'oliva i l'alt consum de fruites i verdures que presents a la dieta mediterrània. És en aquest punt on comença la recerca a escala global per tal de descobrir els beneficis dels polifenols, i de quina manera actuen. Actualment se sap del cert que el polifenols actuen com a grans antioxidants, encara que recentment s'han descrit com a compostos que prevenen i milloren els símptomes de malalties cròniques com ara la síndrome metabòlica. També actuen com a antivirals, antibacterians, antiinflamatoris o inclús anticancerígens.

Actualment s'han descrit més de 8000 compostos fenòlics, que segons la seva estructura química s'han classificat en diversos sub-grups: flavonoids, el grup més abundant i més estudiat, àcids fenòlics, lignans, estilbens i altres compostos fenòlics. La gran majoria de Polifenols són absorbits a l'intestí prim, arribant a plasma sanguini on es distribueixen pels diferents òrgans i teixits, de vegades inclús travessant la barrera hematoencefàlica i arribant al cervell. Petites concentracions de polifenols, també al rang nanomolar son suficients per modular vàries vies metabòliques, fent de la dieta un factor primordial alhora de prevenir o tractar alguna patologia de tipus crònic.

Estudis previs realitzats al nostre grup de recerca van demostrar que els polifenols eren capaços de modular l'expressió de gens centrals en la homeòstasi de zinc. En un experiment *in vivo* amb rates, aquestes foren tractades amb una dosis oral aguda de extracte de procianidines de pinyol de raïm (GSPE) on posteriorment es va analitzar el teixit hepàtic i es va observar un descens significatiu en l'expressió del gen de la metal·lotioneïna (MT). Aquest fenomen va fer pensar que potser hi hauria algun tipus de relació més estreta entre els polifenols i el zinc.

En estudis posteriors *in vitro* fets en una línia cel·lular tumoral d'hepatòcits humans (HepG2), aquests se'ls va tractar amb GSPE, així com amb un extracte pur de galat d'epigalocatequina (EGCG), compost principal del te verd. Els resultats van mostrar que ambdós tractaments exerceixen efectes similars, reduint els nivells intracel·lulars de zinc total, disminuint l'expressió de la MT així com el del transportador ZnT1 però incrementant els nivells de zinc làbil citoplasmàtic. Un altre experiment *in vitro*, en aquest cas realitzat en una línia cel·lular tumoral de ratolí (Hepa 1-6), les cèl·lules foren tractades amb dos compostos purs, quercetina (QCT) i EGCG. Es tracta de dos flavonoids, les propietats fisicoquímiques dels quals són força diferents; mentre EGCG és un compost altament hidrosoluble, QCT es molt insoluble en aigua. Els resultats van mostrar que EGCG segueix exercint efectes similars als observats en cèl·lules HepG2, augmentant el zinc làbil, però disminuint el zinc total i l'expressió dels gens MT i ZnT1, mentre que amb quercetina es va veure un increment en la concentració de zinc làbil citoplasmàtic de la mateixa manera que el EGCG, però també un increment en la concentració de zinc total, així com l'expressió gènica de MT i ZnT1.

Aquesta tesis doctoral es va centrar en l'estudi de la relació entre el zinc i els polifenols, principalment QCT i EGCG, i de quina manera són capaços d'interaccionar i/o modular de manera combinada les diverses vies metabòliques a nivell d'expressió de gens centrals en la homeòstasi del zinc, així com determinar si exerceixen una modulació diferencial en quant a expressió proteica. Tanmateix, es va voler determinar el mecanisme pel qual els polifenols incrementen el zinc làbil citoplasmàtic, així com observar de quina manera són capaços d'afectar la viabilitat cel·lular un cop combinats amb el zinc. Tots els experiments realitzats *in vitro* van ser duts a terme amb cèl·lules Hepa 1-6.

Observant la capacitat dels polifenols per modular la concentració de zinc làbil citoplasmàtic, descrit com a zinc de senyalització, vam proposar determinar si QCT i EGCG són capaços d'interactuar amb cations de zinc no només afectant l'homeòstasi del zinc, sinó també la senyalització cel·lular en cèl·lules Hepa 1-6. Està àmpliament descrit ambdós compostos (polifenols i zinc) exerceixen alguns dels efectes beneficiosos en la prevenció i/o tractament de diverses malalties cròniques a través de la modulació de múltiples vies metabòliques compartides, com ara Akt, implicada en múltiples processos cel·lulars, com la síntesi de proteïnes, supervivència cel·lular, creixement, proliferació, angiogènesi, metabolisme de la glucosa, homeòstasi cardiovascular, neuroprotecció o apoptosi. Els nostres resultats descriuen com la QCT i EGCG, un cop combinats amb el zinc, exerceixen diferents patrons de fosforilació d'Akt en comparació amb els compostos individuals, confirmant la funció de senyalització, així com la modulació diferencial de certes vies metabòliques a través del complex polifenol-zinc. Els resultats també van suggerir que tant QCT com EGCG augmenten les concentracions intracel·lulars de zinc làbil d'una manera similar a la dels ionòfors de zinc com ara el Piritione o el Clioquinol; és a dir, mitjançant la formació de complexos amb zinc capaços de travessar la membrana plasmàtica, que un cop dins de la cèl·lula, es dissocien alliberant els cations de zinc pel citoplasma.

Per determinar si els flavonoides actuen com a ionòfors de zinc, transportant els cations a través de la membrana plasmàtica, QCT, EGCG, Clioquinol (CQ), tant aïllats com en combinació amb zinc, es van afegir a liposomes unilamelelars de dipalmitoil-fosfatidilcolina/colesterol carregats amb el fluorocrom específic per zinc i impermeable a la membrana cel·lular FluoZin-3. Tan sols les combinacions dels quelants combinats amb el zinc van provocar un ràpid augment en la fluorescència del FluoZin-3 dintre dels liposomes, demostrant l'acció ionòfora de QCT, EGCG, i CQ en un sistemes de membranes lipídiques.

Un cop confirmada l'activitat ionòfora de QCT i EGCG, es va presentar un sistema liposomal eficaç per a la detecció de l'activitat ionòfora pel zinc dels polifenols més rellevants. A més, també es va determinar la força de quelació que tenen aquests compostos pel de zinc, en un assaig de competició basat en l'apagament de la fluorescència dependent de zinc emesa pel complex zinc-FluoZin-3. Finalment, es va

demostrar la correlació entre la capacitat de quelació i l'activitat ionòfora, proporcionant un millor coneixement sobre la importància de la conformació estructural envers l'activitat biològica. Els assajos desenvolupats poden ser utilitzats com una eina d'alt rendiment per l'anàlisi de les diferents famílies de polifenols.

La quercetina, un quelant de metalls i ionòfor de zinc, va mostrar efectes diferencials sobre la viabilitat de cel·lular un vegada combinat amb zinc. Els canvis morfològics van suggerir que aquesta combinació podria estar induint apoptosi sobre la línia cel·lular tumoral Hepa 1-6. La fragmentació de l'ADN va ser confirmada mitjançant l'electroforesi en gel.

Per concloure, es podria dir que els polifenols podrien ser afegits a un nou arsenal de fàrmacs per la modulació de l'homeòstasi del zinc, així com la regulació de les vies biològiques senyalitzadores dependents d'aquest.

Summary

Zinc is the most important transition metal after iron in the body. Approximately 75 years ago was discovered the first evidence on the significance of zinc and its relation to the proper functioning of certain types of biological organisms. Subsequently, several decades ago established its biological significance in humans, becoming an essential micronutrient for life. There are a variety of proteins directly dependent on zinc, as well as many others where this element is of great importance for its structure and functionality. The correct regulation and maintenance of concentrations within the physiological range is one of the key points for proper functioning of the body. This regulation comes mainly through the coordination of essential elements for the zinc homeostasis, such as specific transporters (importers and exporters), the metallothionein, and other specific transcription factors. Both excess and deficient levels of zinc can cause pathological processes, sometimes with irreversible consequences. It was also observed that this metal exerts beneficial pharmacological properties against pathological conditions such as immune disorders, metabolic syndrome, diabetes, and certain cancers, inducing programmed cell death (apoptosis) in tumoral cells.

The distinctiveness of zinc, contrasting other metals such as iron or copper, beyond its role as a cofactor of many enzymes and proteins, it can act as a transmitter of information in a similar way as does calcium or other second messengers. Concentrations in the nanomolar range are enough to activate certain metabolic pathways. Therefore, it would be critical to know and to modulate the concentrations of zinc. It is in this new era where more emphasis is being given to the latest investigations, since cell signaling could be used as a new therapeutic tool for the treatment of various diseases. Due to specific zinc fluorophores, it can be easily detected and quantified. In fact, several synthetic drugs have been discovered and designed able to interact with zinc, forming complexes and acting together, modulating the intracellular zinc amounts.

There are natural plant derived compounds able to interact with zinc, although it remains unknown the biological importance of those complexes. Those are the phenolic compounds, commonly known as polyphenols, secondary plant metabolites, widely abundant and distributed in the plant kingdom. These compounds are formed as a defense

mechanism of plants against external stress factors, providing health benefits when consumed in the diet. The consumption of this phytochemicals has been studied for a long time, mainly since the description of the Asian and French paradox respectively. The first one describes how despite the high consumption of tobacco, Asian population have the lowest incidences of arteriosclerosis and lung cancer per capita as a result of the high consumption of green tea in this region, which contains high amounts of polyphenols. On the other hand, the French paradox was first described because of the French population, however now encompasses most Mediterranean countries. It denotes the low incidence of heart diseases despite the consumption of a high fat diet, lack of physical activity and regular consumption of tobacco. One of the features that has been emphasized relates to the high consumption of red wine in the French population and the question as to whether the phenolic antioxidants from this dietary source contribute to the protection from coronary heart disease, beside with the antioxidants consumed in the olive oil and the high intake of antioxidant nutrients from fresh fruits and vegetables found in the Mediterranean diet. This is where the research began on a global scale in order to discover the activity of polyphenols, and their benefits. At the present time, their activity as antioxidants has been well established. However, recently studies have described them as preventive and improver compounds against chronic diseases such as the metabolic syndrome. Moreover it has been strongly suggested their function as antiviral, antibacterial, anti-inflammatory or anticancer agents. Nowadays, more than 8000 phenolic compounds have been identified and described containing several sub-groups according to their chemical structure, such as flavonoids, phenolic acids, lignans, stilbenes and other polyphenols. Most of polyphenols are absorbed in the small intestine, reaching blood plasma and distributed to different organs and tissues, sometimes even crossing the blood brain barrier. Low concentrations of polyphenols, also in the nanomolar range, are enough to modulate several metabolic pathways, making the diet a major factor in preventing or treating different chronic pathologies.

Previous studies in our research group showed that polyphenols are able to modulate the expression of central zinc homeostasis genes. Moreover, in an *in vivo* experiment, rats treated with an oral acute dose of a grape seed procyanidin extract (GSPE), the liver tissue of which was later analyzed, presented a significant down-regulation in the gene expression of metallothionein (MT). This phenomenon made us think that maybe there

would be some deeper relationship between polyphenols and zinc. Further *in vitro* studies in a human hepatoma cell line (HepG2), these were treated with a procyanidin extract (GSPE), as well as with epigallocatechin-3-gallate (EGCG), the main green tea flavonoid. The results showed that both treatments exert similar effects, reducing the intracellular amounts of total zinc, down-regulating the expression of MT and the zinc transporter ZnT1 but increasing the intracellular labile zinc concentration. In a mouse hepatoma cell line (Hepa 1-6), cells were treated with two pure compounds, quercetin (QCT) and EGCG, two flavonoids with different physicochemical properties; EGCG is a highly water-soluble compound, while QCT is highly insoluble in water. The results showed that EGCG still exert similar effects to those observed in HepG2 cells, increasing the labile zinc levels, down-regulating the MT and ZnT1 gene expression and reducing the total zinc concentrations. On the other hand, QCT increases the intracellular labile zinc concentrations, as EGCG, but also increases the total zinc amounts and MT gene expression and ZnT1.

This thesis is focused on the study of the relationship between zinc and polyphenols, principally QCT and EGCG, and their ability to interact and form complexes in order to modulate several metabolic pathways, as well as zinc homeostasis genes and the differential effects on protein expression. Conversely, we wanted to determine the mechanism by which polyphenols increase the intracellular labile zinc, and how they affect cell viability once combined with zinc. All *in vitro* experiments were carried out with Hepa 1-6 cell line. Observing the ability of polyphenols to modulate the labile cytoplasmic zinc concentration, which has been described as a signaling pool of zinc, we proposed to determine whether QCT and EGCG interacts with zinc cations not only affecting zinc homeostasis, but also affecting cellular signaling in Hepa 1-6 cell line. It has been widely reported how both dietary phenolic compounds and zinc exert their beneficial effects on health, preventing and ameliorating several chronic diseases through the modulation of multiple and shared metabolic pathways, such as Akt, which is involved in multiple cellular processes like protein synthesis, cell survival, growth, proliferation, angiogenesis, glucose metabolism, cardiovascular homeostasis, neuroprotection or apoptosis.

Our results described that QCT and EGCG once combined with zinc, exert different Akt phosphorylation patterns compared to individual compounds, confirming the signaling

function and differential modulation of metabolic pathways by polyphenols-zinc complexation. Results also suggested that both QCT and EGCG increase intracellular labile zinc concentrations in a similar way to the well known ionophores Pyrithione (Pyr) or CQ, i.e. by forming complexes with zinc that are able to traverse the plasma membrane and, once inside the cell, dissociate liberating zinc cations to the cytoplasm.

To determine whether flavonoids act as zinc ionophores, transporting zinc cations through the plasma membrane, QCT, EGCG, and the ionophore drug Clioquinol (CQ), alone and combined with zinc, were added to unilamellar dipalmitoylphosphocholine/cholesterol liposomes loaded with membrane-impermeant FluoZin-3, a specific zinc fluorophore. Only the combinations of the chelators with zinc triggered a rapid increase of FluoZin-3 fluorescence within the liposomes, thus demonstrating the ionophore action of QCT, EGCG, and CQ on lipid membrane systems.

Once confirmed the ionophore activity of QCT and EGCG, we presented an efficient liposomal system for screening the zinc ionophore activity of a selected library consisting of the most relevant dietary polyphenols. In addition, the zinc chelation strength of the polyphenols was also tested in a competition assay based on the fluorescence quenching of zinc-dependent fluorescence emitted by zinc-FluoZin-3 complex. Finally, the correlation between the chelation capacity and ionophore activity was demonstrated, thus underlining the sequestering or ionophoric activity that the phenolic compounds can display, providing better knowledge of the importance of the structural conformation versus their biological activity. Furthermore, the developed assays can be used as tools for rapid, high-throughput screening of families of polyphenols.

Quercetin, a metal chelator and zinc ionophore, exert differential effects on cell viability when combined with zinc. Morphological changes suggest that this combination might be inducing apoptosis to the mouse hepatoma Hepa 1-6 cell line. DNA fragmentation has been confirmed by electrophoresis analysis.

Conclusively, polyphenols could be added to a new arsenal of drugs for the modulation of zinc homeostasis and the regulation of zinc dependent biological signaling pathways.

I. INTRODUCTION

1. Zinc

1.1 Zinc History and background

Zinc (Zn), belongs to group II B of the transition metal elements, with an atomic number 30 and atomic weight of 65.39. It was firstly isolated by the German chemist Andreas Sigismund Marggraf in 1746, and named by the Swiss-German physician and alchemist Theophrastus Bombastus von Hohenheim, more commonly known as Paracelsus. It is the most important transition metal after iron, and during the last decades has been classified as an essential micronutrient present in all tissues, organs and fluids in the organism (Kozlowski et al. 2009). The importance of zinc was first reported in 1869 due to its importance in the normal growth of the fungus *Aspergillus niger,* the common bread mold (Raulin 1869). Few years later, in the 1930's decade, several reports confirmed the essentiality of zinc as a nutrient for the normal development of mice and rats (O'Dell & Reeves 1989). In 1939 Keilin and Mann established for the first time the importance of zinc in the activity of an enzyme. They demonstrated that carbonic anhydrase was a zinc protein compound, where Zn formed the active site of the enzyme. This establishes the basis of the physiological function of zinc in living organisms (Keilin & Mann 1940). It passed more than fifteen years till the discovery of a second zinc dependent enzyme, the bovine pancreatic carboxypeptidase (Prasad et al. 1961). Its essentiality was also demonstrated for pigs in 1955 (O'Dell & Reeves 1989). Zinc was not recognized to be important for human life until 1963 when deficiency of this transition metal was discovered as a major contributing factor in nutritional dwarfism syndrome and hypogonadism (Prasad et al. 1961). Furthermore, it was demonstrated that the zinc content of whole blood was subnormal in cancer patients, with a mean decrease of about 20%, suggesting that zinc dishomeostasis could be critical in the correct working of immune system (Addink & Frank 1968).

The beginning of a second phase, and a milestone in the biochemistry of zinc, was the discovery of zinc fingers (Miller et al. 1985). It extended the role of zinc in biology tremendously as it demonstrated that zinc is not only important for protein structure, but for organizing protein domains that interact with proteins and other biomolecules, such as DNA, RNA, and lipids. The third and most recent level of knowledge in the biological zinc, began when it became evident that Zn ions exert roles beyond those as a permanent

cofactor in proteins and may function in information transfer as signaling ions in cellular regulation in a similar way of calcium ions. This advances only became possible with the development of new tools, such as chelating and fluorescent agents when binding zinc, and allow measurement of Zn ions in cells with a sensitivity exceeding that of most other analytical techniques (Maret 2013). (Fig.1) The chemical properties of zinc different from other transition metals, such as copper and iron which display several different oxidation states in biological systems, is that zinc exists as a redox inert Zn^{2+} cation, which does not undergo redox reactions at physiological redox potentials (Eide 2011; Laitaoja et al. 2013). Additionally, zinc can induce the expression and maintaining the levels of potentially radical scavenging proteins such as metallothionein (MT), the major zinc binding protein associated with zinc homeostasis (Quesada et al. 2011), DNA protection, oxidative stress, and apoptosis (Higashimoto et al. 2009; Tapiero & Tew 2003). It can act through stabilization of cell membranes (Powell 2000) or as a structural component of antioxidant enzymes (Klotz et al. 2003). Although it has been studied for many decades, the molecular bases are still not so clear.

During the past 40 years, zinc role in biological processes has been extensively studied. Nevertheless, there is still a long way to go in order to elucidate the effects and molecular mechanisms by which this transition metal exert its activity, and also to find the answers about its importance in health and disease.

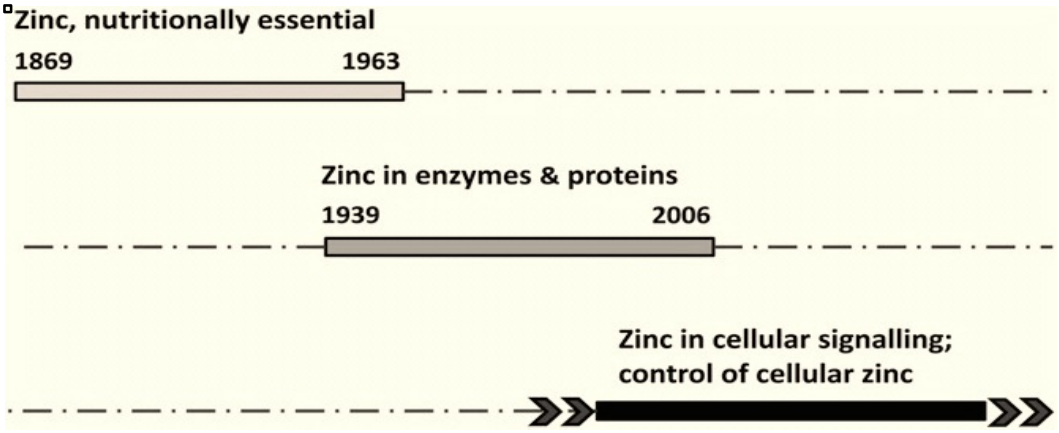


Figure 1. Chronology of events in Zinc: 1) the recognition that zinc is essential for all forms of life, 2) the detection of zinc in enzymes and other proteins as a basis of its catalytic and structural functions and 3) a role of zinc ions in cellular regulation. (Maret 2013)

1.2 Sources and metabolism

There are three major routes of entry for zinc into the human body; by inhalation, through the skin, or by ingestion, which is the main way of zinc uptake via dietary sources (Nickolette 2005). Each exposure type affects specific parts of the body and allows the uptake of different amounts of zinc (Plum et al. 2010).

Zinc can be found in several food sources, specially in red meats, sea products, cocoa, dairy products and nuts (Dreosti 1993). Lean red meat, whole-grain cereals, pulses, and legumes provide the highest concentrations of zinc 25-50 mg/kg (380-760 µmol/kg) raw weight. Processed cereals with low extraction rates, polished rice, and lean meat or meat with high fat content have a moderate zinc content 10-25 mg/kg (150-380 µmol/kg). Fish, green leafy vegetables, fruits, roots and tubers are only modest sources of zinc <10 mg/kg (<150 µmol/kg) (20). Inositol hexaphosphates and pentaphosphates (phytic acid), a natural zinc chelator, binds zinc and forms poorly soluble complexes that result in reduced zinc assimilation. Phytate is present in staple foods includes cereals, corn, and rice, which exerts a highly negative effect on zinc absorption (Arsenault & Brown 2003). Sugar, alcohol, separated fats and oils have very low zinc content. The dietary intake of Zn is estimated at approximately 10–15 mg/day (Table 1), mostly absorbed by the small intestine, duodenum and jejunum (Menard & Cousins 1983) by transferrin (Evans 1976), cysteine-rich intestinal proteins (Hempe & Cousins 1992) and other Zn transporters.

Recommended dietary allowance (RDA) for zinc	
	RDA (mg/day)
Infants	5
Children 1–10 years old	10
Males > 10 years old	15
Females > 10 years old	12
Pregnant Woman	15
Lactating Women in 1st Trimester	19
Lactating Women in 2nd Trimester	16

Table 1. Recommended dietary allowance (RDA) for zinc. (Chien et al. 2006)

As Hempe and Cousins (Hempe & Cousins 1992) have illustrated (Fig.2), the trace mineral is probably bound to an intestinal binding protein after entrance into the cell. In the case of zinc, this intestinal binding protein has been specifically identified as Cysteine-rich

Intestinal Protein (CRIP). The function of this binding protein is to act as both a protective mechanism for the cell by binding to free metal in the cytosol and as a specific carrier to chaperone the mineral across the cell to the basolateral membrane. If an intestinal binding protein fails to bind the trace mineral, it will most likely be bound to a non-specific binding protein or in the case of zinc to metallothionein (MT). The primary function of metallothionein is to maintain homeostasis of zinc (Pattison & Cousins 1986). Once the bound trace mineral crosses through the cytosol and arrives at the basolateral membrane it is removed from the binding protein and transferred across the membrane via a poorly understood, but saturable, transport mechanism (Oestreicher & Cousins 1985). The trace mineral is then bound to albumin as it enters circulation (Smith et al. 1979). The albumin remains bound to the trace element until it reaches the liver and the trace mineral is further metabolized before being released for transport to other body tissues (Richards & Cousins 1976). Of the total plasma Zn concentration, over 90% is associated with albumin, less than 10% with alpha-2 macroglobulin, and a very tiny fraction (< 1%) with amino acids and other low molecular weight species (Cousins 1986). As has been shown by Menard *et al.* (1981), as metallothionein levels increase, the absorption of zinc decreases, and as dietary zinc concentrations increase, an up-regulation in metallothionein mRNA is also observed.

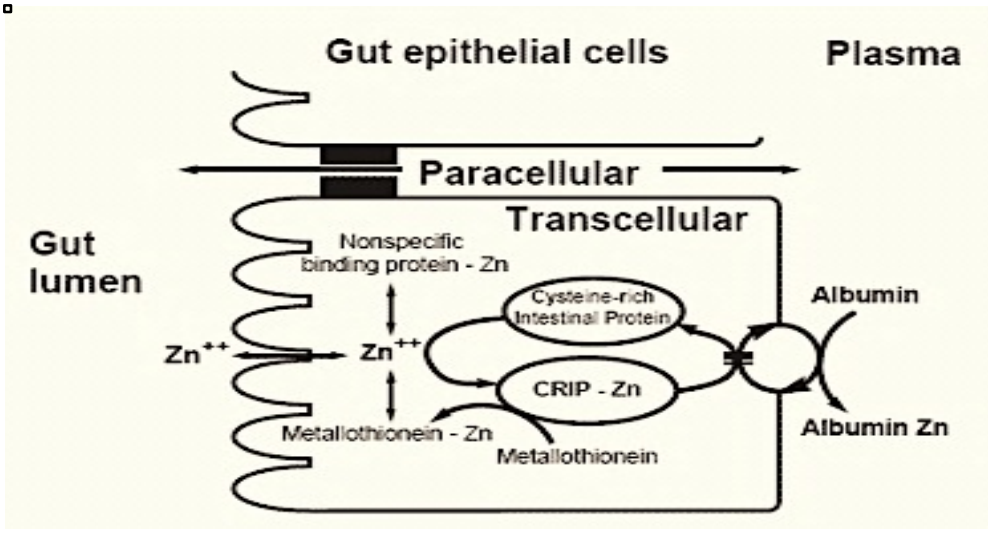


Figure 2. Proposed mechanism for intestinal zinc absorption. (Hempe & Cousins 1992)

The human body contains between 2-3 grams of zinc, most of them gathered in brain and pancreatic cells (Kozlowski et al. 2009). About 95% of total zinc is the intracellular one,

distributed between nucleus (30-40%), membrane (10%) and cytoplasm (50%) (Plum et al. 2010; Vallee & Falchuk 1993). Since the body has no zinc stores in the conventional sense and it may not be accumulated in the organism, a permanent consumption is needed. In conditions of bone resorption and tissue catabolism, zinc is released and may be re-utilised to some extent. Human experimental studies with low-zinc diets 2.6-3.6 mg/day (40-55 µmol/day) have shown that circulating zinc levels and activities of zinc-containing enzymes can be maintained within normal range over several months (Lukaski et al. 1984; Milne et al. 1987) which highlights the efficiency of the zinc homeostasis mechanism. (Fig.3)

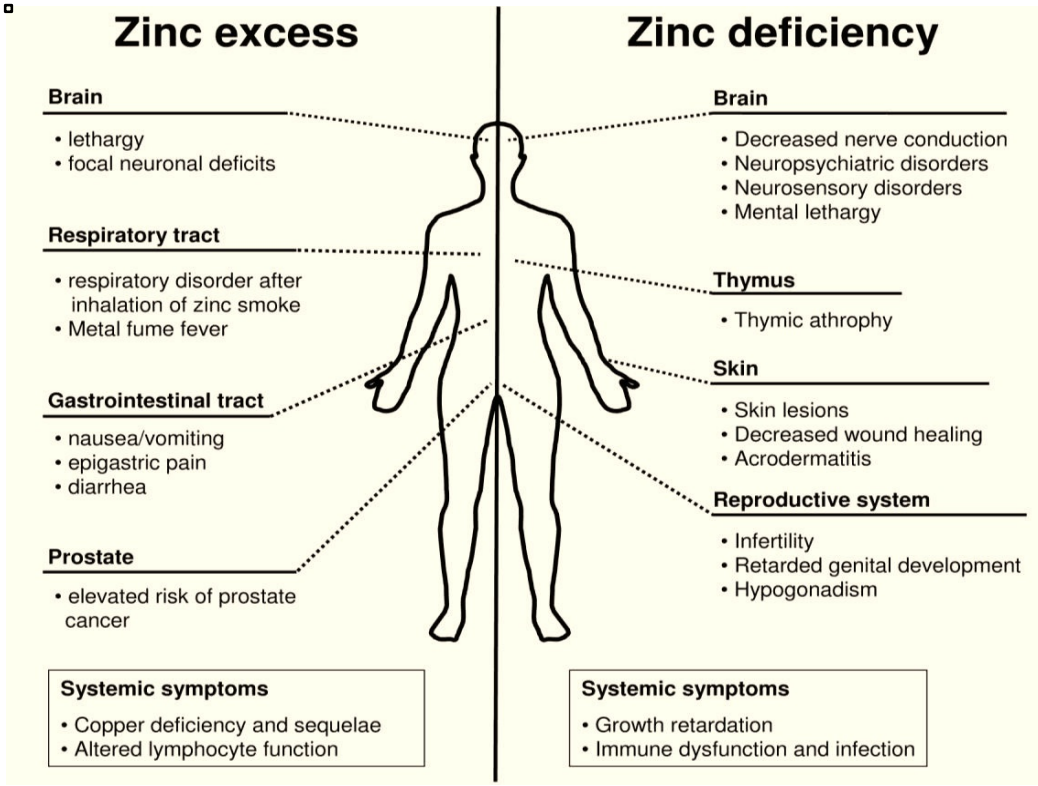


Figure 3. Effects of excessive intake of zinc (left side), and deprivation of zinc (right hand side). Effects that could not be attributed to a certain organ or affect several organs are classified as systemic symptoms. (Plum et al. 2010)

Zinc is lost from the body through the kidneys, skin, and intestine. The endogenous intestinal losses can vary from 7 µmol/day (0.5 mg/day) to more than 45 µmol/day

(3mg/day), depending on zinc intake (Mills 1989). Urinary and skin losses are of the order of 7-10 µmol/day (0.5-0.7 mg/day) each and depend less on normal variations in zinc intake (Mills 1989). Starvation and muscle catabolism also could increase zinc losses in urine. Strenuous exercise and elevated ambient temperatures could lead to losses by perspiration. There is hardly any cellular process not influenced by zinc. In humans, major signs of severe zinc deficiency are growth retardation, hypogonadism in males, skin and neurosensory disorders, and impairment of immunity and cognition. Different stages of zinc deficiency are thought to affect 30% of the world's population, a problem with at least the scope of that of iron deficiency. Among the groups at risk are the elderly. The World Health Organization (WHO) has identified zinc deficiency as the fifth most important risk factor for morbidity and mortality in developing countries. It can be concluded that zinc is an essential micronutrient which should be maintained under a rigorous homeostasis and is required for many biological processes including growth and development, neurological function, reproduction, and immunity (Wong & Ho 2012). (Fig.4)

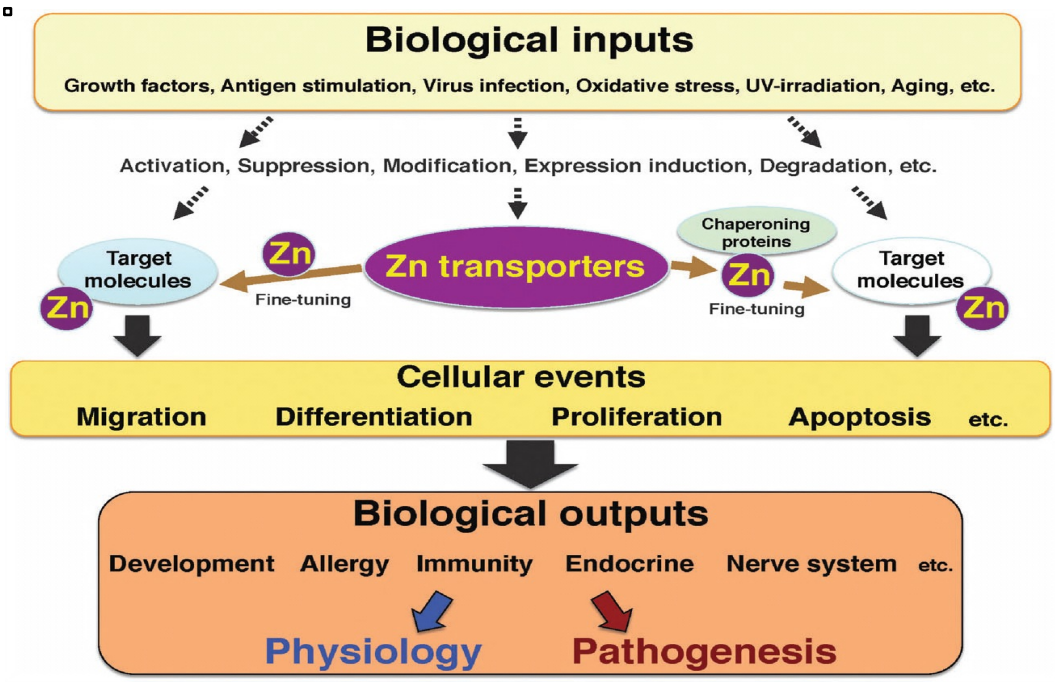


Figure 4. Zinc homeostasis fine-tune biological inputs leading to the control of biological outputs. (Fukada & Kambe 2011)

1.3 Zinc biology

Zinc is one of the most abundant and important metals in biology. Using bioinformatics tools, it has been recently estimated that human proteome includes about 15% of zinc proteins, many more than iron or copper (Andreini et al. 2009; Harding et al. 2010; Brylinski & Skolnick 2011). This bioinformatics approach combines the information from databases for protein structures (Maret 2013) and for protein domains (Finn et al. 2010) to scan gene databases. Three data sets were obtained: metalloproteins with known signatures and unknown domains (2406 proteins), metalloproteins with known signatures and known domains (2506), and metalloproteins with unknown signatures and known domains (2407) (Maret 2013; Bertini & Cavallaro 2010). The total number of identified zinc proteins is 3207, but 2430 were identified by at least two methods and 1684 zinc proteins were identified by all three methods, thus including signatures, domains and annotations (Maret 2013). Identified zinc proteins can be classified and counted as: 397 hydrolases, 302 ligases, 167 transferases, 43 oxidoreductases, 24 lyases/isomerases, 957 transcription factors, 221 signaling proteins, 141 transport/storage proteins, 53 proteins with structural metal sites, 19 proteins involved in DNA repair, replication, and translation, 427 zinc finger proteins of undetermined function and 456 proteins of unknown role. Moreover, zinc is involved in the stabilization and catalysis of over 300 enzymes and a higher number of metalloproteins (Parkin 2004; Vallee & Falchuk 1993). Other zinc proteins whose importance emerged more recently include proteins for zinc signaling, transport, buffering, and storage. As the molecular mechanisms of cellular zinc homeostasis are just beginning to be elucidated, the number of these proteins and thus the size of zinc proteomes is likely to be larger than what is currently realized (Maret 2013; Eide 2009).

The main reason for the selection of zinc as a catalytic cofactor lies in its distinctive chemical properties, which combine Lewis acid strength, lack of redox reactivity, and fast ligand exchange (Andreini et al. 2008). As a reflection of the prevalent use and the remarkable versatility of zinc in biological catalysis, zinc enzymes are present in all six major classes of enzymes, thus oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases (Vallee & Galdes 1984; Andreini et al. 2008). (Table 2) Additionally, zinc ions are also required in many proteins for the right folding of the polypeptide chain, such as zinc finger proteins.

Name	Source	Role
Class I, Oxidoreductases		
Alcohol dehydrogenase	Yeast	c, s
Alcohol dehydrogenase	Vertebrates, plants	c, s
Sorbitol dehydrogenase	Vertebrates	c
D-Lactate dehydrogenase	Barnacle, bacteria	?
D-Lactate cytochrome reductase	Yeast	?
Superoxide dismutase	Vertebrates, plants, fungi, bacteria	ca
Class II, Transferases		
Transcarboxylase	*P. shermanii*	?
Aspartate transcarbamylase	*E. coli*	s
Phosphoglucomutase	Yeast	?
RNA polymerase	Wheat germ, bacteria, viruses	c
Reverse transcriptase	Oncogenic viruses	c
Nuclear poly(A) polymerase	Rat liver, virus	c
Terminal deoxyribonucleotidyl transferase	Calf thymus	?
Mercaptopyruvate sulfur transferase	*E. coli*	?
Class III, Hydrolases		
Leukotriene A_4 hydrolase	Human	c
Alkaline phosphatase	Mammals, bacteria	c, ca
5′-Nucleotidase	Bacteria, lymphoblast, plasma	?
Fructose-1,6-bisphosphatase	Mammals	ca
Phosphodiesterase (exonuclease)	Snake venom	c
Phospholipase C	*B. cereus*	c, ca
Cyclic nucleotide phosphodiesterase	Yeast	?
Nuclease	Microbes	?
α-Amylase	*B. subtilis*	s
α-D-Mannosidase	Mammals, plants	?
Aminopeptidase	Mammals, fungi, bacteria	c, ca
Aminotripeptidase	Rabbit intestine	c
Astacin	Crustacea	c
Meprin	Mammals	?
Enkephalinase	Mammals	?
Thermolysin	Bacteria	c
Dipeptidase	Mammals, bacteria	c
Class III, Hydrolases (*continued*)		
Angiotensin-converting enzyme	Mammals, bacteria	c
Carboxypeptidase A	Vertebrates, crustacea	c
Carboxypeptidase B	Mammals, crustacea	c
Carboxypeptidase (other)	Mammals, plants, bacteria	c
Carboxypeptidase DD	*S. albus*	c
Elastase	*P. aeruginosa*	c
Neutral protease	Vertebrates, fungi, bacteria	c
Collagenase	Mammals, bacteria	c
Protein kinase C	Mammals	s
Hemorrhagic protease	Snake venom	c
Aminoacylase	Pig kidney, microbes	?
Dihydropyrimidine aminohydrolase	Bovine liver	?
Dihydroorotase	*Clostridium oroticum*	?
β-Lactamase II	*B. cereus, P. maltophila*	c
Creatininase	*P. putida*	?
AMP deaminase	Rabbit muscle	?
Inorganic pyrophosphatase	Yeast	?
Nucleotide pyrophosphatase	Yeast	c
Adenosine deaminase	*E. coli*, mammals	?
Class IV, Lyases		
Fructose-bisphosphate aldolase	Yeast, bacteria	c
1-Rhamnulose-1-phosphate aldolase	*E. coli*	c
Carbonic anhydrase	Animals, plants	c
δ-Aminolevulinic acid dehydratase	Mammalian liver, erythrocytes	c
Glyoxalase I	Mammals, yeast	c
Class V, Isomerases		
Phosphomannose isomerase	Yeast	?
DNA topoisomerase I	*E. coli*	?
Class VI, Ligases		
tRNA synthetase	*E. coli, B. stearothermophilus*	c
Pyruvate carboxylase	Yeast, bacteria	?

Table 2. Zinc enzymes comprise all 6 classes of enzymes established by the IUPAC. C, catalytic role; s, structural role; ca, coactive role; ?, lacking information to make an assignment. (Vallee & Falchuk 1993)

Four main groups have been described for zinc coordination environments in proteins: catalytic, cocatalytic (or coactive), structural and protein interface (Auld 2001). (Fig. 5) A catalytic role specifies that the metal contribute directly in enzyme catalysis. If the metal is removed by chelating or by other agents, the enzyme becomes inactive. This abolition of activity is attributed primarily to the fact that zinc itself participates directly in the catalytic process; this does not exclude the possibility that there may also be a concomitant structural change (e.g., in local conformation and/or that of the ligands). A coactive (or cocatalytic) zinc atom enhances or diminishes catalytic function in conjunction with another active site zinc atom in the same enzyme, but is not indispensable of itself for either enzyme activity or stability. Structural zinc atoms are required solely for structural stability

of the protein but do not directly participate in catalysis, and can help stabilize the quaternary structure of oligomeric holoenzymes. Alcohol dehydrogenases of vertebrates contain both a catalytic and a structural zinc atom. Protein interface zinc sites have an impact on the quaternary structure of a protein. They are composed of amino acid ligands that reside in the binding surface between two protein subunits or interacting proteins and generally have the coordination properties of catalytic or structural zinc binding sites (Maret & Li 2009).

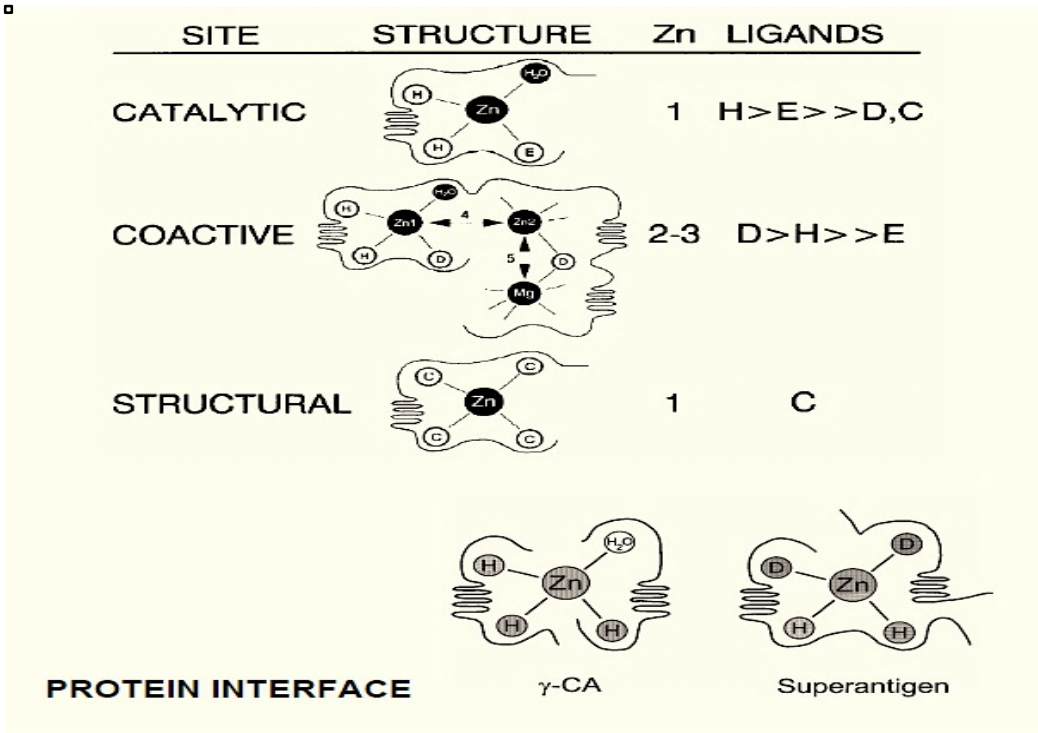


Figure 5. Three amino acids and a water molecule are the zinc ligands in the catalytic zinc site. Enzymes with a coactive zinc site display both catalytic zinc site, Zn1 and a separate coactive site Zn2, which modulate but are not essential for catalysis. These 2 Zn atoms can bind to 1 amino acid, usually aspartic acid, sharing it as a bridge. Furthermore, one of the amino acid ligands of a Zn2 site can additionally form a bridge to another metal, i.e. magnesium. In structural zinc, metal is coordinated tetrahedrally to 4 Cys residues that prevent access of other ligands to the coordination sphere. Protein interface zinc binding sites normally have the coordination properties of catalytic or structural zinc binding sites. (Auld 2001)

In enzymes, there is almost invariably one coordination site occupied by a water molecule, which can easily be displaced to create a catalytically active species for an incoming substrate/ inhibitor molecule (Laitaoja et al. 2013).

Catalytic zinc sites are generally composed of a bound water molecule and three aminoacids, two of which come from a short amino acid spacer, with His being the most common, followed by Glu, Asp, and Cys residues that coordinate the zinc by imidazole and carboxyl groups. The zinc dissociation constant for these protein sites is in the nanomolar to picomolar range. Normally one catalytic zinc atom per subunit of enzyme is found. Zinc is bound to three His in human carbonic anhydrases I and II and It's bound to two His and one Glu in carboxypeptidases A and B. The only exception to the above thus far is alcohol dehydrogenase, the catalytic zinc site that uniquely contains just one His as well as two Cys. However, in this case, the third zinc ligand at the active site appears to be variable and has been deduced to be Cys, Glu, or Asp in different enzymes within the alcohol dehydrogenase superfamily. A water molecule is the fourth ligand at all catalytic sites. Mechanistically, the water molecule can be ionized, polarized, or displaced. Ionization or polarization provides hydroxide ions at neutral pH, while the displacement of the water leads to Lewis acid catalysis.

Cocatalytic or coactive zinc sites are found in enzymes containing two or more zinc or other transition metals in neighbouring proximity to each other that operate in concert as a catalytic unit. The additional zinc (or other metal) site has been named coactive. The distance between the metals is determined by type of amino acid (Asp, Glu, His, or a carboxylated Lys) that bridges the two metals. Sometimes a water molecule forms a bridge between the metal atoms in a cocatalytic site. Asp and His are the most frequent ligands in this type of site. The ligands to these sites often come from nearly the whole length of the protein and the metals are important not only to catalytic function but to protein folding.

Structural zinc sites contain four protein ligands and no metal bound water. While Cys is the most frequent ligand of these sites, any combination of four Cys, His, Glu, and Asp residues in principle can form this type of zinc site. Twelve combinations of the 22 permutations of these four ligands have been observed so far. The role of the structural zinc site is to maintain the localized structure of the protein which could in turn influence

protein folding or function by supplying residues involved in catalysis that arise from within the spacer arms. Structural zinc atoms have been best characterized in three enzymes, protein kinase C, aspartate transcarbamylase, and alcohol dehydrogenase (Vallee & Falchuk 1993). The structural motif in protein kinase C contains 4 atoms of zinc per mole of protein located in the non-catalytic domain of the enzyme. Each zinc atom is fully coordinated tetrahedrally to three cysteines and one histidine. In aspartate transcarbamylase and alcohol dehydrogenase the structural zinc is fully coordinated tetrahedrally to four cysteines (Auld & Bergman 2008).

Protein interface zinc sites have an impact on the quaternary structure of a protein. They are composed of amino acid ligands that reside in the binding surface between two protein subunits or interacting proteins and generally have the coordination properties of catalytic or structural zinc binding sites. Carbonic anhydrase, or insulin are examples of proteins that use zinc to form quaternary structures (Auld 2009; Maret & Li 2009).

There is a functional diversification of the eukaryotic and prokaryotic zinc proteomes. Prokaryotes use zinc proteins to perform enzymatic catalysis, whereas in eukaryotes the zinc proteome is almost equally involved in performing catalysis and in regulating DNA transcription. (Chart 1) This broad difference in function has a correspondence with the organization of the zinc-binding patterns. Indeed, the patterns containing four ligands are associated with structural sites where zinc contributes to the stability of protein structures, whereas zinc-binding patterns containing three protein ligands are associated with catalytic sites, that is, zinc actively participates in the reaction mechanism of the enzyme (Vallee & Auld 1990). The number of zinc proteins correlates linearly with the number of genes in a particular genome and is higher in eukarya (8.8%) than in bacteria and archaea (5-6%) (Andreini et al. 2006). In eukaryotes but not in prokaryotes a big portion of zinc proteins is involved in the regulation of gene expression, pointing out that the biological importance of zinc increased as increasingly complex cellular, and in particular multicellular, systems evolved. Many of these proteins contain one or more so-called zinc fingers, which are small protein domains stabilized by a zinc ion playing a structural role (Klug & Schwabe 1995).

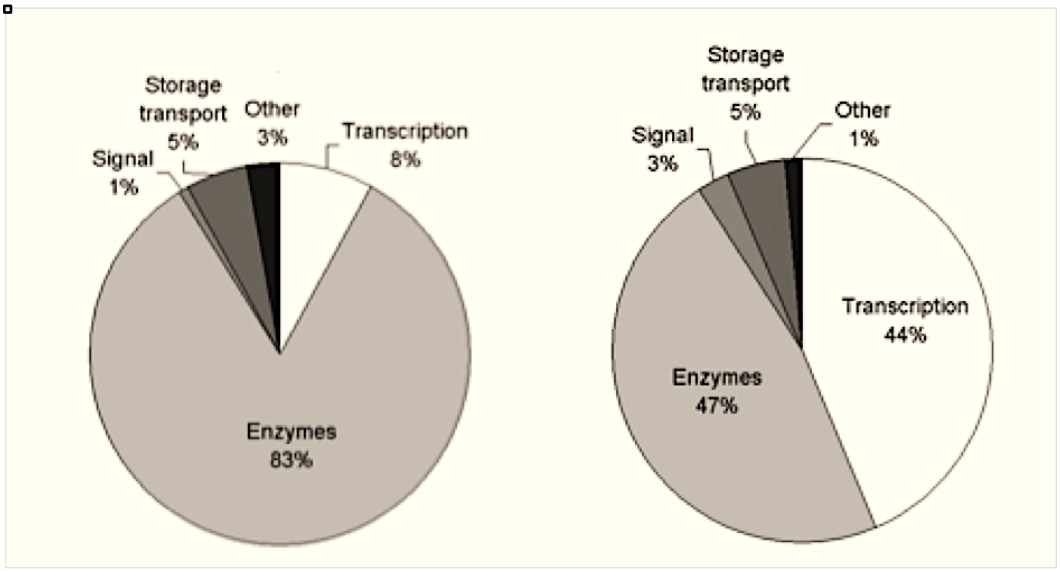


Chart 1. Distribution of the roles of zinc proteins in prokaryotes (left) and eukaryotes (right). Only the proteins with known function are included, which represent about 90% of the total. (Andreini et al. 2006)

1.4 Zinc homeostasis

As explained heretofore, zinc ions play a unique and essential role of an important number of biological processes, including DNA synthesis, gene expression, enzymatic catalysis, neurotransmission, and apoptosis (Krizkova et al. 2012). Approximately 90% of intracellular zinc ions are tightly bound, mainly by cysteine, histidine, and asparagine residues of peptides and proteins, making that zinc pool hard to interact with other molecules (Wilson et al. 2012). The rest, about 10% of total zinc, is weakly bound with relatively low affinities, forming a reactive Zn pool able to interact with other intracellular substances and compartments (Franklin & Costello 2009). Another small fraction of zinc, which comprises less than 0,01% of total intracellular zinc amount, is the free zinc pool, ranging from picomolar to a few nanomolar concentrations, therefore, maintaining an adequate but tightly regulated intracellular zinc level is fundamental to the survival of living organisms including humans (Huang & Tepaamorndech 2013). Changes in intracellular zinc levels have been associated with tumour growth as zinc is a cofactor of enzymes involved in angiogenesis, cell proliferation, and metastasis of cancer, such as matrix metalloproteases and carbonic anhydrase. Altered expression of zinc transporters has been observed in many cancers and these changes may play causal roles in cancer progression. (Taylor et al. 2007; Jeong & Eide 2003) Zinc transporters are involved in many other physiological pathways including insulin synthesis and secretion in the pancreas, bone development, heart health, cognitive function, and body adiposity (Huang & Tepaamorndech 2013).

At least three dozen of proteins are directly involved in the control of human cellular zinc homeostasis and in the regulation of the cellular availability and re-distribution of zinc ions (Maret 2009). Some of the central functions of those proteins are remove the excess of zinc ions to prevent unwanted side reactions, supply the metalloproteins with zinc, safeguard zinc ions during the transport through membranes and sense if the concentrations are the adequate for the cell. There are two main families of zinc transporters of the solute carrier superfamily involved in zinc homeostasis in the body. (Fig.6) The SLC30, also known as ZnT family consisting of ten members in mammals (ZnT1-ZnT10), mediates Zn efflux from cells or influx into intracellular vesicles from the cytosol (Fig.7). Evolutionary and basic sequence relationships among the ZnT genes have

been reviewed (Palmiter & Huang 2004). The SLC39 family, more commonly known as Zip family, comprise fourteen members in mammals (Zip1- Zip14), which promote zinc influx from the extracellular fluid or intracellular vesicles into the cytoplasm (Fig.8) (Fukada et al. 2011).

ZnT proteins contribute to the cytoplasmic zinc balance by exporting zinc out to the extracellular space or by sequestrating cytoplasmic zinc into intracellular compartments when cellular zinc levels are elevated. In contrast, ZIP proteins function to increase cytoplasmic zinc concentrations when cellular zinc is depleted (Huang & Tepaamorndech 2013).

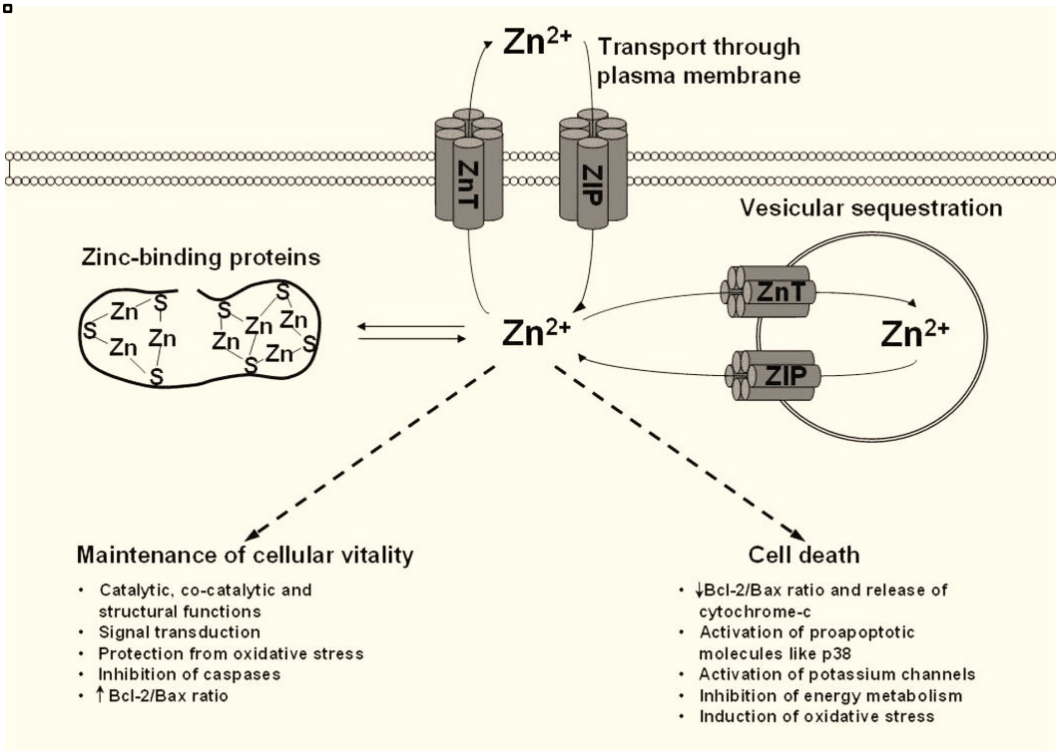


Figure 6. Cellular zinc homeostasis is mediated by three main mechanisms. Transport across the plasma membrane by importers from the Zip family, and exporters from the ZnT family. Zinc binding specific proteins. Transporter mediated sequestration into intracellular organelles, including Golgi, endoplasmic reticulum, and lysosomes. Dishomeostasis of zinc affects cellular viability, frequently leading to cell death. (Plum et al. 2010)

The ZnT family, which include more than 100 members, are found in organisms at all phylogenetic levels. This family is divided into three subfamilies (Gaither & Eide 2001). Subfamily I contains a large proportion of prokaryotic members, while subfamilies II and III contain eukaryotic and prokaryotic members in a similar proportion. Most ZnT proteins have six transmembrane domains (TMDs). The biological distribution of those transporters exert a specific molecular role and is linked to different pathologies. (Table 3)

ZnT1 predominantly performs regulation of zinc export, in particular, to plasma at base-lateral surface of enterocytes in the duodenum and jejunum (Sekler et al. 2002). ZnT2 and ZnT4 sequesters zinc in cytoplasmic vesicles, ZnT3 performs specific function, predominantly in the brain and sequesters zinc in presynaptic glutamate containing secretion granules under the influence of potential dependent chloride channel (Zalewski et al. 2005). ZnT5 is located in membranes of secretion granule of insulin, pancreas cell enriched in zinc. ZnT6 and ZnT7 exert their activity mainly in Golgi apparatus (Huang et al. 2002; Kirschke & Huang 2003), ZnT8 in endocrine pancreas and ZnT10 in the liver and brain (Zalewski et al. 2005)

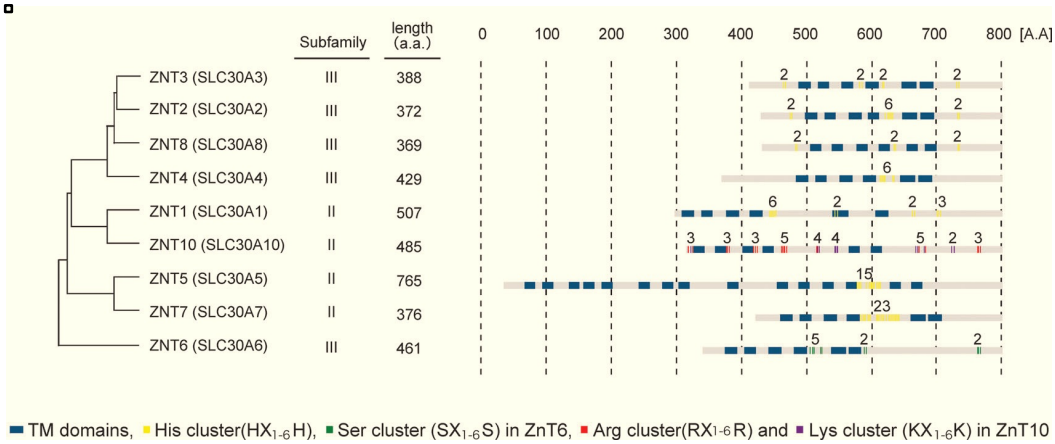


Figure 7. Summary of the structural features of ZnT transporters. A dendrogram showing the sequence similarity among ZnT transporters is shown at the left. The generalized domain structure of ZnT such as the TM domains, His cluster (HX1–6H), Ser cluster (SX1–6S), Arg cluster (RX1–6R), and Lys cluster (KX1–6K) are shown in the right panels. Arabic numbers above the domain structure of each protein means the number of His/Ser/Arg/Lys residues in the clusters. (Fukada & Kambe 2011)

I. INTRODUCTION

At least 86 members of the Zip family have been described (Cousins et al. 2006). Evolutionary and basic sequence relationships of the Zip genes have been reviewed in detail (Jeong & Eide 2003). Zip proteins have been divided into two subfamilies: subfamily I consists mostly of fungal and plant sequences, and subfamily II, composed of insect, nematode, and mammalian sequences (Guerinot 2000). Most Zip proteins are predicted to have eight TMDs with extracellular (or intravesicular) amino and carboxy termini (Gaither & Eide 2001). ZIP transporters play diverse roles in the physiology of cells and organisms. Their genes are expressed in various tissues and cell types, and their proteins are localized to distinct subcellular compartments. (Table 4) Recently, bioinformatic analyses revealed similarities between LIV-1 transporters subfamily members and prion genes (Schmitt-Ulms et al. 2009). It was shown that prion-like protein sequences were present in the N-terminal domains of ZIP5, ZIP6, and ZIP10, suggesting that prion proteins may be evolutionarily descended from ZIP proteins.

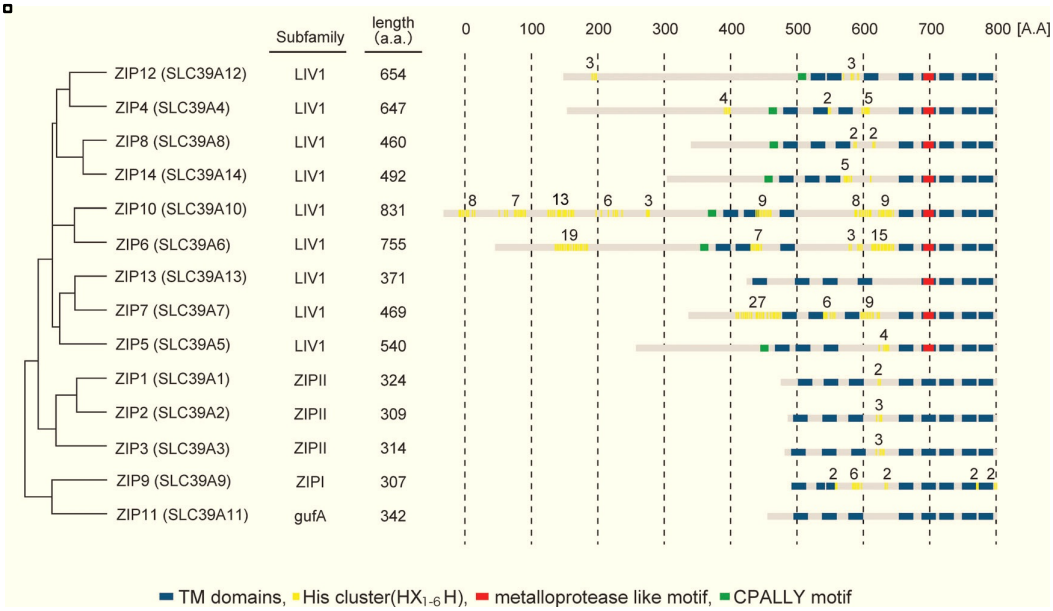


Figure 8. Summary of the structural features of ZIP transporters. A dendrogram showing the sequence similarity among ZIP transporters is shown on the left. The generalized domain structure of ZIP such as the TM domains, His cluster (HX1–6H), metalloprotease like motif, and the CPALLY motif are shown on the right panels. Arabic numbers above the domain structure of each protein means the number of His residue in the His cluster. (Fukada & Kambe 2011)

Gene Name	Alias	Protein (No. of amino acid)	Tissue/cell distribution	Subcellular localization	Link to disease	Mouse KO model
SLC30A1	ZnT1	507	Widespread	Plasma membrane	Embryonic lethal[a]	Yes
SLC30A2	ZnT2	Isoform 1 (372)	Mammary gland, prostate, retina, pancreas, small intestine, kidney	Endosomal/ lysosomal/secretory vesicle (isoform 1)[a,b] Plasma membrane (isoform 2)[b], Mitochondria (isoform 1)[a]	Reduced zinc content in nursing women[b]	No
		Isoform 2 (323)				
SLC30A3	ZnT3	388	Brain, testes, pancreas	Synaptic vesicle[a]	Seizures[a], learning deficit[a], memory loss[a]	Yes
SLC30A4	ZnT4	429	Widespread, predominant in mammary gland, placenta, prostate, brain, kidney	Endosomal/secretory vesicle[a], plasma membrane[b]	Lethal milk[a]	Yes
SLC30A5	ZnT5	Isoform a (765) Isoform b(523)	Widespread, predominant in heart, placenta, pancreas, prostate, ovary, testis, small intestine, thymus, bone	Golgi, unknown vesicles (isoform a)[a,b], Plasma membrane (isoform b)[b]	Bone[a], abnormalities[a], heart failure[a]	Yes
SLC30A6	ZnT6	501/461	Widespread, predominant in brain, lung, intestine	Golgi, unknown vesicles[a,b]	Alzheimer[b]	No
SLC30A7	ZnT7	376	Widespread, predominant intestine, stomach, prostate, retina, pancreas, testis, muscle	Golgi, unknown vesicles[a,b]	Prostate cancer[a], low adiposity[a], diet-induced diabetes[a]	Yes
SLC30A8	ZnT8	369/320	Pancreas, thyroid, adrenal gland, testis	Secretory granule[a,b]	Diabetes[a,b]	Yes
SLC30A9	ZnT9	568/413	Widespread	Cytoplasm[b], nucleus[b]	None known	No
SLC30A10	ZnT10	485/260	Brain, retina, liver	Unknown	None known	No

Table 3. Summary of the SLC30 family members. (Huang & Tepaamorndech 2013)

a: Rodent

b: Human

Gene/ subfamily	Protein/ alias	Substrates	Tissue distribution in humans/ response to Zn or hormones[a]	Subcellular localization	Disease/ pathology	Human gene locus
SLC39A1/ subfamily II	ZIP1/ ZIRTL	Zn	Widespread/ Prolactin (+), testosterone (+), IL-6 (+), cell differentiation (+)	Plasma membrane, intracellular vesicles	Prostate cancer, neurodegeneration[b]	1q21
SLC39A2/ subfamily II	ZIP2/ Eti-1, 6A1	Zn	Widespread	Plasma membrane		14q11.1
SLC39A3/ subfamily II	ZIP3	Zn, not specific	Widespread, mammary cells, testis/ High Zn (-), prolactin (+)	Plasma membrane, lysosomes	Neurodegeneration[b]	19p13.3
SLC39A4/ LIV-1	ZIP4	Zn	Gastrointestinal tract, kidney hippocampal neurons/ Low Zn (+)	Plasma membrane Apical surface of enterocytes, lysosomes	AE, pancreatic cancer, liver cancer[c]	8q24.3
SLC39A5/ LIV-1	ZIP5/ LZT-Hs7	Zn	Pancreas, kidney, liver, stomach, intestine/ IL-6 (+)	Plasma membrane Basolateral surface of enterocytes		12q13.13
SLC39A6/ LIV-1	ZIP6/ LIV-1	Zn	Widespread/ IL-6 (+), IL-1 (+), LPS(+)	Plasma membrane	Breast, pancreatic[d], cervical[e], prostate cancer[f], neuroblastoma[g]	18q12.1
SLC39A7/ LIV-1	ZIP7/HKE4, RING5	Zn, Mn	Widespread	ER, Golgi, intracellular vesicles	Breast cancer	6p21.3
SLC39A8/ LIV-1	ZIP8/ BIGM103, LZT-Hs6	Zn, Cd, Mn	Widespread, T-cells, erythroid, testis/ LPS (+), immune activation (+), TNFα (+)	Plasma membrane, Lysosomes, mitochondria	Inflammation, Breast cancer	4q22-q24
SLC39A9/ subfamily I	ZIP9			trans-Golgi[h]		14q24.1
SLC39A10/ LIV-1	ZIP10/ LZT-Hs2	Zn	Brain, liver, erythroid, kidney/ Low Zn (+), thyroid hormone (+), regulated by MTF-1[i]	Plasma membrane	Breast cancer	2q33.1
SLC39A11/ gufA	ZIP11					17q25.1
SLC39A12/ LIV-1	ZIP12[j]/ LZT-Hs8	Zn	Brain, lung, testis, retina			10p12.33
SLC39A13/ LIV-1	ZIP13/ LZT-Hs9	Zn	Widespread	Intracellular vesicles, Golgi	SCD-EDS	11p.11.12
SLC39A14/ LIV-1	ZIP14/ LZT-Hs4	Zn, Fe, Mn, Cd	Widespread, liver/ IL-1 (+), IL-6 (+), NO (+)	Plasma membrane	Asthma, inflammation, colorectal cancer[k]	8p21.2

Table 4. Summary of the SLC39 family members. a (+), up-regulation; (-), down-regulation. (A. Hediger et al. 2013)

b (Qian et al. 2011)
c (Weaver et al. 2010)
d (Unno et al. 2009)
e (Zhao et al. 2007)
f (Lue et al. 2011)
g (Chowanadisai et al. 2008)
h (Matsuura et al. 2014)
i (Wimmer et al. 2005)
j (Bly 2006)
k (Thorsen et al. 2011)

The expression of specific zinc transporters (and the rate of zinc uptake and excretion) is responsive to dietary zinc levels and is coupled to regulated expression and action of intracellular proteins that associate with zinc, particularly metallothioneins (Haq et al. 2003). For instance, metallothioneins, whose genes are well-known targets of metal-responsive transcription factor-1 (MTF-1), acts as zinc storage proteins and thereby plays an essential role in cellular zinc homeostasis (Laity & Andrews 2007; Colvin et al. 2010; Günther et al. 2012). (Fig. 9) These proteins were first discovered by Margoshes and Valee as cadmium-binding proteins isolated from horse kidney in 1957 (Margoshes & Vallee 1957). Interactions of MTs with zinc ions are influenced by three main conditions: 1) redox state of MT; 2) pH of the environment; and 3) the presence of NO. If these conditions support the formation of the zinc/metallothionein complex, which is, under the healthy physiological state, MTs can serve as "administrators" of Zn^{2+} ions for transporters, enzymes, storage proteins, and transcription factors (Krizkova et al. 2012).

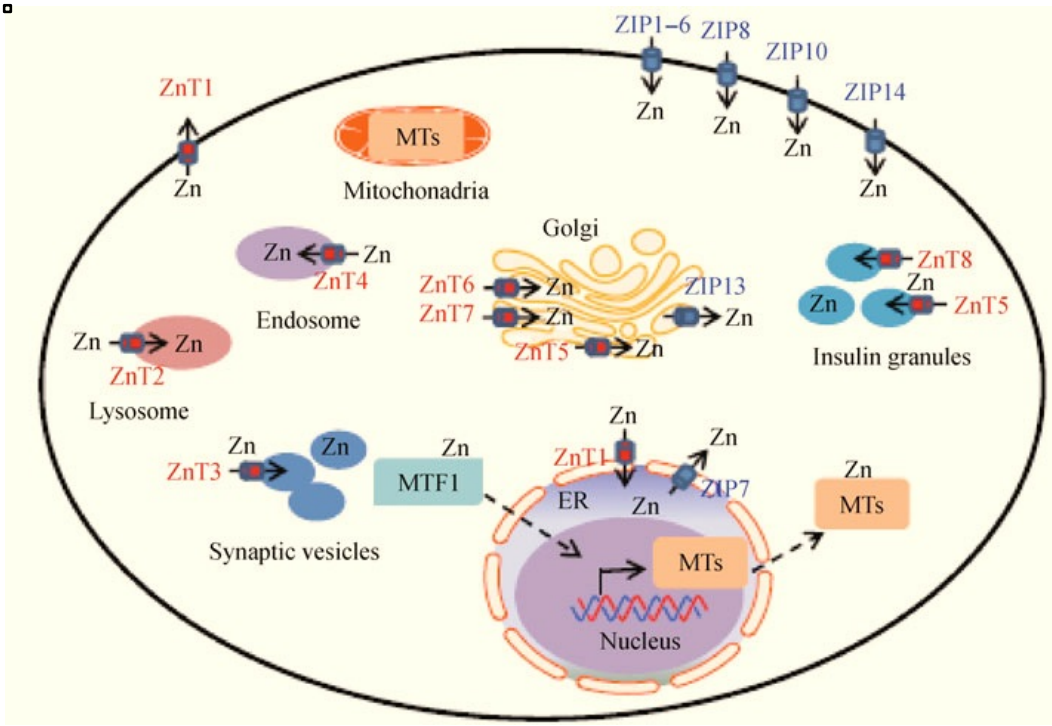


Figure 9. Subcellular localization of Zn transporters and MTs. Localization and potential functions of Zn transporters from the Slc39/ZIP (blue) and Slc30/ZnT (red) families, MT, and metal response element (MRE) binding MTF-1 within the cell. Arrows show the predicted direction of Zn mobilization. ER, endoplasmic reticulum. (Fukada et al. 2011)

Metallothioneins comprise a family of small (normally less than 10 kDa), cysteine-rich, metal-binding proteins found in all eukaryotes. All MTs contain 61-62 amino acids organized into single α and β domains (one α and one β domain per MT molecule). The high number of cysteine residues (11 in the C-terminal α domain and 9 in the N-terminal β domain) are responsible for a high capacity of MTs to coordinate covalent binding of divalent metal cations in metal/thiolate clusters (Haq et al. 2003). Metallothioneins can bind up to a total of seven zinc or cadmium ions, and up to 12 copper ions, in aggregate between the two domains. (Fig.10)

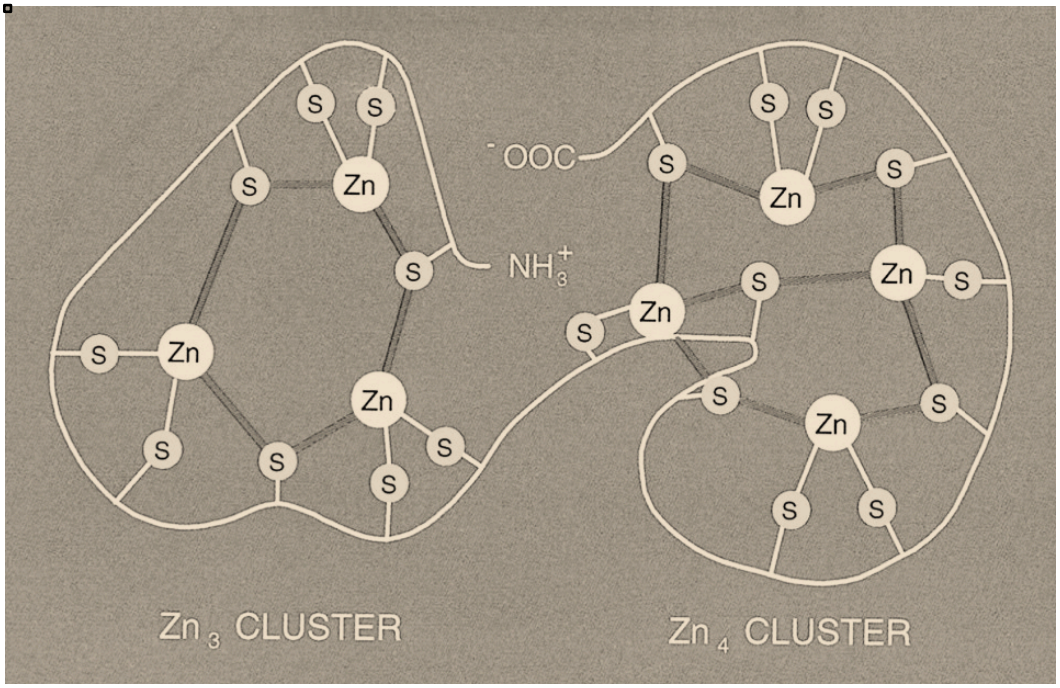


Figure 10. Zinc thiolate clusters of mammalian metallothionein. Each molecule is composed of two clusters, the NH2- and COOH-terminal regions containing 3 (Zn3) and 4 (Zn4) zinc atoms respectively, in each cluster. Each metal is tetrahedrally coordinated to 4 thiolate bonds with some of the thiolate ligands sharing the zinc ion. (Vallee & Falchuk 1993)

In mammals have been described four different MT isoforms (MT-1, MT-2, MT-3, and MT-4). MT-1 and MT-2 are present in practically all mammalian cell types and organs, with the highest expression in liver tissue. MT-3 and MT-4 are limited to specific cells and tissues. MT-3 was first identified as a molecule that suppressed growth of rat neuronal cells in

culture (Uchida et al. 1991) and later as an MT (Palmiter et al. 1992). MT-3 is expressed in brain and primarily in glutaminergic neurons, with additional very low amounts reported in pancreas and intestine (Ebadi et al. 1995). MT-4 is expressed, exclusively in squamous epithelial cells in skin and tongue (Meloni et al. 2006).

Although only four MT proteins have been identified in mammals (including humans), at least 11 MT-1 genes have been described in humans (MT1A, B, E, F, G, H, I, J, K, L, and X), some of which appear to encode RNAs incapable of directing the production of MT protein. The 11 MT-1 genes are clustered within the q13 region of chromosome 16, along with one MT-2 gene (also known as MT-2A), one MT-3 gene, and one MT-4 gene.
In humans, MTs (MT-1 and MT-2) have been described to have aberrant expression in a number of human tumors. For those occurring in breast, kidney, lung, salivary gland, ovary, testes, leukaemia, urinary bladder, nasopharyngeal tissue, and non-Hodgkin lymphoma, an association with increased MT levels has been reported (Cherian et al. 2003; Theocharis et al. 2004; Thirumoorthy et al. 2007). On the other hand, decreased levels of MT-1 and MT-2 in human liver, prostate, thyroid, central nervous system, and testicular tumors have been linked to increased malignancy and poorer prognosis (Pedersen et al. 2009; Cherian et al. 2003; Meijer et al. 2000; Ferrario et al. 2008). The overall role of MTs in tumors, therefore, is not completely clear. (Fig.11)

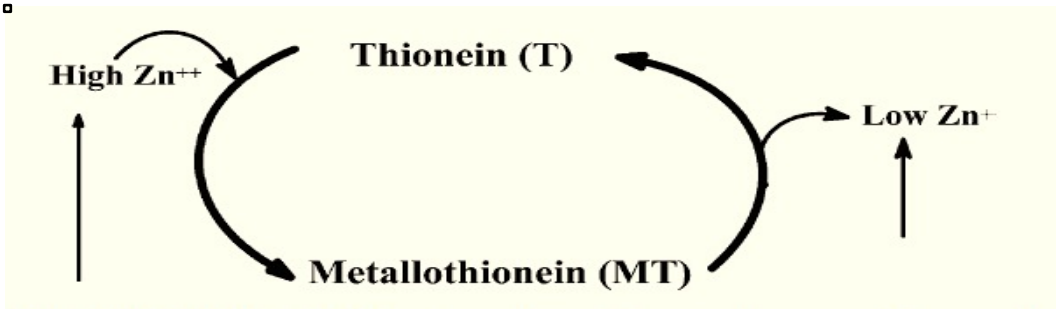


Figure 11. Metallothionein/thionein couple as a homeostatic system. Increased available zinc induces the synthesis of thionein (T) and leads to the formation of MT. Zn is released from MT when the amount available zinc is low. (Krezel & Maret 2008)

1.5 Zinc signaling

Like in the case of the chelatable iron pool, for which terms such as “labile iron,” “intracellular transit iron,” “free iron,” “low molecular weight iron,” “exchangeable iron,” and “metabolically active iron” have been used, a similar terminology has been adopted to methodically define pools of zinc ions, which are believed to be the responsible for signaling effectors (Petrat et al. 2002).

It has been suggested as early as 1984 that Zn ions may have a function in signal transduction, comparable to the second messenger Ca^{2+} (Williams 1984). A second messenger is defined as a molecule whose intracellular status is directly altered by extracellular stimuli and that can transduce the extracellular stimuli into intracellular signaling events (Yamasaki et al. 2007). Only a few years later, it was shown that activation of T cells causes an intracellular redistribution of Zn^{2+}, and that this ions activate protein kinase C (Csermely et al. 1987; Csermely et al. 1988). Since then, zinc signals have been observed in many different cell types, mostly in the form of changes of the free cytoplasmic zinc concentration (Haase & Rink 2009).
From many years, zinc ions have been proposed to act as neurotransmitters (Colvin et al. 2003; Frederickson 2003). Furthermore, it was suggested that Zn has the ability to mimic the actions of hormones, growth factors, and cytokines, which proposes that zinc may act on intracellular signaling molecules (Beyersmann & Haase 2001). In fact, zinc is a known inhibitor of protein tyrosine phosphatases (Brautigan et al. 1981), with a constant of inhibition in the nanomolar range (Maret et al. 1999). In addition, zinc affects the regulation of transcription factors, and can induce the expression of some genes, including those coding for molecules involved in zinc homeostasis, like zinc transporters and metallothioneins (Palmiter & Huang 2004). The gene expression of metallothioneins by zinc is regulated by metal response element–binding transcription factor-1 (Lichtlen & Schaffner 2001).

Zinc stabilizes structural zinc-binding motifs that influence the protein’s biological activity, such as DNA binding and transcriptional activation. Moreover, it can modulate the enzymatic activity of signaling proteins, such as the inhibition of protein tyrosine

phosphatases (PTPs). It cans also affect the assembly of signaling complexes through binding at interfaces between proteins (Haase & Maret 2010). (Fig.12)

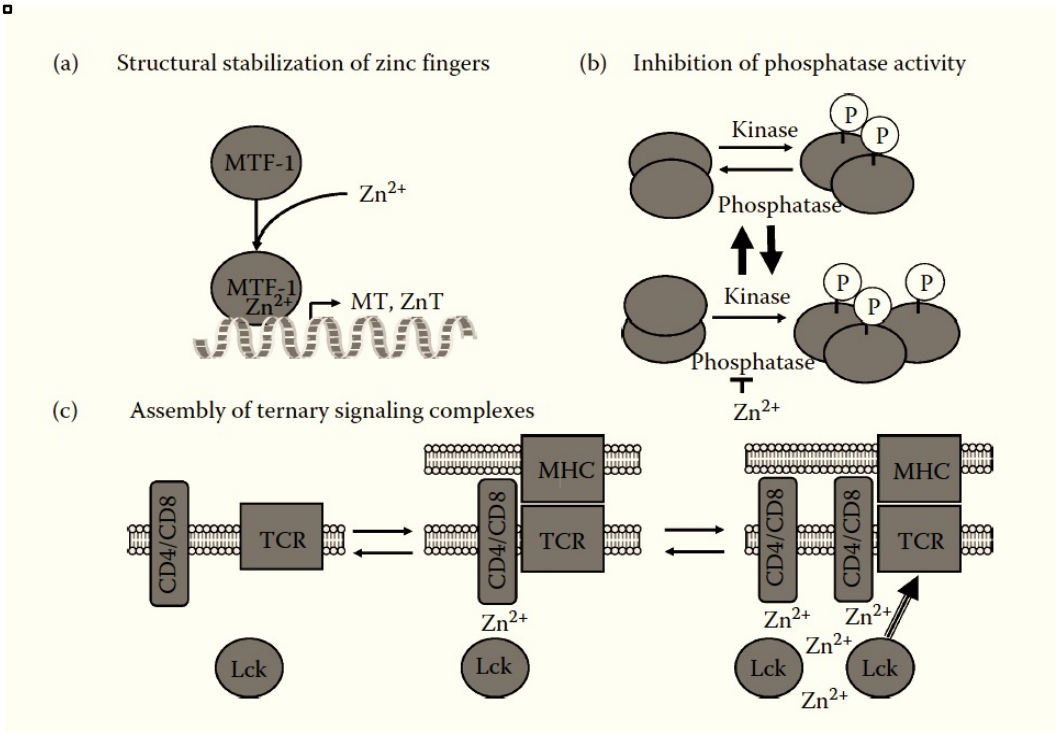


Figure 12. Three molecular mechanisms of how low nanomolar concentrations of zinc ions reversibly affect cellular signaling are known. (a) Structural stabilization. Zinc ions bind to the zinc fingers of the sensor MTF-1, which is translocated to the nucleus and activates gene transcription. (b) Regulation of enzymatic activity. Zinc ions inhibit PTPs and affect phosphorylation cascades. (c) Assembly of ternary complexes. Two zinc-dependent steps are essential for early TCR activation. First, upon contact with the MHC on an antigen-presenting cell, the kinase Lck is recruited to the complex of the TCR by binding to the co-stimulatory molecules CD4 or CD8 via a zinc ion at the interface of the two proteins. Second, a zinc-dependent dimerization through the interfaces between the SH3 domains brings Lck in close proximity, thereby promoting autocatalytic activation. (Haase & Maret 2010)

Several reports have proposed classifying intracellular zinc signals into early Zn signals which are non transcription dependent, and late Zn signals, depending on transcriptional factors taking place in the cell nucleus (Hirano et al. 2008). (Fig.13) On the one hand, early signals take place in cells where Zn levels change rapidly (several minutes) upon

extracellular stimulation (Yamasaki et al. 2007). On the other hand, late signals occur when Zn levels are for altered several hours after extracellular stimulation, through changes in Zn transporters expression. The involvement of zinc signals in biological processes is treated in a rather extensive body of literature on the role of zinc in cellular proliferation, differentiation, and apoptosis (Murakami & Hirano 2008; Hirano et al. 2008; Fukada et al. 2011).

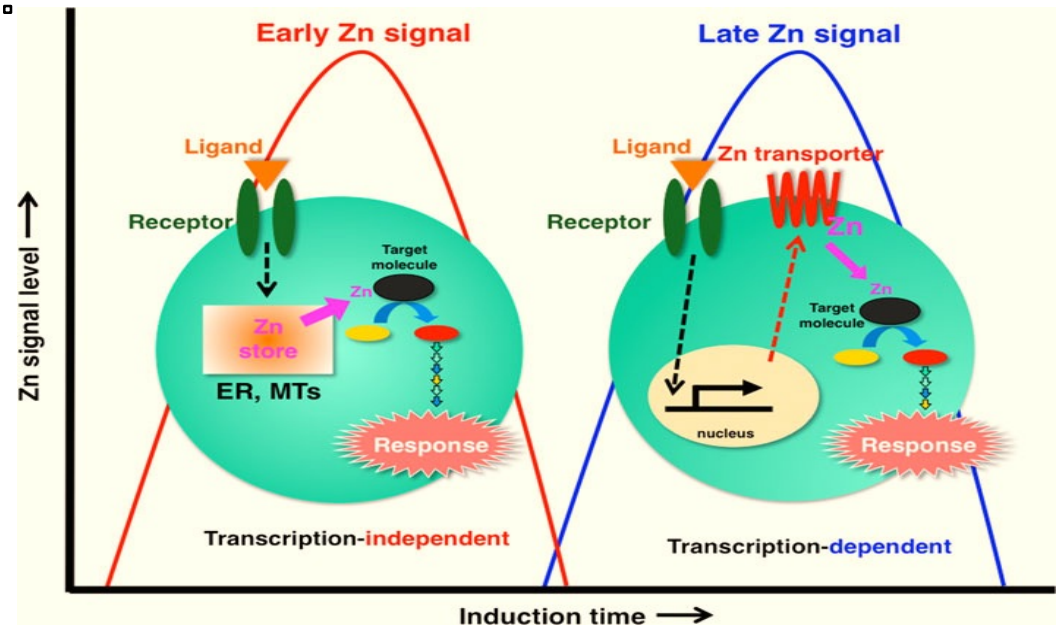


Figure 13. Intracellular Zn signaling falls into two types: early Zn signaling (left), in which an extracellular stimulus directly induces elevated Zn levels within several minutes by releasing Zn from a Zn store such as ER or MTs, and late Zn signaling (right), which is induced several hours after stimulation and is dependent on a transcriptional change in Zn transporter expression. (Fukada et al. 2011)

The total cellular zinc concentration of a few hundred micromolar is an average value that includes all cellular compartments. Significant variations in zinc concentrations of tissues have been reported, indicating that zinc is not distributed randomly. A special aspect of the cell biology of zinc is the role of zinc ions that are not tightly bound to proteins (labile zinc pool) and can be detected with fluorescent indicators such as Zinquin or FluoZin-3.

I. INTRODUCTION

Some cellular compartments, such as secretory vesicles, lysosomes, and vesicles that may or may not be identical to lysosomes and have been called “zincosomes,” the concentrations of free zinc ions can be relatively higher (millimolar range). Free zinc ions are present in synaptic vesicles of zinc-enriched nerve terminals, secretory vesicles of somatotrophic cells in the pituitary gland, zymogene granules in pancreatic acinar cells, β-cells of the islets of Langerhans, Paneth cells of the crypts of Lieberkühn, and secretory cells of the tubuloacinar glands of the prostate, the epithelium of parts of the epididymal ducts, and osteoblasts (Haase & Maret 2010; Danscher & Stoltenberg 2005). (Fig.14)

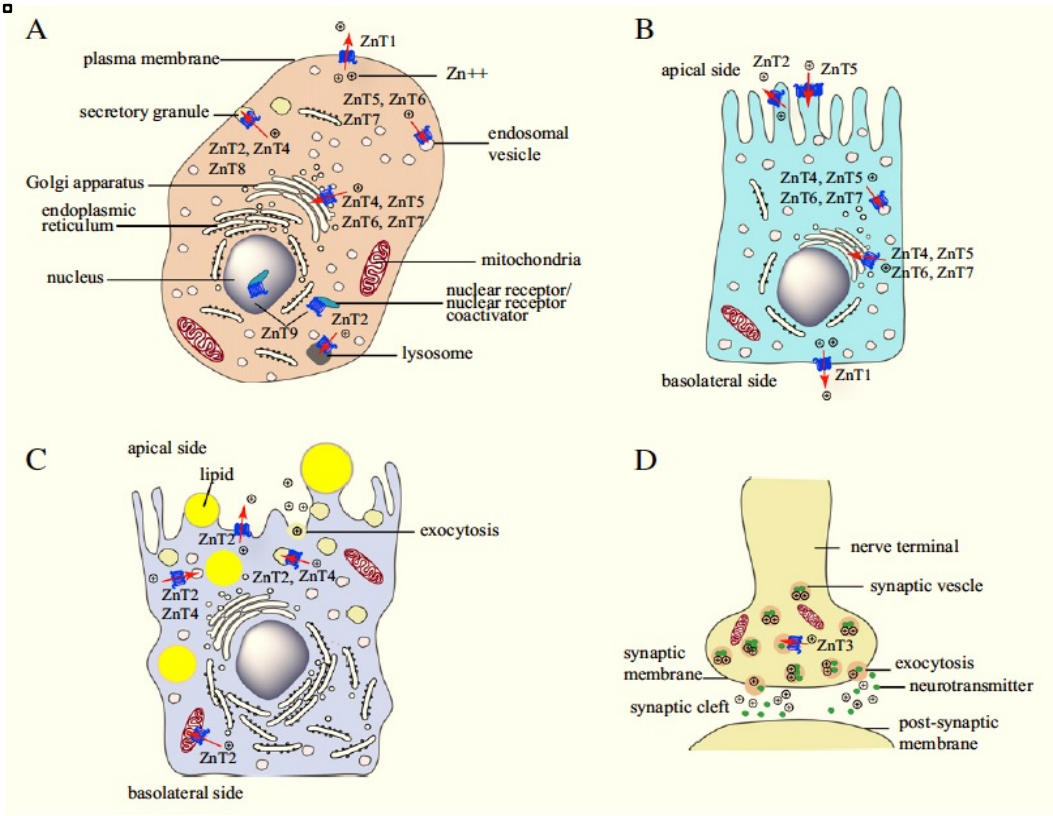


Figure 14. Cellular localization of ZnT transporters. (A) Localization of ZnT transporters in a generalized mammalian cell. (B) Localization of ZnT transporters in an intestinal epithelial cell. (C) Localization of ZnT transporters in a secreting mammary epithelial cell. (D) Localization of ZnT3 in an axon terminal. (Huang & Tepaamorndech 2013)

Pancreas needs elevated amounts of zinc for its normal physiological functions. The highest concentrations of zinc is focused in the β-cells, even though is also present in α-cells and in the zymogen granules of acinar cells (Kristiansen et al. 2001). Zn inhibition may be a mechanism to control the activity of secreted pancreatic proteins. The only protein ligand involved in binding the inhibitory zinc is glutamate. On the other hand, in the islets of Langerhans, β-cells produce insulin and release it via exocytosis in response to elevated blood glucose, thus resulting in a stimulus of glucose uptake and decreasing plasmatic glucose. (Fig.15)

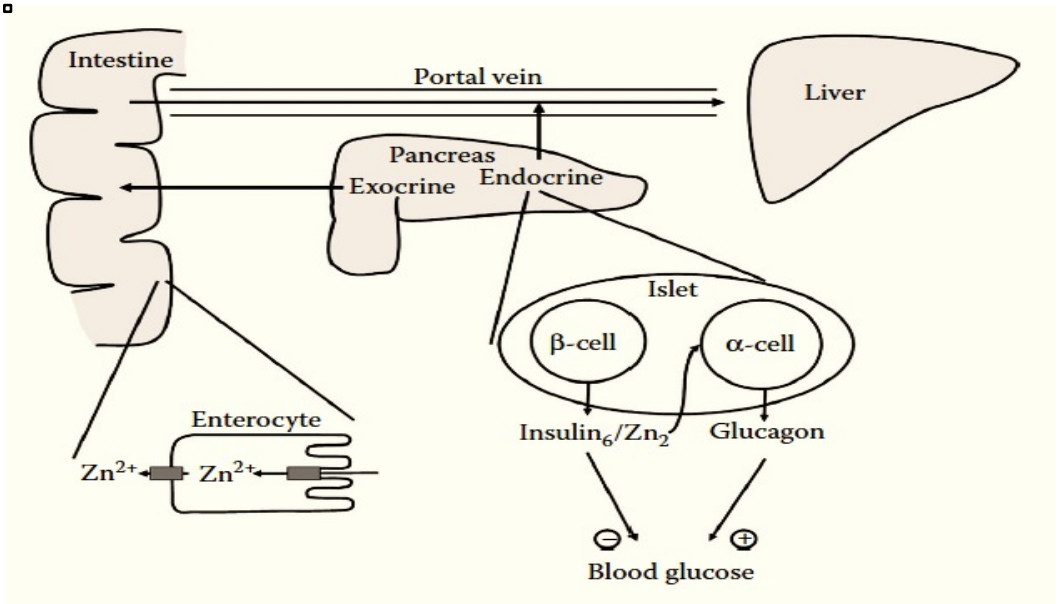


Figure 15. The exocrine pancreas stores zinc in acinar cells and secretes zinc-dependent proteases. In the endocrine pancreas (islets), insulin is stored as a crystalline Zn2–insulin hexamer in β-cells and secreted together with zinc. Secreted zinc ions inhibit the secretion of glucagon from α-cells. In enterocytes, zinc is taken up from the apical side by Zip4 and secreted by ZnT-1 at the basolateral membrane into the blood. (Haase & Maret 2010).

Prostate epithelial cells contain high amounts of Zn, being this organ one with the highest zinc concentration (9.2 mg/g) (Schroeder et al. 1970). Zinc ions are localized to the secretory granules in the lateral lobe and secretory ducts of the epithelial cells. They are secreted into the prostatic fluid in a range of millimolar concentration, which is considerably

higher than the one found in the blood. One of the functions of the secreted zinc may be to provide sufficiently high concentrations for the inhibition of proteolytic enzymes.

An exchangeable Zn ion in the brain was first documented in 1955 (Fukada et al. 2011). In neurons, exocytose stimuli induces zinc release into the surrounding milieu and its uptake into the cytoplasm through specific zinc channels on neighbouring cells. Synaptically released zinc probably travels to adjacent cells such as postsynaptic neurons and glial cells and functions as a modulator and mediator of cell-to-cell signaling (Xie & Smart 1994; Hershfinkel et al. 2001; Li et al. 2001). In this context, zinc acts as an autocrine or paracrine, cellular signaling factor, in a similar way of a neurotransmitter. (Fig.16)

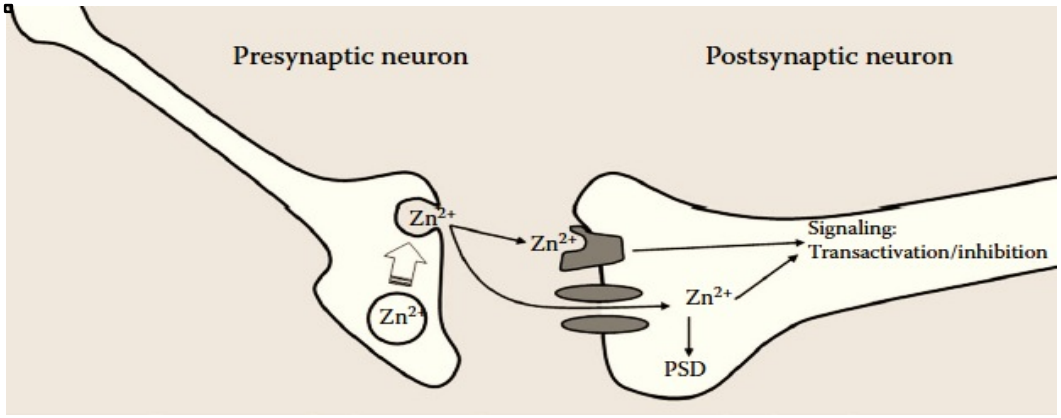


Figure 16. Specialized neurons secrete zinc ions into the synaptic cleft (presynaptic neuron). The released zinc ions affect proteins on the cytoplasmic membrane of dendrites (postsynaptic neuron) and are translocated into the cytosol where they can affect gene transcription, signaling, and assembly of the scaffolding proteins of the postsynaptic density. (Haase & Maret 2010)

Zinc is essential for formation of skeletal tissue, and it stimulates osteoblastic bone formation and inhibits osteoclastic bone resorption (Yamaguchi & Fukagawa 2005). Bone tissue contains about the 29% of total human body zinc, specially in matrix vesicles and regulates the onset of mineralization (Wuthier et al. 1992).

Paneth cells are located at the bases of the crypts of Lieberkühn in the small intestine containing significant amounts of granular zinc (Giblin et al. 2006). Paneth cells secrete microbicidal peptides called cryptidins, and zinc is thought to be an “adjuvant” to this role. Eyes contain particularly high amounts of zinc, specifically gathered in retina (464 µg/g)

and choroid (472 µg/g dry) (Grahn et al. 2001; Ugarte & Osborne 2001). Zinc is essential for vision, not only for its essential role in vitamin A metabolism, but also for its function in neural processing at the first visual synapse in vertebrates, where it's released from photoreceptor terminals (Redenti et al. 2007). Mammary Gland Epithelial Cells are also another important tissue where zinc ions play a relevant role. The zinc concentration in human milk is 3-5 mg/L, while concentrations in the colostrum are about 5 times higher (Underwood 1977). Thus, in a lactating woman, about 0.5-1 mg Zn are secreted daily into the milk (Kelleher & Lönnerdal 2005). Modulation of zinc transporters in the cell is believed to be a mechanism for controlling zinc secretion, a process in which the hormone prolactin is involved.

The zinc physiology of skin reveals another principle, namely the control of extracellular zinc by chelation. Psoriasin is secreted from keratinocytes and protects the human skin from E. coli infections, where Its antimicrobial activity has been linked to zinc chelation (Gläser et al. 2005). Furthermore, it has been demonstrated that zinc homeostasis is crucial for the function of immune cells. In addition to its role in the generation and effectiveness of immune cells, zinc is also vital for the communication between them by soluble factors, such as cytokines (Haase & Rink 2009).

New fluorescent probes and sensors such as Zinquin or FluoZin-3 made possible unprecedented temporal and spatial resolution of cellular zinc localization, mobilization, compartmentalization, and redistribution. The probes provided evidence of transients of cellular zinc ion concentrations in the picomolar to low nanomolar range and secretion of zinc ions from several cell types by vesicular exocytosis. Both probes and sensors were developed from similar technology in the calcium field and they were instrumental in developing the concepts that zinc ions are used for biological control (signaling) when fluxes are induced and that they are extensively compartmentalized in the cell.

As seen previously, the concentrations of signaling zinc ions are many orders of magnitude lower than the total concentrations of structural and catalytic zinc sites in proteins. Therefore, their characterization continues to be a huge challenge, although remarkable improvement has been done recently through the applications of novel fluorescent chelating agents.

1.6 Zinc Chelators: Sequestrant and ionophore agents

Several compounds can chelate or interact with zinc ions forming different complexes with new chemical and biological properties. Some of them bind zinc ions making them non available for the cell acting as sequestrants. In this group we can include sequestering agents such as TPEN (N,N,N′,N′-Tetrakis (2-pyridylmethyl) ethylenediamine), a membrane-permeable zinc chelator or DTPA (Diethylene triamine pentaacetic acid), a hydrophobic and membrane-impermeable chelator which chelate zinc ions making them non available for other biological functions, EGTA (Ethylene glicol-bis(2-aminoethylesther)-N,N,N',N' tetra-acetic acid), EDTA (Ethylenediamineteraacetic acid) or BAPTA (1,2-bis(2-aminophenoxy) ethane N,N N',N' tetra-acetic acid). If those complexes cross the cell membrane in an independent way of membrane channels or transporters and release the metal ions in the cytoplasmic environment, they would not only act as zinc chelators (sequestrants), but also as molecules which enhances the zinc transport within the cell, also known as zinc ionophores. (Fig.15) Some of the most important studied zinc ionophore agents are Pyrithione, Pyrrolidine dithiocarbamate (PDTC), and A23187. Other synthetic molecules known to act as zinc ionophores molecules are able to modulate intracellular labile zinc concentrations, and some of those are being tested as good candidates to treat some carcinogenic processes, such as the drug Clioquinol (Yu et al. 2009; Ding et al. 2005; Magda et al. 2008; Ding & Lind 2009).

Some studies have associated the ionophore activity involvement of two important cell survival signaling pathways; Akt and NF-κB. It has been reported that Clioquinol and PDTC, presenting more pronounced effects in the presence of extra zinc, target both Akt and NF-κB signaling pathways. Whether zinc ionophores primarily affect Akt and subsequently down-regulate NF-κB, or target both pathways simultaneously, requires further investigation. Both Akt and NF-kappa B signaling pathways are overexpressed in many types of malignant cells, and are being considered as cellular targets for chemotherapy. Further studies support the development of zinc ionophores as a novel group of anticancer agents.

Natural occurring compounds can also interact with zinc forming complexes. The medical properties of naturally occurring compounds such as chromones, coumarins and

particularly polyphenols have been well described from many years (Le Nest et al. 2004). However, the discovery that the complexes of phenolic natural occurring compounds with zinc ions are more effective than the non-complexed single molecules changed the course of drug research. Many studies have shown that these new formations could be successfully be used in a range of multiple diseases such as diabetes, neurodegenerative diseases, some bacterial infections, obesity or even cancers (Grazul & Budzisz 2009). Additionally, those complexes may play an important role in the modulation of the intracellular zinc homeostasis, which is an essential factor that affects several biological functions, such as cell signaling.

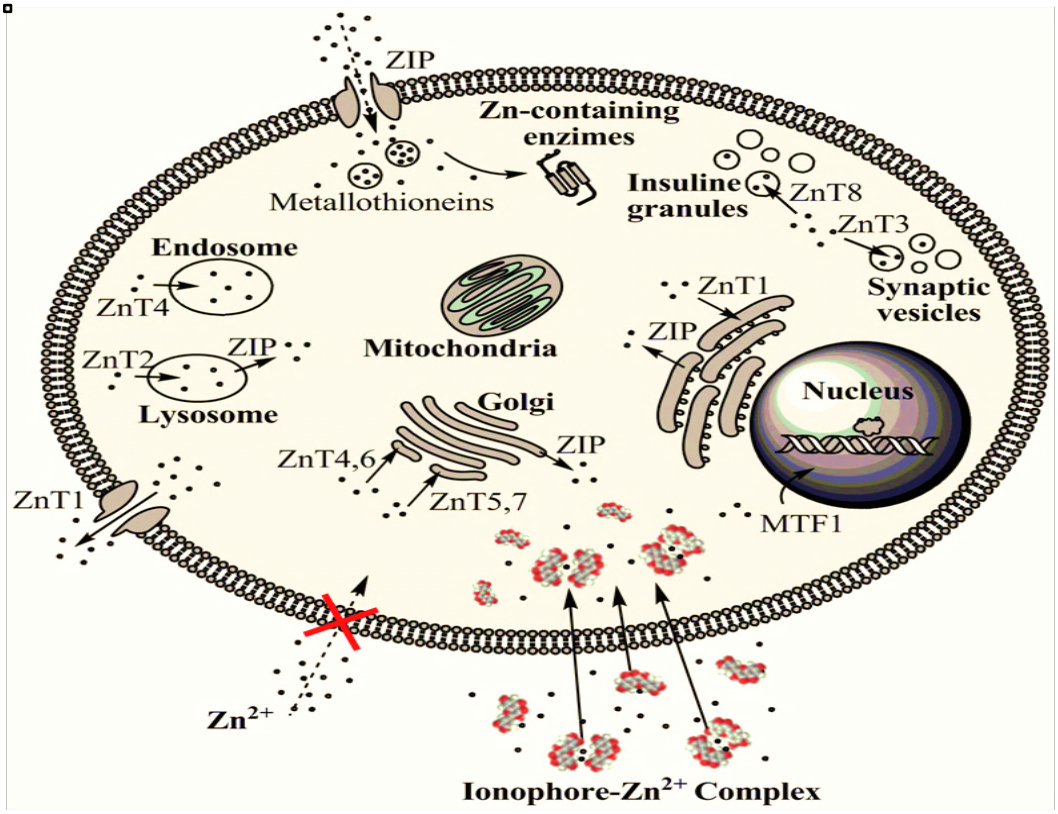


Figure 18. Natural or synthetic ionophore agents may influence zinc homeostasis providing zinc ions within the cell in an independent way of other specific zinc transporters (ZnTs and Zips). Zinc ions cannot cross the cell membrane as free molecules. (Dabbagh-Bazarbachi et al. 2014)

1.7 References

A. Hediger, M., Jeong, J. & Eide, D.J., 2013. The SLC39 family of zinc transporters. *Molecular Aspects of Medicine*, 34(2), pp.612–619.

Addink, N.W. & Frank, L.J., 1968. Remarks apropos of analysis of trace elements in human tissues. *Cancer*, 12(3), pp.544–51.

Andreini, C. et al., 2008. Metal ions in biological catalysis: from enzyme databases to general principles. *Journal of biological inorganic chemistry : JBIC : a publication of the Society of Biological Inorganic Chemistry*, 13(8), pp.1205–18.

Andreini, C. et al., 2006. Zinc through the three domains of life. *Journal of proteome research*, 5(11), pp.3173–8.

Andreini, C., Bertini, I. & Rosato, A., 2009. Metalloproteomes: a bioinformatic approach. *Accounts of chemical research*, 42(10), pp.1471–9.

Arsenault, J.E. & Brown, K.H., 2003. Zinc intake of US preschool children exceeds new dietary reference intakes. *Am J Clin Nutr*, 78(5), pp.1011–1017.

Auld, D.S., 2009. The ins and outs of biological zinc sites. *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine*, 22(1), pp.141–8.

Auld, D.S., 2001. Zinc coordination sphere in biochemical zinc sites. *Biometals*, 14(3-4), pp.271–313.

Auld, D.S. & Bergman, T., 2008. Medium- and short-chain dehydrogenase/reductase gene and protein families : The role of zinc for alcohol dehydrogenase structure and function. *Cellular and molecular life sciences : CMLS*, 65(24), pp.3961–70.

Barthel, A. et al., 1999. Regulation of GLUT1 gene transcription by the serine/threonine kinase Akt1. *The Journal of biological chemistry*, 274(29), pp.20281–6.

Bertini, I. & Cavallaro, G., 2010. Bioinformatics in bioinorganic chemistry. *Metallomics : integrated biometal science*, 2(1), pp.39–51.

Beyersmann, D. & Haase, H., 2001. Functions of zinc in signaling, proliferation and differentiation of mammalian cells. *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine*, 14(3-4), pp.331–41.

Bly, M., 2006. Examination of the zinc transporter gene, SLC39A12. *Schizophrenia research*, 81(2-3), pp.321–2.

Brautigan, D.L., Bornstein, P. & Gallis, B., 1981. Phosphotyrosyl-protein phosphatase. Specific inhibition by Zn. *The Journal of biological chemistry*, 256(13), pp.6519–22.

Brylinski, M. & Skolnick, J., 2011. FINDSITE-metal: integrating evolutionary information and machine learning for structure-based metal-binding site prediction at the proteome level. *Proteins*, 79(3), pp.735–51.

Colvin, R.A. et al., 2010. Cytosolic zinc buffering and muffling: their role in intracellular zinc homeostasis. *Metallomics : integrated biometal science*, 2(5), pp.306–17.

Colvin, R.A. et al., 2003. Zn2+ transporters and Zn2+ homeostasis in neurons. *European journal of pharmacology*, 479(1-3), pp.171–85.

Cousins, R.J., 1986. Toward a molecular understanding of zinc metabolism. *Clinical physiology and biochemistry*, 4(1), pp.20–30.

Cousins, R.J., Liuzzi, J.P. & Lichten, L.A., 2006. Mammalian zinc transport, trafficking, and signals. *The Journal of biological chemistry*, 281(34), pp.24085–9.

Csermely, P. et al., 1988. Zinc can increase the activity of protein kinase C and contributes to its binding to plasma membranes in T lymphocytes. *The Journal of biological chemistry*, 263(14), pp.6487–90.

Csermely, P., Gueth, S. & Somogyi, J., 1987. The tumor promoter tetradecanoyl-phorbol-acetate (TPA) elicits the redistribution of zinc in subcellular fractions of rabbit thymocytes measured by X-ray fluorescence. *Biochemical and biophysical research communications*, 144(2), pp.863–8.

Cherian, M.G., Jayasurya, A. & Bay, B.-H., 2003. Metallothioneins in human tumors and potential roles in carcinogenesis. *Mutation research*, 533(1-2), pp.201–9.

Chien, X.X. et al., 2006. Bioavailability, antioxidant and immune-enhancing properties of zinc methionine. *BioFactors (Oxford, England)*, 27(1-4), pp.231–44.

Chowanadisai, W., Lönnerdal, B. & Kelleher, S.L., 2008. Zip6 (LIV-1) regulates zinc uptake in neuroblastoma cells under resting but not depolarizing conditions. *Brain research*, 1199, pp.10–9.

Dabbagh-Bazarbachi, H. et al., 2014. Zinc Ionophore Activity of Quercetin and Epigallocatechin-gallate: From Hepa 1-6 Cells to a Liposome Model. *Journal of agricultural and food chemistry*.

Danscher, G. & Stoltenberg, M., 2005. Zinc-specific autometallographic in vivo selenium methods: tracing of zinc-enriched (ZEN) terminals, ZEN pathways, and pools of zinc ions in a multitude of other ZEN cells. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*, 53(2), pp.141–53.

Datta, S.R., Brunet, A. & Greenberg, M.E., 1999. Cellular survival: a play in three Akts. *Genes & development*, 13(22), pp.2905–27.

Ding, W.-Q. et al., 2005. Anticancer activity of the antibiotic clioquinol. *Cancer research*, 65(8), pp.3389–95.

Ding, W.-Q. & Lind, S.E., 2009. Metal ionophores - an emerging class of anticancer drugs. *IUBMB life*, 61(11), pp.1013–8.

Dreosti, I.E., 1993. Recommended dietary intakes of iron, zinc, and other inorganic nutrients and their chemical form and bioavailability. *Nutrition (Burbank, Los Angeles County, Calif.)*, 9(6), pp.542–5.

Ebadi, M. et al., 1995. Expression and regulation of brain metallothionein. *Neurochemistry International*, 27(1), pp.1–22.

Eide, D.J., 2009. Homeostatic and adaptive responses to zinc deficiency in Saccharomyces cerevisiae. *The Journal of biological chemistry*, 284(28), pp.18565–9.

Eide, D.J., 2011. The oxidative stress of zinc deficiency. *Metallomics : integrated biometal science*, 3(11), pp.1124–9.

Evans, G.W., 1976. Transferrin function in zinc absorption and transport. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.)*, 151(4), pp.775–8.

Ferrario, C. et al., 2008. Metallothionein 1G acts as an oncosupressor in papillary thyroid carcinoma. *Laboratory investigation; a journal of technical methods and pathology*, 88(5), pp.474–81.

Finn, R.D. et al., 2010. The Pfam protein families database. *Nucleic acids research*, 38(Database issue), pp.D211–22.

Franklin, R.B. & Costello, L.C., 2009. The important role of the apoptotic effects of zinc in the development of cancers. *Journal of cellular biochemistry*, 106(5), pp.750–7.

Frederickson, C., 2003. Imaging zinc: old and new tools. *Science's STKE : signal transduction knowledge environment*, 2003(182), p.pe18.

Fukada, T. et al., 2011. Zinc homeostasis and signaling in health and diseases: Zinc signaling. *Journal of biological inorganic chemistry : JBIC : a publication of the Society of Biological Inorganic Chemistry*, 16(7), pp.1123–34.

Fukada, T. & Kambe, T., 2011. Molecular and genetic features of zinc transporters in physiology and pathogenesis. *Metallomics : integrated biometal science*, 3(7), pp.662–74.

Gaither, L.A. & Eide, D.J., 2001. Eukaryotic zinc transporters and their regulation. *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine*, 14(3-4), pp.251–70.

Giblin, L.J. et al., 2006. Zinc-secreting Paneth cells studied by ZP fluorescence. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*, 54(3), pp.311–6.

Gläser, R. et al., 2005. Antimicrobial psoriasin (S100A7) protects human skin from Escherichia coli infection. *Nature immunology*, 6(1), pp.57–64.

Grahn, B.H. et al., 2001. Zinc and the Eye. *Journal of the American College of Nutrition*, 20(2), pp.106–118.

Grazul, M. & Budzisz, E., 2009. Biological activity of metal ions complexes of chromones, coumarins and flavones. *Coordination Chemistry Reviews*, 253(21), pp.2588–2598.

Guerinot, M.L., 2000. The ZIP family of metal transporters. *Biochimica et biophysica acta*, 1465(1-2), pp.190–8.

Günther, V., Lindert, U. & Schaffner, W., 2012. The taste of heavy metals: gene regulation by MTF-1. *Biochimica et biophysica acta*, 1823(9), pp.1416–25.

Haase, H. & Maret, W., 2010. The Regulatory and Signaling Functions of Zinc Ions in Human Cellular Physiology.

Haase, H. & Rink, L., 2009. Functional significance of zinc-related signaling pathways in immune cells. *Annual review of nutrition*, 29, pp.133–52.

Hajduch, E. et al., 1998. Constitutive activation of protein kinase B alpha by membrane targeting promotes glucose and system A amino acid transport, protein synthesis, and inactivation of glycogen synthase kinase 3 in L6 muscle cells. *Diabetes*, 47(7), pp.1006–13.

Haq, F., Mahoney, M. & Koropatnick, J., 2003. Signaling events for metallothionein induction. *Mutation research*, 533(1-2), pp.211–26.

Harding, M.M., Nowicki, M.W. & Walkinshaw, M.D., 2010. Metals in protein structures: a review of their principal features. *Crystallography Reviews*, 16(4), pp.247–302.

Hemmings, B.A. & Restuccia, D.F., 2012. PI3K-PKB/Akt pathway. *Cold Spring Harbor perspectives in biology*, 4(9), p.a011189.

Hempe, J.M. & Cousins, R.J., 1992. Cysteine-rich intestinal protein and intestinal metallothionein: an inverse relationship as a conceptual model for zinc absorption in rats. *The Journal of nutrition*, 122(1), pp.89–95.

Hershfinkel, M. et al., 2001. A zinc-sensing receptor triggers the release of intracellular Ca2+ and regulates ion transport. *Proceedings of the National Academy of Sciences of the United States of America*, 98(20), pp.11749–54.

Higashimoto, M. et al., 2009. Tissue-dependent preventive effect of metallothionein against DNA damage in dyslipidemic mice under repeated stresses of fasting or restraint. *Life sciences*, 84(17-18), pp.569–75.

Hirano, T. et al., 2008. Roles of zinc and zinc signaling in immunity: zinc as an intracellular signaling molecule. *Advances in immunology*, 97, pp.149–76.

Huang, L., Kirschke, C.P. & Gitschier, J., 2002. Functional characterization of a novel mammalian zinc transporter, ZnT6. *The Journal of biological chemistry*, 277(29), pp.26389–95.

Huang, L. & Tepaamorndech, S., 2013. The SLC30 family of zinc transporters - a review of current understanding of their biological and pathophysiological roles. *Molecular aspects of medicine*, 34(2-3), pp.548–60.

Jeong, J. & Eide, D.J., 2003. The SLC39 family of zinc transporters. *Molecular aspects of medicine*, 34(2-3), pp.612–9.

Keilin, D. & Mann, T., 1940. Carbonic anhydrase. Purification and nature of the enzyme. *The Biochemical journal*, 34(8-9), pp.1163–76.

Kelleher, S.L. & Lönnerdal, B., 2005. Zip3 plays a major role in zinc uptake into mammary epithelial cells and is regulated by prolactin. *American journal of physiology. Cell physiology*, 288(5), pp.C1042–7.

Kirschke, C.P. & Huang, L., 2003. ZnT7, a novel mammalian zinc transporter, accumulates zinc in the Golgi apparatus. *The Journal of biological chemistry*, 278(6), pp.4096–102.

Kloet, D.E.A. & Burgering, B.M.T., 2011. The PKB/FOXO switch in aging and cancer. *Biochimica et biophysica acta*, 1813(11), pp.1926–37.

Klotz, L.-O. et al., 2003. Role of copper, zinc, selenium and tellurium in the cellular defense against oxidative and nitrosative stress. *The Journal of nutrition*, 133(5 Suppl 1), p.1448S–51S.

Klug, A. & Schwabe, J.W., 1995. Protein motifs 5. Zinc fingers. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 9(8), pp.597–604.

Kozlowski, H. et al., 2009. Copper, iron, and zinc ions homeostasis and their role in neurodegenerative disorders (metal uptake, transport, distribution and regulation). *Coordination Chemistry Reviews*, 253(21), pp.2665–2685.

Krezel, A. & Maret, W., 2008. Thionein/metallothionein control Zn(II) availability and the activity of enzymes. *Journal of biological inorganic chemistry : JBIC : a publication of the Society of Biological Inorganic Chemistry*, 13(3), pp.401–9.

Kristiansen, L.H. et al., 2001. Autometallography allows ultrastructural monitoring of zinc in the endocrine pancreas. *Histochemistry and Cell Biology*, 115(2), pp.125–129.

Krizkova, S. et al., 2012. Metallothioneins and zinc in cancer diagnosis and therapy. *Drug metabolism reviews*, 44(4), pp.287–301.

Laitaoja, M., Valjakka, J. & Jänis, J., 2013. Zinc coordination spheres in protein structures. *Inorganic chemistry*, 52(19), pp.10983–91.

Laity, J.H. & Andrews, G.K., 2007. Understanding the mechanisms of zinc-sensing by metal-response element binding transcription factor-1 (MTF-1). *Archives of biochemistry and biophysics*, 463(2), pp.201–10.

Lawlor, M.A. & Alessi, D.R., 2001. PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *Journal of cell science*, 114(Pt 16), pp.2903–10.

Li, Y. et al., 2001. Rapid translocation of Zn(2+) from presynaptic terminals into postsynaptic hippocampal neurons after physiological stimulation. *Journal of neurophysiology*, 86(5), pp.2597–604.

Lichtlen, P. & Schaffner, W., 2001. Putting its fingers on stressful situations: the heavy metal-regulatory transcription factor MTF-1. *BioEssays : news and reviews in molecular, cellular and developmental biology*, 23(11), pp.1010–7.

Lue, H.-W. et al., 2011. LIV-1 promotes prostate cancer epithelial-to-mesenchymal transition and metastasis through HB-EGF shedding and EGFR-mediated ERK signaling. *PloS one*, 6(11), p.e27720.

Lukaski, H.C. et al., 1984. Changes in plasma zinc content after exercise in men fed a low-zinc diet. *The American journal of physiology*, 247(1 Pt 1), pp.E88–93.

Magda, D. et al., 2008. Synthesis and anticancer properties of water-soluble zinc ionophores. *Cancer research*, 68(13), pp.5318–25.

Manning, B.D. & Cantley, L.C., 2007. AKT/PKB signaling: navigating downstream. *Cell*, 129(7), pp.1261–74.

Maret, W. et al., 1999. Inhibitory sites in enzymes: zinc removal and reactivation by thionein. *Proceedings of the National Academy of Sciences of the United States of America*, 96(5), pp.1936–40.

Maret, W., 2009. Molecular aspects of human cellular zinc homeostasis: redox control of zinc potentials and zinc signals. *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine*, 22(1), pp.149–57.

Maret, W., 2013. Zinc biochemistry: from a single zinc enzyme to a key element of life. *Advances in nutrition (Bethesda, Md.)*, 4(1), pp.82–91.

Maret, W., 2006. Zinc coordination environments in proteins as redox sensors and signal transducers. *Antioxidants & redox signaling*, 8(9-10), pp.1419–41.

Maret, W. & Li, Y., 2009. Coordination dynamics of zinc in proteins. *Chemical reviews*, 109(10), pp.4682–707.

Margoshes, M. & Vallee, B.L., 1957. A cadmium protein from equine kidney cortex. *Journal of the American Chemical Society*, 79(17), pp.4813–4814.

Matsuura, W. et al., 2014. SLC39A9 (ZIP9) Regulates Zinc Homeostasis in the Secretory Pathway: Characterization of the ZIP Subfamily I Protein in Vertebrate Cells. *Bioscience, Biotechnology and Biochemistry*, 73(5), pp.1142–1148.

Meijer, C. et al., 2000. Role of metallothionein in cisplatin sensitivity of germ-cell tumours. *International journal of cancer. Journal international du cancer*, 85(6), pp.777–81.

Meloni, G. et al., 2006. Organization and assembly of metal-thiolate clusters in epithelium-specific metallothionein-4. *The Journal of biological chemistry*, 281(21), pp.14588–95.

Menard, M.P. & Cousins, R.J., 1983. Zinc transport by brush border membrane vesicles from rat intestine. *The Journal of nutrition*, 113(7), pp.1434–42.

Miao, X. et al., 2013. Zinc homeostasis in the metabolic syndrome and diabetes. *Frontiers of medicine*, 7(1), pp.31–52.

Milne, D.B. et al., 1987. Ethanol metabolism in postmenopausal women fed a diet marginal in zinc. *The American journal of clinical nutrition*, 46(4), pp.688–93.

Miller, J., McLachlan, A.D. & Klug, A., 1985. Repetitive zinc-binding domains in the protein transcription factor IIIA from Xenopus oocytes. *The EMBO journal*, 4(6), pp.1609–14.

Mills, C.F. ed., 1989. *Zinc in Human Biology*, London: Springer London.

Murakami, M. & Hirano, T., 2008. Intracellular zinc homeostasis and zinc signaling. *Cancer science*, 99(8), pp.1515–22.

Le Nest, G. et al., 2004. Zn–polyphenol chelation: complexes with quercetin, (+)-catechin, and derivatives: II Electrochemical and EPR studies. *Inorganica Chimica Acta*, 357(7), pp.2027–2037.

Nickolette, R., 2005. Toxicological profile for zinc. *Environmental Protection Agency.; Syracuse Research Corporation; United States Agency for Toxic Substances and Diseases*.

O'Dell, B. & Reeves, P., 1989. Zinc status and food intake. *Zinc in human biology*.

Oestreicher, P. & Cousins, R.J., 1985. Copper and zinc absorption in the rat: mechanism of mutual antagonism. *The Journal of nutrition*, 115(2), pp.159–66.

Palmiter, R.D. et al., 1992. MT-III, a brain-specific member of the metallothionein gene family. *Proceedings of the National Academy of Sciences*, 89(14), pp.6333–6337.

Palmiter, R.D. & Huang, L., 2004. Efflux and compartmentalization of zinc by members of the SLC30 family of solute carriers. *Pflügers Archiv : European journal of physiology*, 447(5), pp.744–51.

Parkin, G., 2004. Synthetic analogues relevant to the structure and function of zinc enzymes. *Chemical reviews*, 104(2), pp.699–767.

Pattison, S.E. & Cousins, R.J., 1986. Zinc uptake and metabolism by hepatocytes. *Federation proceedings*, 45(12), pp.2805–9.

Pedersen, M.Ø. et al., 2009. The role of metallothionein in oncogenesis and cancer prognosis. *Progress in histochemistry and cytochemistry*, 44(1), pp.29–64.

Petrat, F. et al., 2002. The Chelatable Iron Pool in Living Cells: A Methodically Defined Quantity. *Biological Chemistry*, 383(3-4), pp.489–502.

Plum, L.M., Rink, L. & Haase, H., 2010. The essential toxin: impact of zinc on human health. *International journal of environmental research and public health*, 7(4), pp.1342–65.

Powell, S.R., 2000. The antioxidant properties of zinc. *The Journal of nutrition*, 130(5S Suppl), p.1447S–54S.

Prasad, A.S., Halsted, J.A. & Nadimi, M., 1961. Syndrome of iron deficiency anemia, hepatosplenomegaly, hypogonadism, dwarfism and geophagia. *The American Journal of Medicine*, 31(4), pp.532–546.

Qian, J. et al., 2011. Knockout of Zn transporters Zip-1 and Zip-3 attenuates seizure-induced CA1 neurodegeneration. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 31(1), pp.97–104.

Quesada, I.M. et al., 2011. Dietary catechins and procyanidins modulate zinc homeostasis in human HepG2 cells. *The Journal of nutritional biochemistry*, 22(2), pp.153–63.

Raulin, J., 1869. Chemical studies on vegetation. *Ann Sci Nat*.

Redenti, S., Ripps, H. & Chappell, R.L., 2007. Zinc release at the synaptic terminals of rod photoreceptors. *Experimental eye research*, 85(4), pp.580–4.

Richards, M.P. & Cousins, R.J., 1976. Metallothionein and its relationship to the metabolism of dietary zinc in rats. *The Journal of nutrition*, 106(11), pp.1591–9.

Schmitt-Ulms, G. et al., 2009. Evolutionary descent of prion genes from the ZIP family of metal ion transporters. A. F. Y. Poon, ed. *PloS one*, 4(9), p.e7208.

Schroeder, H.A., Frost, D. V. & Balassa, J.J., 1970. Essential trace metals in man: Selenium. *Journal of Chronic Diseases*, 23(4), pp.227–243.

Sekler, I. et al., 2002. Distribution of the zinc transporter ZnT-1 in comparison with chelatable zinc in the mouse brain. *The Journal of comparative neurology*, 447(3), pp.201–9.

Smith, J.C., Butrimovitz, G.P. & Purdy, W.C., 1979. Direct measurement of zinc in plasma by atomic absorption spectroscopy. *Clinical chemistry*, 25(8), pp.1487–91.

Tapiero, H. & Tew, K.D., 2003. Trace elements in human physiology and pathology: zinc and metallothioneins. *Biomedicine & pharmacotherapy = Biomédecine & pharmacothérapie*, 57(9), pp.399–411.

Taylor, K.M. et al., 2007. The emerging role of the LIV-1 subfamily of zinc transporters in breast cancer. *Molecular medicine (Cambridge, Mass.)*, 13(7-8), pp.396–406.

Theocharis, S.E. et al., 2004. Metallothionein expression in human neoplasia. *Histopathology*, 45(2), pp.103–18.

Thirumoorthy, N. et al., 2007. Metallothionein: an overview. *World journal of gastroenterology : WJG*, 13(7), pp.993–6.

Thorsen, K. et al., 2011. Alternative splicing of SLC39A14 in colorectal cancer is regulated by the Wnt pathway. *Molecular & cellular proteomics : MCP*, 10(1), p.M110.002998.

Uchida, Y. et al., 1991. The growth inhibitory factor that is deficient in the Alzheimer's disease brain is a 68 amino acid metallothionein-like protein. *Neuron*, 7(2), pp.337–347.

Ugarte, M. & Osborne, N.N., 2001. Zinc in the retina. *Progress in Neurobiology*, 64(3), pp.219–249.

Underwood, E.J., 1977. Trace Elements In Human and Animal Nutrition. *Canadian Journal of Comparative Medicine and Veterinary Science*, 20(9), p.347.

Unno, J. et al., 2009. LIV-1 enhances the aggressive phenotype through the induction of epithelial to mesenchymal transition in human pancreatic carcinoma cells. *International Journal of Oncology*, 35(4), pp.813–821.

Vallee, B.L. & Auld, D.S., 1990. Active-site zinc ligands and activated H2O of zinc enzymes. *Proceedings of the National Academy of Sciences of the United States of America*, 87(1), pp.220–4.

Vallee, B.L. & Falchuk, K.H., 1993. The biochemical basis of zinc physiology. *Physiological reviews*, 73(1), pp.79–118.

Vallee, B.L. & Galdes, A., 1984. The metallobiochemistry of zinc enzymes. *Advances in enzymology and related areas of molecular biology*, 56, pp.283–430.

Weaver, B.P. et al., 2010. Zip4 (Slc39a4) expression is activated in hepatocellular carcinomas and functions to repress apoptosis, enhance cell cycle and increase migration. X. Guan, ed. *PloS one*, 5(10), p.11.

Wilson, M., Hogstrand, C. & Maret, W., 2012. Picomolar concentrations of free zinc(II) ions regulate receptor protein-tyrosine phosphatase β activity. *The Journal of biological chemistry*, 287(12), pp.9322–6.

Williams, R.J.P., 1984. Zinc: what is its role in biology? *Endeavour*, 8(2), pp.65–70.

Wimmer, U. et al., 2005. Two major branches of anti-cadmium defense in the mouse: MTF-1/metallothioneins and glutathione. *Nucleic acids research*, 33(18), pp.5715–27.

Wong, C.P. & Ho, E., 2012. Zinc and its role in age-related inflammation and immune dysfunction. *Molecular nutrition & food research*, 56(1), pp.77–87.

Wuthier, R.E. et al., 1992. Mechanism of matrix vesicle calcification: characterization of ion channels and the nucleational core of growth plate vesicles. *Bone and Mineral*, 17(2), pp.290–295.

Xie, X. & Smart, T.G., 1994. Modulation of long-term potentiation in rat hippocampal pyramidal neurons by zinc. *Pflügers Archiv : European journal of physiology*, 427(5-6), pp.481–6.

Yamaguchi, M. & Fukagawa, M., 2005. Role of zinc in regulation of protein tyrosine phosphatase activity in osteoblastic MC3T3-E1 cells: zinc modulation of insulin-like growth factor-I's effect. *Calcified tissue international*, 76(1), pp.32–8.

Yamasaki, S. et al., 2007. Zinc is a novel intracellular second messenger. *The Journal of cell biology*, 177(4), pp.637–45.

Yu, H. et al., 2009. Clioquinol targets zinc to lysosomes in human cancer cells. *The Biochemical journal*, 417(1), pp.133–9.

Zalewski, P.D. et al., 2005. Zinc metabolism in airway epithelium and airway inflammation: basic mechanisms and clinical targets. A review. *Pharmacology & therapeutics*, 105(2), pp.127–49.

Zhao, L. et al., 2007. LIV-1 suppression inhibits HeLa cell invasion by targeting ERK1/2-Snail/Slug pathway. *Biochemical and biophysical research communications*, 363(1), pp.82–8.

2. Polyphenols

2.1 Overview

Polyphenols (PFs) are natural occurring phytochemicals derived from phenylalanine and containing an aromatic ring with a reactive hydroxyl group. (Signorelli & Ghidoni 2005) They are mainly found in fruits, vegetables, cereals, some beverages and seeds, such as grapes, berries, wine, green tea or cocoa. This secondary metabolites of plants constitute one of the most extensively group of chemicals found in the plant kingdom and consumed in the diet. From several decades, their consumption has been associated with healthy benefits, preventing and ameliorating several diseases. One hypothesis for this behaviour between plants and animals could be explained by a phenomenon called xenohormesis. As the prefix *xeno* comes from the Greek word meaning stranger or foreigner, xenohormesis describes the phenomenon of a “foreign” organism's stress response producing chemicals that yield benefits to another organism. (Fig. 19)

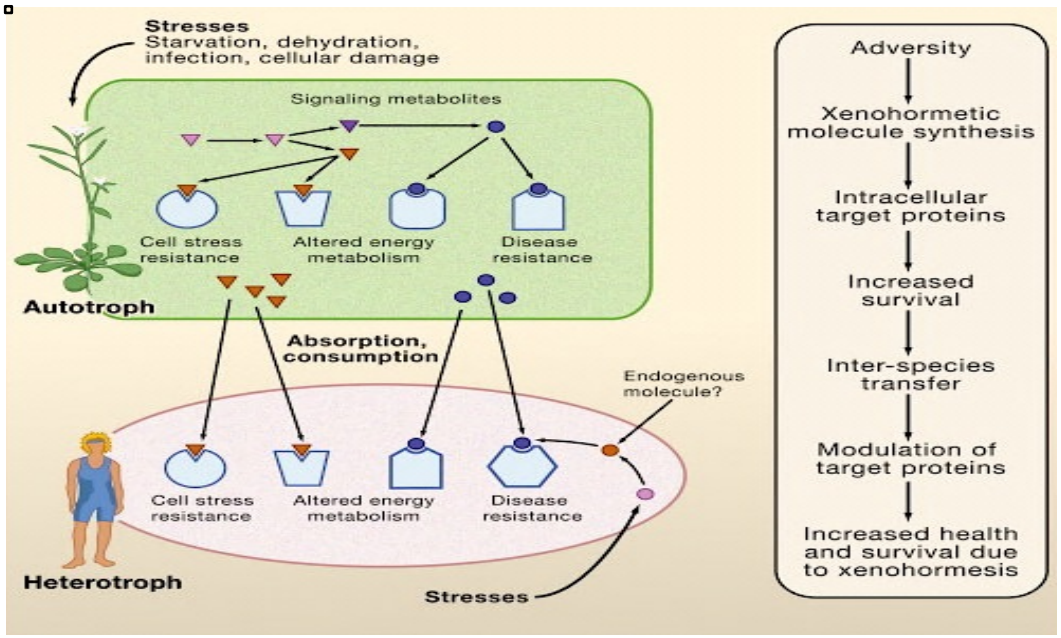


Figure 19. Xenohormesis hypothesis. The theory predicts that many key mammalian enzymes and receptors will have evolved binding pockets that allow modulation by molecules produced by other species. (Howitz & Sinclair 2008)

The word most often refers to the ability of stressed plants to confer stress tolerance to animals that consume them. This singularity was first named by Howitz and Sinclair (Howitz & Sinclair 2008; Hooper et al. 2010). Although the toxic properties of xenohormetic phytochemicals are harmful to microorganisms or insects, once ingested by humans as part of their diet, the same compounds are considered to induce mild cellular stress responses (Son et al. 2008; Mattson et al. 2007). Additionally, it has been described that after an oral ingestion of this compounds, there is an activation of adaptive stress response signaling pathways, leading to an increased expression of genes mostly encoding cytoprotective proteins including antioxidant enzymes, phase-2 detoxifying enzymes, protein chaperones, growth factors, mitochondrial proteins, etc. (Hooper et al. 2010). As xenohormetic phytochemicals can improve our body's functions by stimulating our cellular stress response, they can be applied in drug development and the nutritional enhancement of diet (Hooper et al. 2010).

One of the most important evidence between the regular consumption of polyphenols in the diet and the benefits in health was reported in France, with the phenomena called "French paradox". This dietary anomaly was first observed in French population and found later in other Mediterranean populations. It has been observed that the Southern French had a very low incidence of coronary heart disease despite their high fat diet, low exercise, and smoking habits (de Lange et al. 2007; Renaud & de Lorgeril 1992). The exact molecular mechanism underlying this prevention still poorly understood. One of the features that has been emphasized relates to the high consumption of red wine in the French population and the question as to whether the phenolic antioxidants from this dietary source (mainly resveratrol) contribute to the protection from coronary heart disease, beside with the antioxidants consumed in the olive oil and the high intake of antioxidant nutrients from the fresh fruits and vegetables found in the Mediterranean diet (Rice-Evans & Miller 1995). Similarly, Hertog et al. (Hertog et al. 1993) observed an inverse association of the flavonol intake (mainly quercetin) with the mortality from coronary heart disease in the Zutphen Elderly Study (Netherlands).

On the other hand, another phenomena related to health benefits of phenolic compounds is the "Asian paradox", which is considerable older than the French one. Archaeological findings had revealed that infusions of leaves from various wild plants, including the tea plant, might have been consumed for more than five thousand years. Some legends from China and India suggest that use of tea occurred as far back as 2737 years BC when the

Chinese Emperor Shen Nung, found himself with a beverage holding a pleasant aroma and refreshing taste after dried leaves accidentally blew into hot boiling water. This ancient practice rapidly was associated with curative purposes. Nowadays tea is the most widely consumed beverage in the world, second only to water (Yang et al. 2004; Vinson et al. 2004) with a worldwide per capita consumption of 40 liters per year (Vinson et al. 2004). Next to green tea, one of the most highly consumed products in Asia is tobacco. Evidence strongly associates cigarette smoking with cardiovascular events, including myocardial infarction, stroke, peripheral vascular disease, aggravation of stable angina pectoris, re-thrombosis after thrombolysis, restenosis after angioplasty, and even sudden death, and cancer (Yun et al. 2005; Kilaru et al. 2001). Despite the high consumption of tobacco, Asian population, and Japan in particular have among the lowest incidences of arteriosclerosis and lung cancer per capita (table 5). It has been postulated that this paradox, the "Asian Paradox," exists as a result of the high consumption of green tea in this region, which contains high amounts of flavonols (Dulloo et al. 1999).

	Annual CAD mortality/100,000 population		Lung cancer mortality/100,000 population		Cigarette smokers (%)†	Cigarettes (per adult/y)‡	Tea consumption (kg/person/y)§
Country	Men	Women	Men	Women	Total	Total	Total
Ireland	422	183	50.0	29.0	—	2,316	1.5
China	401	276	34.9	17.4	35.6	1,780	—
United Kingdom	382	178	74.8	44.3	—	1,553	2.3
US	348	177	66.6	45.1	27.0	2,092	0.2
New Zealand	318	139	48.3	29.6	—	1,038	1.0
Canada	280	133	71.4	43.2	25.0	1,820	0.2
Rep of Korea	261	149	39.6	13.0	36.9	2,668	—
Italy	255	108	97.1	19.6	24.9	2,041	0.1
Australia	237	103	50.8	22.9	19.5	1,708	0.8
France	208	74	75.0	14.6	34.5	1,757	0.2
Japan	186	81	66.1	23.5	33.1	2,950	0.9

Table 5. Lifestyle Factors and Mortality from Cardiovascular Disease and Lung Cancer. †Percentage of total population who smoked at least one cigarette a day. ‡Data refers to estimates of apparent consumption based on cigarette production, imports, and exports moving average between 1992 and 2000. §1 kg= approximately 311 cups. CAD, coronary artery disease. (Sumpio et al. 2006)

I. INTRODUCTION

In the last years significant number of studies have focused in dietary phenolic compounds as natural improvers of health. (Fig.20) More than 8000 dietary polyphenols have been identified (Araújo et al. 2011). This growing interest for this compounds resides in the accumulating evidence for their ability to trigger several cellular pathways leading to the prevention and/or amelioration of pathological conditions acting as Antioxidants (Leopoldini et al. 2011), anticarcinogenics (Čipák et al. 2003; Ramos 2008), anti-inflammatories (Bravo 1998), neuroprotectors (Russo et al. 2012), antilipidemic and vasorelaxing among others (Araújo et al. 2011). There is an emerging view that flavonoids and their *in vivo* metabolites do not act only as conventional antioxidants but may also exert modulatory actions on cellular system through direct action on various signaling pathways (Mansuri et al. 2014).

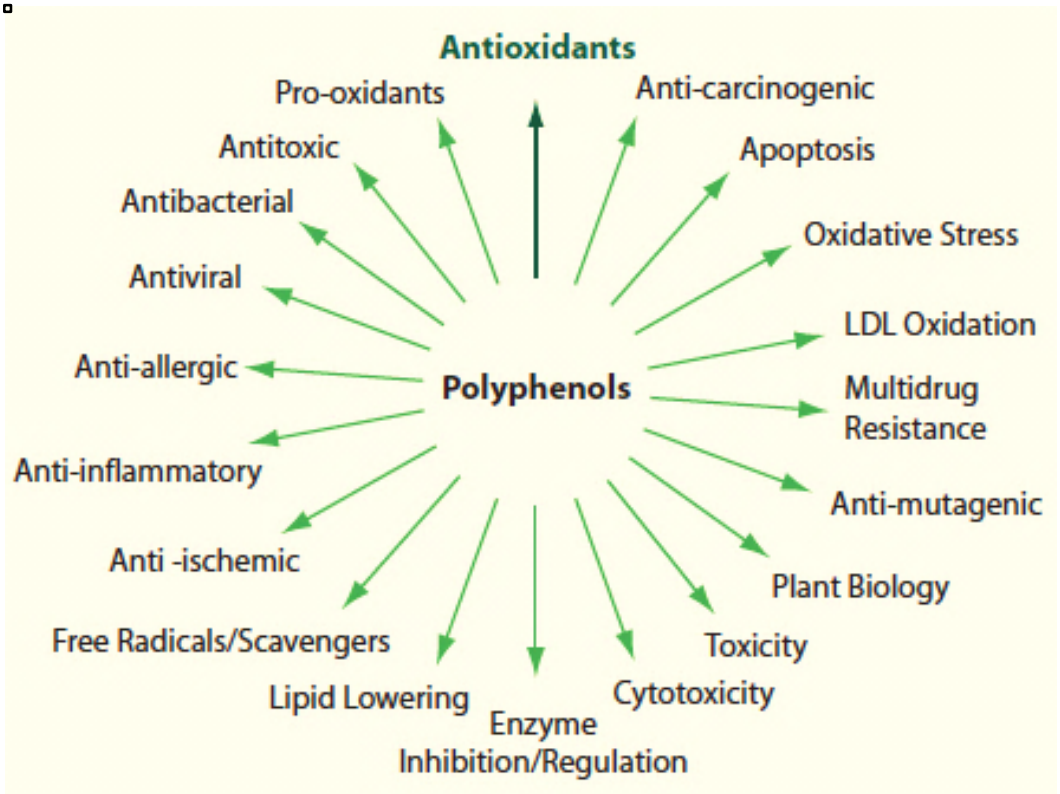


Figure 20. Polyphenols have attracted scientific attention as a potential nutritional strategy to prevent a broad range of chronic disorders. Based on (Mansuri et al. 2014; Araújo et al. 2011)

2.2 Structure and classification of polyphenols

In the last decades polyphenols have been extensively studied due to their properties and beneficial effects when taken as a dietary supplement against several chronic diseases, such as cardiovascular diseases, ageing or cancer (Hu 2007). Since then, more than 8000 phenolic compounds have been identified and described containing several sub-groups according to their chemical structure, such as flavonoids, phenolic acids, lignans, stilbenes and other polyphenols (Cheynier, 2005; Harborne & Williams, 2000; Ito et al., 2005).

By far flavonoids are the most abundant polyphenols found in food and the most studied sub-group with over 4000 (Bravo 1998). The representative structure of flavonoids (Fig.21) contains at least two aromatic rings, bearing one or more hydroxyl groups linked together through a carbon bridge (Lotito & Frei 2006; Beecher 2003). According to the position of the B ring relative to the C ring, as well as the functional groups (ketones, hydroxyls) and presence of a double bond or not in the C ring, flavonoids may be further divided into 7 subclasses: anthocyanins, chalcones, dihydrochalcones, dihydroflavonols, flavan-3-ols, flavanones, flavones, flavonols and isoflavonoids (Ramos 2008). (Table 6)

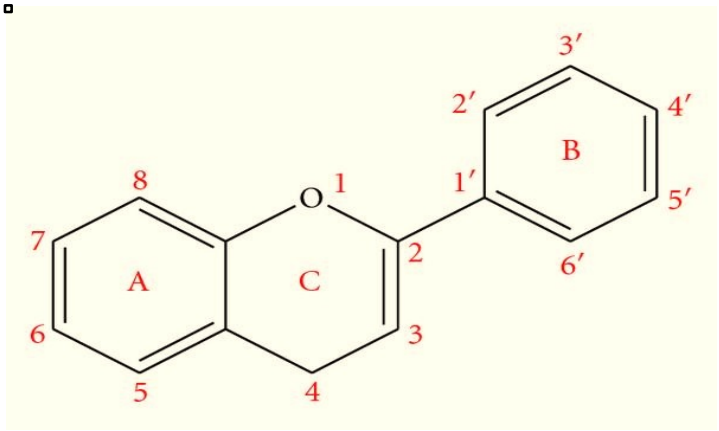


Figure 21. Common Flavonoid Carbon Skeleton Structure. One aromatic ring (A) is connected to the second aromatic ring (B) by a carbon bridge, which consists of three carbon atoms. When the three-carbon chain is connected to a hydroxyl group from A, the formed structure becomes cyclic (C). (Jackson et al. 2011)

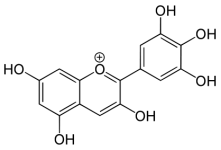
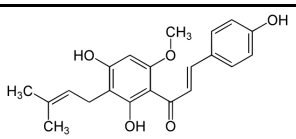
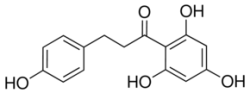
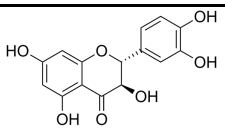
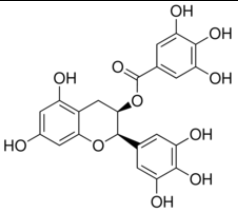
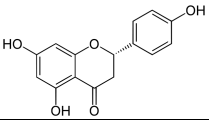
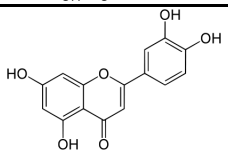
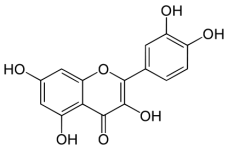
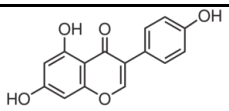
FLAVONOIDS	EXAMPLE	STRUCTURE	DIETARY SOURCE
Anthocyanins	Delphinidin		-Pommegranate -Red wine -Common bean -Black grape
Chalcones	Xanthohumol		-Beer -Hops
Dihydrochalcones	Phloretin		-Apple -Apricot
Dihydroflavonols	Dihydroquercetin (Taxifolin)		-Mexican oregano -Acai berry
Flavanols (Flavan-3-ols)	(−)-Epigallocatechin gallate (EGCG)		-Green tea -Black tea -Apple -Blackberry -Hazelnut -Pecan nut
Flavanones	Naringenin		-Mexican oregano -Grapefruit -Orange -Tomato
Flavones	Luteolin		-Olive -Mexican oregano -Globe artichoke -Green pepper
Flavonols	Quercetin		-Cocoa -Onion -Elderberry -Wine -Black plum -Kale
Isoflavonoids	Genistein		-Soy -Red clover

Table 6. Classification, structure, and dietary sources of flavonoids. Based on (Rothwell et al. 2012)

Phenolic acids can be divided into four main types; benzoic acid, cinnamic acid, phenylacetic acid and phenylpropanoic acid derivatives (table 7). Benzoic and cinnamic acids are predominantly found in dietary sources, while the others are less common. The hydroxybenzoic acid content of edible plants is generally low, with the exception of certain red fruits, black radish and onions, which can have concentrations of several tens of milligrams per kilogram fresh weight (Shahidi & Naczk 1995). The hydroxycinnamic acids are more common than hydroxybenzoic acids and consist principally of coumaric, caffeic, ferulic and sinapic acids. While fruits and vegetables contain many free phenolic acids, in grains and seeds (particularly in the bran or hull) phenolic acids are often in the bound form (Groot & Rauen 1998; Adlercreutz & Mazur 2013; Wink 1997).

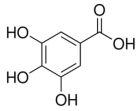
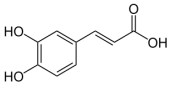
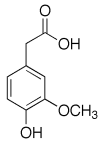
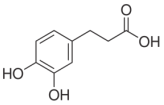
PHENOLIC ACIDS	EXAMPLE	STRUCTURE	DIETARY SOURCE
Hydroxybenzoic acids	Gallic acid		-Chestnut -Black tea -Blackberry -Green chicory
Hydroxycinnamic acids	Caffeic acid		-Coffee -Black chokeberry -Plum
Hydroxyphenylacetic acids	Homovanillic acid		-Green olive -Black olive -Beer
Hydroxyphenylpropanoic acids	Dihydrocaffeic acid		-Green olive -Black olive

Table 7. Phenolic acids, structures and main dietary sources. Based on (Rothwell et al. 2012)

Lignans are diphenolic compounds that contain a 2,3-dibenzylbutane structure that is formed by the dimerization of two cinnamic acid residues (Table 8). Several lignans, such as pinoresinol are considered to be phytoestrogens (Meagher et al. 1999). Moreover, some studies have reported that lignans can potentially reduce the risk of certain cancers and cardiovascular diseases (Milder et al. 2005). One of the richest food source is sesame seed, which contains up to 29,331 μg/100g. Brassica vegetables also contain high levels of lignans (Milder et al. 2005).

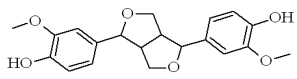
LIGNANS	EXAMPLE	STRUCTURE	DIETARY SOURCE
Lignans	Pinoresinol		-Olive oil -Sesame seed -Brassica

Table 8. Pinoresinol as a lignan example. Structure and main dietary sources.

Based on (Rothwell et al. 2012)

Stilbenes contain two phenyl moieties connected by a two-carbon methylene bridge. (Table 9) Occurrence of stilbenes in the human diet is relatively low. Most stilbenes in plants act as antifungal compounds that are synthesized only in response to infection or injury. One of the best studied, naturally occurring stilbene is resveratrol (3,4',5-trihydroxystilbene), largely found in grapes and red wine. This polyphenol has been widely studied since the discovery of the "French paradox", and several beneficial health effects had been reported (Gliemann et al. 2013; Valenzuela 2014; Timmers et al. 2013).

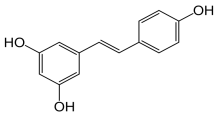
STILBENES	EXAMPLE	STRUCTURE	DIETARY SOURCE
Stilbenes	Resveratrol		-Muscadine grape -Lingonberry -Cranberry -Red wine

Table 9. Resveratrol as a stilbene example. Structure and main dietary sources.

Based on (Rothwell et al. 2012)

In addition to flavonoids, phenolic acids, lignans or stilbenes, there are some polyphenols found in diet that are also considered to be important for human health. They are classified as "other polyphenols" and include several compounds with different multiples phenolic structural units. The number and characteristics of these phenol structures provide exclusive physical, chemical, and biological properties. (Table 10)

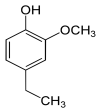
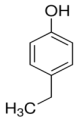
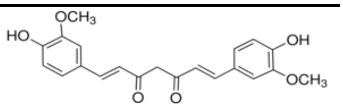
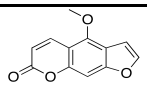
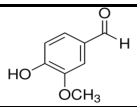
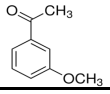
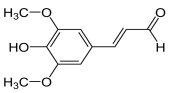
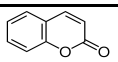
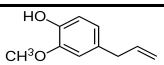
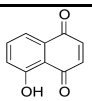
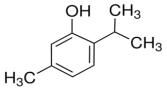
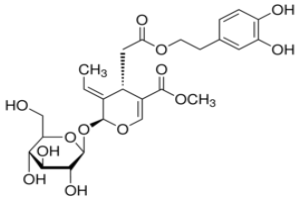
OTHER POLYPHENOLS	EXAMPLE	STRUCTURE
Alkylmethoxyphenols	4-Ethylguaiacol	
Alkylphenols	4-Ethylphenol	
Curcuminoids	Curcumin	
Furanocoumarins	Bergapten	
Hydroxybenzaldehydes	Vanillin	
Hydroxybenzoketones	3-Methoxyacetophenone	
Hydroxycinnamaldehydes	Sinapaldehyde	
Hydroxycoumarins	Coumarin	
Hydroxyphenylpropenes	Eugenol	
Methoxyphenols	Guaiacol	
Naphtoquinones	Juglone	
Phenolic terpenes	Thymol	
Tyrosols	Oleuropein	
Other polyphenols	Catechol	

Table 10. Other Polyphenols. Structures and examples. Based on (Rothwell et al. 2012)

2.3 Absorption and metabolism

The daily consumption of flavonoids depends on the diet and varies from tenths of milligrams to a few grams (Manach et al. 2004). In order to understand the mechanisms of action of dietary polyphenols, it is essential to know their release and absorption from food, their metabolism and their bioavailability. Bioavailability is the correlation between the nutrient that has been digested, absorbed and metabolized (Pandey & Rizvi 2009). Before dietary polyphenols can be absorbed from the gut, they must be released from food sources by the mechanic digestion (chewing), action of the digestive juices in the gastrointestinal tract, and finally the microorganisms of the colon (Hollman & Katan 1997). For polyphenols to become bioavailable, the following barriers must be overcome: solubility, permeability, metabolism, excretion, target tissue uptake and disposition (Hu 2007; Scalbert & Williamson 2000).

In the intestinal lumen, flavonoid-O-glycosides are attacked by hydrolases displaying multiple enzymatic activities, which results in the release of flavonoid aglycones. The aglycones are transported to the human body through the membranes of the intestinal epithelium, which covers more than 90% of the intestinal surface (Day et al. 2000). The bioavailability of flavonoids is very low. Less than 1% of the consumed flavonoids enter the blood circulation (Manach et al. 2005). The hydrolysis of flavonoid glycosides by β-glucosidase and the following attachment of glucuronic acid occur after the diffusion of the glycosides into the cytoplasm of enterocytes, which are the cells of the intestinal epithelium (Gee et al. 2000). Furthermore, the portal vein transports these substances to the liver, where they are methylated and sulfated with appropriate transferases (Alvarez et al. 2010). (Fig.22)

Although the aglycones can be absorbed from the small intestine, dietary polyphenols are mainly present in the form of esters, glycosides, or polymers that cannot be absorbed in their native form (Hu 2007). In order to be absorbed, these substances need to be hydrolyzed by the intestinal enzymes or by the colonic microflora. This process reduces the absorption efficiency because aglycones also are affected by degradation producing many simple aromatic acids. During the course of absorption, polyphenols are conjugated in the small intestine and later in the liver. This phase mainly includes methylation,

sulfation, and glucuronidation. The conjugation mechanisms are highly efficient, and aglycones are generally either absent in blood or present in very low concentrations after consumption of nutritional doses. In the blood, only 5-10% of the flavonoids are not modified (Clifford 2004), whereas glucuronides are predominant (Zhang et al. 2007). (Fig.23)

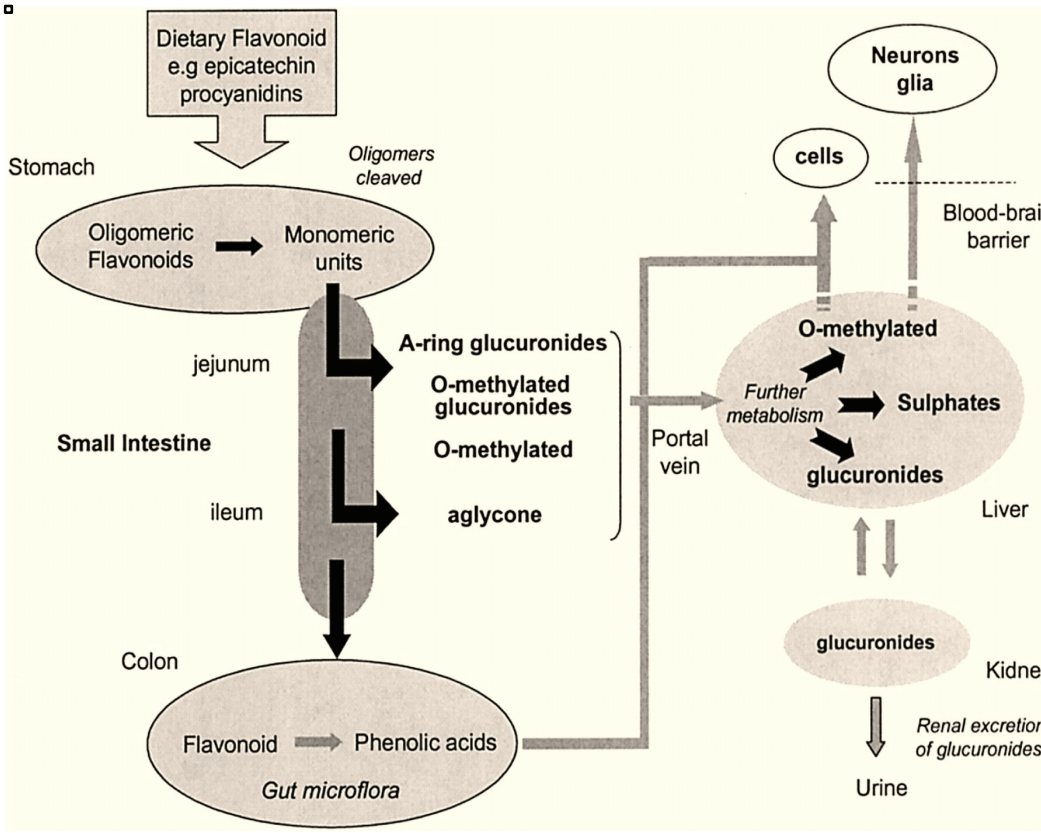


Figure 22. Metabolism of dietary flavonoids in the gastrointestinal tract. Schematic of relevant features concerning to the absorption, metabolism, distribution, and excretion of polyphenols. (Spencer 2003)

Plasma concentration reached after polyphenol ingestion varies highly according to the nature of the polyphenol and the food source. It is on the order of 0,3-0,75 μmol/L after consumption of 80-100 mg quercetin equivalent administered in the form of apples, onions, or meals rich in plant products (Graefe et al., 2001; P. C. Hollman et al., 1997; Manach et al., 1998). When ingested in the form of green tea (0,1-0,7 μmol/L for an intake of 90-150

mg), cocoa (0,25-0,7 μmol/L for an intake of 70-165 mg) (Lee et al. 1995; Unno et al. 1996; Rein et al. 2000; Wang et al. 2000), or red wine (0,09 μmol/L for an intake of 35 mg) (Donovan et al. 1999), catechin and epicatechin are as effectively absorbed, as is quercetin (Manach et al. 2004).

However polyphenol metabolites are not circulating free in the blood. *In vitro* studies incubating quercetin in normal human plasma revealed that quercetin is widely bound to plasma proteins (Boulton et al. 1998). Metabolites of quercetin are also extensively bound to plasma proteins in rats fed with a quercetin-enriched diet (Manach et al. 1995), where albumin is the main responsible protein for that binding. The affinity of polyphenols for albumin varies according to their chemical structure (Manach et al. 1998).

Determination of the bioavailability of polyphenols in tissues may be much more important than the plasma concentrations (Manach et al. 2004; Hollman & Katan 1997; Hu 2007). It has been reported that plasma concentrations are not directly correlated with concentrations in target tissues and that the distribution between blood and tissues differs between different polyphenols (Manach et al. 2004). Experimental studies have demonstrated using single doses of radiolabeled polyphenols (quercetin, epigallocatechin-3-gallate or resveratrol) given to rats or mice and sacrificed 1-6 hours later, that phenolic compounds are mainly located in blood and tissues of the digestive system, such as the stomach, intestine, and liver (Ueno et al. 1983; Suganuma et al. 1998; Mullen et al. 2002; Vitrac et al. 2003). Conversely, polyphenols have been also detected through HPLC analysis in a wide range of tissues in rodents, including endothelial cells (Youdim et al. 2000), heart, kidney, spleen, pancreas, prostate, uterus, ovary, mammary gland, testes, bladder, bone, and skin (Suganuma et al. 1998; Chang et al. 2000; Kim et al. 2000; Coldham & Sauer 2000).

Several studies have also proposed that polyphenols could cross the blood-brain barrier and arrive into the brain, both as its native or metabolized form (Williams & Spencer 2012). For example, (-)-epicatechin and some of its principle metabolites have been detected in rodent brain after oral administration (van Praag et al. 2007; Abd El Mohsen et al. 2002) and the brain levels of catechin and (-)-epicatechin have been found to increase upon repetitive dosing with a grape seed polyphenolic extract (Ferruzzi et al. 2009). Similar

observations have been made with EGCG (Lin et al. 2007; Suganuma et al. 1998), flavanones (Peng et al. 1998), flavonols (Rangel-Ordóñez et al. 2010), and anthocyanins (Talavéra et al. 2005; El Mohsen et al. 2006).

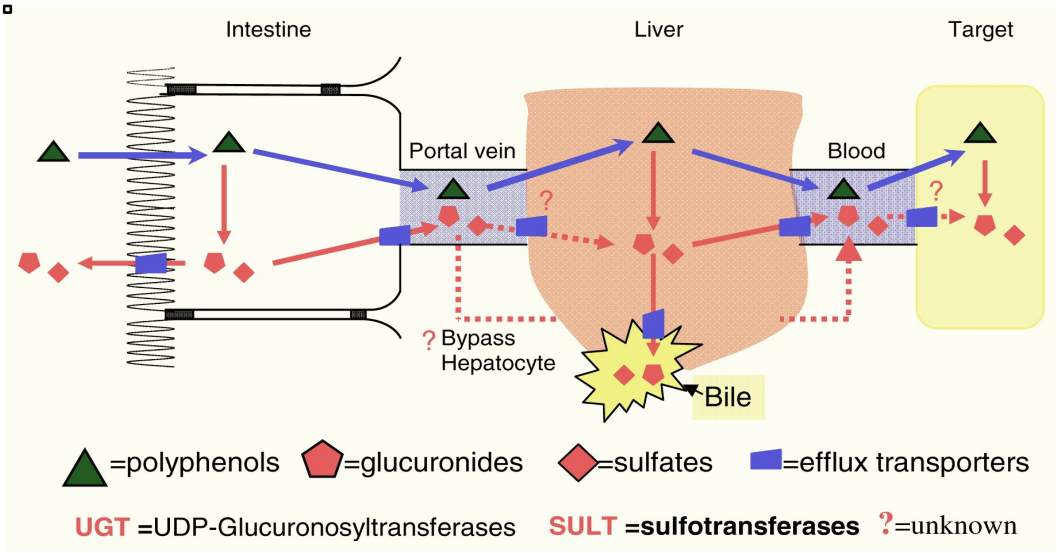


Figure 23. Schematic representation of metabolic barriers to polyphenol bioavailability. (Hu 2007)

Polyphenols and their derivatives are eliminated chiefly in urine and bile. Large, extensively conjugated metabolites are more likely to be eliminated in the bile, whereas small conjugates such as monosulfates are preferentially excreted in urine. When polyphenols are secreted via the biliary route into the duodenum, they are subjected to the action of bacterial enzymes, especially ß-glucuronidase in the distal segments of the intestine, after which they may be reabsorbed. This enterohepatic recycling may lead to a longer presence of polyphenols within the body (Manach et al. 2004). Biliary excretion of polyphenols in humans may differ greatly from that in rats because of the existence of the gall bladder in humans; however, this has never been examined.

Partial information is available on the amounts of polyphenols that are consumed daily throughout the world. These data have been obtained through analysis of the main

aglycones (after hydrolysis of their glycosides and esters) in the nutrients most widely consumed by humans.

In 1976 Kühnau (Kühnau 1976) calculated that dietary flavonoid intake in the United States of America was 1 gram per day distributed as follows: 16% flavonols, flavones, and flavanones; 17% anthocyanins and 20% catechins. Although these figures were obtained under poorly detailed conditions, they continue to serve as reference data. In Spain the total consumption of catechins and proanthocyanidin dimers and trimers has been estimated at 18-31 mg/d, and the main sources are apples, pears, grapes, and red wine (Manach et al. 2004).

Another important issue is how phenolic compounds or their metabolites move across different biological membranes, assuming that some of these are active or can be converted into active parent compounds at target organs. Transport of lipophilic conjugates out of the main metabolic organs such as liver and intestine has only been studied recently, and available evidence suggests that a variety of organic transporters may be involved in the transport of these conjugates in and out of cells (Hu 2007).

2.4 Interaction with metals

In the last decades, phenolic compounds were known to interact with different metals, and because of their distinctive chemical structure, they can easily form complexes through metal ion chelation (Hider et al. 2001) in a comparable way of other well-known metal chelators such as the anticancer drug Clioquinol, which also exerts a ionophore activity (Fig.24). First evidence of those complexes was reported in 1962 between aluminium ions and flavonoids. Since then, and specially in the 80's, scientist have investigated more than 40 metal-flavonoid complexes (Grazul & Budzisz 2009).

All types of flavonoids possess three main domains able to react with metal ions; the 3,4-dihydroxy motif located on the B ring, the 3-hydroxy or 5-hydroxy group in the C ring and the 4-carbonyl group also found in the C ring. The 3- or 5-hydroxypyran-4-one (sometimes the ortho-hydroxyl) groups in the B flavonoid ring also has an important role in chelating (Grazul & Budzisz 2009). Furthermore, it is still not clear which element of the ligand plays a central role in metal ion chelation. Through IR spectroscopy of the Pd(II)-quercetin complex and UO_2-rutin complexes, Malesev and Kunti (Malešev & Kuntić 2007) showed that the benzoyl moiety is the basic site for metal chelation. Moreover, Cornard and Merlin (Cornard & Merlin 2002) proved that the ortho-dihydroxyl system of quercetin is unable to bind Al(III) in acidic media. However, they indicated that the Al(III)-quercetin complexes possess two binding sides: 3-hydroxychromone, by which the complex is formed, and the ortho-hydroxy groups, which are the active groups depending on the medium and pH.

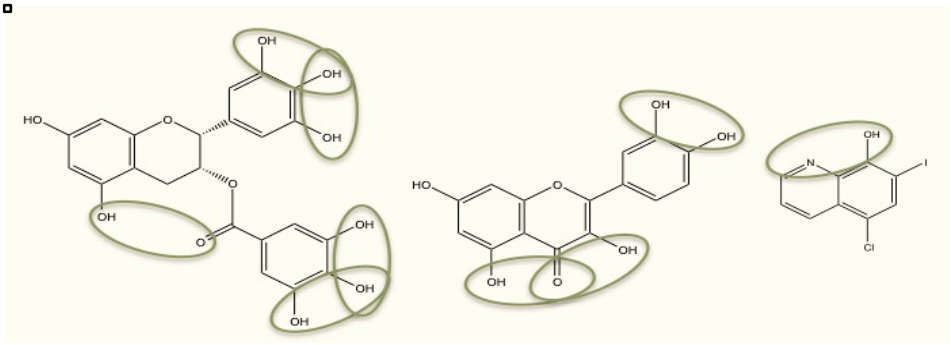


Figure 24. Proposed structures and potential metal-binding sites of EGCG (left) and Quercetin (central) compared to Clioquinol (right). Based on (Hider et al. 2001)

Due to steric reasons, the complexes usually include no more than two flavonoid molecules. Many reports have suggested that the flavonoids predominantly form complexes with a metal-ligand ratio of 1:2 with good stability constants (Ansari & Sharma 2009; Chen et al. 2009; Pękal et al. 2011; Dowling et al. 2010; Malešev & Kuntić 2007). (Fig.25) However Zhou and co-workers through fluorescence spectroscopy found 3:1 complexes of quercetin with eight rare-earth metal ions (Zhou 2001). Literature also reveals that the binding efficiency of flavonoids to the metal ions is closely associated with the nature and state of the transition metal ions. The structure of a flavonoid–metal ion complex is dependent upon numerous factors including the coordination number and oxidation state of the central metal ion, the number of electron donors in the flavonoid, the proximity of the electron donors in the flavonoid, and chelating conditions such as temperature and pH (Malešev & Kuntić 2007). Flavonoids act as weak polybasic acids, playing an important role in complex formation. The optimal pH for complex formation is around 6, although it strongly depends on the metal ion. At pH below 3.0, flavonoids remain undissociated, which is unfavourable for complex formation and high pH values are known to deprotonate flavonoids and form more complex species (Grazul & Budzisz 2009).

On the other hand, metal chelation is thought to be the most important mechanism of flavonoids to exert their antioxidant activity beside with many other biological effects that can be altered by those interactions (de Souza & De Giovani 2005). The antioxidant activity results from binding metal ions like Fe(II), Fe(III), Cu(I) or Zn(II) which participate in free radical-generating reactions. Therefore, they act on two antioxidant pathways; on one hand there is a direct reaction with free radicals and on the other hand the chelation of metal ions involved in production of reactive oxygen species (Afanas'ev, Dcrozhko, Brodskii, Kostyuk, & Potapovitch, 1989; Malešev & Kuntić, 2007; S. A. B. van Acker, van Balen, van den Berg, Bast, & van der Vijgh, 1998; S. A. van Acker et al., 1996). Experimental data indicated that the chelated compounds were more effective free radical scavengers than flavonoids alone. These results suggest that the metal-flavonoid complexes, not only exert singular biological properties, but also can enhance the effects of both compounds individually.

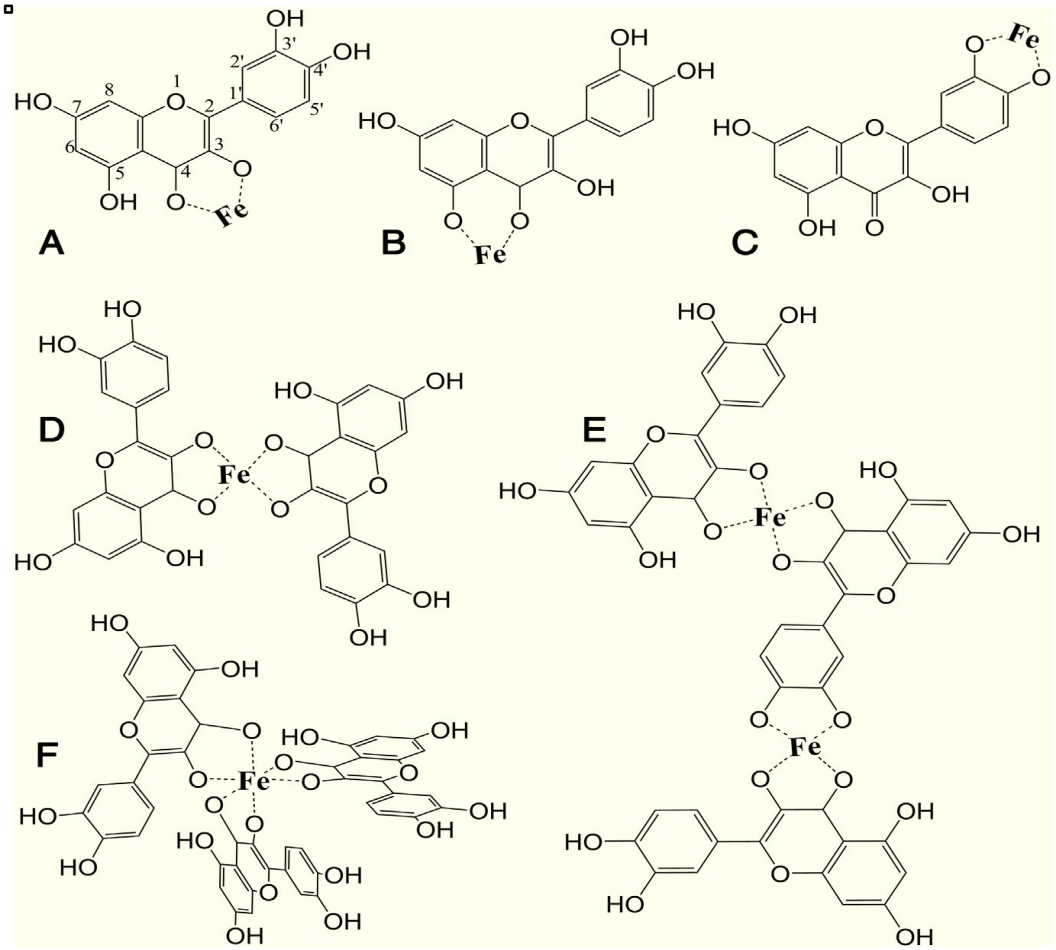


Figure 25. Quercetin–Fe complexes with stoichiometries of 1:1 (a–c), 2:1 (d), 3:2 (e), and 3:1 (f). Similar structures may be formed with other metal ions such as zinc. (Guo et al. 2007; Ren et al. 2008; Tarahovsky et al. 2014)

In fact, one of the mechanisms by which flavonoids exert their antioxidant activity is by chelating redox-active transition metals, mainly iron and copper (Thompson et al. 1976) which are known to catalyze many biological processes leading to the production of free radicals (Mladenka et al. 2010). The essential sites for metal chelation are hydroxyl groups, and the most suitable cations for chelation are Fe(II), Fe(III), Cu(II) and Zn(II) because of they high charge density, stimulating the interaction with the phenoxide groups which have a high negative charge density (Hider et al. 2001). The structure of the formed complexes depends on the type of flavonoid and metal ion involved, which in turn can

influence its biological interactions that may be different from the single flavonoid (Aherne & O'Brien 2000; Afanas'eva et al. 2001; Fernandez et al. 2002; Mira et al. 2002; Payán-Gómez et al. 2011). Moreover, some of the formed complexes could exert many other biological effects, such as molecular signaling, particularly if the metal ion exerts that effect by itself. For example the interaction of flavonoids with zinc ions could enhance the effect of both molecules lonely.

Further studies have revealed that polyphenols not only interact with metal ions, but they deeply modulate expression of MTs, cellular zinc transporters, extracellular zinc carriers, and intracellular zinc accumulation which are key factors in zinc homeostasis (Quesada et al. 2011). In addition, an increase of Zinquin (fluorescent specific zinc indicator)-detectable cytoplasmic levels of zinc in HepG2 cell line was monitored when treated with phenolic compounds (Quesada et al. 2011). This increment in intracellular zinc levels have been reported to induce apoptosis of tumour cells (Ding et al. 2005; Feng et al. 2008), suggesting that zinc ionophores may serve as anticancer agents (Liang et al. 1999).

The enzymatic separation of polyphenols and their metabolites between aqueous and lipid phases is by far supporting the aqueous phase because of their hydrophilicity and binding to albumin. However, in some lipophilic membrane models, some polyphenols penetrate the membrane to various extents (Saija et al. 1995; Castelli et al. 1999; Nakayama et al. 1998; Movileanu et al. 2000; Ollila et al. 2002). Quercetin showed the deepest interaction, probably because of its ability to assume a planar conformation (van Acker et al. 1996). At physiologic pH, most polyphenols interact with the polar head groups of phospholipids at the membrane surface via the formation of hydrogen bonds that involve the hydroxyl groups of the polyphenols (Verstraeten et al. 2003). A high number of hydroxyl groups on the polyphenol structure and an increase in pH that leads to deprotonation of the hydroxyl groups would thus enhance interactions between the polyphenols and the membrane surface.

For instance, transition metal ion complexes of quercetin and genistein significantly alter the chemical properties of their corresponding parent flavonoids (Bukhari et al. 2009; Chen et al. 2009; Dowling et al. 2010).

The complexation of metal ions to flavonoids gives a specific spatial orientation, and this could be the responsible for the pharmacological activity. These metal ion complexes therefore can exhibit similar characteristics as their parent flavonoids and also display unique features distinct from their parent flavonoid due to their structural characteristics (Selvaraj et al. 2013). Several recent reports have indicated that the flavonoid–metal ion complexes possess more potent biological activities than the parent. Many pharmacological effects have been identified for flavonoid-metal ion complexes. (Fig.26)

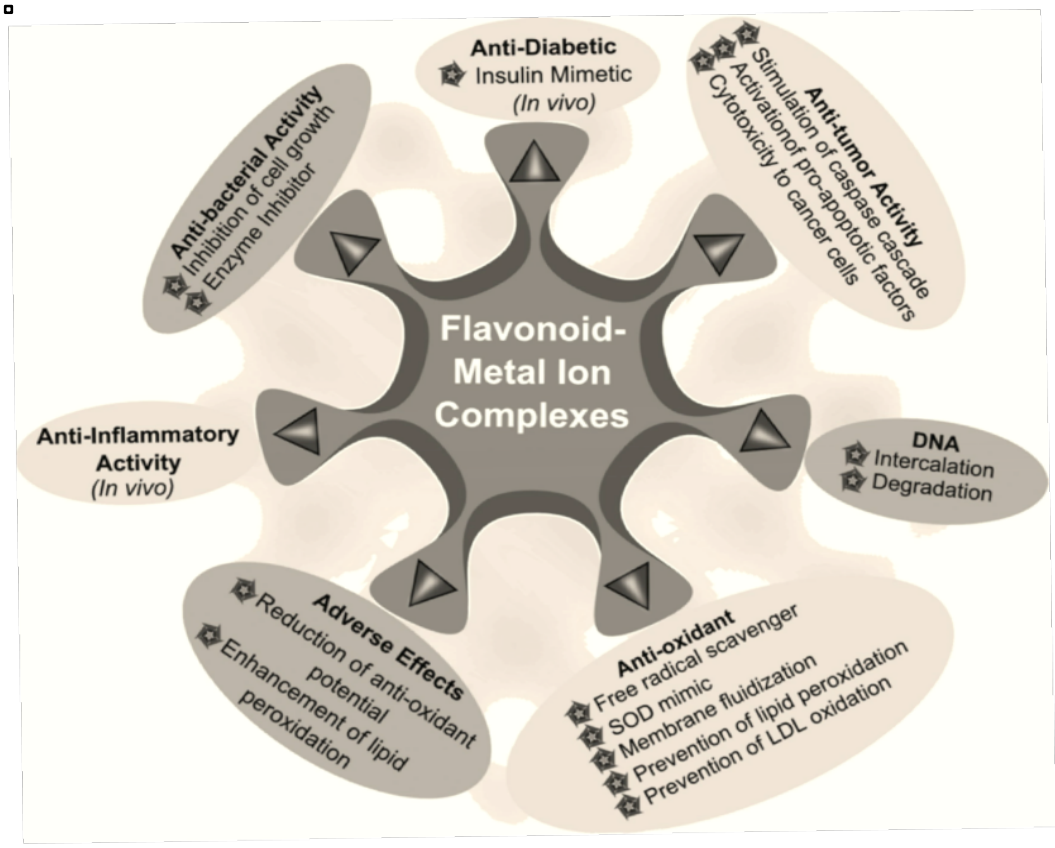


Figure 26. Potential biological activities of flavonoid–metal ion complexes both *in vitro* and *in vivo.* (Selvaraj et al. 2013)

Flavonoids exert their antimicrobial activity due to their ability to affect three important biological processes that are essential for the survival of bacterial cells. These involve blocking of transcription and translation processes, inhibition of cytoplasm and membrane functions, and finally inhibition of energy metabolism (Havsteen 2002). In a similar way,

metal ion complexes may exert antibacterial activity due to their ability to interact with a different set of cellular targets in the microorganism when compared with the single flavonoids as a result of a change in their structure as well as binding affinities to the various intracellular targets. (Fig.27) The alteration of membrane morphology and fluidity of the cells under exposure to flavonoid-metal ion complexes may also play a role in their antibacterial activity (Liu et al. 2012).

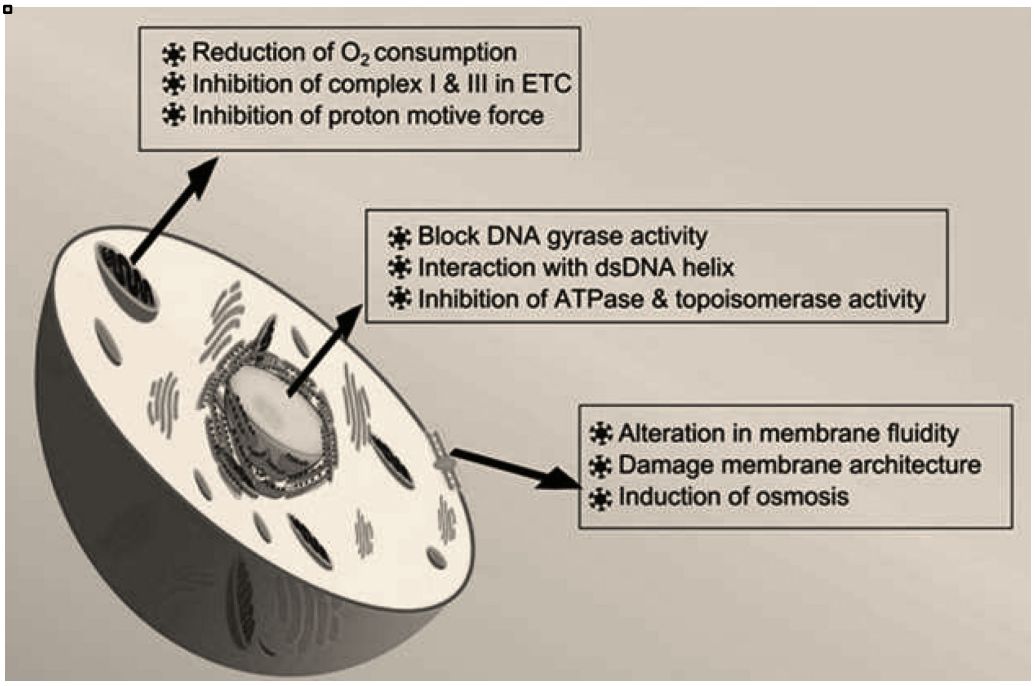


Figure 27. Probable molecular mechanisms for antimicrobial action of flavonoid–metal ion complexes. Based on (Malešev & Kuntić 2007; Kopacz et al. n.d.; Bravo & Anacona 2001)

Considerable amount of work has been focused on the interactions of flavonoids and their metal ion complexes with DNA. Several *in vitro* studies using plasmid DNA have been widely employed to investigate the propensity of flavonoid-metal ion complexes to bind with the DNA double helix. Those complexes such as morin-europium (Woźnicka et al. 2007), rutin–iron (Selvaraj et al. 2012b), quercetin-copper (Tan, Wang, et al. 2009a), quercetin-manganese (Jun et al. 2007), quercetin-nickel (Tan, Zhu, et al. 2009), and quercetin-zinc (Tan, Wang, et al. 2009b) have been reported to show better DNA intercalation when compared with their corresponding parent flavonoid. Flavonoids intercalate between the

double-stranded DNA through hydrophobic interactions with the nitrogenous bases similar to ethidium bromide (Bi et al. 2006). Recent reports have suggested that the isoflavone genistein intercalates with the double-stranded DNA in adenine-rich regions compared to guanine-rich grooves (Hyz et al. 2011; Chen et al. 2009). Conversely, the larger flavonoid-metal ion complexes are expected to exhibit a different mode of interaction with the DNA (Tan et al. 2009; Selvaraj et al. 2012b; Pereira et al. 2007). Aside from hydrophobic forces, the presence of charged transition metal ions in the complexes also favours electrostatic interactions with the negatively charged phosphate backbone in the double-stranded DNA. However, further studies need to be carried out to derive the structure-activity relationships (Selvaraj et al. 2013)

The anticancer activity of flavonoids is has been under research for many decades in order to identify the most potent flavonoid or its derivatives against different forms of cancer as well as to elucidate their mechanism of action. Recent data have revealed that flavonoids show a three to five fold enhancement in the activation of all the proapoptotic factors and caspase-mediated pathways in cancer cells (Choi 2007; Havsteen 2002; Kanadaswami et al. 2005). An important issue for the antitumor activity of flavonoids is due to their ability to directly interact with DNA. They alter gene expression or induce DNA fragmentation, which leads to activation of apoptotic pathways. One of the major causes for the transformation of a normal cell into a cancer cell is due to unregulated activation of key enzymes involved in the cell cycle (Choi 2007). Generally, flavonoids activate the caspase-mediated signal transduction pathways, which activate the tumour suppressor protein p53, thus inhibiting cell proliferation.

Numerous flavonoid-metal ion complexes have been also found to exhibit anticancer properties, which may be mediated through arrest of cell cycle events, modifications in the DNA architecture, or alteration of membrane fluidity (Tan et al. 2009; Selvaraj et al. 2012a; Etcheverry et al. 2008; Tan, Wang, et al. 2009b). The anticancer potentiality of the flavonoid-metal ion complexes in many cases has been found to be greater than the parent flavonoids. These improvements may be due to their better DNA intercalation, pro-oxidant nature, and alterations in the membrane fluidity (Selvaraj et al. 2013). (Fig.28) Several reports have highlighted the efficiency of the flavonoid-metal ion complexes against human cervical carcinoma cells (Durgo et al. 2011), gastric cancer cells (Tan et al. 2009), human

hepatocellular liver carcinoma cells (Tan et al. 2009), leukaemia cells (Pereira et al. 2007), human colon adenocarcinoma cells (Etcheverry et al. 2008), human hepatoma cells and osteoblast cancer cells (Etcheverry et al. 2008). Quercetin-zinc complexes, using different tumour cell lines, described potential antitumor activity. The complex showed significant cytotoxicity, and nuclear staining assays revealed that HepG2 cells underwent the typical morphologic changes of apoptosis characterized by nuclear shrinkage, chromatin condensation, or fragmentation after exposure to the complex (Tan, Wang, et al. 2009b).

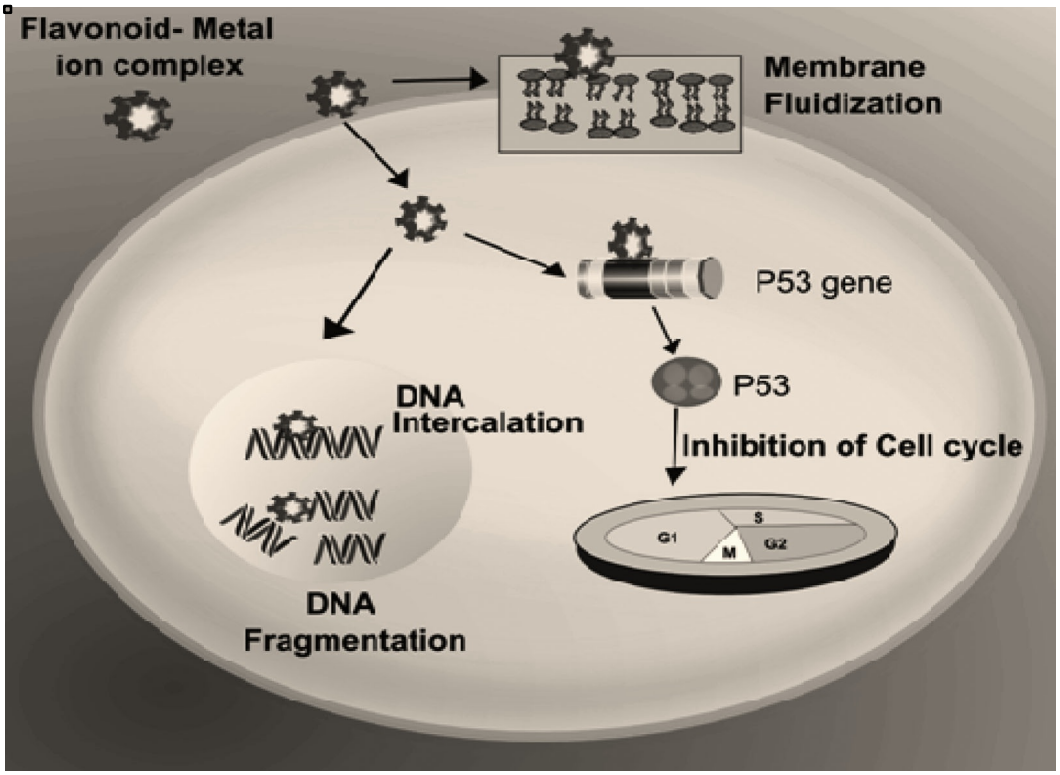


Figure 28. Proposed signal cascade for anticancer activity of flavonoid–metal ion complexes. Based on (Tan, Wang, et al. 2009a; Tan et al. 2009; Etcheverry et al. 2008; Durgo et al. 2011)

Many reports in the literature have suggested the excellent antidiabetic potential of flavonoids, especially for the treatment of insulin-dependent diabetes mellitus. The antidiabetic efficiency of flavonoids is mainly attributed to their ability to alter the gene expression in glycolysis and glycogenesis metabolic pathways. A recent investigation has ascribed the antidiabetic activity of flavonoids to their insulin-mimic activity due to the

activation of the insulin receptor substrate, thereby stimulating protein phosphatase resulting in the conversion of glucose to glycogen (Müller 2000). There are also some evidences about the insulin-mimic property of the complex with metal ions, however the mechanism is not yet elucidated. Probably the complex might also act on similar targets as the parent flavonoids, or maybe it can act promoting the activation of multiple targets enhancing the insulin-mimic effect. (Fig.29)

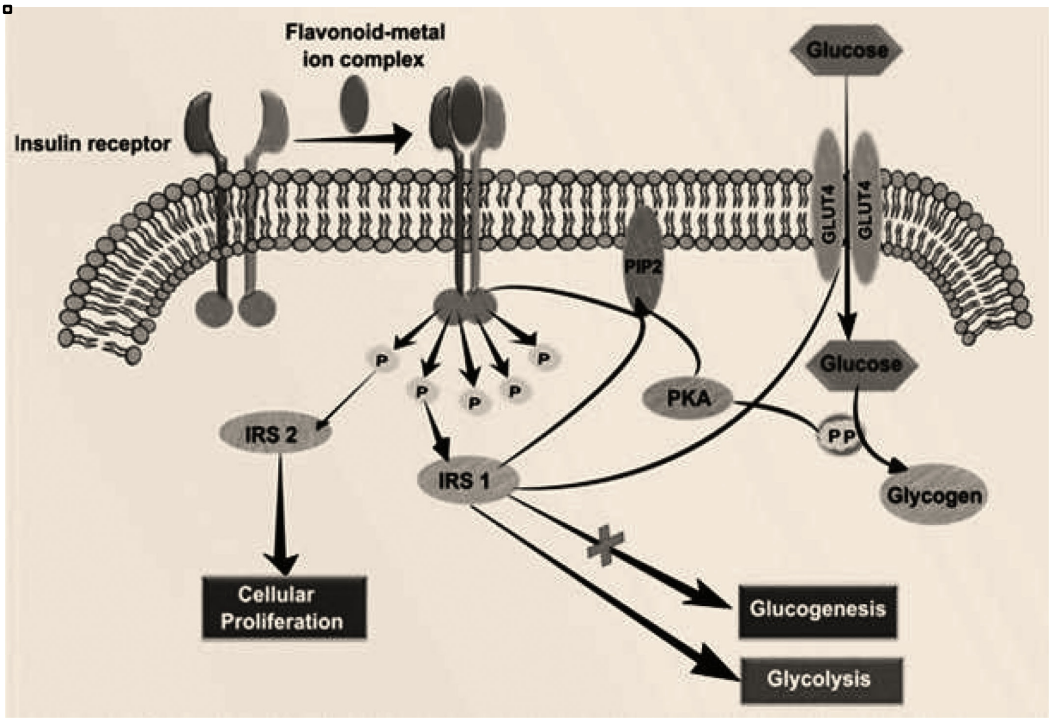


Figure 29. Proposed Insulin-mimetic events of flavonoid–metal ion complexes. IRS, insulin receptor substrate; PP, protein phosphatase; PKA, protein kinase A; PIP2, phosphatidyl inositol pyrophosphate; GLUT, glucose transporter. Based on (Müller 2000; Cazarolli et al. 2006; Badea et al. 2009)

The anti-inflammatory activities of many flavonoids are widely documented in literature (Pan et al. 2010; Dannenberg & Berger 2013; González et al. 2011). They exert their anti-inflammatory effect by blocking the inflammation-mediated pathways and stimulating the secretion of anti-inflammatory molecules. As with most anti-inflammatory phytochemicals, flavonoids are also known to inhibit the plasma protein inflammation cascade system,

block the secretion of inflammatory mediators, and reduce vascular permeability (Jung et al. 2014). Metal chelation of flavonoids has also been demonstrated to enhance their anti-inflammatory activity by blocking the inflammation-mediated pathways. Flavonoid-metal ion complexes modulates the lipooxygenase pathway, aside from the cyclooxygenase pathway to suppress the secretion of pro-inflammatory molecules. (Fig.30) This difference may be attributed to structural changes and cellular localization of the complexes promoting the activation of different targets. However, this mechanism remains poorly explored.

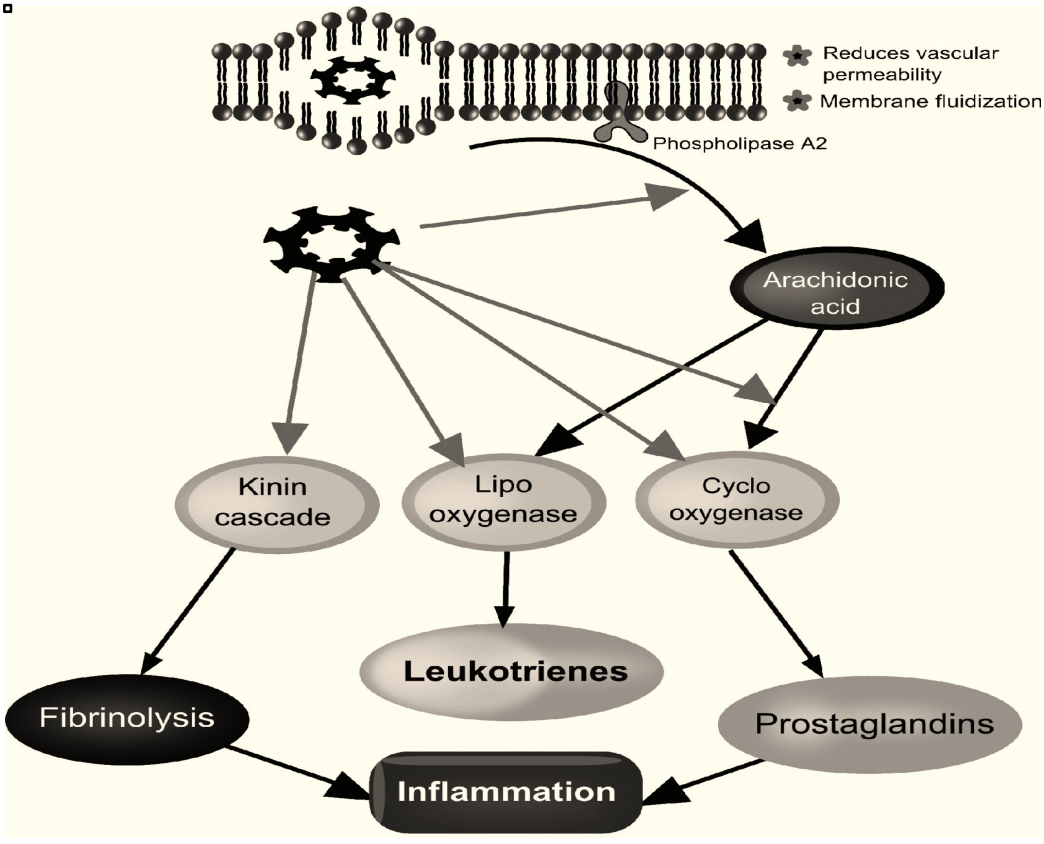


Figure 30. Proposed Anti-inflammatory role of flavonoid–metal ion complexes. Based on (Li et al. 2009; Pan et al. 2010)

2.5 References

Abd El Mohsen, M.M. et al., 2002. Uptake and metabolism of epicatechin and its access to the brain after oral ingestion. *Free radical biology & medicine*, 33(12), pp.1693–702.

Van Acker, S.A. et al., 1996. A quantum chemical explanation of the antioxidant activity of flavonoids. *Chemical research in toxicology*, 9(8), pp.1305–12.

Van Acker, S.A.B.. et al., 1998. Influence of iron chelation on the antioxidant activity of flavonoids. *Biochemical Pharmacology*, 56(8), pp.935–943.

Adlercreutz, H. & Mazur, W., 2013. Phyto-oestrogens and Western Diseases.

Afanas'ev, I.B. et al., 1989. Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. *Biochemical Pharmacology*, 38(11), pp.1763–1769.

Afanas'eva, I.B. et al., 2001. Enhancement of antioxidant and anti-inflammatory activities of bioflavonoid rutin by complexation with transition metals. *Biochemical Pharmacology*, 61(6), pp.677–684.

Aherne, S.A. & O'Brien, N.M., 2000. Mechanism of protection by the flavonoids, quercetin and rutin, against tert-butylhydroperoxide- and menadione-induced DNA single strand breaks in Caco-2 cells. *Free radical biology & medicine*, 29(6), pp.507–14.

Alvarez, A.I. et al., 2010. Modulation of the activity of ABC transporters (P-glycoprotein, MRP2, BCRP) by flavonoids and drug response. *Journal of pharmaceutical sciences*, 99(2), pp.598–617.

Ansari, A.A. & Sharma, R.K., 2009. Synthesis and Characterization of a Biologically Active Lanthanum(III)–Catechin Complex and DNA Binding Spectroscopic Studies. *Spectroscopy Letters*, 42(4), pp.178–185.

Araújo, J.R., Gonçalves, P. & Martel, F., 2011. Chemopreventive effect of dietary polyphenols in colorectal cancer cell lines. *Nutrition research (New York, N.Y.)*, 31(2), pp.77–87.

Badea, M. et al., 2009. Thermal stability of new vanadyl complexes with flavonoid derivatives as potential insulin-mimetic agents. *Journal of Thermal Analysis and Calorimetry*, 99(3), pp.823–827.

Beecher, G.R., 2003. Overview of Dietary Flavonoids: Nomenclature, Occurrence and Intake. *J. Nutr.*, 133(10), p.3248S–3254.

Bi, S. et al., 2006. Study of interactions of flavonoids with DNA using acridine orange as a fluorescence probe. *Sensors and Actuators B: Chemical*, 119(1), pp.199–208.

Boulton, D.W., Walle, U.K. & Walle, T., 1998. Extensive binding of the bioflavonoid quercetin to human plasma proteins. *The Journal of pharmacy and pharmacology*, 50(2), pp.243–9.

Bravo, A. & Anacona, J.R., 2001. Metal complexes of the flavonoid quercetin: antibacterial properties. *Transition Metal Chemistry*, 26(1-2), pp.20–23.

Bravo, L., 1998. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition reviews*, 56(11), pp.317–33.

Bukhari, S.B. et al., 2009. Synthesis, characterization and antioxidant activity copper-quercetin complex. *Spectrochimica acta. Part A, Molecular and biomolecular spectroscopy*, 71(5), pp.1901–6.

Castelli, F. et al., 1999. Differences between coumaric and cinnamic acids in membrane permeation as evidenced by time-dependent calorimetry. *Journal of agricultural and food chemistry*, 47(3), pp.991–5.

Cazarolli, L.H. et al., 2006. Follow-up studies on glycosylated flavonoids and their complexes with vanadium: their anti-hyperglycemic potential role in diabetes. *Chemico-biological interactions*, 163(3), pp.177–91.

Čipák, L. et al., 2003. Effects of flavonoids on cisplatin-induced apoptosis of HL-60 and L1210 leukemia cells. *Leukemia Research*, 27(1), pp.65–72.

Clifford, M.N., 2004. Diet-derived phenols in plasma and tissues and their implications for health. *Planta medica*, 70(12), pp.1103–14.

Coldham, N.G. & Sauer, M.J., 2000. Pharmacokinetics of [(14)C]Genistein in the rat: gender-related differences, potential mechanisms of biological action, and implications for human health. *Toxicology and applied pharmacology*, 164(2), pp.206–15.

Cornard, J.. & Merlin, J.., 2002. Spectroscopic and structural study of complexes of quercetin with Al(III). *Journal of Inorganic Biochemistry*, 92(1), pp.19–27.

Chang, H.C. et al., 2000. Mass spectrometric determination of Genistein tissue distribution in diet-exposed Sprague-Dawley rats. *The Journal of nutrition*, 130(8), pp.1963–70.

Chen, W. et al., 2009. Antioxidant property of quercetin–Cr(III) complex: The role of Cr(III) ion. *Journal of Molecular Structure*, 918(1-3), pp.194–197.

Chen, Z.P. et al., 1998. Green tea epigallocatechin gallate shows a pronounced growth inhibitory effect on cancerous cells but not on their normal counterparts. *Cancer Letters*, 129(2), pp.173–179.

Cheynier, V., 2005. Polyphenols in foods are more complex than often thought. *The American journal of clinical nutrition*, 81(1 Suppl), p.223S–229S.

Choi, E.J., 2007. Hesperetin induced G1-phase cell cycle arrest in human breast cancer MCF-7 cells: involvement of CDK4 and p21. *Nutrition and cancer*, 59(1), pp.115–9.

Dannenberg, A.J. & Berger, N.A. eds., 2013. *Obesity, Inflammation and Cancer*, New York, NY: Springer New York.

Day, A.J. et al., 2000. Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. *FEBS letters*, 468(2-3), pp.166–70.

Ding, W.-Q. et al., 2005. Anticancer activity of the antibiotic clioquinol. *Cancer research*, 65(8), pp.3389–95.

Donovan, J.L. et al., 1999. Catechin Is Present as Metabolites in Human Plasma after Consumption of Red Wine. *J. Nutr.*, 129(9), pp.1662–1668.

Dowling, S., Regan, F. & Hughes, H., 2010. The characterisation of structural and antioxidant properties of isoflavone metal chelates. *Journal of inorganic biochemistry*, 104(10), pp.1091–8.

Dulloo, A.G. et al., 1999. Efficacy of a green tea extract rich in catechin polyphenols and caffeine in increasing 24-h energy expenditure and fat oxidation in humans. *Am J Clin Nutr*, 70(6), pp.1040–1045.

Durgo, K. et al., 2011. Cytotoxic and Genotoxic Effects of the Quercetin/Lanthanum Complex on Human Cervical Carcinoma Cells In Vitro. *Archives of Industrial Hygiene and Toxicology*, 62(3), pp.221–227.

Etcheverry, S.B. et al., 2008. Antioxidant effects of the VO(IV) hesperidin complex and its role in cancer chemoprevention. *Journal of biological inorganic chemistry : JBIC : a publication of the Society of Biological Inorganic Chemistry*, 13(3), pp.435–47.

Feng, P. et al., 2008. The involvement of Bax in zinc-induced mitochondrial apoptogenesis in malignant prostate cells. *Molecular cancer*, 7(1), p.25.

Fernandez, M.T. et al., 2002. Iron and copper chelation by flavonoids: an electrospray mass spectrometry study. *Journal of inorganic biochemistry*, 92(2), pp.105–11.

Ferruzzi, M.G. et al., 2009. Bioavailability of gallic acid and catechins from grape seed polyphenol extract is improved by repeated dosing in rats: implications for treatment in Alzheimer's disease. *Journal of Alzheimer's disease : JAD*, 18(1), pp.113–24.

Gee, J.M. et al., 2000. Intestinal Transport of Quercetin Glycosides in Rats Involves Both Deglycosylation and Interaction with the Hexose Transport Pathway. *J. Nutr.*, 130(11), pp.2765–2771.

Gliemann, L. et al., 2013. Resveratrol blunts the positive effects of exercise training on cardiovascular health in aged men. *The Journal of physiology*, 591(Pt 20), pp.5047–59.

González, R. et al., 2011. Effects of flavonoids and other polyphenols on inflammation. *Critical reviews in food science and nutrition*, 51(4), pp.331–62.

Graefe, E.U. et al., 2001. Pharmacokinetics and bioavailability of quercetin glycosides in humans. *Journal of clinical pharmacology*, 41(5), pp.492–9.

Grazul, M. & Budzisz, E., 2009. Biological activity of metal ions complexes of chromones, coumarins and flavones. *Coordination Chemistry Reviews*, 253(21), pp.2588–2598.

Groot, H. & Rauen, U., 1998. Tissue injury by reactive oxygen species and the protective effects of flavonoids. *Fundamental & Clinical Pharmacology*, 12(3), pp.249–255.

Guo, M. et al., 2007. Iron-binding properties of plant phenolics and cranberry's bio-effects. *Dalton transactions (Cambridge, England : 2003)*, (43), pp.4951–61.

Harborne, J.B. & Williams, C.A., 2000. Advances in flavonoid research since 1992. *Phytochemistry*, 55(6), pp.481–504.

Havsteen, B.H., 2002. The biochemistry and medical significance of the flavonoids. *Pharmacology & Therapeutics*, 96(2-3), pp.67–202.

Hertog, M.G.. et al., 1993. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *The Lancet*, 342(8878), pp.1007–1011.

Hider, R.C., Liu, Z.D. & Khodr, H.H., 2001. Metal chelation of polyphenols. *Methods in enzymology*, 335, pp.190–203.

Hollman, P.C. & Katan, M.B., 1997. Absorption, metabolism and health effects of dietary flavonoids in man. *Biomedicine & pharmacotherapy = Biomédecine & pharmacothérapie*, 51(8), pp.305–10.

Hollman, P.C.. et al., 1997. Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man. *FEBS Letters*, 418(1-2), pp.152–156.

Hooper, P.L. et al., 2010. Xenohormesis: health benefits from an eon of plant stress response evolution. *Cell stress & chaperones*, 15(6), pp.761–70.

Howitz, K.T. & Sinclair, D.A., 2008. Xenohormesis: sensing the chemical cues of other species. *Cell*, 133(3), pp.387–91.

Hu, M., 2007. Commentary: bioavailability of flavonoids and polyphenols: call to arms. *Molecular pharmaceutics*, 4(6), pp.803–6.

Hyz, K. et al., 2011. Genistein binding mode to doubly nicked dumbbell DNA. Dynamic and diffusion ordered NMR study. *Journal of medicinal chemistry*, 54(24), pp.8386–93.

Ito, H. et al., 2005. Polyphenol levels in human urine after intake of six different polyphenol-rich beverages. *The British journal of nutrition*, 94(4), pp.500–9.

Jackson, R. et al., 2011. Predicting Flavonoid UGT Regioselectivity. *Advances in bioinformatics*, 2011, p.506583.

Jun, T., Bochu, W. & Liancai, Z., 2007. Hydrolytic cleavage of DNA by quercetin manganese(II) complexes. *Colloids and surfaces. B, Biointerfaces*, 55(2), pp.149–52.

Jung, Y.-S. et al., 2014. Anti-inflammatory effect of tricin 4'-O-(threo-β-guaiacylglyceryl) ether, a novel flavonolignan compound isolated from Njavara on in RAW264.7 cells and in ear mice edema. *Toxicology and applied pharmacology*, 277(1), pp.67–76.

Kanadaswami, C. et al., 2005. The Antitumor Activities of Flavonoids. *In Vivo*, 19(5), pp.895–909.

Kilaru, S. et al., 2001. Nicotine: a review of its role in atherosclerosis. *Journal of the American College of Surgeons*, 193(5), pp.538–46.

Kim, S. et al., 2000. Plasma and tissue levels of tea catechins in rats and mice during chronic consumption of green tea polyphenols. *Nutrition and cancer*, 37(1), pp.41–8.

Kopacz, M., Woźnicka, E. & Gruszecka, J., Antibacterial activity of morin and its complexes with La(III), Gd(III) and Lu(III) ions. *Acta poloniae pharmaceutica*, 62(1), pp.65–7.

Kühnau, J., 1976. The flavonoids. A class of semi-essential food components: their role in human nutrition. *World review of nutrition and dietetics*, 24, pp.117–91.

Lambert, J.D. & Yang, C.S., 2003. Mechanisms of cancer prevention by tea constituents. *The Journal of nutrition*, 133(10), p.3262S–3267S.

De Lange, D.W. et al., 2007. Polyphenolic grape extract inhibits platelet activation through PECAM-1: an explanation for the French paradox. *Alcoholism, clinical and experimental research*, 31(8), pp.1308–14.

Lee, M.J. et al., 1995. Analysis of plasma and urinary tea polyphenols in human subjects. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*, 4(4), pp.393–9.

Leopoldini, M., Russo, N. & Toscano, M., 2011. The molecular basis of working mechanism of natural polyphenolic antioxidants. *Food Chemistry*, 125(2), pp.288–306.

Li, J. et al., 2009. Synthesis, characterization, and anti-inflammatory activities of rare earth metal complexes of luteolin. *Medicinal Chemistry Research*, 20(1), pp.88–92.

Liang, J.Y. et al., 1999. Inhibitory effect of zinc on human prostatic carcinoma cell growth. *The Prostate*, 40(3), pp.200–7.

Lin, L.-C. et al., 2007. Pharmacokinetics of (-)-epigallocatechin-3-gallate in conscious and freely moving rats and its brain regional distribution. *Journal of agricultural and food chemistry*, 55(4), pp.1517–24.

Liu, Y. et al., 2012. [Advance of studies on bioactivity of flavonoid-metal complexes]. *Zhongguo Zhong yao za zhi = Zhongguo zhongyao zazhi = China journal of Chinese materia medica*, 37(13), pp.1901–4.

Lotito, S.B. & Frei, B., 2006. Consumption of flavonoid-rich foods and increased plasma antioxidant capacity in humans: cause, consequence, or epiphenomenon? *Free radical biology & medicine*, 41(12), pp.1727–46.

Malešev, D. & Kuntić, V., 2007. Investigation of metal-flavonoid chelates and the determination of flavonoids via metal-flavonoid complexing reactions. *Journal of the Serbian Chemical Society*, 72(10), pp.921–939.

Manach, C. et al., 2005. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am J Clin Nutr*, 81(1), p.230S–242.

Manach, C. et al., 2004. Polyphenols: food sources and bioavailability. *The American journal of clinical nutrition*, 79(5), pp.727–47.

Manach, C. et al., 1998. Quercetin is recovered in human plasma as conjugated derivatives which retain antioxidant properties. *FEBS Letters*, 426(3), pp.331–336.

Manach, C. et al., 1995. Quercetin metabolites in plasma of rats fed diets containing rutin or quercetin. *The Journal of nutrition*, 125(7), pp.1911–22.

Mansuri, M.L. et al., 2014. Flavonoids in modulation of cell survival signalling pathways. *Genes & nutrition*, 9(3), p.400.

Mattson, M.P., Son, T.G. & Camandola, S., 2007. Viewpoint: mechanisms of action and therapeutic potential of neurohormetic phytochemicals. *Dose-response : a publication of International Hormesis Society*, 5(3), pp.174–86.

Meagher, L.P. et al., 1999. Isolation and Characterization of the Lignans, Isolariciresinol and Pinoresinol, in Flaxseed Meal. *Journal of Agricultural and Food Chemistry*, 47(8), pp.3173–3180.

Milder, I.E.J. et al., 2005. Lignan contents of Dutch plant foods: a database including lariciresinol, pinoresinol, secoisolariciresinol and matairesinol. *The British journal of nutrition*, 93(3), pp.393–402.

Mira, L. et al., 2002. Interactions of flavonoids with iron and copper ions: a mechanism for their antioxidant activity. *Free radical research*, 36(11), pp.1199–208.

Mladenka, P. et al., 2010. Cardiovascular effects of flavonoids are not caused only by direct antioxidant activity. *Free radical biology & medicine*, 49(6), pp.963–75.

El Mohsen, M.A. et al., 2006. Absorption, tissue distribution and excretion of pelargonidin and its metabolites following oral administration to rats. *The British journal of nutrition*, 95(1), pp.51–8.

Movileanu, L., Neagoe, I. & Flonta, M.L., 2000. Interaction of the antioxidant flavonoid quercetin with planar lipid bilayers. *International journal of pharmaceutics*, 205(1-2), pp.135–46.

Mullen, W. et al., 2002. Determination of flavonol metabolites in plasma and tissues of rats by HPLC-radiocounting and tandem mass spectrometry following oral ingestion of [2-(14)C]quercetin-4'-glucoside. *Journal of agricultural and food chemistry*, 50(23), pp.6902–9.

Müller, G., 2000. The molecular mechanism of the insulin-mimetic/sensitizing activity of the antidiabetic sulfonylurea drug Amaryl. *Molecular medicine (Cambridge, Mass.)*, 6(11), pp.907–33.

Nakayama, T., Ono, K. & Hashimoto, K., 1998. Affinity of antioxidative polyphenols for lipid bilayers evaluated with a liposome system. *Bioscience, biotechnology, and biochemistry*, 62(5), pp.1005–7.

Nijveldt, R.J. et al., 2001. Flavonoids: a review of probable mechanisms of action and potential applications. *The American journal of clinical nutrition*, 74(4), pp.418–25.

Ollila, F. et al., 2002. Characterization of flavonoid--biomembrane interactions. *Archives of biochemistry and biophysics*, 399(1), pp.103–8.

Pan, M.-H., Lai, C.-S. & Ho, C.-T., 2010. Anti-inflammatory activity of natural dietary flavonoids. *Food & function*, 1(1), pp.15–31.

Pandey, K.B. & Rizvi, S.I., 2009. Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative medicine and cellular longevity*, 2(5), pp.270–8.

Payán-Gómez, S.A. et al., 2011. Computational molecular characterization of the flavonoid Morin and its Pt(II), Pd(II) and Zn(II) complexes. *Journal of molecular modeling*, 17(5), pp.979–85.

Pękal, A., Biesaga, M. & Pyrzynska, K., 2011. Interaction of quercetin with copper ions: complexation, oxidation and reactivity towards radicals. *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine*, 24(1), pp.41–9.

Peng, H.W. et al., 1998. Determination of naringenin and its glucuronide conjugate in rat plasma and brain tissue by high-performance liquid chromatography. *Journal of chromatography. B, Biomedical sciences and applications*, 714(2), pp.369–74.

Pereira, R.M.S. et al., 2007. Synthesis and characterization of a metal complex containing naringin and Cu, and its antioxidant, antimicrobial, antiinflammatory and tumor cell cytotoxicity. *Molecules (Basel, Switzerland)*, 12(7), pp.1352–66.

Van Praag, H. et al., 2007. Plant-derived flavanol (-)epicatechin enhances angiogenesis and retention of spatial memory in mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 27(22), pp.5869–78.

Quesada, I.M. et al., 2011. Dietary catechins and procyanidins modulate zinc homeostasis in human HepG2 cells. *The Journal of nutritional biochemistry*, 22(2), pp.153–63.

Ramos, S., 2008. Cancer chemoprevention and chemotherapy: dietary polyphenols and signalling pathways. *Molecular nutrition & food research*, 52(5), pp.507–26.

Rangel-Ordóñez, L. et al., 2010. Plasma levels and distribution of flavonoids in rat brain after single and repeated doses of standardized Ginkgo biloba extract EGb 761®. *Planta medica*, 76(15), pp.1683–90.

Rein, D. et al., 2000. Epicatechin in human plasma: in vivo determination and effect of chocolate consumption on plasma oxidation status. *The Journal of nutrition*, 130(8S Suppl), p.2109S–14S.

Ren, J. et al., 2008. Complexation of flavonoids with iron: structure and optical signatures. *The journal of physical chemistry. B*, 112(6), pp.1845–50.

Renaud, S. & de Lorgeril, M., 1992. Wine, alcohol, platelets, and the French paradox for coronary heart disease. *The Lancet*, 339(8808), pp.1523–1526.

Rice-Evans, C. & Miller, N.J., 1995. Antioxidants – the case for fruit and vegetables in the diet. *British Food Journal*, 97(9), pp.35–40.

Ross, J.A. & Kasum, C.M., 2002. Dietary flavonoids: bioavailability, metabolic effects, and safety. *Annual review of nutrition*, 22, pp.19–34.

Rothwell, J.A. et al., 2012. Phenol-Explorer 2.0: a major update of the Phenol-Explorer database integrating data on polyphenol metabolism and pharmacokinetics in humans and experimental animals. *Database : the journal of biological databases and curation*, 2012(0), p.bas031.

Russo, M. et al., 2012. The flavonoid quercetin in disease prevention and therapy: facts and fancies. *Biochemical pharmacology*, 83(1), pp.6–15.

Saija, A. et al., 1995. Flavonoids as antioxidant agents: importance of their interaction with biomembranes. *Free radical biology & medicine*, 19(4), pp.481–6.

Scalbert, A. & Williamson, G., 2000. Dietary Intake and Bioavailability of Polyphenols. *J. Nutr.*, 130(8), p.2073S–2085.

Selvaraj, S. et al., 2013. Flavonoid-Metal Ion Complexes: A Novel Class of Therapeutic Agents. *Medicinal research reviews*.

Selvaraj, S. et al., 2012a. Membrane fluidization & eryptotic properties of hesperidin–copper complex. *RSC Advances*, 2(29), p.11138.

Selvaraj, S. et al., 2012b. Synthesis, characterization and DNA binding properties of rutin–iron complex. *RSC Advances*, 2(7), p.2797.

Shahidi, F. & Naczk, M., 1995. *Food Phenolics: Sources, Chemistry, Effects, Applications*, Technomic Publishing Company, Incorporated.

Signorelli, P. & Ghidoni, R., 2005. Resveratrol as an anticancer nutrient: molecular basis, open questions and promises. *The Journal of nutritional biochemistry*, 16(8), pp.449–66.

Son, T.G., Camandola, S. & Mattson, M.P., 2008. Hormetic dietary phytochemicals. *Neuromolecular medicine*, 10(4), pp.236–46.

De Souza, R.F. V & De Giovani, W.F., 2005. Synthesis, spectral and electrochemical properties of Al(III) and Zn(II) complexes with flavonoids. *Spectrochimica acta. Part A, Molecular and biomolecular spectroscopy*, 61(9), pp.1985–90.

Spencer, J.P.E., 2003. Metabolism of tea flavonoids in the gastrointestinal tract. *The Journal of nutrition*, 133(10), p.3255S–3261S.

Suganuma, M. et al., 1998. Wide distribution of [3H](-)-epigallocatechin gallate, a cancer preventive tea polyphenol, in mouse tissue. *Carcinogenesis*, 19(10), pp.1771–6.

Sumpio, B.E. et al., 2006. Green tea, the “Asian paradox,” and cardiovascular disease. *Journal of the American College of Surgeons*, 202(5), pp.813–25.

Talavéra, S. et al., 2005. Anthocyanin metabolism in rats and their distribution to digestive area, kidney, and brain. *Journal of agricultural and food chemistry*, 53(10), pp.3902–8.

Tan, J., Wang, B. & Zhu, L., 2009a. DNA binding and oxidative DNA damage induced by a quercetin copper(II) complex: potential mechanism of its antitumor properties. *Journal of biological inorganic chemistry : JBIC : a publication of the Society of Biological Inorganic Chemistry*, 14(5), pp.727–39.

Tan, J., Wang, B. & Zhu, L., 2009b. DNA binding, cytotoxicity, apoptotic inducing activity, and molecular modeling study of quercetin zinc(II) complex. *Bioorganic & medicinal chemistry*, 17(2), pp.614–20.

Tan, J., Zhu, L. & Wang, B., 2009. DNA binding and cleavage activity of quercetin nickel(II) complex. *Dalton transactions (Cambridge, England : 2003)*, (24), pp.4722–8.

Tan, M. et al., 2009. Synthesis, cytotoxic activity, and DNA binding properties of copper (II) complexes with hesperetin, naringenin, and apigenin. *Bioinorganic chemistry and applications*, p.347872.

Tarahovsky, Y.S. et al., 2014. Flavonoid-membrane interactions: involvement of flavonoid-metal complexes in raft signaling. *Biochimica et biophysica acta*.

Thompson, M., Williams, C.R. & Elliot, G.E., 1976. Stability of flavonoid complexes of copper(II) and flavonoid antioxidant activity. *Analytica chimica acta*, 85(2), pp.375–81.

Timmers, S., Hesselink, M.K.C. & Schrauwen, P., 2013. Therapeutic potential of resveratrol in obesity and type 2 diabetes: new avenues for health benefits? *Annals of the New York Academy of Sciences*, 1290, pp.83–9.

Ueno, I., Nakano, N. & Hirono, I., 1983. Metabolic fate of [14C] quercetin in the ACI rat. *The Japanese journal of experimental medicine*, 53(1), pp.41–50.

Unno, T. et al., 1996. Analysis of (-)-epigallocatechin gallate in human serum obtained after ingesting green tea. *Bioscience, biotechnology, and biochemistry*, 60(12), pp.2066–8.

Valenzuela, H., 2014. Resveratrol Improves Health.

Verstraeten, S. V et al., 2003. Flavan-3-ols and procyanidins protect liposomes against lipid oxidation and disruption of the bilayer structure. *Free radical biology & medicine*, 34(1), pp.84–92.

Vinson, J.A., Teufel, K. & Wu, N., 2004. Green and black teas inhibit atherosclerosis by lipid, antioxidant, and fibrinolytic mechanisms. *Journal of agricultural and food chemistry*, 52(11), pp.3661–5.

Vitrac, X. et al., 2003. Distribution of [14C]-trans-resveratrol, a cancer chemopreventive polyphenol, in mouse tissues after oral administration. *Life sciences*, 72(20), pp.2219–33.

Wang, J.F. et al., 2000. A dose-response effect from chocolate consumption on plasma epicatechin and oxidative damage. *The Journal of nutrition*, 130(8S Suppl), p.2115S–9S.

Williams, R.J. & Spencer, J.P.E., 2012. Flavonoids, cognition, and dementia: actions, mechanisms, and potential therapeutic utility for Alzheimer disease. *Free radical biology & medicine*, 52(1), pp.35–45.

Wink, M., 1997. *Compartmentation of secondary metabolites and xenobiotics in plant vacuoles*, Elsevier.

Woźnicka, E. et al., 2007. New complexes of La(III), Ce(III), Pr(III), Nd(III), Sm(III), Eu(III) and Gd(III) ions with morin. *Journal of inorganic biochemistry*, 101(5), pp.774–82.

Yang, Y.-C. et al., 2004. The protective effect of habitual tea consumption on hypertension. *Archives of internal medicine*, 164(14), pp.1534–40.

Youdim, K.A., Martin, A. & Joseph, J.A., 2000. Incorporation of the elderberry anthocyanins by endothelial cells increases protection against oxidative stress. *Free radical biology & medicine*, 29(1), pp.51–60.

Yun, A.J. et al., 2005. The smoking gun: many conditions associated with tobacco exposure may be attributable to paradoxical compensatory autonomic responses to nicotine. *Medical hypotheses*, 64(6), pp.1073–9.

Zhang, L., Zuo, Z. & Lin, G., 2007. Intestinal and hepatic glucuronidation of flavonoids. *Molecular pharmaceutics*, 4(6), pp.833–45.

Zhou, J., 2001. Synthesis, characterization, antioxidative and antitumor activities of solid quercetin rare earth(III) complexes. *Journal of Inorganic Biochemistry*, 83(1), pp.41–48.

3. The Akt pathway

During the last decade, important advances have been made in our understanding of the molecular events underlying cellular responses to extracellular signals. The cell signaling is a critically important mechanism for the multicellular organisms including human beings. In recent years, there is much emphasis on modulation of signaling pathways especially during various diseases. Therefore, there is a new emerging view to prevent such diseases by modulating the intracellular signaling pathways through the use of various natural compounds. Diet is a lifestyle factor that plays a major role in the primary and secondary prevention of several chronic diseases. It has been widely reported how both dietary phenolic compounds and zinc exert beneficial effects on health, preventing and ameliorating those chronic diseases such as diabetes, hypertension, obesity, neurodegenerative diseases or cancer through the modulation of multiple metabolic pathways. For example, zinc dyshomeostasis has been related to certain disorders such as metabolic syndrome, diabetes and diabetic complications (Shan et al. 2014; Miao et al. 2013; Li et al. 2013; Jansen et al. 2012). Several studies suggest that zinc supplementation could prevent that disorders. (Table 11)

Zn pretreaments	Major mechanisms	Animals & type of diabetes	Outcomes
Injection, i.p. (10 mg/kg)	MT induction	Rats, STZ single dose (75 mg/kg)	++
Drinking (20 mmol/L), 8 weeks	MT(ND)	*ob/ob* mice	++++
Dietary (1000 ppm), 4 weeks	MT(ND)	Pro-diabetic BB Wister rats	++++
Drinking (25 mmol/L), 1 week	MT induction	C57BL/6 & B6SJL/F_1 mice	++++
		5 × 40 mg STZ/kg	
Dietary (300 ppm), 6 weeks	MT(ND)	*db/db* mice	++++
Dietary (1000 ppm), 2 weeks	MT induction	CD-1 mice, ALX (50 mg/kg)	++++
		STZ (5× 40 mg/kg)	++++
Drinking (25 mmol/L), 1 (12) weeks	MT(ND)	C57BL/6, ALX (50 mg/kg)	+++
Drinking (25 mmol/L), 1 week	Inhibiting NF-κB	C57BL/6 mice	++++
	&/or AP1	NOD	
Dietary (15 mg/kg), 2 weeks	MT(ND)	KK-A^y mice	+++
Injection, i.p. (1.5–3 mg/kg), 4 weeks	MT(ND)	KK-A^y mice	+++
Genetic enhancing MT	Zn-MT	MT-TG mice, STZ (1 × 200 mg/kg)	++++

Table 11. Evidence for the preventive effect of Zn supplementation on diabetes. MT(ND), no detecting pancreatic MT content; 1 (12) weeks, 1 week prior to STZ and continued 12 weeks after STZ; inhibiting NF-κB &/or AP1, through inhibition of pancreatic NF-κB and/or AP1 activation; ALX, alloxan; NOD, non-obese diabetes. (Miao et al. 2013)

Some of the insulinomimetic effects of zinc can be explained by the influence of zinc on insulin signaling. (Fig.31) One zinc-dependent molecule, insulin-responsive amino peptidase (IRAP), which is expressed and characterized in fat and muscle as insulin target tissues (Keller et al. 1995), seems to be required for the maintenance of normal glucose transporter (GLUT) 4 levels in order to ensure glucose uptake into tissues (Keller 2004).

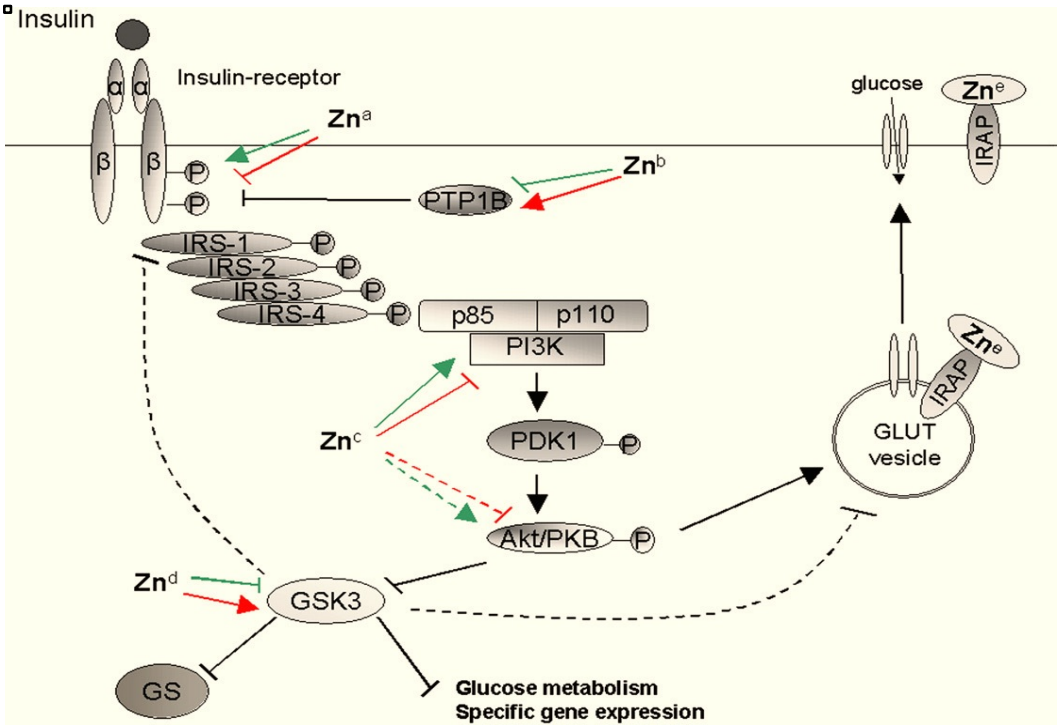


Figure 31. Influence of zinc on the insulin-signaling pathway. PI3K activation triggers the phosphorylation of PDK1, a serine kinase that activates the Akt pathway. Akt leads to stimulation of GLUT 4 translocation in adipocytes and to inhibition of GSK-3, thereby allowing activation of glycogen synthase in adipocytes, translocation of GLUT to the cell surface and induction of glucose metabolism. Akt is activated by zinc in a PI3K-dependent way and zinc inhibits GSK-3, just like insulin. (Jansen et al. 2009)

In a comparable way, several polyphenols have been described to exert an important function against the metabolic syndrome and diabetes, having in some cases, a preventive effect (Basu et al. 2013; Sohrab et al. 2013; Munir et al. 2013).

One of the most studied flavonoids from green tea, EGCG, has been described to inhibit gluconeogenesis in hepatocytes and to stimulate glucose uptake in rat skeletal muscle cells by using a PI3K-dependent mechanism that mimics metabolic actions of insulin (Jung et al. 2008; Waltner-Law et al. 2002). Also, EGCG directly and acutely stimulates production of NO from primary endothelial cells by using a signaling pathway that involves low-level generation of H2O2 and possibly other reactive oxygen species that then goes on to stimulate a signaling cascade including sequential activation of the src family kinase fyn, PI3K, Akt, and eNOS (Kim et al. 2007). Interestingly, this pathway shares features in common with insulin signaling pathways regulating activation of eNOS and NO production in endothelial cells (Kim et al. 2006).

Same similarities have been reported for several diseases, where both zinc or phenolic compounds have been suggested as suitable candidates in order to promote health benefits. Even though the molecular mechanisms of action are still hardly known, they have similar targets and in some cases they may act through the same metabolic pathways. (Fig.32)

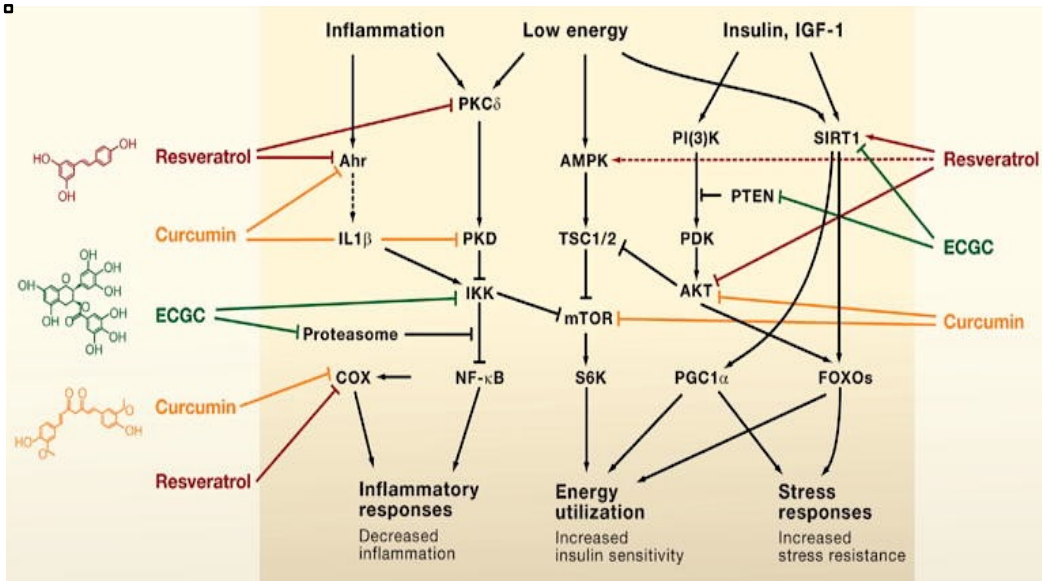


Figure 32. Polyphenols modulate key pathways that control inflammation, the energy status of cells, and cellular stress responses. In many cases, zinc modulates the same pathways exerting similar effects. Some interactions activate signaling pathways (arrows) whereas others inhibit them (bars). (Howitz & Sinclair 2008; Gruber et al. 2013)

An example of a shared target for either zinc or polyphenols is the Akt pathways. Protein kinase B (PKB), also known as Akt, is a Serine/Threonine Kinase, which plays a key role in multiple cellular processes such as protein synthesis, cell survival, growth, proliferation, angiogenesis, glucose metabolism, cardiovascular homeostasis, neuroprotection or apoptosis (Manning & Cantley 2007; Kloet & Burgering 2011). (Fig.33) It is a highly conserved pathway, and its modulation is controlled through multiple receptors (Hemmings & Restuccia 2012). Its identification pathway began in the early 1980s trying to identify the insulin signaling mechanisms and observing its responsibility in the inactivation of glycogen synthase kinase 3 (GSK3) and in the regulation of glycogen synthesis in insulin sensitive tissues (Lawlor & Alessi 2001). It also induced the glucose uptake through different transporters such as GLUT1, GLUT3 and GLUT4 (Hajduch et al. 1998; Barthel et al. 1999). The PI3K/Akt pathway is a critical regulator of cell survival, involved in the regulation of specific proteins such as BCL-2 family, which are critical in the maintenance of cell survival or the induction of apoptosis, depending on the balance between homo- and heterodimers (Datta et al. 1999).

Inhibition of PI3K pathway abolishes cell survival and accelerates apoptosis, whereas an activated form of the Akt/PKB, a downstream effector of PI3K, blocked apoptosis (Kennedy et al. 1997). PI3Ks are enzymes that transfer phosphate to position 3 of the phosphoinositide ring, regulating a variety of cell responses. Based on their primary structure and substrate specificity, PI3Ks are divided into three subclasses, but only the class I enzymes generate phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate (3-poly-PtdIns) products in vivo (Jimenez et al. 2002). Active PI3K catalyses the production of phosphatidylinositol-3,4,5-triphosphate (PIP3) by phosphorylating phosphatidylinositol (PI), phosphatidylinositol- 4-phosphate (PIP), and phosphatidylinositol-4,5-bisphosphate (PIP2). PIP3 may then activate phosphoinositi dependent protein kinase 1 (PDK1), which plays a central role in many signal transduction pathways (Carpenter & Cantley 1996; Simpson & Parsons 2001), activating Akt and the PKC isoenzymes p70 S6 kinase and p90 ribosomal S6 kinase (Neri et al. 2002). All members of Akt/PKB family have structural homology within their catalytic domain and have the similar mechanism of activation. Because of its involvement in various cellular functions, the Akt/PKB plays a central role in the signal transduction pathways. Akt/PKB is activated in response to growth factors or insulin, and it is thought to promote growth

factor-mediated cell survival and to block apoptosis (Zhang & Liu 2011). As seen, natural phenolic compounds are also able to modulate the PKB pathway, as well as changes on intracellular zinc concentrations do. The ability of Akt/PKB to promote cell survival is due to its kinase activity and depends on the activity of its upstream activator PI3K (Kennedy et al. 1997).

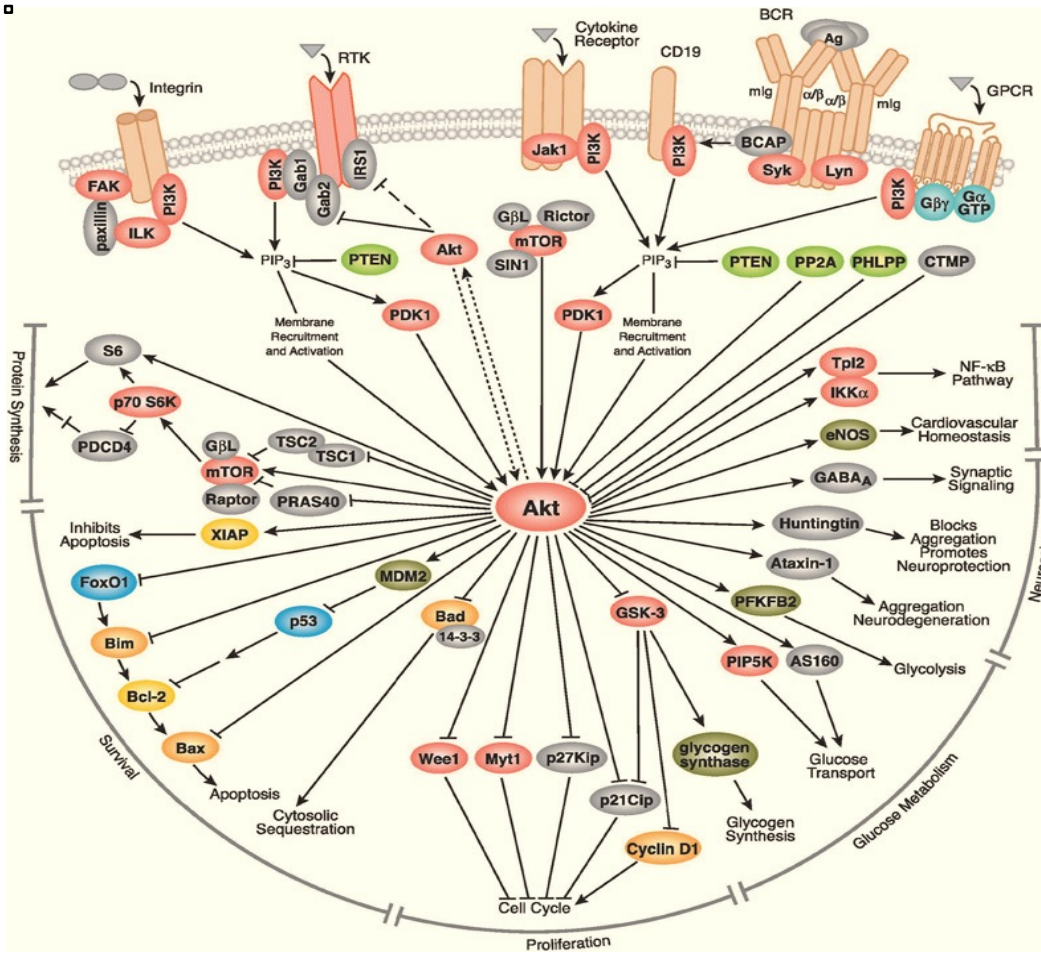


Figure 33. Akt plays a key role in multiple cellular processes such as protein synthesis, cell survival, growth, proliferation, angiogenesis, glucose metabolism, cardiovascular homeostasis, neuroprotection or apoptosis. Downstream targets of Akt include Bad, Caspase9, Caspase3, FoxO, GLUTs, eNOS, PFK2, PFK1, GSK3 or mTOR. (From www.cellsignal.com)

3.1 References

Barthel, A. et al., 1999. Regulation of GLUT1 gene transcription by the serine/threonine kinase Akt1. *The Journal of biological chemistry*, 274(29), pp.20281–6.

Basu, A. et al., 2013. Green tea supplementation increases glutathione and plasma antioxidant capacity in adults with the metabolic syndrome. *Nutrition research (New York, N.Y.)*, 33(3), pp.180–7.

Carpenter, C.L. & Cantley, L.C., 1996. Phosphoinositide kinases. *Current opinion in cell biology*, 8(2), pp.153–8.

Datta, S.R., Brunet, A. & Greenberg, M.E., 1999. Cellular survival: a play in three Akts. *Genes & development*, 13(22), pp.2905–27.

Gruber, K. et al., 2013. The role of zinc in immunity and inflammation. , pp.123–156.

Hajduch, E. et al., 1998. Constitutive activation of protein kinase B alpha by membrane targeting promotes glucose and system A amino acid transport, protein synthesis, and inactivation of glycogen synthase kinase 3 in L6 muscle cells. *Diabetes*, 47(7), pp.1006–13.

Hemmings, B.A. & Restuccia, D.F., 2012. PI3K-PKB/Akt pathway. *Cold Spring Harbor perspectives in biology*, 4(9), p.a011189.

Howitz, K.T. & Sinclair, D.A., 2008. Xenohormesis: sensing the chemical cues of other species. *Cell*, 133(3), pp.387–91.

Jansen, J. et al., 2012. Disturbed zinc homeostasis in diabetic patients by in vitro and in vivo analysis of insulinomimetic activity of zinc. *The Journal of nutritional biochemistry*, 23(11), pp.1458–66.

Jansen, J., Karges, W. & Rink, L., 2009. Zinc and diabetes--clinical links and molecular mechanisms. *The Journal of nutritional biochemistry*, 20(6), pp.399–417.

Jimenez, C. et al., 2002. The p85 regulatory subunit controls sequential activation of phosphoinositide 3-kinase by Tyr kinases and Ras. *The Journal of biological chemistry*, 277(44), pp.41556–62.

Jung, K.H. et al., 2008. Epigallocatechin gallate stimulates glucose uptake through the phosphatidylinositol 3-kinase-mediated pathway in L6 rat skeletal muscle cells. *Journal of medicinal food*, 11(3), pp.429–34.

Keller, S.R. et al., 1995. Cloning and Characterization of a Novel Insulin-regulated Membrane Aminopeptidase from Glut4 Vesicles. *Journal of Biological Chemistry*, 270(40), pp.23612–23618.

Keller, S.R., 2004. Role of the insulin-regulated aminopeptidase IRAP in insulin action and diabetes. *Biological & pharmaceutical bulletin*, 27(6), pp.761–4.

Kennedy, S.G. et al., 1997. The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. *Genes & development*, 11(6), pp.701–13.

Kim, J. et al., 2006. Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms. *Circulation*, 113(15), pp.1888–904.

Kim, J.-A. et al., 2007. Epigallocatechin gallate, a green tea polyphenol, mediates NO-dependent vasodilation using signaling pathways in vascular endothelium requiring reactive oxygen species and Fyn. *The Journal of biological chemistry*, 282(18), pp.13736–45.

Kloet, D.E.A. & Burgering, B.M.T., 2011. The PKB/FOXO switch in aging and cancer. *Biochimica et biophysica acta*, 1813(11), pp.1926–37.

Lawlor, M.A. & Alessi, D.R., 2001. PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *Journal of cell science*, 114(Pt 16), pp.2903–10.

Li, B. et al., 2013. The role of zinc in the prevention of diabetic cardiomyopathy and nephropathy. *Toxicology mechanisms and methods*, 23(1), pp.27–33.

Manning, B.D. & Cantley, L.C., 2007. AKT/PKB signaling: navigating downstream. *Cell*, 129(7), pp.1261–74.

Miao, X. et al., 2013. Zinc homeostasis in the metabolic syndrome and diabetes. *Frontiers of medicine*, 7(1), pp.31–52.

Munir, K.M. et al., 2013. Mechanisms for food polyphenols to ameliorate insulin resistance and endothelial dysfunction: therapeutic implications for diabetes and its cardiovascular complications. *American journal of physiology. Endocrinology and metabolism*, 305(6), pp.E679–86.

Shan, Z. et al., 2014. Interactions between zinc transporter-8 gene (SLC30A8) and plasma zinc concentrations for impaired glucose regulation and type 2 diabetes. *Diabetes*, 63(5), pp.1796–803.

Simpson, L. & Parsons, R., 2001. PTEN: life as a tumor suppressor. *Experimental cell research*, 264(1), pp.29–41.

Sohrab, G. et al., 2013. Dietary polyphenols and metabolic syndrome among Iranian adults. *International journal of food sciences and nutrition*, 64(6), pp.661–7. Available at: http://informahealthcare.com/doi/abs/10.3109/09637486.2013.787397 [Accessed September 16, 2014].

Waltner-Law, M.E. et al., 2002. Epigallocatechin gallate, a constituent of green tea, represses hepatic glucose production. *The Journal of biological chemistry*, 277(38), pp.34933–40.

Zhang, Y. & Liu, D., 2011. Flavonol kaempferol improves chronic hyperglycemia-impaired pancreatic beta-cell viability and insulin secretory function. *European journal of pharmacology*, 670(1), pp.325–32.

II. OBJECTIVES

II. OBJECTIVES

The research work carried out in this PhD Thesis is part of a more general research plan developed by the Nutrigenomics Research Group of the University Rovira i Virgili, which deals with the beneficial effects, and underlying mechanisms of action, of dietary polyphenols, mostly for prevention and amelioration of metabolic disorders associated with the so called Metabolic Syndrome. These disorders include insulin resistance, obesity, atherogenic dyslipidemia, hypertension and pro-inflammatory state, and all of them are associated with a higher risk of cardiovascular diseases (CVD) and Type-2 Diabetes Mellitus.

Initial *in vivo* studies of our research group showed that a single oral dose of a grape seed procyanidin extract (GSPE) to healthy rats was able to lowers plasma triglyceride levels to 50% in the postprandial state. Microarray analysis of liver gene expression profile associated to this GSPE administration identified some previously undiscovered target genes of polyphenols in hepatic cells. Among these where the metallothionein genes, whose mRNAs were 3 fold less abundant in the liver of GSPE-treated rats than in untreated control animals (Quesada et al. 2007). In another study, at the proteome level, zinc exporter ZnT-1 were found to be diminished in the liver of diet-induced obese rats chronically treated with procyanidin extracts (Baiges et al. 2010).

Further *in vitro* research in our group revealed that the green tea flavonoid EGCG was also able to down-regulate MT and ZnT1 expression in the human carcinoma cell line HepG2 (Quesada et al. 2011) whereas another flavonoid, quercetin, was found to up-regulate MT-1, MT2 and ZnT1 in the mouse hepatic carcinoma cell line Hepa1-6 (M. Bustos, personal communication, 2011, Universitat Rovira i Virgili). EGCG was found to diminish intracellular total zinc accumulation, whereas quercetin did just the opposite. However, both EGCG and Quercetin enhanced the level of cytoplasmic labile zinc. To explain this fact, it was hypothesized that both EGCG and quercetin might by acting as zinc ionophores, i.e., they would form complexes with zinc transporting it through the plasma membrane inside the cytoplasm.

Given the relevance of polyphenols by one side, and zinc on the other side, in the control of different metabolic pathways and intracellular signaling, this thesis was aimed to advance in the knowledge of the biological actions of polyphenols on zinc homeostasis and

signaling, and on the biological effects of combining polyphenols with zinc. More specifically, the purpose of this thesis was to characterize how a water soluble polyphenol, namely EGCG, and a hydrophobic polyphenol, namely quercetin, affect zinc signaling in cultured hepatic cancer cells. In addition, it was pursued to found out whether these and other polyphenol classes are true ionophores.

To fulfil these purposes, the following objectives were successively proposed:

1. Characterize the combined effect of Quercetin and zinc cations on Hepa 1-6 viability, cytoplasmic labile zinc levels and Akt phosphorylation.

2. Characterize the combined effect of EGCG and zinc cations on Hepa 1-6 viability, cytoplasmic labile zinc and Akt phosphorylation.

3. Assess whether EGCG, quercetin and other classes of dietary polyphenols may act as true zinc ionophores in a liposomal system.

4. Find out whether combinations of non-toxic quantities of quercetin with non-toxic amounts of zinc may induce apoptosis in the hepatic carcinoma cell line Hepa1-6.

References

Baiges, I. et al., 2010. Lipogenesis is decreased by grape seed proanthocyanidins according to liver proteomics of rats fed a high fat diet. *Molecular & cellular proteomics : MCP*, 9(7), pp.1499–513.

Quesada, I.M. et al., 2011. Dietary catechins and procyanidins modulate zinc homeostasis in human HepG2 cells. *The Journal of nutritional biochemistry*, 22(2), pp.153–63.

Quesada, I.M. et al., 2007. Grape seed procyanidins inhibit the expression of metallothione in genes in human HepG2 cells. *Genes & nutrition*, 2(1), pp.105–9.

III. RESULTS

1. Quercetin affects zinc homeostasis and Akt signaling in liver carcinoma Hepa 1-6 cells

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Zinc, the second most abundant transition metal in humans, is an essential micronutrient with structural, catalytic and signaling function and shows antioxidant action in cells. Quercetin is one of the most abundant polyphenols found in fruits and vegetables distinctive of the Mediterranean Diet. Its consumption is beneficial for prevention of chronic diseases such as obesity, hypertension, diabetes, Alzheimer or cancer. Food polyphenols are known to act as antioxidants and as signaling molecules, and to form complexes with copper and iron thereby affecting their bioavailability.

In this work, we seek to check whether Quercetin interacts with zinc and to evaluate the effect of this interaction on intracellular zinc homeostasis and zinc signaling in an *in vitro* model of hepatic tumour cells (Hepa 1-6). Quercetin strongly binds to zinc cations in solution as shown by the quenching of zinc dependent Zinquin fluorescence. Administration of Quercetin together with supplemental zinc to hepatoma cells results in enhanced expression of the zinc-store protein metallothionein and the zinc-export transporter ZnT1 (as determined by RT-PCR); total intracellular zinc content (assayed by FAAS) and cytoplasmic labile zinc (monitored by Zinquin fluorescence) are increased. Western-blot analyses show that low doses of Quercetin greatly enhance zinc-induced phosphorylation of Akt and, vice versa, low doses of zinc enhance the phosphorylation of Akt induced by Quercetin. These results show that combinations of zinc with Quercetin are more effective that the single compounds in modulating intracellular zinc homeostasis and signaling. They also suggest that Quercetin may act as zinc ionophore.

The LDH assay, the determination of total intracellular zinc concentrations and the gene expression profile study were conducted with my colleague Dr. Mario Bustos. Only the results related to the chelation in solution prove, the MTT viability assay, the intracellular labile zinc determination and the effects on Akt phosphorylation belong to this thesis.

Quercetin affects zinc homeostasis and Akt signaling in liver carcinoma Hepa 1-6 cells

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List of abbreviations:

EGCG, (–)-epigallocatechin-3-gallate; QCT, Quercetin; ClQ, clioquinol; ZnPyr, zinc pyrithione;FluoZin-3, 2-[2-[2-[2-[bis-(carboxylatomethyl)amino]-5-methoxyphenoxy]ethoxy]-4-(2,7-difluoro-3-oxido-6-oxo-4a,9a-dihydroxanthen-9-yl)-anilino]acetate; MRE, metal response element; MT, metallothionein; MTF-1, MRE-binding transcription factor-1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TPEN, N,N,N′,N′-tetrakis(2-phridylmethyl) ethylenediamine; Zinquin, ethyl (2-methyl-8-p-toluenesulfonamido-6-quinolyloxy); ZIP, ZRT/IRT related protein (SLC39 family of zinc transporters); ZnT, zinc transporter (SLC30 family of zinc transporters), Znt1 / Slc30a1, SLC30A1, solute carrier family 30 (zinc transporter) member 1.

Key words:

Clioquinol, Quercetin, flavonoids, labile zinc, zinc transporters, metallothionein, zinc signaling, Akt, hepatocellular carcinoma cell line Hepa 1-6

Running title: Quercetin modulates zinc signaling

Funding: This work was supported by grants AGL2005-04889 and AGL2008-00387 from the Spanish Ministry of Education and Science. H. D-B was granted a predoctoral grant from the University Rovira i Virgili.

Notes: The authors declare no competing financial interest.

ABSTRACT

Quercetin is one of the most abundant flavonoids found in dietary fruits and vegetables, and plays a relevant role in human health by virtue, in part, of its antioxidant actions, which partially derives from its interaction with the redox-active transition metals iron and copper. The interaction of Quercetin with the redox-inert zinc and the biological effects of this interaction are barely studied. The purpose of this study was to determine whether Quercetin chelates zinc cations and affects zinc homeostasis in the mouse liver carcinoma Hepa 1-6 cells. We have previously shown that other type of flavonoids, a grape seed procyanidin extract and green tea (–)-epigallocatechin-3-gallate (EGCG), interact with zinc cations and affect zinc homeostasis in human hepatic carcinoma HepG2 cells. Here we first show that Quercetin binds zinc cations in solution with a similar strength to TPEN and Clioquinol, a zinc-specific chelators and a zinc-ionophore respectively. Accordingly, Quercetin is able to counteract the noxious effects of excess zinc on Hepa 1-6 and vice versa. Quercetin added to Hepa 1-6 cells increases the expression of metallothionein genes MT1 and MT2, as well as the plasma membrane zinc exporter ZnT1 (SLC30A1). Likewise, Quercetin enhances intracellular accumulation of zinc, and also increases the tiny fraction of cytoplasmic labile zinc detectable by the zinc-specific fluorophore Zinquin, i.e., a pool of interchangeable, metabolically active, or signaling zinc. Both Quercetin and zinc are known to modulate phosphorylation of Akt. Here we show that Quercetin activates Akt phosphorylation in a dose and zinc-dependent manner. Taken together, our results show that Quercetin-zinc complexes modulate cellular zinc homeostasis and signaling and strongly suggest that the signaling and metabolic pathways modulated by labile zinc will be also target of this flavonoid. Thus, complexation of Quercetin with zinc cations may explain some of the beneficial health effects ascribed to Quercetin consumption.

1. INTRODUCTION

Quercetin is one of the most abundant flavonoids present in fruits and vegetables. The richest food sources of Quercetin are onions, oranges, leeks, broccoli, apples, and blueberries (Manach, Scalbert et al. 2004). Red wine and tea can also contain a significant amount of Quercetin. This molecule has been thoroughly investigated for its abilities to express mainly antioxidant properties attributed to its chemical structure (Rice-Evans,

Miller et al. 1996; Williams, Spencer et al. 2004). Like other flavonoids, Quercetin also behaves as a signaling molecule, modulating multiple cell signaling and metabolic pathways and gene expression (Kuo, Huang et al. 2001; Williams, Spencer et al. 2004; Scalbert, Johnson et al. 2005; Peluso 2006). Quercetin has strong anti-inflammatory (Orsolic, Knezevic et al. 2004) and anti-carcinogenic (Erlund, Freese et al. 2006) capacity. It has also been demonstrated that Quercetin possesses anti-fibrotic (Phan, Lim et al. 2003), anti-coagulative (Bucki, Pastore et al. 2003), anti-bacterial (Cushnie TP 2005), anti-atherogenic (Perez-Vizcaino, Duarte et al. 2006), anti-hypertensive (Duarte J 2001; Perez-Vizcaino, Duarte et al. 2006) and anti-proliferative properties (Orsolic, Knezevic et al. 2004; Gulati N 2006). Furthermore, Quercetin exerts anti-adipogenic activity activating AMPK signaling pathway in 3T3-L1 pre-adipocytes and induces apoptosis in mature adipocytes by modulating the ERK and JNK pathways (Ahn, Lee et al. 2008). These effects were increased when Quercetin was combined with the non-flavonoid polyphenol resveratrol (Park HJ 2008).

Flavonoids have been widely reported to chelate metals, in particular iron and copper (Hider, Liu et al. 2001; Quesada, Bustos et al. 2011). Quercetin possesses three possible metal chelating sites that can interact with metal ions; the 3,4-dihydroxy group located on the B ring and the 3- or 5-hydroxy and 4-carbonyl group in the C ring (Hider, Liu et al. 2001). The metal-complexing properties of this molecule may contribute to its total antioxidant activity. Thus, many studies have been focused on quelation of redox-active metals copper and iron, which are known to catalyse many biological processes leading to the production of free radicals (Kostyuk, Potapovich et al. 2001; Kostyuk, Potapovich et al. 2007; Ren, Meng et al. 2008; Pekal A 2011). Although much less studied, it is also known that some flavonoids, including Quercetin, can also chelate zinc redox-inert cations (Tan 2009) and affect zinc absorption by intestinal Caco-2 cells Sreenivasulu 2010.

Zinc is an essential cofactor for hundreds of enzymes. It is involved in protein, nucleic acid, carbohydrate, and lipid metabolism, as well as in the control of gene transcription, growth, development, and differentiation (Fuchs, Babusiak et al. 2003; Rudolf, Rudolf et al. 2003; Frassinetti, Bronzetti et al. 2006). At the molecular level, zinc is almost completely bound to proteins or chelated by low molecular weight ligands, leading to a very low concentration (in the nanomolar range) of the free ionized species and acts as a second messenger

modulating multiple signaling and metabolic pathways (Coyle, Zalewski et al. 1994; Beyersmann and Haase 2001; Hirano, Murakami et al. 2008; Murakami and Hirano 2008; Maret 2009). The intracellular labile zinc pool is metabolically important, because it responds to zinc deprivation or supplementation by decreasing or increasing its content, respectively (Zalewski, Forbes et al. 1993). Thus, the cell must maintain a tight regulation of this pool of zinc, so a large number of proteins are dedicated to zinc transport and buffering. Ten proteins of the ZnT family (SLC30A) and 14 proteins of the ZIP family (SLC39A) coordinate zinc transport out of and into the cytosol, respectively (Liuzzi and Cousins 2004; Lichten and Cousins 2009).

Zinc homeostasis is also regulated by zinc sensors, such as metal response element binding transcription factor-1 (MTF-1) (Andrews 2001; Lichtlen and Schaffner 2001; Laity and Andrews 2007), and zinc-binding proteins such as glutathione, and a family of at least 10 functional metallothionein (MT) proteins in human (only four in rodents). MTs are low-molecular weight metal-binding proteins. Within the cytoplasm, zinc is bound by metal-free apo-metallothionein (apo-MT) to generate Zn-MT. The Zn-MT/apoMT ratio functions at a central node in cellular signaling by redistributing cellular zinc, presiding over the availability of zinc, and interconverting redox and zinc signals (Krezel, Hao et al. 2007; Krezel and Maret 2008). MT also serves as a heavy metal chelator and its transcriptional regulation is conferred by MREs (Metal Response Elements), to which MTF-1 binds. The induction of MT with transition metals requires phosphorylation of MTF-1 by a kinases pathway that includes PI3K (phosphoinositol-3 kinase), PKC (protein kinase C) and JNK (c-jun N-terminal kinase) (LaRochelle, Gagne et al. 2001; Saydam, Adams et al. 2002). MTF-1 activates other important genes besides those encoding MTs. ZnT1, the main exporter of zinc out of the cell, was shown to be another in vivo target gene of MTF-1 (Cuajungco and Lees 1997). Thus, MTF-1 is a crucial transcriptional regulator for basal expression of at least three important genes (MT-1, MT-2 and ZnT1) involved in zinc metabolism.

Given that our previous results showed that catechins and proanthocyanidins interact with the redox-inactive metal zinc and modulate zinc homeostasis in the human hepatocarcinoma HepG2 cells (Quesada, Bustos et al. 2011), the aim of this work was to determine the interaction of another type of flavonoid, the flavone Quercetin, with zinc ions

and the modulation of different parameters of zinc homeostasis in the mouse hepatoma Hepa 1-6 cells. We also aimed to elucidate whether Quercetin-zinc complexes with different flavonoid to zinc ratios affect the metabolically active labile pool of zinc and a known target of this zinc pool, Akt, also known as Protein kinase B (PKB). Akt is a Serine/Threonine Kinase, which plays a key role in multiple cellular processes such as protein synthesis, cell survival, growth, proliferation, angiogenesis, glucose metabolism, cardiovascular homeostasis, neuroprotection or apoptosis (Kloet & Burgering, 2011; Manning & Cantley, 2007). Quercetin has been shown to modulate Akt phosphorylation in different cell types (Granado-Serrano, 2006) and zinc-chelators that increase the labile pool of zinc such as pyrithione are also known to affect Akt phosphorylation (Maret, 2013 Haase, 2009). Hence, we aimed to explore how the combination of zinc and Quercetin affect Akt phosphorylation.

2. MATERIALS AND METHODS

2.1. Chemicals

Quercetin, TPEN [N,N,N′,N′-tetrakis(2-phridylmethyl) ethylenediamine], ZnCl2, Zinquin ethyl ester, ethanol, dimethyl sulfoxide (DMSO) and Clioquinol were from Sigma (St. Louis, MO).

2.2. Fluorometric assay for zinc chelation by Quercetin

The zinc-dependent fluorescent emission of Zinquin ethyl ester (ZQEE) and Zinquin acid (ZQA), dissolved in phosphate buffered saline (PBS), pH 7.4, was recorded in a Biotek FLx 800 spectrofluorometer, with 100 µL samples, in COSTAR Corning 96 well, opaque bottom plates, with excitation set at 360/40 and emission at 460/40 nm, at 37ºC. Quenching of zinc-dependent fluorescence of fluorophore-zinc complexes by Quercetin, TPEN or Clioquinol, was monitored 15 min after addition of different amounts of the compounds to the solutions containing various amounts of fluorophores and zinc cations, as specified in the figures.

2.3. Cell cultures and treatments

Hepa 1-6 cells (BW7756 ECACC) were grown in Dulbecco´s Modified Eagle Medium (DMEM; BioWittaker) supplemented with 10% fetal bovine serum (BioWittaker), 2mM

glutamine and 1% non-essential amino acids. This medium contains 3.9 ± 0.3 µM zinc determined by flame atomic absorption spectrometry (FAAS). Cells were incubated at 37ºC in humidified, 5% CO2-enriched atmosphere and routinely splitted at a 1:5 ratio upon reaching 80% confluence. For treatments, cultures at 80% confluence were trypsinized and resuspended at a density of 5x105 cells/ml; 1 ml aliquots of resuspended cells were seeded per well in 12-well plates (Orange Scientific). Twenty-four hours later, medium was replaced with fresh medium containing the different treatment substances or vehicle (final 0.05 % ethanol, 0.1% DMSO).

2.4. In vitro cytotoxicity assays

Cytotoxicity of Quercetin and ZnCl2 was assessed in Hepa 1-6 cells treated 24 h with different concentration of the substances using the lactate dehydrogenase (LDH) leakage assay of plasma membrane integrity as previously described (Puiggros, Llopiz et al. 2005) and the methyl tetrazolium (MTT) assay of mitochondrial functionality and cell viability as previously described (Stern 2006). 104 cells were seeded per well of 96-well plates in 100 *µ*L media for MTT assays.

2.5. Quantification of MT and ZnT-1 gene expression

Total RNA was isolated from Hepa 1-6 cells using RNeasy Mini Kit (Qiagen). To quantify relative mRNA levels of specific genes in different RNA samples, cDNAs were generated from total RNA using TaqMan Reverse Transcription Reagents (Applied Biosystems) and quantitative reverse transcriptase-polymerase chain reactions (RT-PCRs) were performed using the TaqMan PCR Core Reagents Kit and Specific TaqMan Gene expression assay Probes (Applied Biosystems): MT-I (Mm00496660_g1), MT-II (Mm00809556_s1) and Znt1/Slc30a1 (Mm00437377_m1). Cyclophilin peptidylprolyl isomerase A (cyclophilin A, PPIA) (Mm03024003_g1) was used as endogenous control gene for sample input normalization in RT-PCR experiments. Quantitative PCR amplification and detection were performed using the Applied Biosystems Real Time 7000 PCR System termocycler and software.

2.6. Quantification of total intracellular zinc

To quantify total intracellular zinc, cells were thoroughly washed with PBS and lysed with 0.01 M NaOH. Aliquots of the cell lysates were used to quantify zinc by FAAS as

previously described (Reaves, Fanzo et al. 2000), using a Hitachi Z-8200 Polarized Zeeman AA Spectrophotometer. Total zinc amount was normalized per total protein content of the cells, determined by the Bradford method.

2.7. Measurements of intracellular labile zinc

To measure changes in intracellular labile zinc, cells were washed with PBS after treatments, incubated 30 minutes at 37ºC in 20 μM Zinquin ethyl ester in PBS; protein content and Zinquin fluorescence was determined in cell aliquots. Background fluorescence of Zinquin loaded cells was subtracted from readings to derive Zinquin-dependent fluorescence as described (Coyle, Zalewski et al. 1994).

For microscopy visualization, cells were attached to glass coverslips, the medium was discarded after treatments and the cells washed (three times) with PBS. Coverslips were immersed in a solution of Zinquin (20μM) in PBS and incubated for 30 min at 37 ºC. Coverslips were inverted on to microscope slides, and images were captured at 1000x magnification with a Leica DM 4000B microscope using UV light illumination (λex = 340-380 nm) and a blue emission filter (λem ≥ 425nm).

2.8. Changes in phosphorylation of Akt /PKB-1 were monitored by Western blotting

For immunoblotting analysis, Hepa1-6 cell extracts were prepared through cell lysis in RIPA buffer (100 nM Tris-Cl pH 7,4, Tween 10%, Na-Deoxycholate 10%, SDS 0,1%). BioRad Bradford Assay was performed in order to quantify protein concentration, and 30 μg of protein were analysed for each sample. Proteins were separated using 10% SDS-Polyacrylamide Gel Electrophoresis and afterwards transferred into an activated polyvinylidene difluoride membranes (PVDF, Amersham Hybond™-P roll). Membranes were blocked with 5% non-fat milk powder in TBS-Tween 0.05% for 2 hours. Primary antibodies used were: rabbit anti-Phospho-Akt (Ser473) antibody (Cell Signaling Technology, Product # 9271); rabbit anti-Akt antibody (Cell Signaling Technology, Product # 9272) and rabbit β-Actin antibody (Cell Signaling Technology, Product # 4967). β-Actin was used as internal sample control. Anti-rabbit IgG, HRP-linked Antibody was used as secondary antibody in all western analysis (#7074). Primary antibodies were incubated overnight at 4ºC, while secondary antibody was incubated for 2 hours at room temperature. HRP on the membrane was revealed with a chemiluminescent reagent (Amersham ECL Prime Western Blotting Detection Reagent, product RPN2232), and images captured with

SYNGENE G:BOX Chemi XL 1.4. Imaging System. Relative levels of different proteins were obtained analysing the image bands by the Java-based software ImageJ.

2.9. Statistical analysis

For statistical analysis in cytotoxicity assays, zinc and protein quantification, quantitative RT-PCRs, and fluorescence measurements, t-test and one-way ANOVA analyses were performed using SPSS software. All data are the result of at least 3 independent experiments. Differences were considered significant for P values 0.05.

3. RESULTS

3.1. Quercetin chelates zinc cations in solution

In order to test if Quercetin interact with zinc cations in solution, we first characterized the zinc-dependent fluorescence of membrane impermeable Zinquin Acid (ZQA) and of membrane permeable Zinquin Ethyl Ester (ZQEE), which are zinc-specific fluorescent chelators widely employed to measure concentrations of labile (free plus loosely bound) zinc within cells and in the biological fluids (Zalewski, Truong-Tran et al. 2006). The capability of the flavonoid to chelate zinc cations in solution was then measured as the quenching of zinc-dependent fluorescence of ZQEE and of ZQA previously complexed with zinc. Both zinc-specific fluorophores lose fluorescence when are not bound to zinc, indicating that the flavonoid has retrieved zinc cations from the fluorescent fluorophore-zinc complex. We found that Quercetin quenched the zinc-dependent Zinquin fluorescence rapidly, with an efficacy comparable to that of the zinc-specific chelators Clioquinol and TPEN, at a 100:10 Quercetin to Zinquin molar ratio, in the presence of 10, 20 or 40 μM ZnCl2 (Fig. 1). These results imply that Quercetin binds zinc cations retrieving them from Zinquin-zinc complexes.

3.2. Quercetin protects cells from zinc-induced cell death

We next performed LDH and MTT tests to evaluate the effect of increasing amounts of zinc and Quercetin on Hepa 1-6 cells viability. Zinc concentrations above 200 µM resulted in significant LDH leakage, reaching 40% upon incubation of cells with 1000 µM zinc (Fig. 2A), and 14% upon incubation with 600 µM Quercetin during 24 h (Fig. 2B). The noxious

effects of 300 µM zinc were partially reversed by the addition of 300 µM Quercetin (1:1 Quercetin-zinc ratio), and addition of 600 µM Quercetin (2:1 ratio) completely abolished the toxic effect of 300 µM zinc, leaving the cells as untreated cells after the LDH test (Fig. 2C). Thus, 300 µM Quercetin, a dose that is not toxic for the cells, was able to decrease the toxic effects of excessive zinc and 600 µM Quercetin, a dose that is toxic to Hepa 1-6 cells, was able to completely counteract the toxic effects of excess zinc. Vice versa, the toxic effects of 600 µM Quercetin are counteracted by the addition of an amount of zinc that is toxic in the absence of Quercetin. This result was confirmed by the MTT test (Fig. 3). 800 µM Quercetin is able to reverse the lethality of zinc present at concentrations as high as 2500 μM (Fig. 3B) and 3000 µM (Fig. 3C). These results strongly suggest that Quercetin and zinc form complexes that render Quercetin anions and zinc cations unavailable for cells.

3.3. Quercetin enhances the expression of MT and ZnT1 genes in Hepa 1-6 cells

Hepa 1-6 cells were cultured and grown 24 hours in a standard culture medium (3.9 ± 0.3 µM zinc) or in zinc-loaded medium (50 µM added ZnCl2) supplemented with either vehicle or 100 µM Quercetin, and monitored changes in gene expression of MT genes and the plasma membrane zinc exporter, ZnT1 at various times, using quantitative RT-PCR. The results show that both MT genes, the only ones expressed in mouse liver cells, were up-regulated by 100 µM Quercetin with respect to control cells (Fig. 4A and B), reaching maximal values 6 hours after addition of Quercetin. Addition of 50 µM zinc to the culture medium resulted in an induction of MTs with a 12 fold induction of MT-I and a 20 fold induction of MT-II 6 hours after zinc supplementation.

Afterwards, we monitored changes in the expression profile of ZnT1 gene grown in standard culture medium (approximately 4 µM zinc) supplemented with 100 µM Quercetin. The results showed that ZnT1 was slightly up regulated by 100µM Quercetin with respect to control cells 3 hours after addition of Quercetin (Fig. 4C). In zinc-loaded conditions, ZnT1 expression was highly up-regulated reaching maximal values 3 hours after addition of zinc. Addition of 100µM Quercetin to the zinc-loaded medium almost doubled the zinc-induced up-regulation of ZnT1 expression at this time (Fig. 4C). The modification elicited by Quercetin in the expression of MTs and ZnT1 in Hepa 1-6 cells strongly suggest that zinc cations are made more available by the presence of Quercetin, i.e., that Quercetin

increase zinc bioavailability, since MT and ZnT1 expression are known to be up-regulated by increments in zinc concentrations. This is in contrast with the above mentioned sequestering effect of high concentrations of Quercetin and high concentrations of zinc.

3.4. Quercetin increases intracellular total zinc levels

To address this point, we measured the total intracellular zinc content in Hepa 1-6 cells grown in standard (3.9 ± 0.3 μM zinc) and zinc-loaded (50 μM added zinc) media treated with either vehicle or 100 μM Quercetin, at the same times used in gene expression experiments (Fig. 5). In basal conditions, 100 μM Quercetin elicited a slight increase in total intracellular zinc only after 12 hours treatment. When the cultured medium was supplemented with zinc, total zinc accumulation was greatly increased compared with untreated cells, as expected. In this zinc-loaded medium, 100 Quercetin slightly inhibited total zinc accumulation 6 hours after addition of Quercetin, but eventually, after 24 hours, cells accumulated 2 fold as much zinc in Quercetin plus zinc medium than in zinc-only medium. Therefore, Quercetin increases the accumulation of zinc in Hepa 1-6.

3.5. Quercetin elevates cytoplasmic labile zinc in Hepa 1-6 cells

We next measured cytoplasmic labile zinc in standard (3.9 ± 0.3 μM zinc) and zinc-loaded (50 μM zinc) medium treated with vehicle, 10 μM or 100 μM Quercetin in Hepa 1-6 cells. Both doses of Quercetin tested produced no effect on Zinquin-detectable labile zinc in cells cultured in basal medium. When the medium was supplemented with 50μM zinc, intracellular labile zinc augmented markedly with respect to control cells, as expected. Addition of 10μM Quercetin to this zinc-loaded medium decreased cytoplasmic labile zinc temporally, at 6 hours of treatment. In contrast, 100μM Quercetin highly increased cytoplasmic labile zinc at 3, 6 and 12 hours of treatment when combined with 50 μM Zn (Fig. 6A). Microscopic observation of Zinquin-loaded Hepa 1-6 cells was done to visualize this effect of Quercetin on cytoplasmic labile zinc in Hepa 1-6 cells grown in basal zinc concentration as well as in condition of added zinc (Fig. 6B). As a control, Hepa 1-6 were also treated with Clioquinol, a well-known zinc ionophore that increases labile zinc concentrations when applied together with additional zinc. Both Quercetin and Clioquinol produced a significant elevation of Zinquin-detectable labile zinc, only when combined with zinc and only in a subpopulation of the cultured cells, producing a similar pattern of intracellular fluorescence.

3.6. Quercetin modulates Akt phosphorylation in a zinc-dependent manner in Hepa 1-6 cells

Akt plays a central role in diverse cell signaling pathways that rely on Akt phosphorylation. Quercetin and other polyphenols are known to modulate Akt phosphorylation (Granado-Serrrano, 2006), and in this way affect apoptosis and other cell processes. Likewise, zinc, as a second messenger, modulates Akt phosphorylation and apoptosis (Maret, 2013 Haase, 2009), as shown by the use of the classical zinc ionophore Pyrithione. We wonder whether Quercetin might affect the effect of zinc treatment on Akt phosphorylation and, vice versa, whether the addition of supplemental zinc would affect the effect of Quercetin on Akt phosphorylation. Akt signals through phosphorylation of two residues, Ser427 and Thr345. As a proof of concept, we choose to analyse the changes in phosphorylation of Ser427 by Western blotting. Results show (Fig. 7) that low amounts of QCT (10 μM) by themselves do not affect Akt phosphorylation, whereas when combined with 50 μM zinc elevates S427P-Akt at 6 and 24 hours of treatment. 100 µM QCT increases Akt phosphorylation slightly 24 hours after treatment and drastically (6 fold over basal levels) when combined with 50 µM zinc. Thus, QCT enhances Akt phosphorylation in a way that positively correlates with zinc concentrations in the culture medium. Likewise, zinc alone triggers a much lower enhancement of Akt phosphorylation than when combined with Quercetin. This strongly suggests that the effect of Quercetin on Akt phosphorylation (and hence signaling) is mediated by intracellular zinc.

4. DISCUSSION

We evaluated the effect of Quercetin on zinc homeostasis in an in vitro model for hepatic carcinoma, the Hepa 1-6 cell line. We have previously demonstrated that a grape seed procyanidin extract (GSPE), a mixture of catechins and proanthocyanidins, as well as individual catechins and procianidines display an affinity for zinc cations in solution high enough to make them dissociate from the zinc-specific chelator, Zinquin (Quesada, Bustos et al. 2011). In this study we tested another type of flavonoid, Quercetin and we compared this molecule with a well-known intracellular zinc chelator, TPEN. We have shown here that Quercetin also displaced zinc ions from zinc-Zinquin complex with similar affinity as TPEN at very low concentrations and at molar ratios of 0.5 µM Quercetin to 20 µM Zinquin and 1 µM zinc. Sreenivasulu et al. also showed that Quercetin and tannic acid quenched

zinc-induced Zinquin fluorescence in a dose dependent manner (Sreenivasulu, Raghu et al. 2010). It was also reported that the stilbene resveratrol, chelates zinc in vitro (Zhang, Wu et al. 2009). Our result implies that Quercetin interacts with zinc cations in solution, suggesting that it could be able to modulate the metabolically active labile zinc.

If Quercetin indeed interacts with zinc cations forming Quercetin-zinc complexes, it also would be able to reduce zinc toxicity in Hepa 1-6 cells. To test this, we assessed the viability of Hepa 1-6 cells co-incubated with a toxic dose of zinc and different doses of Quercetin, determined by LDH and MTT. We have shown that Quercetin exerted a reversion of excess zinc toxicity (300 µM zinc) in Quercetin to zinc ratios (1:1) and (2:1). The 2:1 ratio showed major effect in preventing zinc toxicity than 1:1 ratio. This result reflects the importance of the Quercetin-zinc complex stoichiometry. We have also recently demonstrated that GSPE and EGCG are also able to counteract zinc toxicity in HepG2 cells (Quesada, Bustos et al. 2011). Our findings are in agreement with Sun et al. who have shown that EGCG protected PC-3 cells from damages induced by zinc ions (Sun, He et al. 2008).

In order to asses whether the ability of Quercetin to complex with zinc may affect cellular zinc homeostasis, we monitored changes in the expression profile of genes involved in storage and zinc transport (MT and ZnT1 respectively) and total intracellular zinc accumulation in Hepa 1-6 cells grown in standard culture medium (3.9 ± 0.3 µM zinc) and in zinc-loaded medium (50 µM additional zinc). The results showed that modulation elicited by Quercetin was more effective when the medium was supplemented with excess zinc, increasing the expression of MTs and of zinc exporter ZnT1 gene. In this regard, Weng et al. reported that Quercetin increase MTs expression in HepG2 cells producing liver protection against oxidative stress activating MAPK and PI3K pathways and also increasing DNA binding activity of Nrf2 (Weng, Chen et al. 2011). It has been also reported that zinc-induced MT gene expression was up-regulated by Quercetin in CaCo-2 cells (Sreenivasulu, Raghu et al. 2010). Also the isoflavone genistin was shown to increase MT and ZnT1 expression in HepG2 cells (Chung, Kang et al. 2006). However, the effect of these flavonoids on zinc accumulation was not reported. The changes elicited by Quercetin in the expression of MT and ZnT1 genes in different conditions are consistent with an increased availability of zinc and a concerted response of the cellular machinery to get rid

of zinc excess. To address this point we measured total intracellular zinc. We found that total zinc content was increased with 100μM Quercetin in standard and zinc-loaded medium. We have previously demonstrated opposite changes in these parameters with other flavonoids, GSPE and EGCG. These compound down-regulated MTs and ZnT1 expression and also decreased total intracellular zinc accumulation in human hepatic cells, HepG2. GSPE and EGCG were used in a flavonoid to zinc ratio of 1:1; in contrast, in this study we used a Quercetin to zinc ratio 2:1. In this regard, Sun et al. inferred about the importance of the structure and stoichiometry of EGCG-zinc complexes in the bioavailability of the flavonoid, demonstrating that EGCG in the presence of zinc was more effective than EGCG alone in enhancing the permeability of the cell membrane, whereas zinc-EGCG complex had no effect on PC-3 cell membrane permeability (Sun, He et al. 2008).

We also measured the labile pool of zinc in Hepa 1-6 cells grown in a standard and in a zinc-loaded medium supplemented with two doses of Quercetin (10 and 100µM). We found no effects of any doses of Quercetin in the standard medium. However, in zinc-loaded medium, we found that co-treatments with Quercetin and zinc in a 1:5 (Quercetin:zinc) ratio, Zinquin-detectable labile zinc diminished. In contrast, co-treatments in a 2:1 (Quercetin:zinc) ratio markedly increased this pool of labile zinc. This result also reflects the importance of the flavonoid-zinc ratios in the outcomes. The increase of labile zinc elicited by Quercetin in the presence of zinc at certain concentration was here shown to be similar to that elicited by the zinc chelators drug with ionophoric action Clioquinol, suggesting that Quercetin might have, beside a zinc sequestering effect, a zinc ionophoric action. It is suggested that the effect of Quercetin on zinc availability will depend of the stoichiometry and structure of the zinc-Quercetin complex formed.

We have previously shown that GSPE and EGCG, flavonoids that behave opposite to Quercetin in MT and ZnT expression and total zinc content, also augmented the Zinquin-detectable labile pool of zinc in HepG2 cells. It appears that flavonoids may enhance intracellular labile zinc levels independently of their effect on MT and ZnT1 expression and total intracellular zinc content. Other zinc chelators have been reported to increase the labile pool of zinc. Clioquinol, an antibiotic with anti-amyloid and anti-cancer effects, induces autophagy in cultured astrocytes and neurons in a zinc-dependent manner, acting

as a zinc ionophore, increasing labile zinc in the cytosol and in autophagic vacuoles (Park MH 2011). The glycone isoflavone genistin, enhances the proapoptotic effects of zinc in HepG2 cells and up-regulates the expression of MT and ZnT1 concomitantly increasing the labile zinc pool detectable by FluoZin-3. Resveratrol, at physiological concentrations (10 μM), when applied to normal human prostate epithelial cells cultured in 16 or 32 μM zinc, arrests cell growth and enhances Zinquin-detectable zinc, while not affecting total zinc nor MT expression.

We hypothesize that, depending on flavonoid to zinc ratio, absolute concentrations, the chemical structure of the flavonoid, temperature, pH, and the presence of other competing metals, different types of flavonoid-zinc complexes will be formed. In our laboratory conditions, Quercetin will form membrane-permeable complexes with zinc, which will then act as ionophores and therefore transport the metal into the cell. Once within the cell, Quercetin might be metabolized and zinc cations added to the labile pool of zinc. Or it could also be possible that zinc cations bound to Quercetin may be considered as loosely bound zinc without dissociate from Quercetin. Zinquin does not only detect free zinc, but also some of the zinc atoms that are bound to metallothionein, and probably also to other proteins (Coyle, Zalewski et al. 1994). To support this hypothesis, it was reported that Clioquinol acts as a zinc ionophore increasing the labile pool of zinc as mentioned above (Park MH 2011). In addition, resveratrol not only interact with zinc cations in solution, but also elevated the labile pool of zinc in normal human prostate epithelial cells. The authors inferred that the elevation of the labile zinc elicited by resveratrol is due to the cellular uptake of resveratrol-zinc complexes, followed by the intracellular dissociation of the complexes (Zhang, Wu et al. 2009).

Taken together, it appears that flavonoids may enhance intracellular labile zinc levels independently of their effect on MT and ZnT1 expression and total intracellular zinc content. However it is dependent of the flavonoid-zinc ratio of the complexes. We inferred that the proportion of the different types of flavonoid-zinc complexes would affect the bioavailability and biological actions of both the metal and the flavonoid. The labile pool of zinc can reversibly bind to regulatory sites in signaling proteins. Consequently, changes of the labile zinc concentration can affect cell-signaling pathways and might even act as zinc signals.

Summing up, Quercetin is added to catechins and procianidines as polyphenols with zinc-chelating properties and, as a result, affects zinc homeostasis. We forward the hypothesis that elevation of labile zinc by Quercetin may be a relevant mechanism by which affects multiple metabolic and cell signaling pathways that respond to intracellular fluctuations of labile zinc. In this work, we have demonstrated that phosphorylation of Akt (and therefore Akt signaling) elicited by Quercetin is greatly affected by the concentration of zinc in the medium. Further research is necessary to evaluate the mechanisms by which flavonoids enhance cytoplasmic labile zinc and the consequences of this enhancement on modulation of zinc signaling and metabolic pathways.

REFERENCES

[1] Ahn, J., H. Lee, et al. (2008). "The anti-obesity effect of Quercetin is mediated by the AMPK and MAPK signaling pathways." Biochemical and Biophysical Research Communications 373(4): 545-549.

[2] Andrews, G. K. (2001). "Cellular zinc sensors: MTF-1 regulation of gene expression." Biometals 14(3-4): 223-37.

[3] Beyersmann, D. and H. Haase (2001). "Functions of zinc in signaling, proliferation and differentiation of mammalian cells." Biometals 14(3-4): 331-41.

[4] Bucki, R., J. J. Pastore, et al. (2003). "Flavonoid inhibition of platelet procoagulant activity and phosphoinositide synthesis." J Thromb Haemost 1(8): 1820-8.

[5] Chung, M. J., A. Y. Kang, et al. (2006). "Water-soluble genistin glycoside isoflavones up-regulate antioxidant metallothionein expression and scavenge free radicals." J Agric Food Chem 54(11): 3819-26.

[6] Coyle, P., P. D. Zalewski, et al. (1994). "Measurement of zinc in hepatocytes by using a fluorescent probe, Zinquin: relationship to metallothionein and intracellular zinc." Biochem J 303 (Pt 3): 781-6.

[7] Cuajungco, M. P. and G. J. Lees (1997). "Zinc metabolism in the brain: relevance to human neurodegenerative disorders." Neurobiol Dis 4(3-4): 137-69.

[8] Cushnie TP, L. A. (2005). " Antimicrobial activity of flavonoids." Int J Antimicrob Agents. 26(5): 343-56.

[9] Devergnas, S., F. Chimienti, et al. (2004). "Differential regulation of zinc efflux transporters ZnT-1, ZnT-5 and ZnT-7 gene expression by zinc levels: a real-time RT-PCR study." Biochem Pharmacol 68(4): 699-709.

[10] Duarte J, P.-P. R., Vargas F, Ocete MA, Pérez-Vizcaino F, Zarzuelo A, Tamargo J. (2001). "Antihypertensive effects of the flavonoid Quercetin in spontaneously hypertensive rats." Br J Pharmacol. 133(1): 117-24.

[11] Erlund, I., R. Freese, et al. (2006). "Bioavailability of Quercetin from berries and the diet." Nutr Cancer 54(1): 13-7.

[12] Frassinetti, S., G. Bronzetti, et al. (2006). "The role of zinc in life: a review." J Environ Pathol Toxicol Oncol 25(3): 597-610.

[13] Fuchs, O., M. Babusiak, et al. (2003). "Role of zinc in eukaryotic cells, zinc transporters and zinc-containing proteins. Review article." Sb Lek 104(2): 157-70.

[14] Gulati N, L. B., Zohrabian VM, Murali R, Jhanwar-Uniyal M. (2006). "The antiproliferative effect of Quercetin in cancer cells is mediated via inhibition of the PI3K-Akt/PKB pathway." Anticancer Res. 26(2A): 1177-81.

[15] Haase, H. & Rink, L., 2009. Functional significance of zinc-related signaling pathways in immune cells. *Annual review of nutrition*, 29, pp.133–52.

[16] Hider, R. C., Z. D. Liu, et al. (2001). "Metal chelation of polyphenols." Methods Enzymol 335: 190-203.

[17] Hirano, T., M. Murakami, et al. (2008). "Roles of zinc and zinc signaling in immunity: zinc as an intracellular signaling molecule." Adv Immunol 97: 149-76.

[18] Kostyuk, V. A., A. I. Potapovich, et al. (2007). "Metal complexes of dietary flavonoids: evaluation of radical scavenger properties and protective activity against oxidative stress in vivo." Cell Mol Biol (Noisy-le-grand) 53(1): 62-9.

[19] Kostyuk, V. A., A. I. Potapovich, et al. (2001). "Influence of metal ions on flavonoid protection against asbestos-induced cell injury." Arch Biochem Biophys 385(1): 29-37.

[20] Krezel, A., Q. Hao, et al. (2007). "The zinc/thiolate redox biochemistry of metallothionein and the control of zinc ion fluctuations in cell signaling." Arch Biochem Biophys 463(2): 188-200.

[21] Krezel, A. and W. Maret (2008). "Thionein/metallothionein control Zn(II) availability and the activity of enzymes." J Biol Inorg Chem 13(3): 401-9.

[22] Kuo, S. M., C. T. Huang, et al. (2001). "Quercetin cumulatively enhances copper induction of metallothionein in intestinal cells." Biol Trace Elem Res 84(1-3): 1-10.

[23] Laity, J. H. and G. K. Andrews (2007). "Understanding the mechanisms of zinc-sensing by metal-response element binding transcription factor-1 (MTF-1)." Arch Biochem Biophys.

[24] LaRochelle, O., V. Gagne, et al. (2001). "Phosphorylation is involved in the activation of metal-regulatory transcription factor 1 in response to metal ions." J Biol Chem 276(45): 41879-88.

[25] Lichten, L. A. and R. J. Cousins (2009). "Mammalian zinc transporters: nutritional and physiologic regulation." Annu Rev Nutr 29: 153-76.

[26] Lichtlen, P. and W. Schaffner (2001). "The "metal transcription factor" MTF-1: biological facts and medical implications." Swiss Med Wkly 131(45-46): 647-52.

[27] Liuzzi, J. P. and R. J. Cousins (2004). "Mammalian zinc transporters." Annu Rev Nutr 24: 151-72.

[28] Manach, C., A. Scalbert, et al. (2004). "Polyphenols: food sources and bioavailability." Am J Clin Nutr 79(5): 727-47.

[29] Maret, W. (2009). "Molecular aspects of human cellular zinc homeostasis: redox control of zinc potentials and zinc signals." Biometals 22(1): 149-57.

[30] Maret, W. Zinc biochemistry: from a single zinc enzyme to a key element of life. *Adv. Nutr.* 4, 82–91 (2013)

[31] Murakami, M. and T. Hirano (2008). "Intracellular zinc homeostasis and zinc signaling." Cancer Sci 99(8): 1515-22.

[32] Orsolic, N., A. H. Knezevic, et al. (2004). "Immunomodulatory and antimetastatic action of propolis and related polyphenolic compounds." J Ethnopharmacol 94(2-3): 307-15.

[33] Park HJ, Y. J., Ambati S, Della-Fera MA, Hausman DB, Rayalam S, Baile CA. (2008). "Combined effects of genistein, Quercetin, and resveratrol in human and 3T3-L1 adipocytes." Journal of Medicinal Food 11(4).

[34] Park MH, L. S., Byun HR, Kim Y, Oh YJ, Koh JY, Hwang JJ. (2011). "Clioquinol induces autophagy in cultured astrocytes and neurons by acting as a zinc ionophore." Neurobiology of disease 42(3): 242.

[35] Pekal A, B. M., Pyrzynska K. (2011). "Interaction of Quercetin with copper ions: complexation, oxidation and reactivity towards radicals." BioMetals 24(1): 41-9.

[36] Peluso, M. R. (2006). "Flavonoids attenuate cardiovascular disease, inhibit phosphodiesterase, and modulate lipid homeostasis in adipose tissue and liver." Exp Biol Med (Maywood) 231(8): 1287-99.

[37] Perez-Vizcaino, F., J. Duarte, et al. (2006). "Endothelial function and cardiovascular disease: effects of Quercetin and wine polyphenols." Free Radic Res 40(10): 1054-65.

[38] Phan, T. T., I. J. Lim, et al. (2003). "Quercetin inhibits fibronectin production by keloid-derived fibroblasts. implication for the treatment of excessive scars." Journal of Dermatological Science 33(3): 192-194.

[39] Puiggros, F., N. Llopiz, et al. (2005). "Grape seed procyanidins prevent oxidative injury by modulating the expression of antioxidant enzyme systems." J Agric Food Chem 53(15): 6080-6.

[40] Quesada, I. M., M. Bustos, et al. (2011). "Dietary catechins and procyanidins modulate zinc homeostasis in human HepG2 cells." The Journal of Nutritional Biochemistry 22(2): 153-163.

[41] Reaves, S. K., J. C. Fanzo, et al. (2000). "Expression of the p53 tumor suppressor gene is up-regulated by depletion of intracellular zinc in HepG2 cells." J Nutr 130(7): 1688-94.

[42] Ren, J., S. Meng, et al. (2008). "Complexation of Flavonoids with Iron: Structure and Optical Signatures." The Journal of Physical Chemistry B

[43] J. Phys. Chem. B 112(6): 1845-1850.

[44] Rice-Evans, C. A., N. J. Miller, et al. (1996). "Structure-antioxidant activity relationships of flavonoids and phenolic acids." Free Radic Biol Med 20(7): 933-56.

[45] Rudolf, E., K. Rudolf, et al. (2003). "The role of intracellular zinc in modulation of life and death of Hep-2 cells." Biometals 16(2): 295-309.

[46] Saydam, N., T. K. Adams, et al. (2002). "Regulation of metallothionein transcription by the metal-responsive transcription factor MTF-1: identification of signal transduction cascades that control metal-inducible transcription." J Biol Chem 277(23): 20438-45.

[47] Scalbert, A., I. T. Johnson, et al. (2005). "Polyphenols: antioxidants and beyond." Am J Clin Nutr 81(1 Suppl): 215S-217S.

[48] Sreenivasulu, K., P. Raghu, et al. (2010). "Polyphenol-Rich Beverages Enhance Zinc Uptake and Metallothionein Expression in Caco-2 Cells." Journal of Food Science 75(4): H123-H128.

[49] Sun, S. L., G. Q. He, et al. (2008). "Free Zn(2+) enhances inhibitory effects of EGCG on the growth of PC-3 cells." Mol Nutr Food Res 52(4): 465-71.

[50] Weng, C.-J., M.-J. Chen, et al. (2011). "Hepatoprotection of Quercetin against oxidative stress by induction of metallothionein expression through activating MAPK

and PI3K pathways and enhancing Nrf2 DNA-binding activity." New Biotechnology Special issues on Biocatalysis and Agricultural Biotechnology: number 7-9 28.

[51] Williams, R. J., J. P. Spencer, et al. (2004). "Flavonoids: antioxidants or signaling molecules?" Free Radic Biol Med 36(7): 838-49.

[52] Zalewski, P., A. Truong-Tran, et al. (2006). "Use of a zinc fluorophore to measure labile pools of zinc in body fluids and cell-conditioned media." Biotechniques 40(4): 509-20.

[53] Zalewski, P. D., I. J. Forbes, et al. (1993). "Correlation of apoptosis with change in intracellular labile Zn(II) using Zinquin [(2-methyl-8-p-toluenesulphonamido-6-quinolyloxy)acetic acid], a new specific fluorescent probe for Zn(II)." Biochem J 296 (Pt 2): 403-8.

[54] Zhang, J. J., M. Wu, et al. (2009). "Effect of resveratrol and zinc on intracellular zinc status in normal human prostate epithelial cells." Am J Physiol Cell Physiol 297(3): C632-44.

[55] Barthel, A., Okino, S. T., Liao, J., Nakatani, K., Li, J., Whitlock, J. P., & Roth, R. A. (1999). Regulation of GLUT1 gene transcription by the serine/threonine kinase Akt1. The Journal of Biological Chemistry, 274(29), 20281–6.

[56] Datta, S. R., Brunet, A., & Greenberg, M. E. (1999). Cellular survival: a play in three Akts. Genes & Development, 13(22), 2905–27.

[57] Hajduch, E., Alessi, D. R., Hemmings, B. A., & Hundal, H. S. (1998). Constitutive activation of protein kinase B alpha by membrane targeting promotes glucose and system A amino acid transport, protein synthesis, and inactivation of glycogen synthase kinase 3 in L6 muscle cells. Diabetes, 47(7), 1006–13.

[58] Hemmings, B. A., & Restuccia, D. F. (2012). PI3K-PKB/Akt pathway. Cold Spring Harbor Perspectives in Biology, 4(9), a011189.

[59] Kloet, D. E. A., & Burgering, B. M. T. (2011). The PKB/FOXO switch in aging and cancer. Biochimica et Biophysica Acta, 1813(11), 1926–37.

[60] Lawlor, M. A., & Alessi, D. R. (2001). PKB/Akt: a key mediator of cell proliferation, survival and insulin responses Journal of Cell Science, 114(Pt 16), 2903–10.

[61] Manning, B. D., & Cantley, L. C. (2007). AKT/PKB signaling: navigating downstream. Cell, 129(7), 1261–74.

Figures

Quercetin affects zinc homeostasis and Akt signaling in liver carcinoma Hepa 1-6 cells

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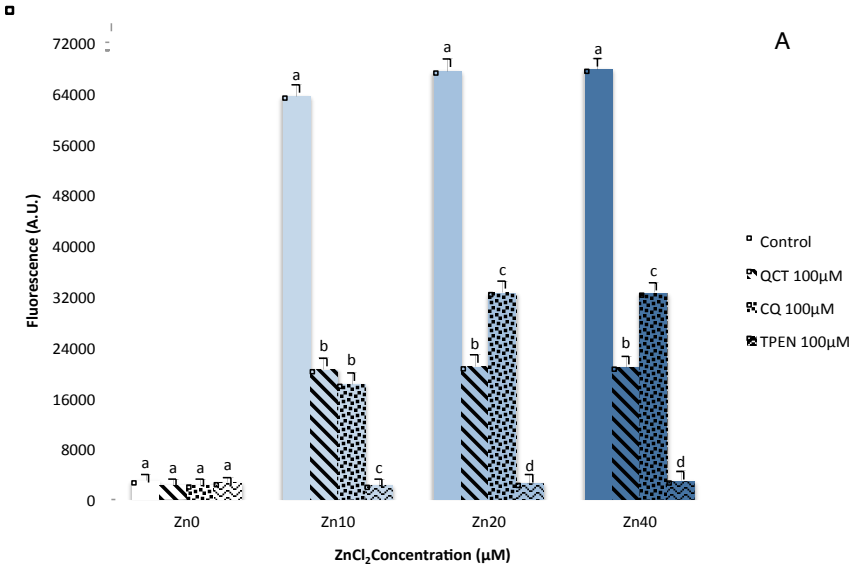
Figure 1. Quercetin chelates zinc cations in solution.
The capability of QCT to chelate zinc cations in solution was measured as the quenching of zinc-dependent fluorescence of Zinquin-EE (Panel A) and of Zinquin Acid (panel B) when complexed with zinc. Both zinc-specific fluorophores lose fluorescence when not bound to zinc, indicating that Quercetin retrieved zinc cations from the fluorescent fluorophore-zinc complex. (A) Decrease of zinc-dependent fluorescence of 10 μM ZQEE by 100 μM Quercetin, TPEN or CQ at various concentrations of zinc cations. (B) Decrease of zinc-dependent fluorescence of 10 μM ZQ Acid by 100 μM Quercetin, TPEN or CQ at various concentrations of zinc cations.



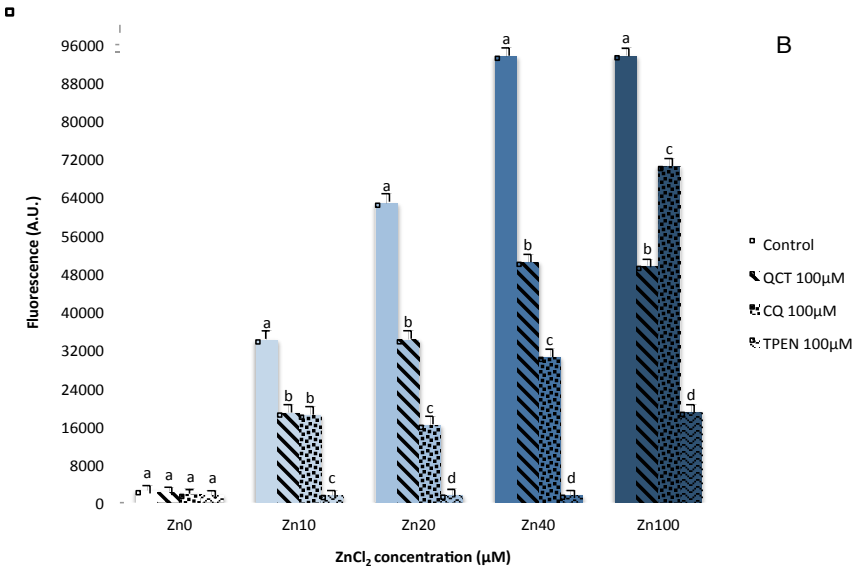


Figure 2. Quercetin protects cells from zinc-induced cell death.
Cell cytotoxicity was measured by the LDH method in cell cultures adding increasing amounts of $ZnCl_2$ (A), QCT (B), and both (C).

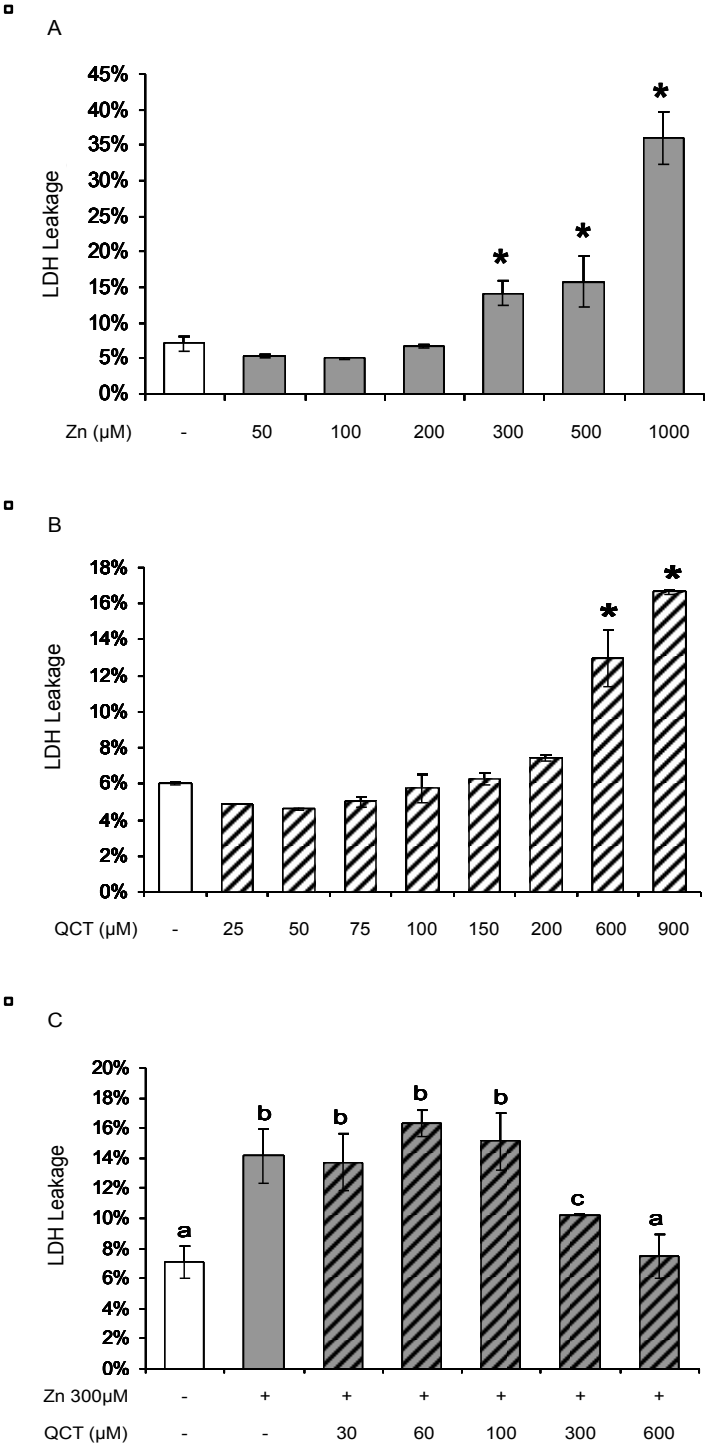


Figure 3. Quercetin promotes a partial reversion of high zinc dose toxicity.
Cell viability was measured by the MTT assay in cell cultures adding increasing amounts of QCT (A) and combining increasing amounts of QCT with 2,5 mM (B) or 3 mM (C) of $ZnCl_2$.

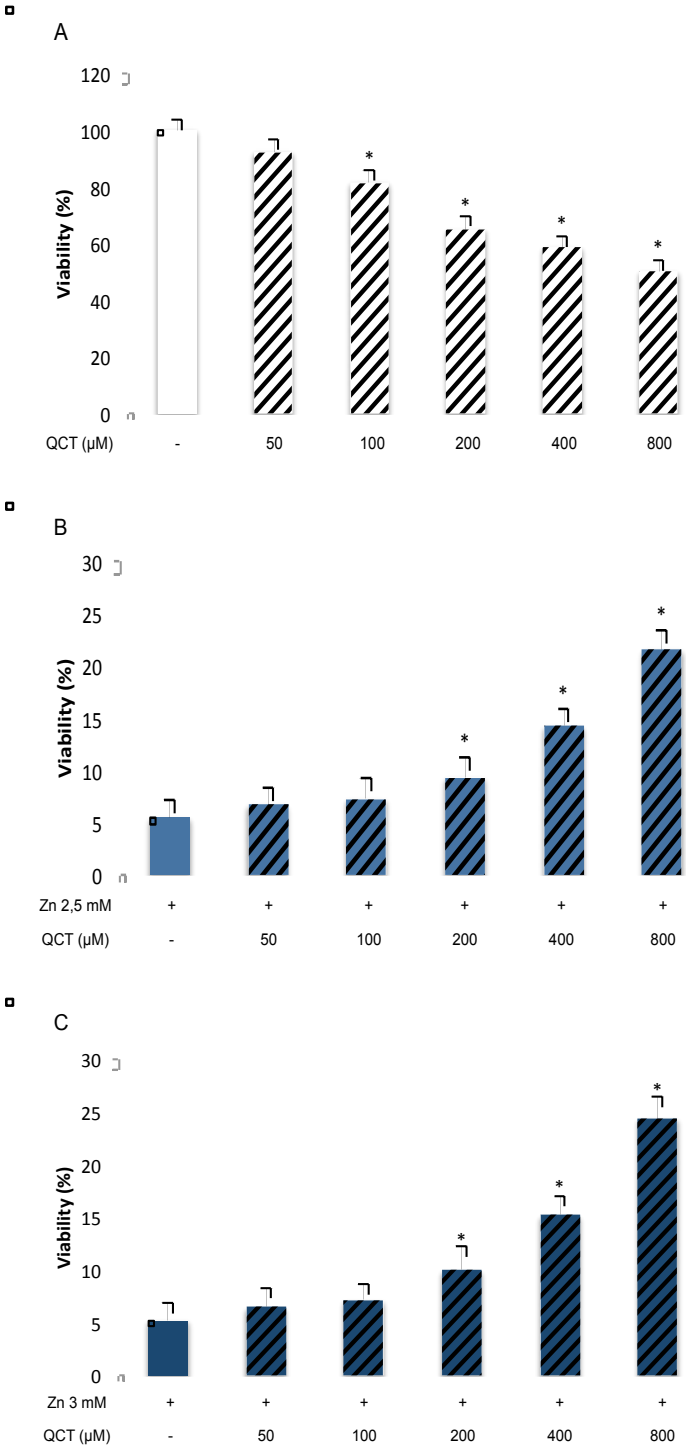


Figure 4. Quercetin enhances expression of Mt1, Mt2 and ZnT1 at the mRNA level. Quercetin enhances the expression of MTI, MTII and ZnT1 mRNA levels relative to control (untreated) cells. Internal standard was PPIA.

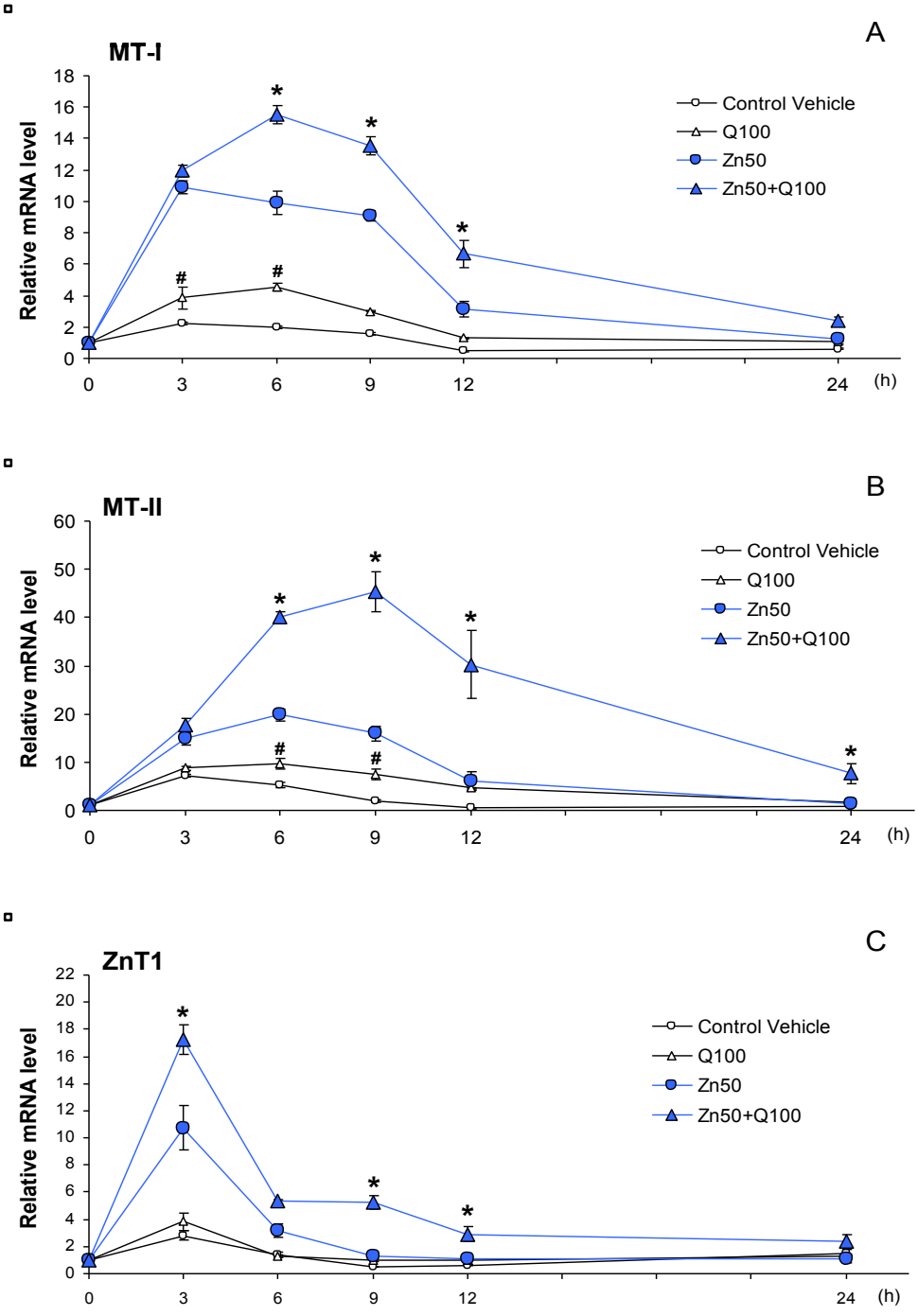


Figure 5. Quercetin increases total intracellular zinc levels in a zinc-dependent way. Cellular zinc was quantified by FAA (Flame Atomic Absorption) and normalized to the protein amount of each cell sample. Results are referred to the content of zinc in cells at the beginning of the treatment.

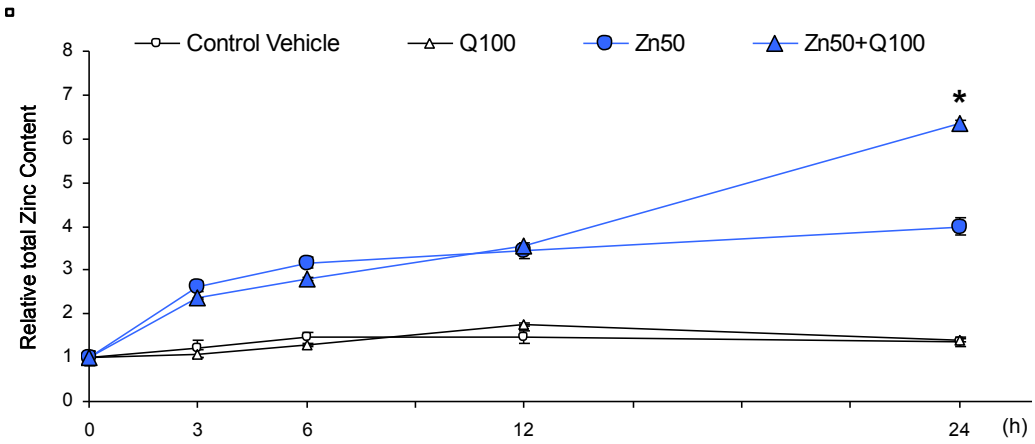


Figure 6. Quercetin raises cytoplasmic labile zinc levels detectable by Zinquin. (A) Evolution of labile Zinquin detectable zinc upon treatment of Hepa 1-6 cells with QCT, zinc or a combination of both. ZQ fluoresce of cultured cells at the beginning of treatment is assigned as a reference value of 1. (B) Effect of QCT and CQ on the cytoplasmic pool of labile zinc in Hepa1-6 cells. Hepa 1-6 cells were first treated with 100 μM QCT or CQ in the presence or absence of 50 μM $ZnCl_2$ for 4 hours. Treated medium was then removed, and 25 μM Zinquin ethyl ester was added. After 30 minutes of incubation at 37ºC in the dark, cells were washed and examined under a confocal fluorescence microscope. Control cells were treated with vehicle. Scale bars represent 10 μm.

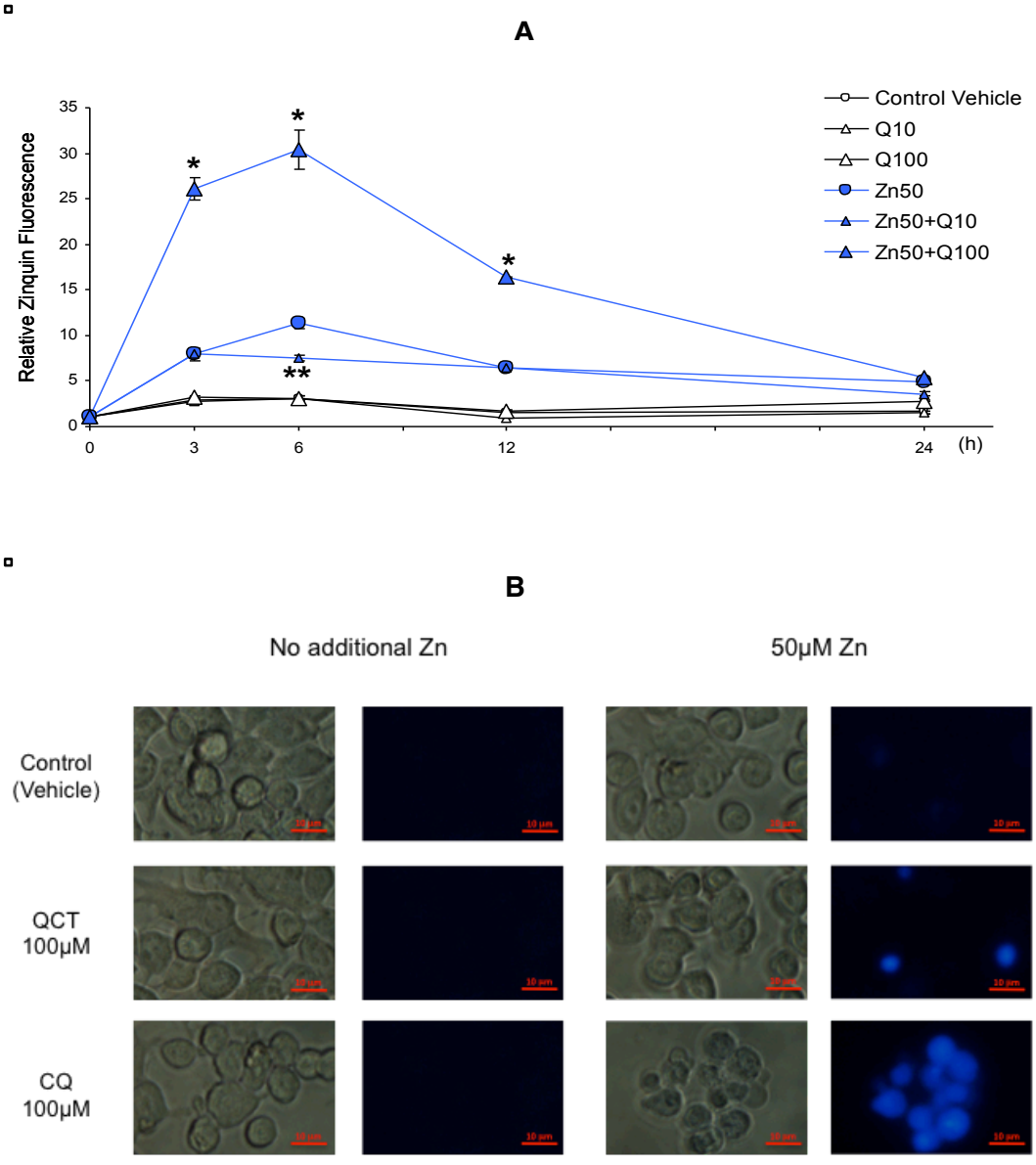
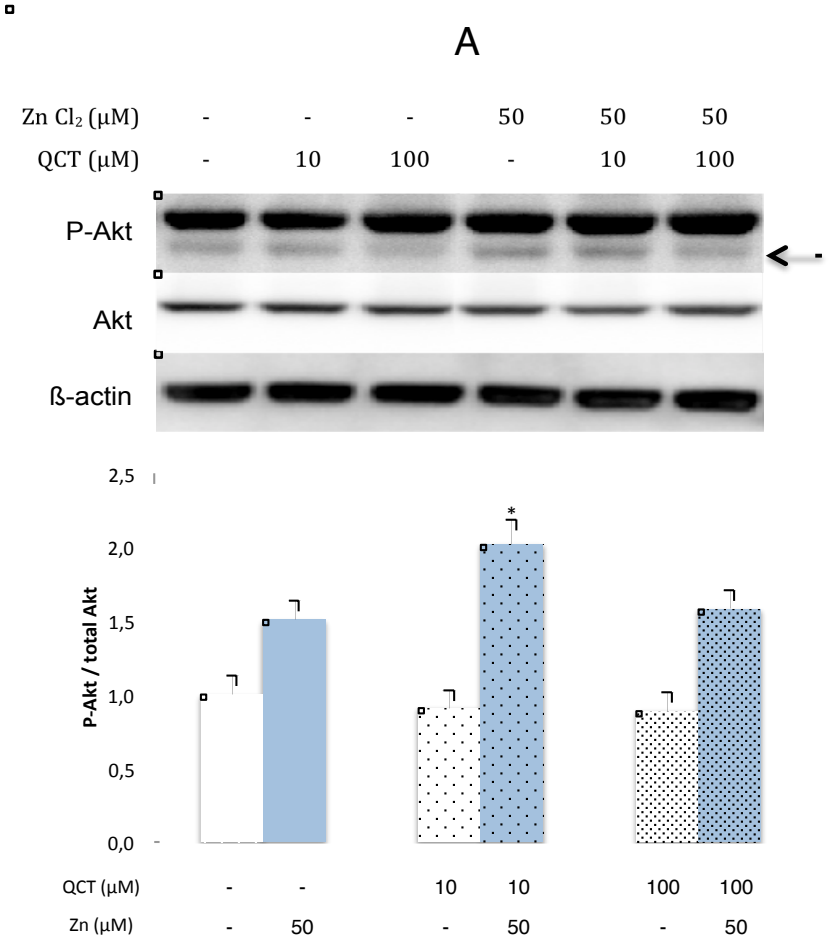
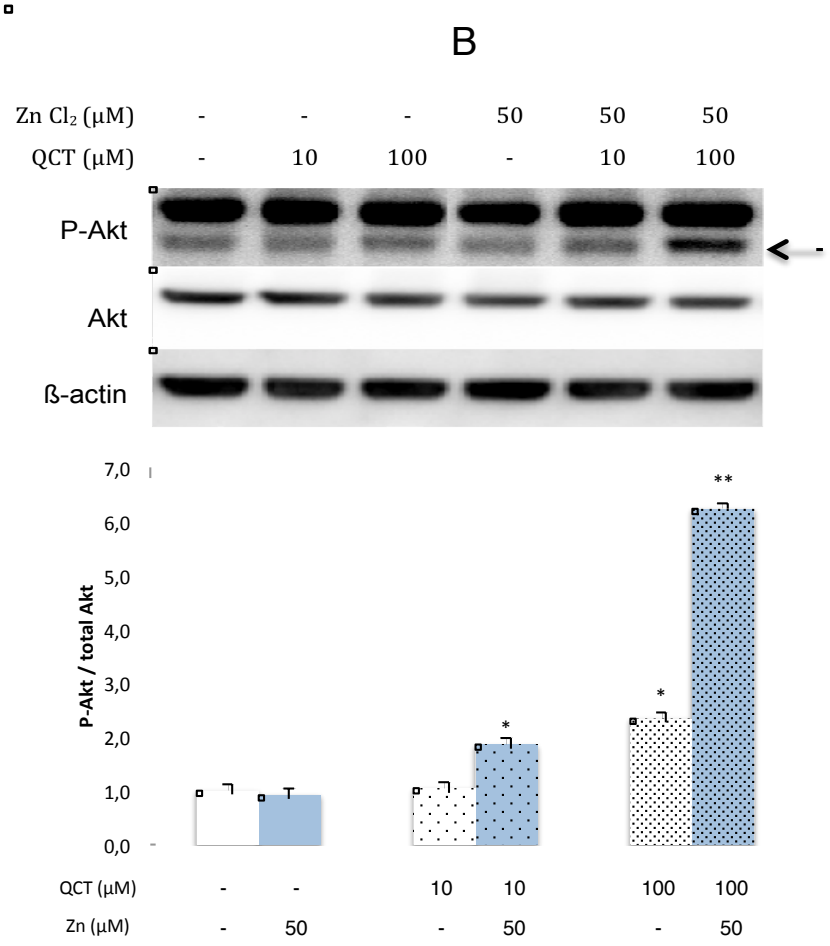


Figure 7. Immunoblotting analysis of the effect of Quercetin, zinc or both on Akt phosphorylation in Hepa 1-6 cells.
Hepa 1-6 cells were treated with vehicle, 10 μM QCT, 100 μM QCT, alone and combined with 10 or 100 μM $ZnCl_2$, for 6 or 24 hours. Immunoblots of cell extracts were first probed with anti-247SerPAkt, and, after stripping off the antibodies, with anti-Akt and anti-β-actin antibodies. Results show that QCT modulates Akt phosphorylation in a zinc-dependent manner. Low doses of Quercetin greatly enhance zinc-induced phosphorylation of Akt at 6 hours treatment (A), and vice versa, low doses of zinc enhance the phosphorylation of Akt induced by Quercetin at 24 hours treatment (B). Arrows indicates 60 kDa bands of P-Akt.




B
Zn Cl2 (µM) - - - 50 50 50
QCT (µM) - 10 100 - 10 100
P-Akt
Akt
ß-actin
P-Akt / total Akt
7,0
6,0
5,0
4,0
3,0
2,0
1,0
0,0
*
*
**
QCT (µM) - - 10 10 100 100
Zn (µM) - 50 - 50 - 50

2. Chelation of zinc cations by green tea epigallocatechin gallate affects zinc homeostasis and Akt signaling in liver carcinoma Hepa 1-6 cells

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Manuscript in preparation

Tea ranks second only to water as a major component of fluid intake worldwide and has been considered a health-promoting beverage since ancient times. For the past two decades, it has been investigated the potential therapeutic effects of green tea and its most abundant catechin, Epigallocatechin-gallate (EGCG), and its importance on the modulation of various signal transduction pathways. Since EGCG has been described to interact with zinc forming different complexes, as well as to modulate the intracellular zinc homeostasis, a new viewpoint has emerged, considering new possibilities of EGCG-zinc complex.

In the present study, we seek to characterize the combined effect of EGCG and zinc cations on Hepa 1-6 viability, cytoplasmic labile zinc and Akt phosphorylation. Administration of EGCG together with supplemental zinc to hepatoma cells results in a decreased expression of the zinc-store protein metallothionein as well as the zinc-export transporter ZnT1 (as determined by RT-PCR) and total intracellular zinc content (assayed by FAAS). Cytoplasmic labile zinc (monitored by Zinquin fluorescence) was increased. EGCG also modulates Akt phosphorylation in a zinc-dependent way, whereas high EGCG/zinc ratio inhibits Akt phosphorylation and low EGCG/zinc ratio enhances Akt phosphorylation. Moreover, toxic concentrations of EGCG have been demonstrated to counteract the toxic effects of excess zinc, and vice versa, toxic amounts of zinc neutralize the noxious effect of excess EGCG.

The determination of total intracellular zinc concentrations and the gene expression profile study were conducted with my colleague Dr. Mario Bustos. Only the results related to the chelation in solution prove, the MTT viability assay, the intracellular labile zinc determination and the effects on Akt phosphorylation belong to this thesis.

Chelation of zinc cations by green tea Epigallocatechin-gallate affects zinc homeostasis and Akt signaling in liver carcinoma Hepa 1-6 cells

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List of abbreviations:

DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; EGCG, (–)-epigallocatechin-3-gallate; FluoZin-3, 2-[2-[2-[2-[bis-(carboxylatomethyl)amino]-5-methoxyphenoxy]ethoxy]-4-(2,7-difluoro-3-oxido-6-oxo-4a,9a-dihydroxanthen-9-yl)-anilino]acetate; MRE, metal response element; MT, metallothionein; MTF-1, MRE-binding transcription factor-1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TPEN, N,N,N′,N′-tetrakis(2-phridylmethyl) ethylenediamine; Zinquin, ethyl (2-methyl-8-p-toluenesulfonamido-6-quinolyloxy); ZIP, ZRT/IRT related protein (SLC39 family of zinc transporters); ZnT, zinc transporter (SLC30 family of zinc transporters), Znt1 / Slc30a1, SLC30A1, solute carrier family 30 (zinc transporter), member 1.

Key words:

Akt, Clioquinol, Epigallocatechin-galate, flavonoids, hepatocellular carcinoma, labile zinc, metallothionein, zinc signaling, zinc transporters.

Running title: Green tea polyphenol modulates zinc signaling

Funding: This work was supported by grants AGL2005-04889 and AGL2008-00387 from the Spanish Ministry of Education and Science. H. D-B was granted a predoctoral grant from the University Rovira i Virgili.

Notes: The authors declare no competing financial interest.

ABSTRACT

(–)-epigallocatechin-3-gallate (EGCG) is one of the most consumed flavonoids by humans since it is present in green tea, and plays a relevant role in human health by virtue of its antioxidant action, which partially derives from its interaction with the redox-active transition metals iron and copper, and also modulates diverse intracellular signaling and metabolic pathways. The interaction of EGCG with the redox-inert zinc, and the biological effects of this interaction are barely studied. Previously, we have shown that a grape seed procyanidin extract (GSPE) and EGCG interact with zinc cations and affect zinc homeostasis in human hepatic carcinoma HepG2 cells. The purpose of this study was to determine how EGCG affects zinc homeostasis and zinc signaling in mouse liver carcinoma Hepa 1-6 cells. Here we first confirm that EGCG binds zinc cations in solution with a similar strength to TPEN, a zinc-specific chelator. Accordingly, EGCG is able to counteract the noxious effects of excess zinc on Hepa 1-6 cells, and vice versa, zinc neutralizes the toxicity of excess EGCG. As it happens in HepG2 cells, EGCG added to Hepa 1-6 cells decreases the expression of metallothionein genes MT1 and MT2 and of the plasma membrane zinc exporter ZnT1 (SLC30A1). Likewise, EGCG decreases the intracellular accumulation of zinc, although it also increases the tiny fraction of cytoplasmic labile zinc detectable by the zinc-specific fluorophore Zinquin, i.e., the pool of interchangeable, metabolically active, or signaling zinc. Both EGCG and zinc are known to modulate phosphorylation of Akt, a key factor in diverse signaling intracellular pathways. Here we show that EGCG activates Akt phosphorylation in a dose and zinc-dependent manner. Taken together, our results describe how EGCG-zinc complexes modulate cellular zinc homeostasis and signaling, and strongly suggest that the signaling and metabolic pathways modulated by labile zinc will also be targets of this polyphenol. Thus, complexation of EGCG with zinc cations may explain some of the beneficial health effects ascribed to EGCG consumption.

1. INTRODUCTION

EGCG is one of the most abundant flavonoids present in fruits and vegetables. The richest source of EGCG is green tea (Manach, Scalbert et al. 2004). Human studies suggest that green tea may contribute to a reduction in the risk of cardiovascular disease and some

forms of cancer, as well as to the promotion of oral health and other physiological functions such as anti-hypertensive effect, body weight control, antibacterial and antiviral activity, solar ultraviolet protection, bone mineral density increase, anti-fibrotic properties, and neuroprotective power (J Am Coll Nutr. 2006). EGCG has a strong anti-inflammatory (Orsolic, Knezevic et al. 2004) and anti-carcinogenic (Erlund, Freese et al. 2006) capacity. It also demonstrates anti-fibrotics (Phan, Lim et al. 2003), anti-coagulatives (Bucki, Pastore et al. 2003), anti-bacterial (Cushnie TP 2005), anti-atherogenic (Perez-Vizcaino, Duarte et al. 2006), anti-hypertensive (Duarte J 2001; Perez-Vizcaino, Duarte et al. 2006) and anti-proliferative properties (Orsolic, Knezevic et al. 2004; Gulati N 2006). Furthermore, EGCG exerts anti-adipogenic activity triggering the AMPK signaling pathway in 3T3-L1 pre-adipocytes. Moreover, it induces apoptosis in mature adipocytes by modulating the ERK and JNK pathways (Ahn, Lee et al. 2008). The beneficial effects of polyphenols in general and flavonoids in particular have been largely attributed to their well-known antioxidant activity (Bagchi et al., 2003; Cos et al., 2003; Dragsted, 2003; Shi et al., 2003; Ariga, 2004; Cos et al., 2004) (Rice-Evans, Miller et al. 1996; Williams, Spencer et al. 2004). Flavonoids exert their antioxidant activity by three mechanisms: (a) direct scavenging of reactive oxygen species (ROS), such as superoxide radical anion, hydroperoxyl radical, hydrogen peroxide, and hydroxyl radical; and reactive nitrogen species, such as peroxinitrite; (b) by chelating redox-active transition metals as iron and copper, that may act as powerful generators of ROS; and (c) by inhibition of ROS producing enzymes, in particular xanthine oxidase, NADPH oxidase and lipoxygenases (Premysl, 2010). By the first two mechanisms, flavonoids act as potent inhibitors of LDL peroxidation triggered by different pro-oxidant systems, both in vitro and in vivo, and hence are active against genesis and progression of inflammation and atherosclerosis (Plumb, 1998; Aviram and Fuhrman, 2002; da Silva Porto et al., 2003; Aviram et al., 2004).

Yet, the bioactivity of flavonoids is not limited to their direct antioxidant actions. They also behave as signaling molecules, modulating multiple cell signaling and metabolic pathways and gene expression (Kuo, Huang et al. 2001; Williams, Spencer et al. 2004; Scalbert, Johnson et al. 2005; Peluso 2006). Several flavonoids have been shown to interact with specific plasma membrane receptors, cytoplasmic signal transduction factors and nuclear receptors, serving themselves as signaling agents and eventually modulating gene expression at the transcriptional level. Actions of flavonoids on phosphoinositide 3-kinase

(PI 3-kinase), Akt/protein kinase B (Akt/PKB), tyrosine kinases, protein kinase C (PKC), and mitogen activated protein kinase (MAP kinase) signaling cascades are well characterized (Williams et al., 2004b). Moreover, recent studies show that flavonoids can directly act on some protein kinases, including Akt/PKB, Fyn, Janus kinase 1 (JAK1), mitogen-activated protein kinase kinase 1 (MEK1), PI3K, mitogen-activated protein (MAP) kinase kinase 4 (MKK4), Raf1, and ζ chain-associated 70-kDa protein (ZAP-70) kinase, and then alter their phosphorylation state to regulate multiple cell signaling pathways in carcinogenesis processes (Hou, 2010).

Flavonoids, including EGCG have been widely reported to chelate metals (Hider, Liu et al. 2001; Quesada, Bustos et al. 2011). EGCG possesses four possible metal chelating sites that can interact with metal ions; the 3,4-dihydroxy group located on the B ring and the 3- or 5-hydroxy and 4-carbonyl group in the C ring (Hider, Liu et al. 2001). The metal-complexing properties of this molecule may contribute to its total antioxidant activity. Thus, many studies have been focused on quelation of redox-active metals such as copper and iron, which are known to catalyze many biological processes leading to the production of free radicals (Kostyuk, Potapovich et al. 2001; Kostyuk, Potapovich et al. 2007; Ren, Meng et al. 2008; Pekal A 2011). Although much less studied, it is known that flavonoids can also chelate the redox-inert zinc cations and affect some aspects of zinc homeostasis. In spite of Zn(II) being a redox-inert cation, many reports have studied Zn complexes to evaluate the antioxidant activity of the flavonoid. For instance, Bodini et al. have reported that the complexation of catechin with Zn(II) ions favours the antioxidant activity of catechin decreasing the oxidation potentials (Bodini et al., 2001). Le Nest et al. have also studied the formation of complexes between Quercetin and catechin with Zn(II) in two media at neutral pH in the absence of oxygen and its influence on their antioxidant properties (Le Nest, 2003). In addition, Sun Sh. et al. described that complexation of Zn(II) with EGCG influences the rate of absorption of EGCG by prostate cancer (PC-3) cells (Sun et al., 2008). Likewise, Kuo et al. found that diverse flavonoids may affect the expression of metallothionein in human intestinal CaCo cells (Kuo et al., 1998; Kuo and Leavitt, 1999; Kuo et al., 2001) an effect that was then attributed to the antioxidant action of the flavonoids, and not to a direct interaction of the flavonoids with metals.

Zinc is an essential cofactor for hundreds of enzymes. It is involved in protein, nucleic acid, carbohydrate, and lipid metabolism, as well as in the control of gene transcription, cell growth, development, and differentiation (Fuchs, Babusiak et al. 2003; Rudolf, Rudolf et al. 2003; Frassinetti, Bronzetti et al. 2006). At the molecular level, zinc is almost completely bound to proteins or chelated by low molecular weight ligands, leading to a very low concentration (in the nanomolar range) of the free ionized species and acts as a second messenger modulating multiple signaling and metabolic pathways (Coyle, Zalewski et al. 1994; Beyersmann and Haase 2001; Hirano, Murakami et al. 2008; Murakami and Hirano 2008; Maret 2009). The intracellular labile zinc pool is metabolically important, because it responds to zinc deprivation or supplementation by decreasing or increasing its content, respectively (Zalewski, Forbes et al. 1993). Thus, the cell must maintain a tight regulation of this pool of zinc, so a large number of proteins are dedicated to zinc transport and buffering. Ten proteins of the ZnT family (SLC30A) and 14 proteins of the ZIP family (SLC39A) coordinate zinc transport out of and into the cytosol, respectively (Liuzzi and Cousins 2004; Lichten and Cousins 2009), The transcription factor MTF-1 (Metal Response Element Binding Transcription Factor-1) co-ordinately up-regulates the transcription of zinc exporter ZnT1, glutathione, and a family of at least 10 functional human metallothionein (MT) proteins, and the four MT genes in mouse and rat in response to increments of cytoplasmic zinc (Andrews 2001; Lichtlen and Schaffner 2001; Laity and Andrews 2007). MTs are low-molecular weight metal-binding proteins. Within the cytoplasm, zinc is bound by metal-free apo-metallothionein (apo-MT) to generate Zn-MT. The Zn-MT/apo-MT ratio functions at a central node in cellular signaling by redistributing cellular zinc, presiding over the availability of zinc, and interconverting redox and zinc signals (Krezel, Hao et al. 2007; Krezel and Maret 2008). MT also serves as a heavy metal chelator and its transcriptional regulation is conferred by MREs (Metal Response Elements), to which MTF-1 binds. The induction of MT with transition metals requires phosphorylation of MTF-1 by a kinases pathway that includes PI3K (phosphoinositol-3 kinase), PKC (protein kinase C) and JNK (c-jun N-terminal kinase) (LaRochelle, Gagne et al. 2001; Saydam, Adams et al. 2002). MTF-1 activates other important genes besides those encoding MTs. ZnT1, the main exporter of zinc out of the cell, was shown to be another in vivo target gene of MTF-1 (Cuajungco and Lees 1997).

Given that our previous results showed that proanthocyanidins interact with the redox-inactive zinc metal and modulate zinc homeostasis in the human hepatocarcinoma HepG2 cells (Quesada, Bustos et al. 2011), resulting in an increment of cytoplasmic Zinquin-detectable labile zinc, the aim of this work was to determine the interaction of the flavonoid Epigallocatechin-gallate with zinc cations and the modulation of different parameters of zinc homeostasis in mouse hepatic carcinoma cells Hepa 1-6. We also aimed to elucidate whether EGCG-zinc complexes with different flavonoid to zinc ratios affect the metabolically active labile pool of zinc, and concomitantly modulate the phosphorylation of Akt, a signaling molecule with a central role in various signaling pathways a processes such as thus leading to apoptosis, in a dependent or modulated way by zinc concentrations.

2. MATERIALS AND METHODS

2.1. Chemicals

EGCG, TPEN [N,N,N′,N′-tetrakis(2-phridylmethyl) ethylenediamine], ZnCl2, Zinquin ethyl ester, ethanol, dimethyl sulfoxide (DMSO) and Clioquinol, were from Sigma (St. Louis, MO).

2.2. Fluorometric assay for chelation of zinc by EGCG

The zinc-dependent fluorescent emission of Zinquin ethyl ester (ZQEE) and Zinquin acid (ZQA) dissolved in phosphate buffered saline (PBS), pH 7.4, was recorded in a Biotek FLx 800 spectrofluorometer, with 100 µL samples, in COSTAR Corning 96 well, opaque bottom plates, at 37ºC, with excitation set at 360/40 and emission at 460/40 nm for ZQEE and ZQA. Quenching of zinc-dependent fluorescence of fluorophore-zinc complexes by EGCG, TPEN or Clioquinol, was monitored 15 min after addition of different amounts of the compounds to the solutions containing various amounts of fluorophores and zinc cations, as specified in the figures.

2.3. Cell cultures and treatments

Hepa 1-6 cells (BW7756 ECACC) were grown in Dulbecco´s Modified Eagle Medium (DMEM; BioWittaker) supplemented with 10% fetal bovine serum (BioWittaker), 2mM glutamine and 1% non-essential amino acids. This medium contains 3.9 ± 0.3 µM zinc

determined by flame atomic absorption spectrometry (FAAS). Cells were incubated at 37ºC in humidified, 5% CO2-enriched atmosphere and routinely splitted at a 1:5 ratio upon reaching 80% confluence. For treatments, cultures at 80% confluence were trypsinized and resuspended at a density of 5x105 cells/ml; 1 ml aliquots of suspended cells were seeded per well in 12-well plates (Orange Scientific). Twenty-four hours later, medium was replaced with fresh medium containing the different treatment substances or vehicle (final 0.05 % ethanol, 0.1% DMSO).

2.4. In vitro cytotoxicity assays

Cytotoxicity of EGCG and ZnCl2 was assessed in Hepa 1-6 cells treated 24 h with different concentration of the test substances using the lactate dehydrogenase (LDH) leakage assay of plasma membrane integrity as previously described (Puiggros, Llopiz et al. 2005) and the methyl tetrazolium (MTT) assay of mitochondrial functionality and cell viability as previously described (Stern 2006). 104 cells were seeded per well of 96-well plates in 100 μL media for MTT assays.

2.5. Quantification of MT and ZnT-1 gene expression

Total RNA was isolated from Hepa 1-6 cells using RNeasy Mini Kit (Qiagen). To quantify relative mRNA levels of specific genes in different RNA samples, cDNAs were generated from total RNA using TaqMan Reverse Transcription Reagents (Applied Biosystems) and quantitative reverse transcriptase-polymerase chain reactions (RT-PCRs) were performed using the TaqMan PCR Core Reagents Kit and Specific TaqMan Gene expression assay Probes (Applied Biosystems): MT-I (Mm00496660_g1), MT-II (Mm00809556_s1) and Znt1/Slc30a1 (Mm00437377_m1). Cyclophilin peptidylprolyl isomerase A (cyclophilin A, PPIA) (Mm03024003_g1) was used as endogenous control gene for sample input normalization in RT-PCR experiments. Quantitative PCR amplification and detection were performed using the Applied Biosystems Real Time 7000 PCR System termocycler and software.

2.6. Measurements of total intracellular zinc

To quantify total intracellular zinc, cells were thoroughly washed with PBS and lysed with 0.01 M NaOH. Aliquots of the cell lysates were used to quantify zinc by FAAS as previously described (Reaves, Fanzo et al. 2000), using an Hitachi Z-8200 Polarized

Zeeman AA Spectrophotometer. Total zinc amount was normalized per total protein content of the cells, determined by the Bradford method.

2.7. Measurements of intracellular labile zinc

To measure changes in intracellular labile zinc, cells were washed with PBS after treatments, incubated 30 minutes at 37ºC in 20 μM Zinquin ethyl ester in PBS; protein content and Zinquin fluorescence was determined in cell aliquots. Background fluorescence of Zinquin unloaded cells was subtracted from readings to derive Zinquin-dependent fluorescence as described (Coyle, Zalewski et al. 1994).

For microscopy visualization, cells were attached to glass coverslips, the medium was discarded after treatments and the cells were washed (three times) with PBS. Coverslips were immersed in a solution of Zinquin (20μM) in PBS and incubated for 30 min at 37 ºC. Coverslips were inverted on to microscope slides, and images were captured at 1000x magnification with a Leica DM 4000B microscope using UV light illumination (λex = 340-380 nm) and a blue emission filer (λem ≥ 425nm).

2.8. Changes in phosphorylation of Akt /PKB-1 were monitored by Western blotting

For Immunoblotting analysis, Hepa1-6 cell extracts were prepared through cell lysis in RIPA buffer (100 nM Tris-Cl pH 7,4, Tween 10%, Na-Deoxycholate 10%, SDS 0,1%). BioRad Bradford Assay was performed in order to quantify protein concentration, and 30 μg of protein were analyzed for each sample. Proteins were separated using 10% SDS-Polyacrylamide Gel Electrophoresis and afterwards transferred into an activated polyvinylidene difluoride membranes (PVDF, Amersham Hybond™-P roll). Membranes were blocked with 5% non-fat milk powder in TBS-Tween 0.05% for 2 hours. Primary antibodies used were: rabbit anti-Phospho-Akt (Ser473) antibody (Cell Signaling Technology, Product # 9271); rabbit anti-Akt antibody (Cell Signaling Technology, Product # 9272) and rabbit β-Actin antibody (Cell Signaling Technology, Product # 4967); β-Actin was used as internal sample control. Anti-rabbit IgG, HRP-linked Antibody was used as secondary antibody in all western analysis (Cell Signaling Technology, Product #7074). Primary antibodies were incubated overnight at 4ºC, while secondary antibody was incubated for 2 hours at room temperature. HRP on the membrane was revealed with a chemiluminescent reagent (Amersham ECL Prime Western Blotting Detection Reagent, product RPN2232), and images captured with SYNGENE G:BOX Chemi XL 1.4. Imaging

System. Relative levels of different proteins were obtained analysing the image bands by the Java-based software ImageJ.

2.9. Statistical analysis

For statistical analysis in cytotoxicity assays, zinc and protein quantification, quantitative RT-PCRs, and fluorescence measurements, t-test and one-way ANOVA analyses were performed using SPSS software. All data are the result of at least 3 independent experiments. Differences were considered significant for P values 0.05.

3. RESULTS

3.1. EGCG interacts with zinc cations in solution

In order to test if EGCG interact with zinc cations in solution, we first characterized the zinc-dependent fluorescence of membrane impermeable Zinquin Acid (ZQA) and of membrane permeable Zinquin Ethyl Ester (ZQEE), which are zinc-specific fluorescent chelators widely employed to measure concentrations of labile (free plus loosely bound) zinc within cells and in the biological fluids (Zalewski, Truong-Tran et al. 2006). The capability of the flavonoid to chelate zinc cations in solution was then measured as the quenching of zinc-dependent fluorescence of ZQEE and of ZQA previously complexed with zinc. Both zinc-specific fluorophores lose fluorescence when are not bound to zinc, indicating that the flavonoid has retrieved zinc cations from the fluorescent fluorophore-zinc complex. We found that EGCG quenched the zinc-dependent Zinquin fluorescence rapidly, with a comparable efficacy as the zinc-specific chelators Clioquinol and TPEN, at EGCG to Zinquin ratios of 0.5:20 in the presence of 1 μM ZnCl2 (Fig. 1). These results imply that EGCG bind zinc cations from Zinquin-zinc complexes.

3.2. EGCG prevents zinc toxicity in Hepa 1-6 cells

We next performed MTT viability assays in order to evaluate the effect of increasing amounts of zinc and EGCG on Hepa 1-6 cells. Zinc concentrations above 300 µM resulted in significant cell death, reaching 50% upon incubation of cells with 400 µM zinc (Fig. 2A). Likewise, 50% cell mortality after 24 h was caused by addition of 400 µM EGCG (Fig. 2B). Strikingly, addition of 400 µM EGCG and 400 µM Zn (1:1 ratio) resulted nearby a 100% viability of cells, implying that the noxious effect of both zinc and EGCG when in excess get

neutralized (Fig. 2C and D). This strongly suggests that chelation of zinc ions by EGCG renders a relevant amount of zinc cations in the culture medium not available for the cells.

3.3. EGCG reduces the expression of MT and the zinc transporter ZnT1 genes in Hepa 1-6 cells

Afterward, Hepa 1-6 cells were cultured and grown 24 hours in standard culture medium (3.9 ± 0.3 μM zinc) or in zinc overload medium (50 μM zinc) supplemented with either vehicle or 100 μM EGCG, and changes in gene expression of MT genes and the plasma membrane zinc exporter, ZnT1 were monitored at various times, using quantitative RT-PCR. The results show that both MT genes tested were down-regulated by 100 μM EGCG with respect to control cells (Fig. 3A and B). Addition of 50 μM zinc to the culture medium, resulted in a maximal induction of MTs with a 12 fold induction of MT-I 3 hours after addition of zinc and a 20 fold induction of MT-II 6 hours after addition zinc. Thereafter, we monitored changes in the expression profile of ZnT1 gene grown in standard culture medium (5 μM zinc) supplemented with 100 μM EGCG. The results show that ZnT1 was down-regulated by 100μM EGCG with respect to control cells 3 hours after addition of EGCG (Fig. 3C). In zinc overload conditions, ZnT1 expression was up-regulated reaching maximal values, 4 hours after addition of zinc. Addition of 100μM EGCG to the zinc overloaded medium down-regulated ZnT1 expression at the same time (Fig. 3C). The modification elicited by EGCG in the expression of MTs and ZnT1 in Hepa 1-6 cells suggest that zinc becomes less available for cells in the presence of EGCG, a consistent result with the previously described reduction of zinc toxicity induced by EGCG.

3.4. EGCG decreases total intracellular zinc

To address this point, we measured the total intracellular zinc content in Hepa 1-6 cells grown in standard (3.9 ± 0.3 μM zinc) and zinc overload medium (50 μM additional zinc) supplemented with either vehicle, or 100 μM EGCG, at the same times used in gene expression (Fig. 4). In basal zinc conditions, 100 μM EGCG elicited a significant decrement in total intracellular zinc 3 hours after treatment. When the cultured medium was supplemented with 50 μM zinc, cell accumulated more zinc than when no addition zinc was added to the medium. The addition of EGCG resulted, as expected, in an important reduction of total zinc intracellular accumulation at all time points compared with cells treated with only 50 μM zinc.

3.5. EGCG elevates cytoplasmic labile zinc in Hepa 1-6 cells

We next measured cytoplasmic labile zinc in standard (3.9 ± 0.3 µM zinc) and zinc overload (50 µM zinc) medium treated with either vehicle, 10 µM and 100 µM EGCG in Hepa 1-6 cells, incubated with 20µM Zinquin ethyl ester. Both doses of EGCG tested produced no effect in basal medium. When the medium was supplemented with 50µM zinc, intracellular labile zinc augmented markedly with respect to control cells, as expected. Addition of 10µM EGCG to this zinc-overload medium decreased cytoplasmic labile zinc at 3 and 6 hours of treatment. In contrast, 100µ M EGCG highly increased cytoplasmic labile zinc at 3, 6 and 12 hours of treatment (Fig. 5A). Microscopic observation of Zinquin-loaded Hepa 1-6 cells were done to visualize this effect of 100 µM EGCG on cytoplasmic labile zinc in Hepa 1-6 cells grown in basal zinc concentrations as well as in conditions of 50 µM added zinc (Fig. 5B). In this experiment, we also used Clioquinol as a reference drug, which a known anticancer agent with zinc-ionophoric effect. EGCG behaves in a similar way to Clioquinol in the sense that it produced an increase in the cytoplasmic pool of labile zinc in Hepa 1-6 cells only when it was applied to the cells together with additional zinc.

3.6. EGCG modulates phosphorylation of Akt in a zinc-dependent way

Several reports have shown that Akt phosphorylation may be modulated by labile zinc in response to zinc chelators such as TPEN or to zinc ionophores such as Pyrithione. On the other hand, Akt has shown to be targeted by several polyphenols in different cell lines. Hence, it is expected that combination of zinc with EGCG will affect Akt phosphorylation, and signaling, in a way that is different from that is different of that caused by EGCG alone or by zinc alone. To test this, we treated Hepa 1-6 with the same amounts of EGCG and zinc used to test the combined effect of these substances on Zinquin-detectable labile zinc, and analyzed the ratio of phosphorylated Akt versus total Akt by Western analysis (Fig. 6). The results show that EGCG alone at low concentration, is not able to modulate Akt phosphorylation; zinc alone at 50 μM, enhances Akt phosphorylation by 50% upon 6 hours of treatment. When 10 μM EGCG is combined with 50 μM Zn, Akt phosphorylation is enhanced up to 200 % of control levels. In contrast, high (100 μM) EGCG concentrations inhibit Akt phosphorylation, and addition of 50 μM zinc is not able to reverse this effect. Consequently, the amount of EGCG and the ratio EGCG/zinc dictates different phosphorylation states of Akt. When compared with the amount of Zinquin-detectable labile zinc that results from these same treatments it look like Akt phosphorylation correlates

inversely with the level of labile zinc. Phosphorylation of Akt also correlates directly with the amount of total intracellular zinc. The meaning of these correlation remains to be elucidated.

4. DISCUSSION

We evaluated the effect of EGCG on zinc homeostasis in an *in vitro* cell line that serves as a model for hepatic carcinoma cells. We have previously demonstrated that a grape seed procyanidin extract (GSPE), a mixture of catechins and proanthocyanidins display an affinity for zinc cations in solution high enough to make them dissociate from the zinc-specific chelators Zinquin (Quesada, Bustos et al. 2011). In this study we tested another type of flavonoid, EGCG and we compared this molecule with the well-known zinc chelator TPEN. We have shown here that EGCG also displaced zinc ions from zinc-Zinquin complex with similar affinity as TPEN at very low concentrations and at molar ratios of 0.5 μM EGCG to 20 μM Zinquin and 1 μM zinc. Sreenivasulu et al. also showed that Quercetin and tannic acid quenched zinc-induced Zinquin fluorescence in a test tube in a dose dependent manner (Sreenivasulu, Raghu et al. 2010). It was also reported that the stilbene resveratrol, chelates zinc in vitro (Zhang, Wu et al. 2009). Our result implies that EGCG interacts with zinc cations in solution and strongly suggest that it could be able to modulate the metabolically active labile zinc. If EGCG indeed interacts with zinc cations forming EGCG-zinc complexes, it also would be able to reduce zinc toxicity in Hepa 1-6 cells. To test this, we assessed the viability of Hepa 1-6 cells co-incubated with a toxic dose of zinc and different doses of EGCG, determined by the MTT assay for mitochondrial viability. We have shown that EGCG exerted a reversion of excess zinc toxicity (400 μM zinc) in EGCG to zinc ratios (1:1). Our findings are in agreement with Sun et al. who have shown that EGCG protected PC-3 cells from damages induced by zinc ions (Sun, He et al. 2008).

In order to asses whether the ability of EGCG to complex with zinc may affect cellular zinc homeostasis, we monitored changes in the expression profile of genes involved in storage and export of zinc (MT and ZnT1 respectively) and total intracellular zinc accumulation in Hepa 1-6 cells grown in standard culture medium (3.9 ± 0.3 μM zinc) and in zinc-loaded medium (50 μM zinc). The results show that repression of these genes elicited by EGCG was more effective when the medium was supplemented with excess zinc decreasing the

expression of MT and the zinc transporter ZnT1 genes with a concomitant decrease in zinc accumulation in Hepa 1-6 cells. This results contrast with others reported for other flavonoid, Quercetin. In this regard, Weng et al. reported that the flavonoid Quercetin increase MTs expression in HepG2 cells, promoting a liver protection against oxidative stress thus activating MAPK and PI3K pathways and also increasing DNA binding activity of Nrf2 (Weng, Chen et al. 2011). It was also reported that zinc-induced MT gene expression was up-regulated by Quercetin in CaCo-2 cells (Sreenivasulu, Raghu et al. 2010). The isoflavone genistin was shown to increase MT and ZnT1 expression in HepG2 cells (Chung, Kang et al. 2006). However, the effect of these flavonoids on zinc accumulation was not reported. The changes elicited by EGCG in the expression of MT and ZnT1 genes in different conditions are consistent with a decreased availability of zinc, and reversion of toxicity of high amounts of zinc. To address this point we measured total intracellular zinc. We found that total zinc content was decreased with 100 μM EGCG in a standard and in a zinc-loaded medium. In this regard, Sun et al. inferred about the importance of the structure and stoichiometry of EGCG-zinc complexes in the bioavailability of the flavonoid, demonstrating that EGCG in the presence of zinc was more effective than EGCG alone in enhancing the permeability of the cell membrane, whereas zinc-EGCG complex had no effect on PC-3 cell membrane permeability (Sun, He et al. 2008).

We next measured the labile pool of zinc in Hepa 1-6 cells grown in standard and in zinc-loaded medium supplemented with two doses of EGCG (10 and 100µM). We found no effects of any doses of EGCG in basal medium. However, in zinc-loaded medium, we found that co-treatments with EGCG and zinc in a 1:5 (EGCG:zinc) ratio, Zinquin-detectable labile zinc diminished. In contrast, co-treatments in a 2:1 (EGCG:zinc) ratio markedly increased this pool of labile zinc. This result reflects the importance of the flavonoid-zinc ratios in the outcomes of biological effects of EGCG.

Other zinc chelators have been reported to increase the labile pool of zinc. Clioquinol, a drug with anti-amyloid and anti-tumour effects, induces autophagy in cultured astrocytes and neurons in a zinc-dependent manner, acting as a zinc ionophore agent, increasing labile zinc in the cytosol and in autophagic vacuoles (Park MH 2011). The glycone isoflavone genistin enhances the proapoptotic effects of zinc in HepG2 cells and up-

regulate the expression of MT and ZnT1 concomitantly increasing the labile zinc pool detectable by FluoZin-3. Resveratrol, at physiological concentrations (10 μM), when applied to normal human prostate epithelial cells cultured in 16 or 32 μM zinc, arrests cell growth and enhances Zinquin-detectable zinc, while not affecting total zinc nor MT expression.

It may be postulated that, depending on flavonoid to zinc ratio, absolute concentrations, the chemical structure of the flavonoid, temperature, pH, and the presence of other competing metals, different types of flavonoid-zinc complexes will be formed. In our laboratory conditions, EGCG would form membrane-permeable complexes with zinc that will then act as ionophores and therefore transport the metal into the cell. Once within the cell, EGCG might be metabolized and zinc cations added to the labile pool of zinc. It could also be possible that zinc cations bound to EGCG may be considered as loosely bound zinc without dissociating from EGCG. Zinquin does not only detect free zinc, but also some of the zinc atoms that are bound to metallothionein, and also to other proteins (Coyle, Zalewski et al. 1994). To support this hypothesis, it was reported that Clioquinol acts as a zinc ionophore, enhancing the labile pool of zinc as mentioned above (Park MH 2011). In addition, resveratrol not only interact with zinc cations in solution, but also elevated the labile pool of zinc in normal human prostate epithelial cells. The authors inferred that the elevation of the labile zinc elicited by resveratrol is due to the cellular uptake of resveratrol-zinc complexes, followed by the intracellular dissociation of the complexes (Zhang, Wu et al. 2009).

The labile pool of zinc can reversibly bind to regulatory sites in signaling proteins. Consequently, changes of the labile concentration can affect cell-signaling pathways and might even act as zinc signals. To test this possibility, we have here tested whether the changes elicited by EGCG-zinc complexes correlate with changes in phosphorylation state of Akt, reported to be regulated by labile zinc on the one side, and by different polyphenols on other reports. We have here shown that EGCG modulates Akt phosphorylation in a zinc-dependent way. Whereas high (100/50) EGCG/zinc ratios inhibit Akt phosphorylation, whereas low (10/50) EGCG/zinc ratios enhance Akt phosphorylation.

To sum up, EGCG is added to polyphenols with zinc-chelating properties and, as a result, affects zinc homeostasis. We forward the hypothesis that elevation of labile zinc by EGCG may be a relevant mechanism by which EGCG affects multiple metabolic and cell signaling pathways that respond to intracellular fluctuations of labile zinc. Further research is needed to assess the mechanisms by which flavonoids enhance cytoplasmic labile zinc and the consequences of this enhancement on modulation of zinc signaling and metabolic pathways.

REFERENCES

[1] Ahn, J., H. Lee, et al. (2008). "The anti-obesity effect of Quercetin is mediated by the AMPK and MAPK signaling pathways." Biochemical and Biophysical Research Communications 373(4): 545-549.

[2] Andrews, G. K. (2001). "Cellular zinc sensors: MTF-1 regulation of gene expression." Biometals 14(3-4): 223-37.

[3] Beyersmann, D. and H. Haase (2001). "Functions of zinc in signaling, proliferation and differentiation of mammalian cells." Biometals 14(3-4): 331-41.

[4] Bucki, R., J. J. Pastore, et al. (2003). "Flavonoid inhibition of platelet procoagulant activity and phosphoinositide synthesis." J Thromb Haemost 1(8): 1820-8.

[5] Chung, M. J., A. Y. Kang, et al. (2006). "Water-soluble genistin glycoside isoflavones up-regulate antioxidant metallothionein expression and scavenge free radicals." J Agric Food Chem 54(11): 3819-26.

[6] Coyle, P., P. D. Zalewski, et al. (1994). "Measurement of zinc in hepatocytes by using a fluorescent probe, Zinquin: relationship to metallothionein and intracellular zinc." Biochem J 303 (Pt 3): 781-6.

[7] Cuajungco, M. P. and G. J. Lees (1997). "Zinc metabolism in the brain: relevance to human neurodegenerative disorders." Neurobiol Dis 4(3-4): 137-69.

[8] Cushnie TP, L. A. (2005). " Antimicrobial activity of flavonoids." Int J Antimicrob Agents. 26(5): 343-56.

[9] Devergnas, S., F. Chimienti, et al. (2004). "Differential regulation of zinc efflux transporters ZnT-1, ZnT-5 and ZnT-7 gene expression by zinc levels: a real-time RT-PCR study." Biochem Pharmacol 68(4): 699-709.

[10] Duarte J, P.-P. R., Vargas F, Ocete MA, Pérez-Vizcaino F, Zarzuelo A, Tamargo J. (2001). "Antihypertensive effects of the flavonoid Quercetin in spontaneously hypertensive rats." Br J Pharmacol. 133(1): 117-24.

[11] Erlund, I., R. Freese, et al. (2006). "Bioavailability of Quercetin from berries and the diet." Nutr Cancer 54(1): 13-7.

[12] Frassinetti, S., G. Bronzetti, et al. (2006). "The role of zinc in life: a review." J Environ Pathol Toxicol Oncol 25(3): 597-610.

[13] Fuchs, O., M. Babusiak, et al. (2003). "Role of zinc in eukaryotic cells, zinc transporters and zinc-containing proteins. Review article." Sb Lek 104(2): 157-70.

[14] Gulati N, L. B., Zohrabian VM, Murali R, Jhanwar-Uniyal M. (2006). "The antiproliferative effect of Quercetin in cancer cells is mediated via inhibition of the PI3K-Akt/PKB pathway." Anticancer Res. 26(2A): 1177-81.

[15] Hider, R. C., Z. D. Liu, et al. (2001). "Metal chelation of polyphenols." Methods Enzymol 335: 190-203.

[16] Hirano, T., M. Murakami, et al. (2008). "Roles of zinc and zinc signaling in immunity: zinc as an intracellular signaling molecule." Adv Immunol 97: 149-76.

[17] Kostyuk, V. A., A. I. Potapovich, et al. (2007). "Metal complexes of dietary flavonoids: evaluation of radical scavenger properties and protective activity against oxidative stress in vivo." Cell Mol Biol (Noisy-le-grand) 53(1): 62-9.

[18] Kostyuk, V. A., A. I. Potapovich, et al. (2001). "Influence of metal ions on flavonoid protection against asbestos-induced cell injury." Arch Biochem Biophys 385(1): 29-37.

[19] Krezel, A., Q. Hao, et al. (2007). "The zinc/thiolate redox biochemistry of metallothionein and the control of zinc ion fluctuations in cell signaling." Arch Biochem Biophys 463(2): 188-200.

[20] Krezel, A. and W. Maret (2008). "Thionein/metallothionein control Zn(II) availability and the activity of enzymes." J Biol Inorg Chem 13(3): 401-9.

[21] Kuo, S. M., C. T. Huang, et al. (2001). "Quercetin cumulatively enhances copper induction of metallothionein in intestinal cells." Biol Trace Elem Res 84(1-3): 1-10.

[22] Laity, J. H. and G. K. Andrews (2007). "Understanding the mechanisms of zinc-sensing by metal-response element binding transcription factor-1 (MTF-1)." Arch Biochem Biophys.

[23] LaRochelle, O., V. Gagne, et al. (2001). "Phosphorylation is involved in the activation of metal-regulatory transcription factor 1 in response to metal ions." J Biol Chem 276(45): 41879-88.

[24] Lichten, L. A. and R. J. Cousins (2009). "Mammalian zinc transporters: nutritional and physiologic regulation." Annu Rev Nutr 29: 153-76.

[25] Lichtlen, P. and W. Schaffner (2001). "The "metal transcription factor" MTF-1: biological facts and medical implications." Swiss Med Wkly 131(45-46): 647-52.

[26] Liuzzi, J. P. and R. J. Cousins (2004). "Mammalian zinc transporters." Annu Rev Nutr 24: 151-72.

[27] Manach, C., A. Scalbert, et al. (2004). "Polyphenols: food sources and bioavailability." Am J Clin Nutr 79(5): 727-47.

[28] Maret, W. (2009). "Molecular aspects of human cellular zinc homeostasis: redox control of zinc potentials and zinc signals." Biometals 22(1): 149-57.

[29] Murakami, M. and T. Hirano (2008). "Intracellular zinc homeostasis and zinc signaling." Cancer Sci 99(8): 1515-22.

[30] Orsolic, N., A. H. Knezevic, et al. (2004). "Immunomodulatory and antimetastatic action of propolis and related polyphenolic compounds." J Ethnopharmacol 94(2-3): 307-15.

[31] Park HJ, Y. J., Ambati S, Della-Fera MA, Hausman DB, Rayalam S, Baile CA. (2008). "Combined effects of genistein, Quercetin, and resveratrol in human and 3T3-L1 adipocytes." Journal of Medicinal Food 11(4).

[32] Park MH, L. S., Byun HR, Kim Y, Oh YJ, Koh JY, Hwang JJ. (2011). "Clioquinol induces autophagy in cultured astrocytes and neurons by acting as a zinc ionophore." Neurobiology of disease 42(3): 242.

[33] Pekal A, B. M., Pyrzynska K. (2011). "Interaction of Quercetin with copper ions: complexation, oxidation and reactivity towards radicals." BioMetals 24(1): 41-9.

[34] Peluso, M. R. (2006). "Flavonoids attenuate cardiovascular disease, inhibit phosphodiesterase, and modulate lipid homeostasis in adipose tissue and liver." Exp Biol Med (Maywood) 231(8): 1287-99.

[35] Perez-Vizcaino, F., J. Duarte, et al. (2006). "Endothelial function and cardiovascular disease: effects of Quercetin and wine polyphenols." Free Radic Res 40(10): 1054-65.

[36] Phan, T. T., I. J. Lim, et al. (2003). "Quercetin inhibits fibronectin production by keloid-derived fibroblasts. Implication for the treatment of excessive scars." Journal of Dermatological Science 33(3): 192-194.

[37] Puiggros, F., N. Llopiz, et al. (2005). "Grape seed procyanidins prevent oxidative injury by modulating the expression of antioxidant enzyme systems." J Agric Food Chem 53(15): 6080-6.

[38] Quesada, I. M., M. Bustos, et al. (2011). "Dietary catechins and procyanidins modulate zinc homeostasis in human HepG2 cells." The Journal of Nutritional Biochemistry 22(2): 153-163.

[39] Reaves, S. K., J. C. Fanzo, et al. (2000). "Expression of the p53 tumor suppressor gene is up-regulated by depletion of intracellular zinc in HepG2 cells." J Nutr 130(7): 1688-94.

[40] Ren, J., S. Meng, et al. (2008). "Complexation of Flavonoids with Iron: Structure and Optical Signatures." The Journal of Physical Chemistry B

[41] J. Phys. Chem. B 112(6): 1845-1850.

[42] Rice-Evans, C. A., N. J. Miller, et al. (1996). "Structure-antioxidant activity relationships of flavonoids and phenolic acids." Free Radic Biol Med 20(7): 933-56.

[43] Rudolf, E., K. Rudolf, et al. (2003). "The role of intracellular zinc in modulation of life and death of Hep-2 cells." Biometals 16(2): 295-309.

[44] Saydam, N., T. K. Adams, et al. (2002). "Regulation of metallothionein transcription by the metal-responsive transcription factor MTF-1: identification of signal transduction cascades that control metal-inducible transcription." J Biol Chem 277(23): 20438-45.

[45] Scalbert, A., I. T. Johnson, et al. (2005). "Polyphenols: antioxidants and beyond." Am J Clin Nutr 81(1 Suppl): 215S-217S.

[46] Sreenivasulu, K., P. Raghu, et al. (2010). "Polyphenol-Rich Beverages Enhance Zinc Uptake and Metallothionein Expression in Caco-2 Cells." Journal of Food Science 75(4): H123-H128.

[47] Sun, S. L., G. Q. He, et al. (2008). "Free Zn(2+) enhances inhibitory effects of EGCG on the growth of PC-3 cells." Mol Nutr Food Res 52(4): 465-71.

[48] Weng, C.-J., M.-J. Chen, et al. (2011). "Hepatoprotection of Quercetin against oxidative stress by induction of metallothionein expression through activating MAPK and PI3K pathways and enhancing Nrf2 DNA-binding activity." New Biotechnology

[49] Special issues on Biocatalysis and Agricultural Biotechnology: number 7-9 28(6): 767-777.

[50] Williams, R. J., J. P. Spencer, et al. (2004). "Flavonoids: antioxidants or signaling molecules?" Free Radic Biol Med 36(7): 838-49.

[51] Zalewski, P., A. Truong-Tran, et al. (2006). "Use of a zinc fluorophore to measure labile pools of zinc in body fluids and cell-conditioned media." Biotechniques 40(4): 509-20.

[52] Zalewski, P. D., I. J. Forbes, et al. (1993). "Correlation of apoptosis with change in intracellular labile Zn(II) using Zinquin [(2-methyl-8-p-toluenesulphonamido-6-quinolyloxy)acetic acid], a new specific fluorescent probe for Zn(II)." Biochem J 296 (Pt 2): 403-8.

[53] Zhang, J. J., M. Wu, et al. (2009). "Effect of resveratrol and zinc on intracellular zinc status in normal human prostate epithelial cells." Am J Physiol Cell Physiol 297(3): C632-44.

Figures

Chelation of zinc cations by green tea Epigallocatechin-gallate affects zinc homeostasis and Akt signaling in liver carcinoma Hepa 1-6 cells

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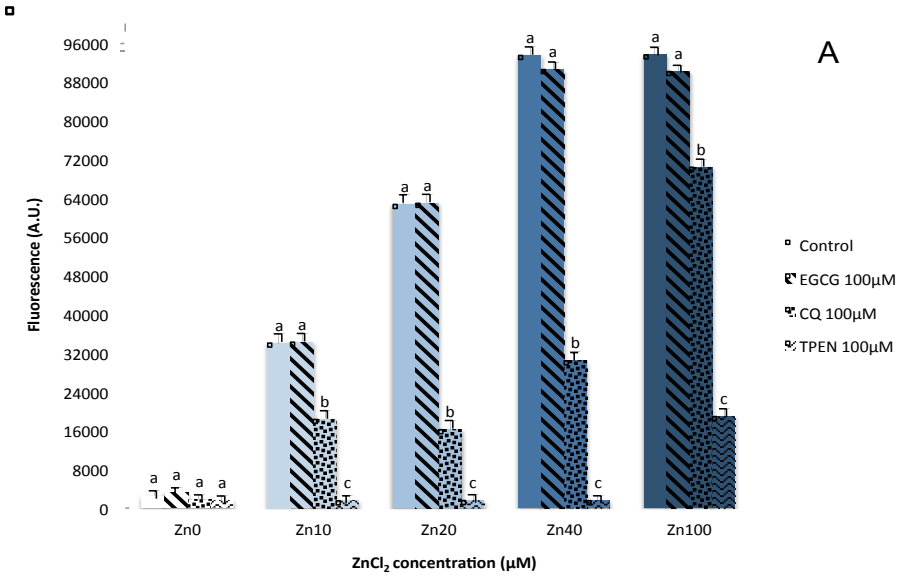
Figure 1. EGCG chelates zinc cations in solution.

Decrease of zinc-dependent fluorescence of 10 μM ZQ Acid (A) and 10 μM ZQEE (B) by 100 μM EGCG, TPEN or CQ at increasing concentrations of zinc cations.



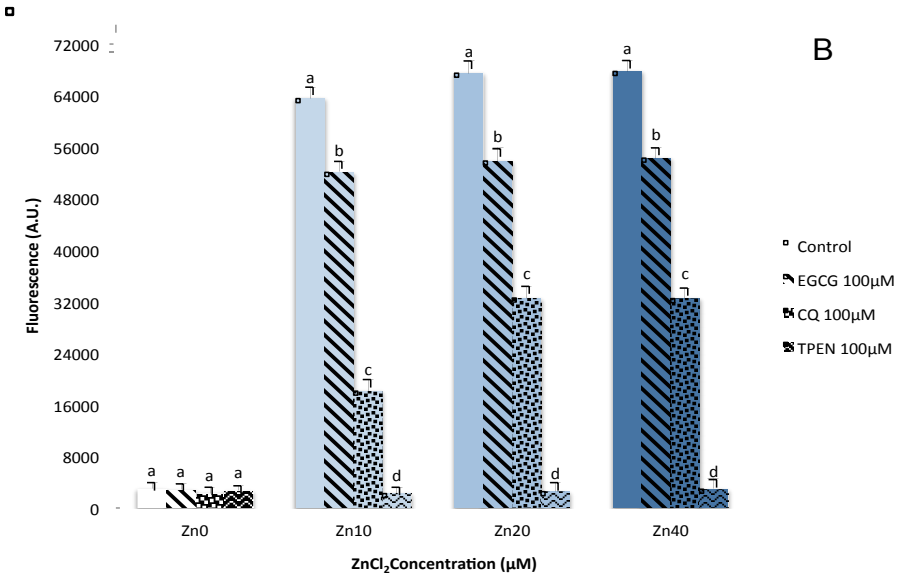
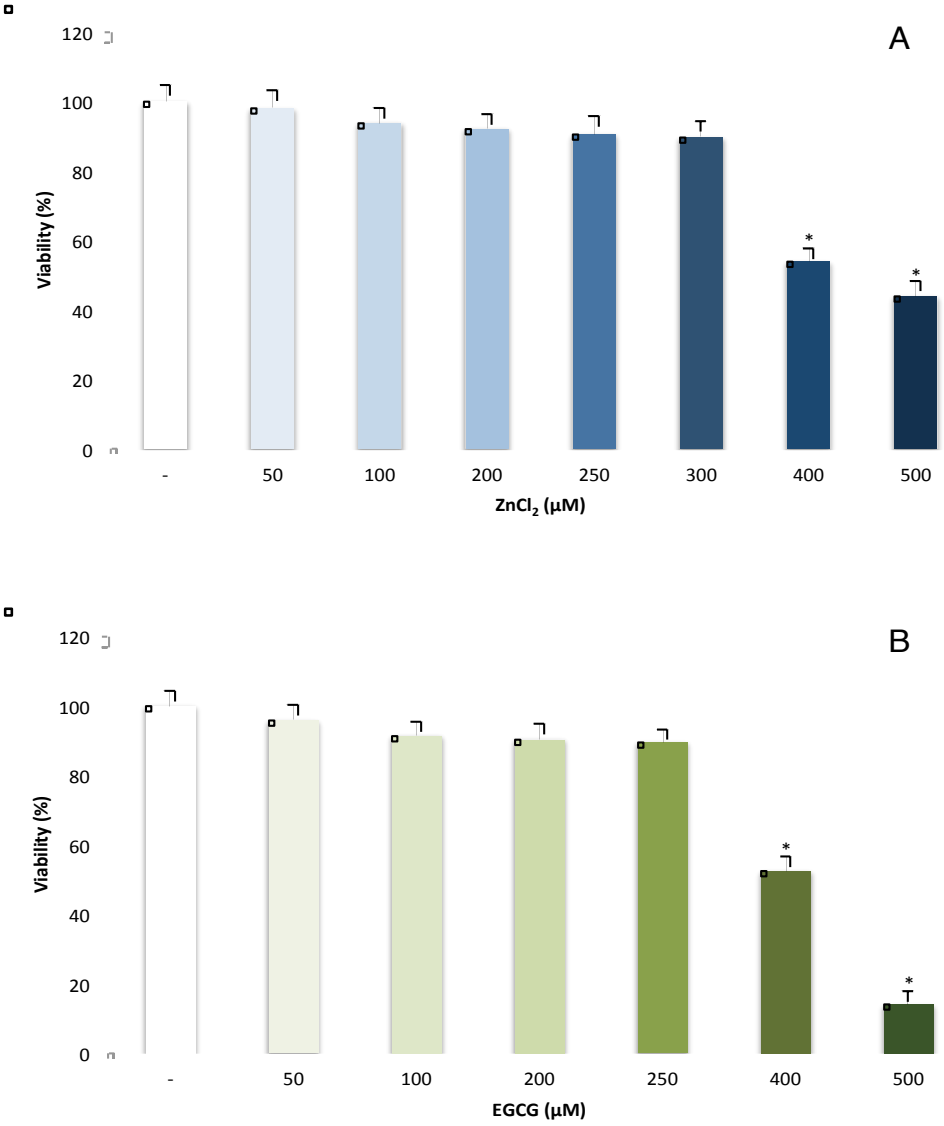
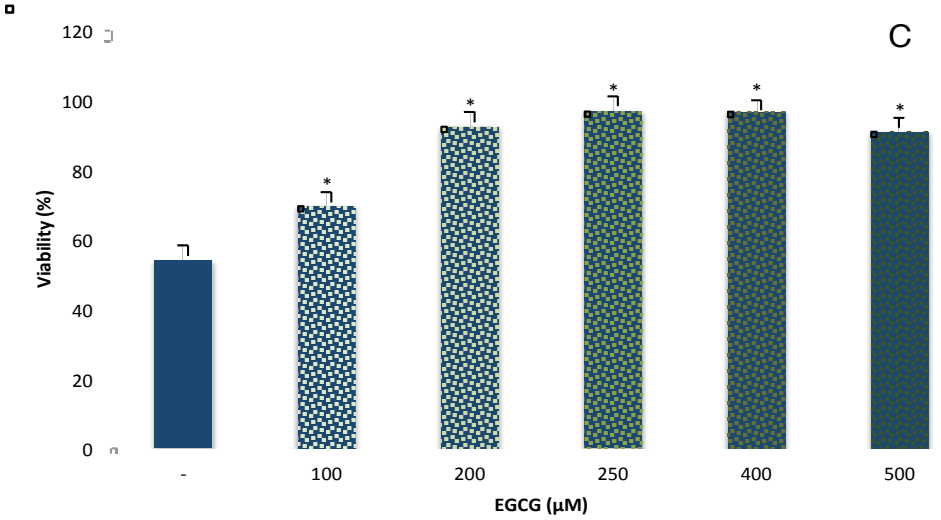


Figure 2. EGCG protects cells from zinc-induced cell death.

Cell viability was measured by the MTT assay in cultures added increasing concentrations of zinc cations (A) or increasing concentrations of EGCG (B), and after combining 400 μM zinc cations with increasing amounts of EGCG (C) or vice versa, 400 μM EGCG with increasing amounts of zinc cations (D). For statistical analysis t-test and one-way ANOVA were performed using SPSS software. All data are the result of at least 3 independent experiments. Differences were considered significant for P values 0.05.




C
120
100
80
60
40
20
0
Viability (%)
*
-
100
200
250
400
500
EGCG (µM)

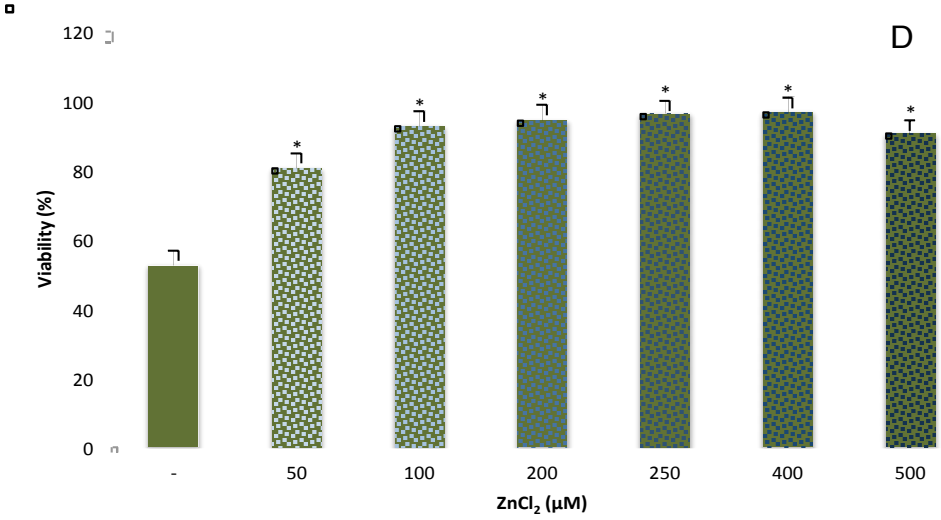

D
120
100
80
60
40
20
0
Viability (%)
*
-
50
100
200
250
400
500
ZnCl2 (µM)

Figure 3. EGCG reduces the expression of Mt1, Mt2 and ZnT.

EGCG enhances the expression of MTI, MTII and ZnT1mRNA levels relative to control (untreated) cells. Internal standard gene for RT-PCR reactions was PPIA.

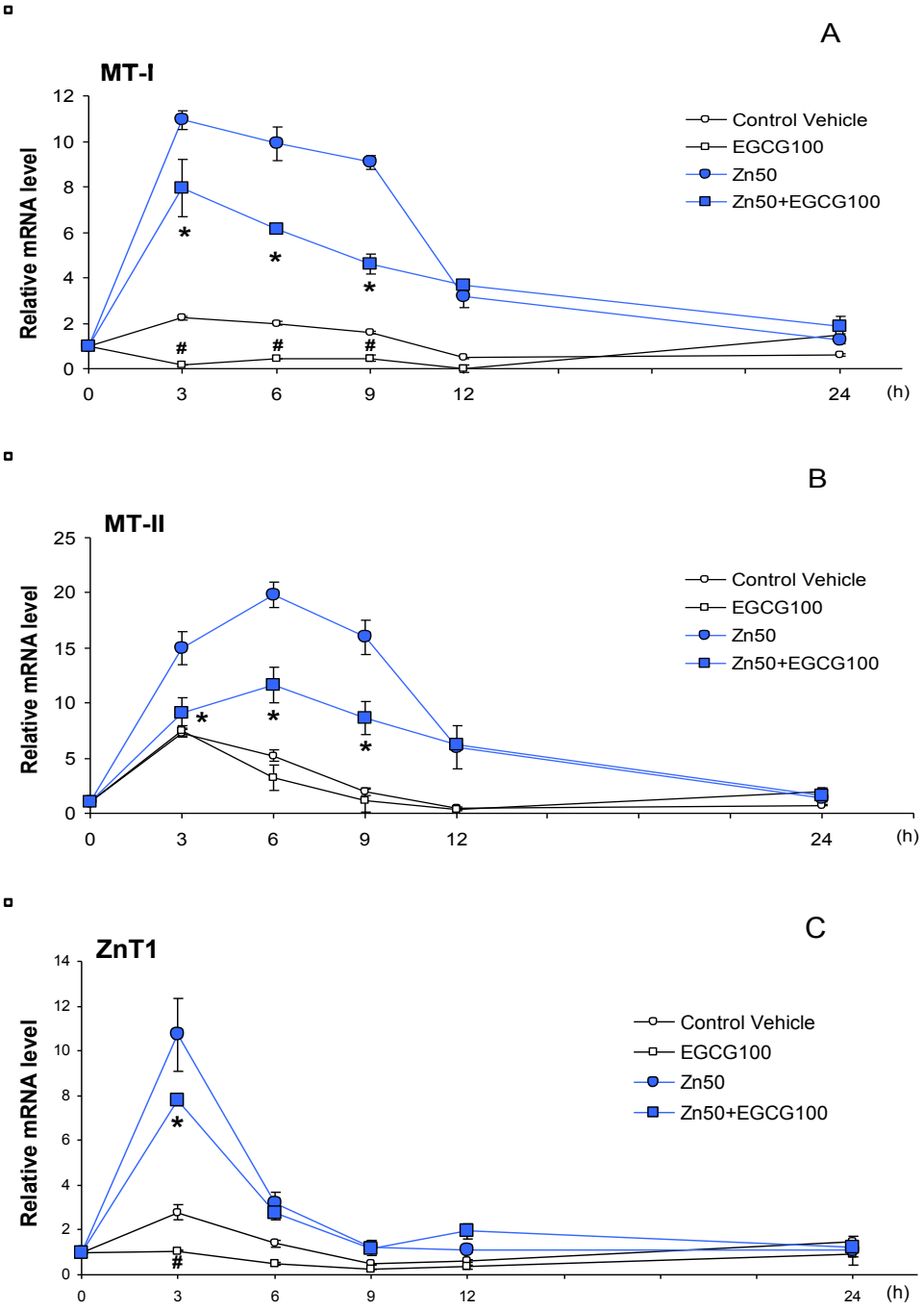


Figure 4. EGCG decreases total intracellular zinc levels.

Cellular zinc was quantified by FAA (Flame Atomic Absorption) and normalized to the protein amount of each cell sample. Results are referred to the content of zinc in cells at the beginning of the treatment.

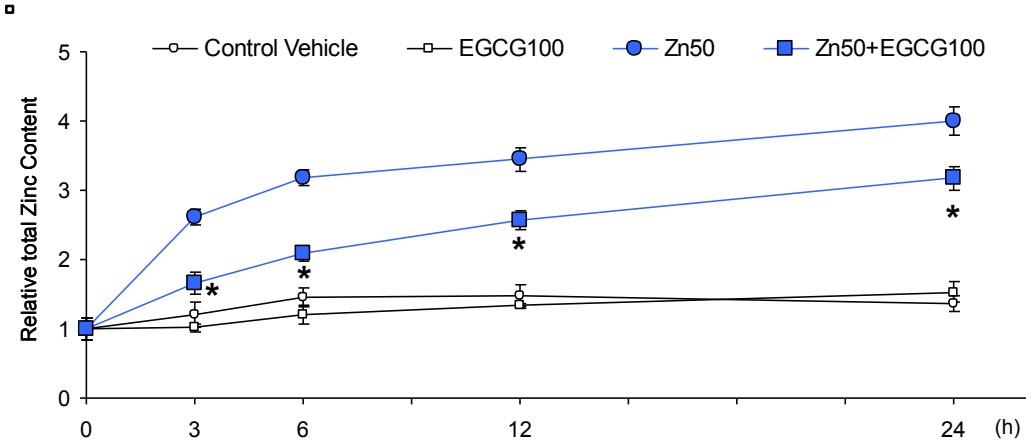
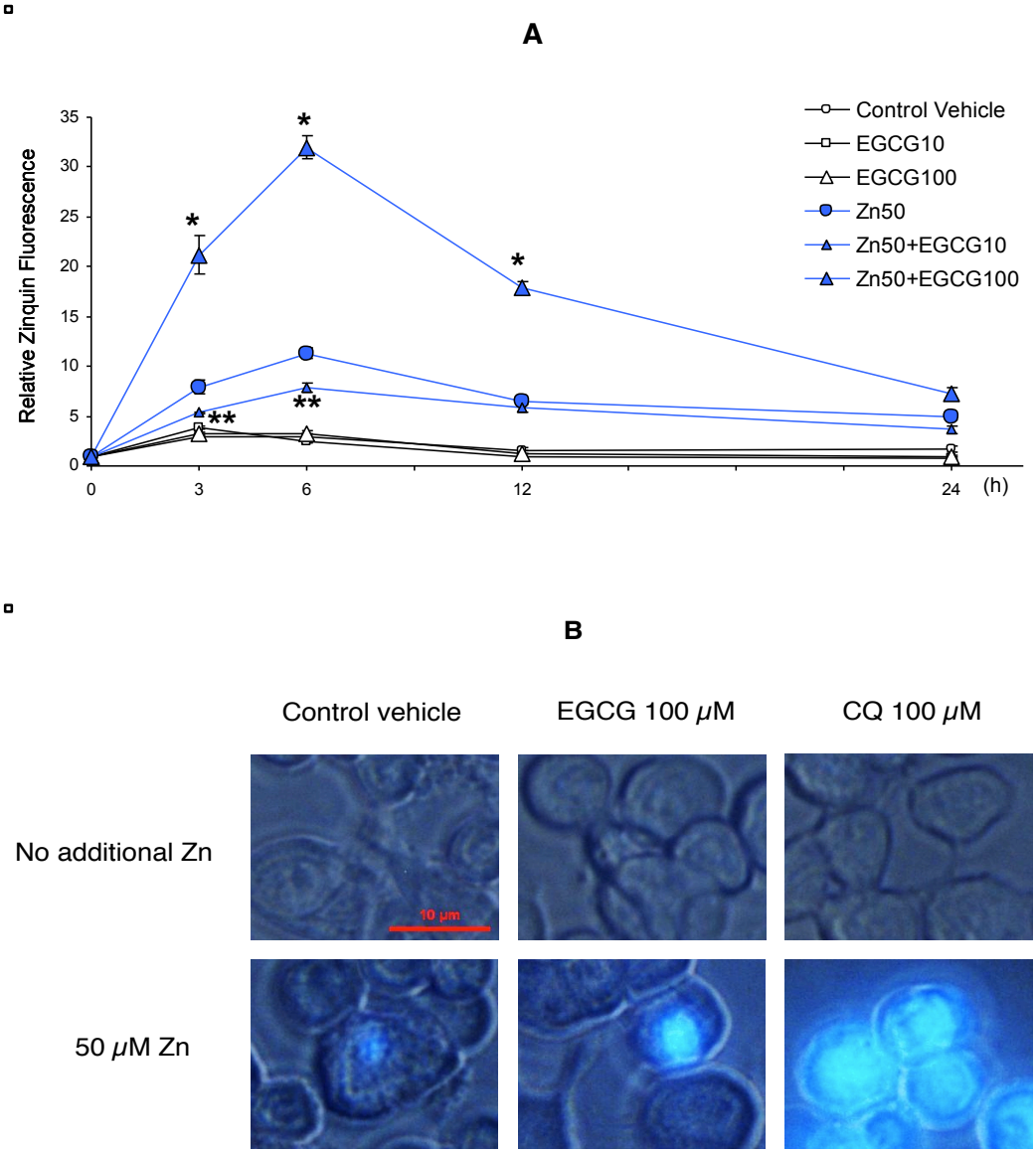


Figure 5. EGCG modulates cytoplasmic labile zinc levels detectable by Zinquin. Kinetics of variation in ZQ-detectable cytoplasmic zinc in Hepa 1-6 upon treatments with vehicle, 50 μM ZnCl2, 10 and 100 μM EGCG alone or combined with 50 μM Zn (A). Micrographs of Hepa 1-6 loaded with 25 μM ZQEE after 4 h treatments with vehicle, 50 μM ZnCl2, 100 μM EGCG or 100 μM Clioquinol, or pairwise combination of Zinc with the chelators (B). Quantification of labile zinc measured as Zinquin fluorescence in the samples of panel B (C). Scale bar indicates 10 μm.



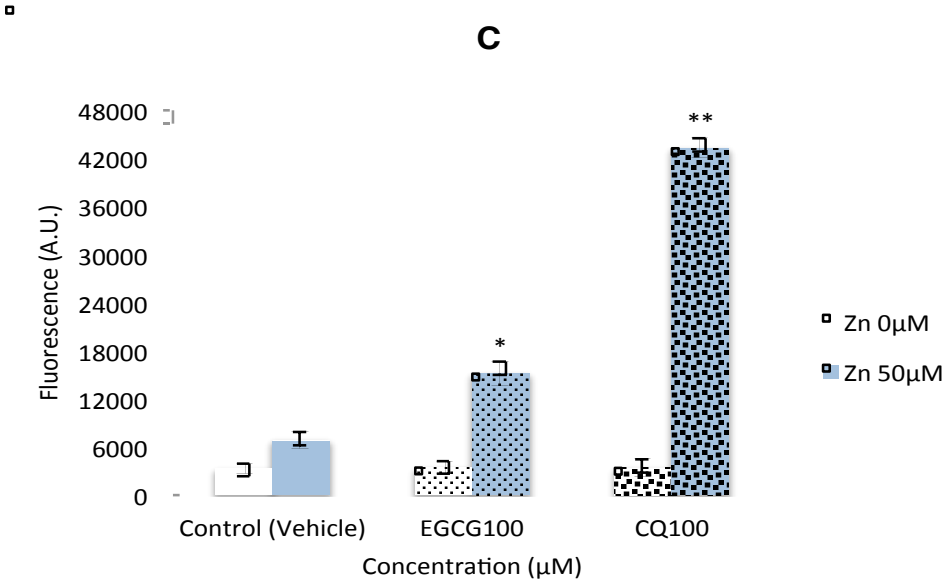
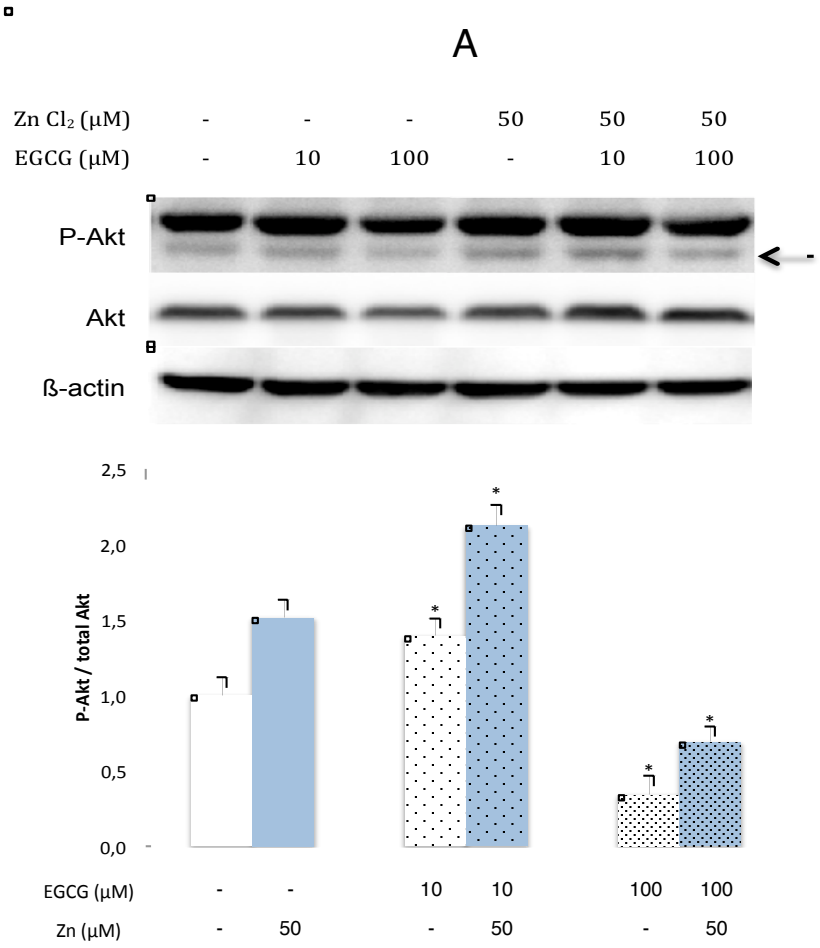
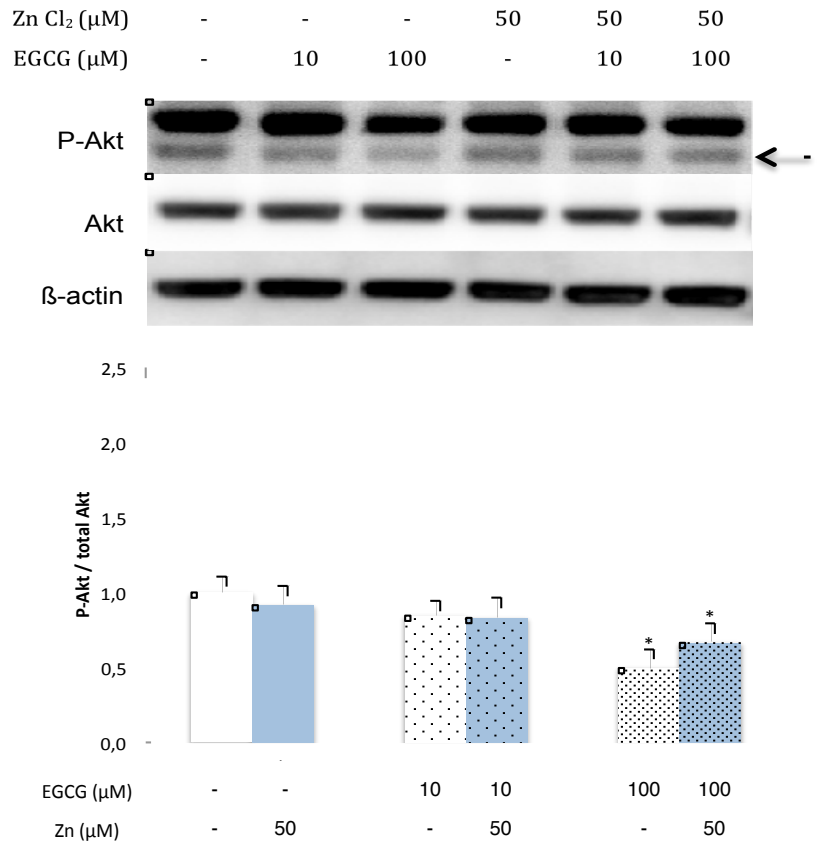


Figure 6. EGCG modulates Akt phosphorylation in a zinc-dependent manner.

Hepa 1-6 cells were treated with vehicle, 10 μM EGCG, 100 μM EGCG, alone and combined with 10 or 100 μM $ZnCl_2$, for 6 or 24 hours. Immunoblots of cell extracts were first probed with anti-247SerPAkt, and, after stripping off the antibodies, with anti-Akt and anti-β-actin antibodies. Results at 6 hours treatment (A) and 24 hours treatment (B) show that high EGCG/zinc ratios inhibit Akt phosphorylation, whilst low EGCG/zinc ration enhance Akt phosphorylation. Arrows indicates 60 kDa bands of P-Akt.



B


Zn Cl2 (µM) - - - 50 50 50
EGCG (µM) - 10 100 - 10 100
P-Akt
Akt
ß-actin
P-Akt / total Akt
2,5
2,0
1,5
1,0
0,5
0,0
EGCG (µM) - - 10 10 100 100
Zn (µM) - 50 - 50 - 50

3. Zinc ionophore activity of Quercetin and Epigallocatechin-gallate: From Hepa 1-6 cells to a Liposome Model

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Published in Journal of Agricultural and Food Chemistry

Zinc is an essential micronutrient with structural and catalytic functions in all cells. Labile zinc, a tiny fraction of total intracellular zinc that is loosely bound to proteins, modulates the activity of numerous signaling and metabolic pathways. Dietary consumption of plant polyphenols such as Quercetin (QCT) and epigallocatechin gallate (EGCG) is beneficial for prevention and cure of obesity, hypertension, diabetes, and cancer. Remarkably, the activity of numerous enzymes targeted by polyphenols is dependent on zinc levels.

We have previously reported that EGCG chelates zinc and that, in human hepatocarcinoma cells, decreases the expression of the zinc-storage protein metallothionein concomitantly decreasing total intracellular zinc whilst increasing Zinquin-detectable labile zinc. We hypothesized that EGCG and other polyphenols might be acting as zinc ionophores. To prove this hypothesis, in this work we first quantified the capacity of QCT and EGCG to chelate zinc cations by measuring their capacity to quench the fluorescence of FluoZin-3-Zinc complexes. Both polyphenols showed a chelating strength similar to that of Clioquinol (CQ), a zinc-ionophore drug with antitumor activity. Secondly, we confirmed that treatments of mouse hepatoma (Hepa 1-6) cells with QCT, EGCG or CQ, results in a rapid and dose-dependent rise of FluoZin-3 AM-detectable labile zinc when the chelators are combined with zinc cations.

Finally, in order to confirm that polyphenols are able to transport zinc across the plasma membrane independently of zinc transporters, we added QCT, EGCG or CQ, alone and combined with zinc, to unilamellar DPPC/cholesterol liposomes loaded with cell-impermeant FluoZin-3. Only the combinations of the chelators with zinc triggered a rapid increase of FluoZin-3 fluorescence, thus clearly demonstrating the ionophoric action of QCT, EGCG and CQ. This report establishes for the first time that dietary polyphenols display ionophore activity. This activity may underlay the raising of labile zinc levels triggered in cells by polyphenols and, thus, many of their biological actions.

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Zinc Ionophore Activity of Quercetin and Epigallocatechin-gallate: From Hepa 1-6 Cells to a Liposome Model

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ABSTRACT: Labile zinc, a tiny fraction of total intracellular zinc that is loosely bound to proteins and easily interchangeable, modulates the activity of numerous signaling and metabolic pathways. Dietary plant polyphenols such as the flavonoids quercetin (QCT) and epigallocatechin-gallate act as antioxidants and as signaling molecules. Remarkably, the activities of numerous enzymes that are targeted by polyphenols are dependent on zinc. We have previously shown that these polyphenols chelate zinc cations and hypothesized that these flavonoids might be also acting as zinc ionophores, transporting zinc cations through the plasma membrane. To prove this hypothesis, herein, we have demonstrated the capacity of QCT and epigallocatechin-gallate to rapidly increase labile zinc in mouse hepatocarcinoma Hepa 1-6 cells as well as, for the first time, in liposomes. In order to confirm that the polyphenols transport zinc cations across the plasma membrane independently of plasma membrane zinc transporters, QCT, epigallocatechin-gallate, or clioquinol (CQ), alone and combined with zinc, were added to unilamellar dipalmitoylphosphocholine/cholesterol liposomes loaded with membrane-impermeant FluoZin-3. Only the combinations of the chelators with zinc triggered a rapid increase of FluoZin-3 fluorescence within the liposomes, thus demonstrating the ionophore action of QCT, epigallocatechin-gallate, and CQ on lipid membrane systems. The ionophore activity of dietary polyphenols may underlay the raising of labile zinc levels triggered in cells by polyphenols and thus many of their biological actions.

KEYWORDS: *clioquinol, epigallocatechin-gallate, flavonoids, liposomes, quercetin, zinc ionophores*

1. INTRODUCTION

Quercetin (QCT), a water-insoluble flavonoid present in onions, nuts, and many other vegetables, and epigallocatechin-3-gallate (EGCG), a water-soluble flavonoid present in green tea, are among the most consumed and most studied polyphenols present in the human diet.[1] Flavonoids are considered bioactive micronutrients whose regular consumption, either as food components, or as dietary supplements and nutraceuticals,[2] entails benefits for human health, including prevention and amelioration of cancers,[3] diabetes, and cardiovascular[4] and neurodegenerative[5] diseases. Many of the health benefits of flavonoids have historically been ascribed to their antioxidant activity, which they exert directly by scavenging reactive oxygen species (ROS) and by chelating the redox-active transition metals iron and copper, which may act as ROS generators in biological systems.[6] Flavonoids also act as antioxidants indirectly by inhibiting redox-sensitive transcription factors and pro-oxidant enzymes as well as through induction of phase II and antioxidant enzymes.[7] However, it is currently believed that the levels of polyphenols achieved through ingestion are not enough to justify their wide array of biological actions. Beyond their antioxidant actions, flavonoids are also known to act as signaling molecules that, either directly or indirectly, interact with proteins and nucleic acids, thus modulating multiple cell signaling pathways, gene transcription, metabolic fluxes, and cell fate including apoptosis.[8,9]

Diverse polyphenols have been shown able to form complexes with the redox-inactive transition metal zinc.[10] Zinc is an essential micronutrient for humans, the deficiency of which causes multiple dysfunctions, including alterations of glucidic and lipidic metabolisms.[11] Within cells, the vast majority of zinc cations (in concentrations usually ranging from 100 to 300 μM for most cells) are tightly bound to proteins, functioning as a catalytic or structural component of an estimated 3000 mammalian proteins involved in virtually all cellular processes.[12] A minor fraction of intracellular zinc, termed labile zinc, exists in its free ionic form (picomolar concentrations) or loosely bound to proteins (in nanomolar concentrations). This pool of zinc is detectable by specific fluorophores with very high affinities for zinc cations at neutral pH such as Zinquin and FluoZin-3. Zinc ionophores such as pyrithione and clioquinol (CQ) have been used to increment labile zinc within cells and determine the fundamental roles that this zinc pool plays in cellular biology. Thus, free and labile zinc acts as second messenger molecule, which modulates the

Received: April 1, 2014
Revised: July 22, 2014
Accepted: July 22, 2014

   dx.doi.org/10.1021/jf5014633 | J. Agric. Food Chem. XXXX, XXX, XXX−XXX

activity of many enzymes and thus signaling and metabolic pathways and cellular processes, including cell fate and apoptosis.[13,14] While many enzymes are inhibited by small elevations of zinc concentrations, others are activated.[15,16] Mammalian cells tightly control the subcellular distribution of zinc cations and the levels of labile zinc through the coordinated action of dedicated transmembrane zinc transporters and zinc-chelating metallothioneins.

Zinc from the extracellular milieu and from intracellular compartments enters the cytoplasm through 14 specialized transmembrane proteins of the ZIP/SLC39 family, whereas cytoplasmic extrusion of zinc toward organelles or the extracellular environment is performed by 10 transporters of the ZnT/SLC30 family, being ZnT1, located at the plasma membrane, the main regulator of cellular zinc efflux and export of excess zinc in most cells.[17] Within the cytoplasm, zinc may bind to metal free apo-metallothionein (apo-MT) to generate Zn−MT complexes. The apo-MT/Zn−MT ratio controls free and labile zinc concentrations. MT also serves as a ROS scavenger and heavy metal chelator, and the transcription of MT responds, in addition to zinc, to stress stimuli such as ROS, heavy metals, and proinflammatory cytokines.[18] In response to elevations of intracellular zinc, the zinc-sensor transcription factor MTF1 coordinately upregulates the expression of MT and ZnT1,[19] thus keeping zinc levels within a functional range. Excessively high levels of labile zinc are associated with cellular death through apoptosis.[20] Dysfunctions of MT and zinc transporters are promoting factors in cardiovascular diseases,[21] diabetes,[22] Alzheimer's disease,[23] and cancer.[24]

Several studies have shown that flavonoids affect zinc metabolism. For instance, rats fed during long periods with bicalain and rutin showed reduced hepatic levels of total zinc, as well as iron and copper, implying that flavonoids may sequester these metals and render them unavailable for absorption in a similar way as phytate.[25] Consistent with this, feeding obese rats with proanthocyanidins reverse dyslipidemia and lower protein levels of ZnT1 in the liver, reflecting lower levels of hepatic zinc.[26] Early in vitro studies showed that, in human intestinal Caco-2 cells, genistein enhanced the expression of MT, here regarded as an antioxidant enzyme,[27] while QCT increased the copper-induced expression of MT.[28] More recently, QCT was shown to enhance zinc uptake by Caco-2 cells, increasing total zinc accumulation and MT expression.[29] In contrast, grape seed flavonoids, produced a reduction in apical zinc uptake in Caco-2 cells, similar to that produced by phytate, whereas EGCG did not alter zinc absorption.[30] In prostate cancer cells, EGCG accelerated the accumulation of total zinc in the cytosol and mitochondria.[31] Two reports have shown that polyphenols may produce an increase of intracellular labile zinc. A water-soluble glycoside of the isoflavone genistin enhanced MT expression in human hepatocarcinoma HepG2 cells concomitantly increasing labile zinc and cellular death.[32] Furthermore, the stilbene resveratrol was shown to enhance total and labile zinc in normal human prostate epithelial cells, while not significantly affecting MT expression, and this was accompanied by increased cellular death. These authors suggested that the increment of labile zinc elicited by resveratrol might be due to the uptake of resveratrol−zinc complexes, followed by the dissociation of the complexes in the cytoplasm.[33]

Conversely, zinc may affect the bioactivity of flavonoids, as detailed in a few reports, including one that outlines the stimulating effect of zinc on the apoptotic effect of genistein in osteoclastic cells.[34] Zinc also yields EGCG effective in protecting cultured rat hepatocytes against hepatotoxin-induced cell injury[35] and enhances the antiproliferative, proapoptotic effects of EGCG on various lines of prostate cancer cells.[36] Zinc was also shown to affect the uptake of EGCG by prostate carcinoma cells, where Zn−EGCG chelates were less internalized by cells than EGCG alone, while mixtures of EGCG with zinc enhanced the transport of EGCG into the cells. These authors also showed that zinc enhances the incorporation of EGCG into liposomes.[37]

We have previously reported that the water-soluble flavonoid EGCG and the water-insoluble flavonoid QCT profoundly alter zinc homeostasis in cultured human and mouse hepatocarcinoma cells. Whereas EGCG reduced the levels of total intracellular zinc and the expression of MT and ZnT1,[38] QCT enhanced total zinc accumulation as well as MT and ZnT1 expression (M. Bustos, personal communication, 2011, Universitat Rovira i Virgili). However, both QCT and EGCG dose-dependently prevented zinc-induced toxicity, suggesting that most zinc cations in the culture medium are rendered unavailable to cells due to their chelation by flavonoids and the formation of flavonoid−zinc concatemers, as shown for iron and copper complexed with diverse polyphenols.[39] In addition, both polyphenols enhanced cytoplasmic levels of Zinquin-detectable labile zinc, suggesting that a fraction of the flavonoid molecules in the culture medium formed complexes with zinc that cross the plasma membrane; that is, the flavonoids may also act as zinc ionophores, transporting zinc cations across the plasma membrane independently from zinc transporters.

The aim of this work was to evaluate the capacity of QCT and EGCG to act as zinc ionophores. Clioquinol (CQ) was also tested in this study as it is a synthetic antitumor drug recently reported to induce apoptosis in diverse cells lines by enhancing intracellular labile zinc and therefore inferred to act as a water-soluble zinc ionophore.[40] We evaluated the ability of QCT, EGCG, and CQ to chelate zinc cations and the subsequent formation of a complex with FluoZin-3, a fluorophore that displays a very high affinity for zinc cations (K_d = 15 nM).[41] The uptake of zinc by mouse hepatocarcinoma cells was measured fluorescently using FluoZin-3 in the presence and absence of QCT, EGCG, and CQ. This study was repeated using unilamellar liposomes with encapsulated FluoZin-3 to investigate whether the transport of the zinc cations across the cytoplasmic membrane to form a complex with FluoZin-3 was indeed enhanced by the presence of QCT, EGCG, or CQ or was simply due to the activity of cellular zinc transporters.

2. MATERIALS AND METHODS

2.1. Chemicals. The lipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), cholesterol, zinc chloride ($ZnCl_2$), QCT, EGCG, CQ, dimethyl sulfoxide (DMSO), ethanol, and phosphate-buffered saline (0.01 M PBS, pH 7.4) were bought from Sigma−Aldrich. Cell-impermeant FluoZin-3 tetrapotassium salt and cell permeant FluoZin-3 AM were bought from Molecular Probes. A Simplicity 185 Millipore water system was used to obtain Milli-Q water (18.2 mΩ·cm^{-1}) for the preparation of buffers and liposomes. The compounds QCT, EGCG, and CQ were dissolved as 100 μM solutions in 100% DMSO, aliquoted, and stored at −20 °C. $ZnCl_2$ was stored as 1 M solution in ethanol/PBS (50%/50% v/v). FluoZin-3 indicators were used at 10 μM in 100% DMSO.

2.2. Cell Cultures and Treatments. The mouse hepatoma cell line Hepa 1-6 was obtained from the European Collection of Cell Cultures (BW7756 ECACC) and propagated in Dulbecco's Modified Eagle medium (DMEM; BioWittaker) supplemented with 10% fetal

dx.doi.org/10.1021/jf5014633 | J. Agric. Food Chem. XXXX, XXX, XXX−XXX

bovine serum (BioWittaker), 2 mM glutamine in 0.85% NaCl, 1000 U/mL penicillin/streptomycin, and 1.25 M HEPES. This medium contains 4.9 ± 0.2 μM zinc, as determined by flame atomic absorption spectroscopy (FAAS). Cells were cultured at 37 °C in a humidified, 5% CO_2-enriched atmosphere and routinely split every 3−4 days at a 1:5 ratio upon reaching approximately 80% confluence. For treatments, cells at 80% confluence were detached with Accutase (Sigma−Aldrich) and resuspended at a density of 5×10^5 cells/mL; 500 μL of this cell suspension (25×10^4 cells) was then seeded per well in 24-well plates (Orange Scientific). Twenty four hours after plating, medium was removed, and the cells were treated by adding 100 μL of fresh medium containing either 50 μM $ZnCl_2$, 100 μM QCT, 100 μM EGCG, 100 μM CQ, or the combination of 50 μM $ZnCl_2$ with each chelator for 1 and 4 h, respectively. As a control experiment, untreated cells were incubated just with medium and vehicle (final 0.1% DMSO and 0.05% ethanol).

2.3. Measurements of Cytoplasmic Labile Zinc in Hepa 1-6 Cells. The intracellular levels of free and labile zinc cations were measured as the fluorescence emission of cells upon loading them with the membrane-permeant zinc specific detector FluoZin-3, using fluorescence microscopy as described.[42] Briefly, following cell treatment (Section 2.2), culture media were replaced with a fresh one containing 1.5 μM FluoZin-3 (AM, cell permeant) and incubated for 30 min at 37 °C. This medium was then removed, and the cells were washed three times with PBS, and the zinc-dependent FluoZin-3 fluorescence within cells was measured using a Nikon Eclipse TE2000-S microscope, with excitation set at 494 nm and emission at 516 nm. Fluorescent intensities were quantified using the NIS-Elements AR software (Nikon Instruments) and the software ImageJ, a Java-based image processing program developed at the NIH (National Institutes of Health).[43]

2.4. Liposomes as Cell Membrane Models. Homogeneous populations of liposomes were prepared using a previously reported method.[44] FluoZin-3 in a final concentration of 3 μM was mixed with 5 mL of PBS (0.01 M, pH 7.4) in a glass reactor protected from light-induced degradation, under stirring conditions and a blanket of argon gas. After 15 min, a mixture of DPPC and cholesterol (9:1 molar ratio) was added and maintained under stirring conditions and argon at 25 °C for another 15 min. The homogeneous mixture was then treated with a rapid pH jump from pH 7.4 to pH 11 and then back to pH 7.4 within a 3 s frame, followed by an equilibration step of 25 min where lipid clusters curl into FluoZin-3 encapsulating liposomes. The resulting FluoZin-3-loaded liposomes were purified using a Sephadex G-100 size-separation column and used immediately.

Liposomes with encapsulated FluoZin-3 were separately incubated with 10 μM QCT, EGCG, or CQ, in the presence and absence of 10 μM $ZnCl_2$. All the solutions were allowed to incubate at 25 °C for 30 min before measuring their fluorescence. For the kinetic experiment, liposomes loaded with FluoZin-3 were added to three different cuvettes, the fluorescence was measured for 15 min, followed by addition of $ZnCl_2$ (final 10 μM) to each cuvette, and the fluorescence emission was measured for another 15 min. Finally, QCT, EGCG, or CQ was added (final 10 μM) to each sample, and fluorescence emission was monitored over the duration of 1 h until the fluorescent intensity reached a plateau.

3. RESULTS

3.1. QCT, EGCG, and CQ Increase the Cytoplasmic Labile Zinc in Hepa 1-6 Cells. The increase of cytoplasmic labile zinc is modulated by the cellular zinc transporters, where the zinc ions are transported to the cytoplasm through specific channels of the ZIP family, bound to ionophore molecules that independently cross the lipid bilayer, or liberated from zinc-binding proteins such as metallothioneins (Figure 1).

In order to assess the effect of QCT, EGCG, CQ and zinc on cytoplasmic labile zinc, Hepa 1-6 cells were treated for 1 and 4 h with the chelators and supplemental zinc, and variations in the intracellular levels of labile zinc were measured as changes

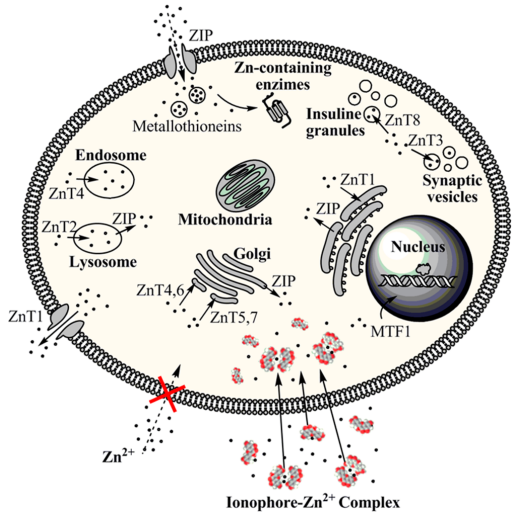

Figure 1. Schematic representation of zinc homeostasis. Intracellular labile zinc is modulated by the coordinated activity of a large family of zinc transporters (ZnT and ZIP) and zinc-binding proteins, such as metallothionein or ionophore molecules.

in the FluoZin-3 fluorescence intensity as described in Section 2.3. Figure 2 shows the fluorescent images of the Hepa 1-6 cells after 1 and 4 h of treatment. No significant increase in fluorescence was obtained when QCT or EGCG were added to the culture medium without additional zinc. Only CQ insignificantly enhanced the fluorescence in these conditions, that is, with basal zinc concentration in the culture medium, which is roughly 5 μM.

However, when 50 μM $ZnCl_2$ was added, both QCT and EGCG doubled the amount of FluoZin-3-detectable zinc after 1 h, and CQ increased this pool of zinc 10-fold with respect to the control (50 μM $ZnCl_2$ in the absence of any of QCT, EGCG, or CQ) (Figure 3), suggesting a slower ionophore action of the flavonoids as compared to CQ. After 4 h treatment with additional 50 μM $ZnCl_2$, all treatments triggered a significant increase in fluorescence intensity. In the case of the control, the increase of the cytoplasmic labile zinc is associated with the plasma membrane ZIP transporters, whereby zinc ions are transported into the cell. QCT doubled the amount of labile zinc attained with only 50 μM zinc, EGCG quadrupled this value, and CQ increased it 7-fold.

A closer view of intracellular distribution of FluoZin-3 fluorescence (Figure 4) after 4 h of treatment shows a similar punctuated pattern of labile zinc for CQ, EGCG, and QCT, suggesting similar ways of action for the three compounds.

3.2. Zinc Ionophore Activity of QCT, EGCG, and CQ Using Liposomes as Membrane Models. Increases of cytoplasmic labile zinc levels triggered by CQ and pyrithione in a variety of cell lines have been attributed to their ionophore activity, that is, to the capacity of CQ−zinc and pyrithione−zinc complexes to cross the plasma membrane. To our knowledge, however, the classification of CQ and pyrithione as zinc ionophores is based on their functional effect in cells, that is, the rapid increase in Zinquin-detectable or Fluozin-3-detectable intracellular zinc, but no direct biochemical assay has

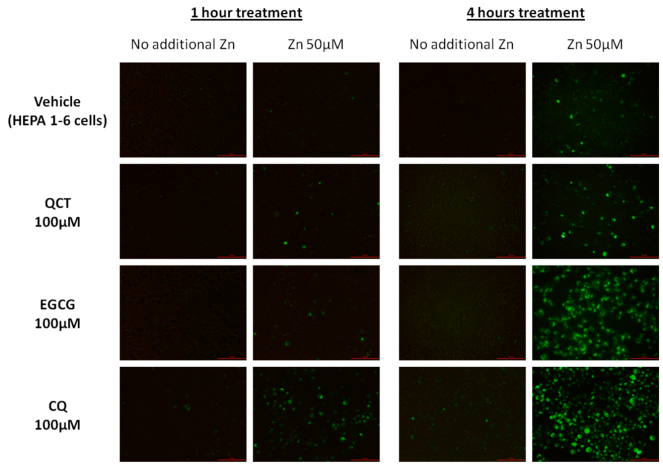

Figure 2. Effect of QCT, EGCG, and CQ on the cytoplasmic pool of labile zinc in Hepa 1-6 cells. Hepa 1-6 cells were first treated with 100 μM QCT, EGCG, or CQ, in the presence or absence of 50 μM $ZnCl_2$ for 1 and 4 h. The medium was then removed, and 3 μM FluoZin-3 (AM, cell permeant) was added. After 30 min incubation, cells were washed and examined using a confocal fluorescence microscope. Control cells were treated with vehicle (final 0.05% ethanol, 0.1% DMSO). Scale bars are 50 μm.

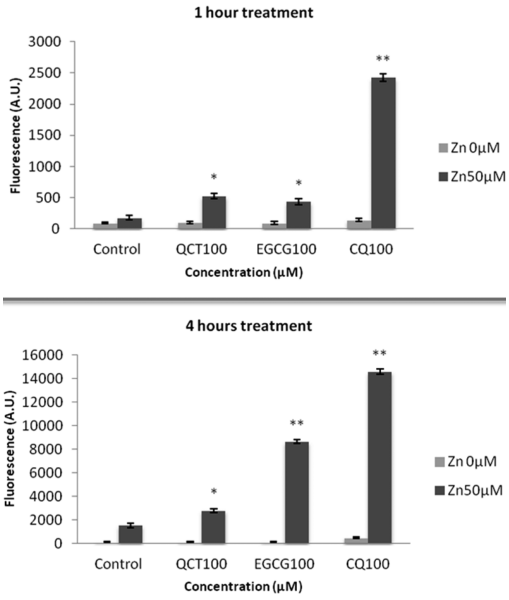

Figure 3. Intensity of FluoZin-3 fluorescence signal from images in Figure 2 was quantified using quanta program and considering an equal number of cells in each field. All values are mean ± SEM of three independent experiments. Significant differences between treatments were determined using one-way ANOVA (Tukey test). *$P \leq 0.05$; **$P \leq 0.01$.

been performed to discard the involvement of plasma membrane zinc importers or the origin of labile zinc from intracellular components on this effect. Furthermore, there is no report confirming that polyphenols are able to transport zinc across the plasma membrane independently of cell transport mechanisms, such as zinc transporters or endocytosis. To directly prove the ionophore action of the flavonoids and CQ, we tested their capacity to transport zinc cations across the lipid bilayer of protein-free liposomes as a model system that mimics a cell membrane devoid of protein and polysaccharide fractions. Taking advantage of our previously reported method for the rapid preparation of liposomes,[44] 3 μM concentration of cell-impermeant FluoZin-3 was encapsulated within unilamellar liposomes composed of DPPC/cholesterol in a 9:1 molar ratio. The resulting FluoZin-3-loaded liposomes were purified by passing the sample through a Sephadex G-100 size-separation column to remove the unencapsulated FluoZin-3 molecules. Dynamic light scattering (DLS) and ζ potential analysis were performed to clearly confirm the presence of stable liposomes within a size range 1−2 μm and surface charge around zero (Table 1).

The zinc ionophore activity of polyphenols was then tested as their capacity to transport zinc cations into the liposome cavity, interacting with the encapsulated zinc-dependent FluoZin-3 and consequently increasing the fluorescence signal within the liposomes (Figure 5). $ZnCl_2$ (10 μM) was added to the liposomal suspension in the absence and presence of 10 μM QCT, EGCG, and CQ, respectively, and zinc-dependent fluorescence intensity was measured over time. Following the addition of QCT, EGCG, or CQ, the Zn−polyphenol chelation complex is formed and transported across the bilayer, followed by interaction with the encapsulated FluoZin-3 probe, resulting in a significant and immediate increase in the fluorescence intensity.

Following the addition of 10 μM $ZnCl_2$ to the liposomes, a very small increase in the fluorescence appears due to the presence of a few free FluoZin-3 molecules that were not removed during the purification process and represent the background fluorescent signal. Even so, the fluorescent signal remains very low as zinc ions alone cannot cross the liposome membrane. The results from the kinetic experiment demon-

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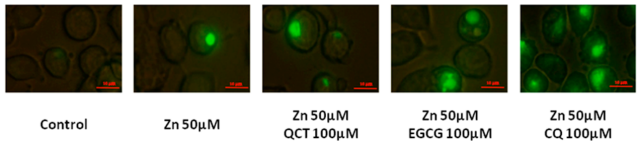

Figure 4. Subcellular localization of FluoZin-3 detectable zinc in Hepa 1-6 cells after 4 h treatment in the same samples as in Figure 2 showed at a greater magnification. Scale bars are 10 μm.

Table 1. Dynamic Light Scattering and ζ Potential Measurements of Liposomes Loaded with FluoZin-3 before and after Treatments with 10 μM Quercetin (QCT10), 10 μM Epigallocatechin-3-gallate (EGCG10), or 10 μM Clioquinol (CQ10) in the Presence and Absence of 10 μM Zinc Chloride (Zn10)[a]

sample	size average (μm)	ζ potential (mV)
FluoZin-3-loaded liposomes	1.4 ± 0.3	−4.7 ± 2.5
FluoZin-3-loaded liposomes + Zn10	1.1 ± 0.7	−5.0 ± 5.9
FluoZin-3-loaded liposomes + QCT10	1.2 ± 0.5	−1.9 ± 4.1
FluoZin-3-loaded liposomes + EGCG10	1.5 ± 0.4	−6.9 ± 7.6
FluoZin-3-loaded liposomes + CQ10	1.4 ± 0.2	−3.0 ± 3.7
FluoZin-3-loaded liposomes + Zn10 + QCT10	1.8 ± 0.2	−1.2 ± 2.5
FluoZin-3-loaded liposomes + Zn10 + EGCG10	1.6 ± 0.3	−3.5 ± 3.2
FluoZin-3-loaded liposomes + Zn10 + CQ10	1.4 ± 0.2	−9.0 ± 2.7

[a]Final concentrations of solvents in the samples were 0.05% ethanol and 0.1% DMSO. Standard deviations were calculated from the mean data of a series of experiments ($n \geq 3$).

strate that QCT, EGCG, and CQ present different ionophore properties. CQ showed the strongest ionophore activity, producing a 35-fold increase in the zinc-dependent FluoZin-3 fluorescence intensity. Moreover, the maximum fluorescence is achieved rapidly after reaching the equilibrium in less than 15 min. QCT and EGCG also display a high ionophore activity in the system, although to a lesser extent as compared to the strong ionophore CQ, with 8- and 16-fold increases in fluorescence signal observed for QCT and EGCG, respectively. It can also be observed that both QCT and EGCG required more time (>60 min) to achieve the plateau phase, displaying slower chelation and transport kinetics (Figure 6).

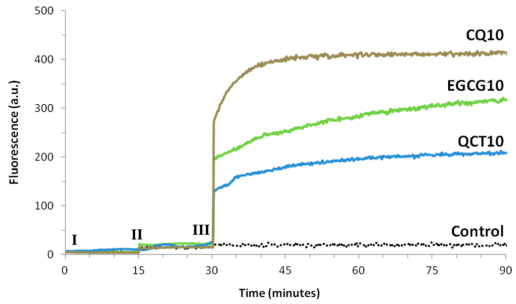

Figure 6. Effect of QCT, EGCG, and CQ on the uptake of zinc cations by liposomes. Zinc-dependent fluorescence emission of FluoZin-3 encapsulated within liposomes treated with zinc cations, polyphenols, and CQ. The fluorescence emission (λ_{ex} = 494 nm; λ_{em} = 516 nm) of purified FluoZin-3-loaded liposomes was recorded continuously. Background fluorescence (0−15 min) was negligible (I). Upon the addition of 10 μM $ZnCl_2$ to the liposomal suspensions (II), a small fluorescence signal was detected, presumably due to the presence of trace amounts of unencapsulated FluoZin-3 in the liposomal solutions. At time point 30 min, 10 μM quercetin (QCT10), epigallocatechin-3-gallate (EGCG10), clioquinol (CQ10), or vehicle (control, final 0.1% DMSO) were added to the liposomal solutions, and the fluorescence was monitored for one additional hour (III).

Confocal microscopy analysis was also performed in order to visualize and corroborate that the fluorescence produced by the interaction between zinc and FluoZin-3 was atributable to fluorescence in the inner part of the liposomes. As can be seen in Figure 7, fluorescence is only observed when the combination of $ZnCl_2$ with QCT, EGCG, or CQ is present, and the fluorescent signal comes from the inside part of the liposomes and not from the lipid membrane or the background

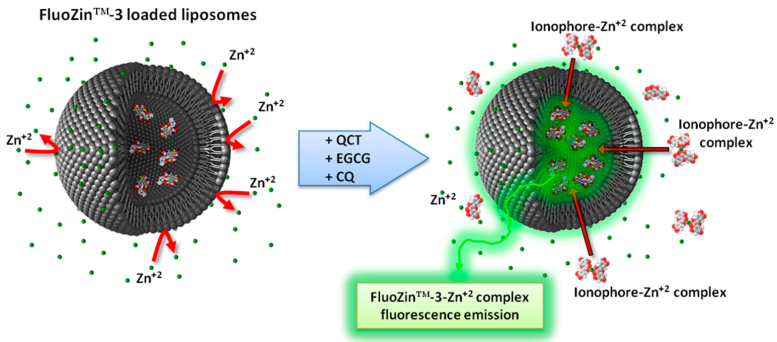

Figure 5. Schematic design of the FluoZin-3-loaded liposomes and the ionophore-like effect interpretation.

dx.doi.org/10.1021/jf5014633 | J. Agric. Food Chem. XXXX, XXX, XXX−XXX

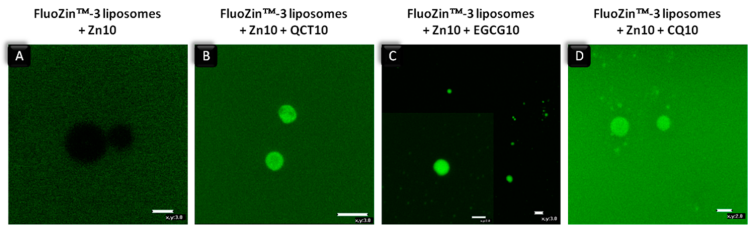

Figure 7. Effect of QCT, EGCG, and CQ on the uptake of zinc cations by liposomes. Three-dimensional confocal microscopy images of zinc-dependent fluorescence emission of FluoZin-3 loaded within liposomes treated with zinc cations, polyphenols, and CQ.

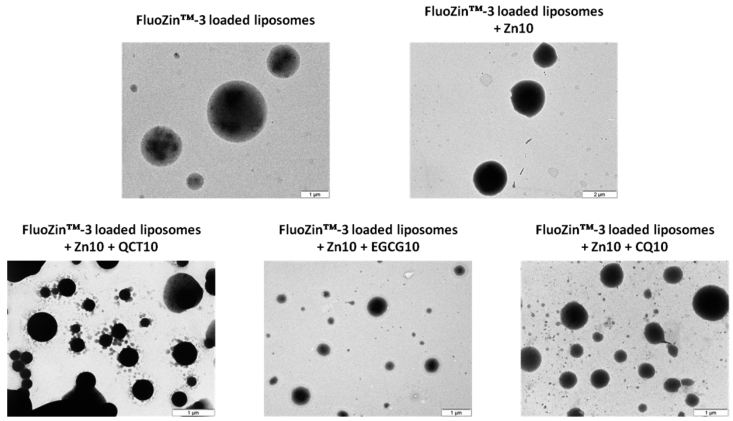

Figure 8. Effect of QCT, EGCG, and CQ on the uptake of zinc cations by liposomes. Transmission electron micrographs of liposomes with encapsulated FluoZin-3 after treatment with $ZnCl_2$, QCT, EGCG, and CQ.

solution. To further support this, stability studies were carried out in order to check whether the QCT, EGCG, or CQ can destabilize and break the lipid vesicles. All the liposomes were characterized using transmission electron microscopy (TEM), DLS, and ζ potential to check that their stability was maintained following exposure.

As shown in Figure 8, TEM images of the liposomes before and after treatments clearly demonstrate that liposome stability was not affected with the morphology and mean size (1−2 μm) being maintained. Moreover, the DLS and ζ potential results presented in Table 1, confirmed that the size of the liposomes and their surface charge were not significantly affected by the addition of zinc and/or the ionophores, thus demonstrating that FluoZin-3-loaded liposomes were not destabilized and their around-zero charge, due to the zwitterionic nature of main lipid component DPPC, was maintained, thus confirming that the fluorescence signal is due to transport of the Zn−QCT/EGCG/CQ complex across the lipid membrane.

4. DISCUSSION

The consequences that zinc chelation by flavonoids may have on zinc availability to the cells may in principle be dual: sequestering or ionophore, as shown for other well-characterized zinc-binding compounds.[45] On the one hand, the formation of zinc−flavonoid complexes may render zinc unavailable for cells, as do other dietary phytochemicals such as phytatesc[46] and zinc-chelator drugs such as TPEN that induce zinc deficiency in vitro and in vivo.[47] Metal chelating therapy using CQ has been proposed for neurodegenerative disorders that course with high levels of metal accumulation such as Alzheimer's and Parkinson.[48] On the other hand, flavonoids may form water-insoluble membrane-permeant complexes with zinc that cross the plasma membrane, and thereby act as zinc ionophores. The ionophore effect of zinc-binding compounds has been characterized for pyrithione and CQ. Both drugs chelate zinc cations and, when applied to cells, trigger a rapid increase of the intracellular pool of zinc that is detectable with different fluorophores such as Zinquin or FluoZin-3. Thereafter, it is assumed that these chelators form membrane-permeable complexes that are transported into the cell and that, once within the cell, chelator−zinc complexes dissociate into the single compounds due to the low concentration of intracellular free and labile zinc, thus providing labile zinc cations. Although FluoZin-3 has been widely accepted as a fluorophore that specifically interacts with zinc,[49−52] a recent report has indicated that this marker may in fact suffer from a lack of specificity.[53] However, this has no impact on the proof-of-concept study reported here as zinc is the only ion present and studied and thus no interfering effect from other ions will occur. We have shown here that treatment of Hepa1-6 cells with zinc together with QCT, EGCG, or CQ elicits a rapid and drastic increase in FluoZin-3-detectable intracellular zinc. The same effect was previously observed using Zinquin upon treatments of HepG2 cells with combinations of zinc and EGCG or zinc and QCT.[38] In these cells, the upregulation of MT and ZnT1 by zinc was

dx.doi.org/10.1021/jf5014633 | J. Agric. Food Chem. XXXX, XXX, XXX−XXX

enhanced by QCT. In contrast, EGCG decreased the intracellular zinc accumulation. Similar to QCT, the stilbene resveratrol efficiently chelates zinc in solution and enhances total and Zinquin-detectable cytoplasmic zinc in cultured human prostate epithelial cells, and this correlated with the antiproliferative action of resveratrol on cells.[33]

While an increasing effort has been made to understand the interaction of flavonoids with lipid bilayers,[54] no report has been published reporting the use of liposomes to demonstrate the zinc ionophore activity of polyphenols. Liposomes have been widely used as the simplest cell membrane systems in order to study the ionophore activity of molecules across the lipidic bilayer.[55] We have herein used a liposomal system to prove that zinc can transverse lipid bilayers when combined with flavonoids. It is not necessary to evoke the intervention of zinc transporters in the plasma membrane or the mobilization of zinc from intracellular compartments to account for the elevation of intracellular zinc levels in cells treated with flavonoids. The flavonoid-dependent transport of zinc cations into the liposomal cavity also implies that polyphenols may cross biological membranes when conjugated with metal cations. The mechanisms by which polyphenols enter the cells are largely unknown, but our results imply that complexation with metals may increase the bioavailability of polyphenols to cells.

There are several reports that strongly suggest that the demonstrated zinc ionophore effect on polyphenols will be observed in real physiological conditions if studied in vivo. Lee et al.[56] in 2002 has reported that a minor part of the EGCG found in plasma conserved its native form, and it has also been reported that the polyphenol metabolites still maintain their ability to chelate and form complexes with metal ions[30] and furthermore that nanomolar concentrations of polyphenols or their metabolites are able to modulate some metabolic pathways,[5,57,58] as does labile zinc in a picomolar to nanomolar concentration range.[14] While all these reports point toward the same ionophore effect being observed under physiological conditions, in a recent report by Oyama et al.,[59] the authors suggest the dual effect of CQ depending on the extracellular zinc concentration, where the known ionophore effect of CQ was only observed when extracellular zinc was available, and when zinc was not available in the extracellular environment, CQ could cross the membrane and chelate the intracellular zinc ions. To this end, ongoing work is looking at extending the proof-of-concept reported and demonstrated here with both liposomes and cellular models to true physiological conditions exploring the interactions between a range of polyphenols and polyphenol metabolites and zinc in a lower concentration range of picomolar to micromolar.

In conclusion, we have demonstrated that QCT, EGCG, and CQ rapidly increase intracellular labile zinc in Hepa 1-6 cells and that they function as ionophores for zinc in a liposomal system. Thus, natural flavonoids can be added to an arsenal of drugs that may be used to modulate zinc homeostasis and regulate zinc-dependent biological pathways.

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Funding

This work was supported by grant AGL2008-00387 from the Spanish Ministry of Education and Science. H.D. was granted a predoctoral grant from the University Rovira i Virgili.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; EGCG, (−)-epigallocatechin-3-gallate; FluoZin-3, 2-[2-[2-[2-[bis-(carboxylatomethyl)amino]-5-methoxyphenoxy]ethoxy]-4-(2,7-difluoro-3-oxido-6-oxo-4*a*,9*a*-dihydroxanthen-9-yl)-anilino]acetate; MRE, metal response element; MT, metallothionein; MTF-1, MRE-binding transcription factor-1; TPEN, *N*,*N*,*N*′,*N*′-tetrakis(2-phridylmethyl) ethylenediamine; Zinquin, ethyl (2-methyl-8-*p*-toluenesulfonamido-6-quinolyloxy); ZIP, ZRT/IRT related protein (SLC39 family of zinc transporters); ZnT, zinc transporter (SLC30 family of zinc transporters)

REFERENCES

(1) Aron, P. M.; Kennedy, J. A. Flavan-3-ols: Nature, occurrence and biological activity. *Mol. Nutr. Food Res.* **2008**, *52*, 79−104.

(2) Egert, S.; Rimbach, G. Which sources of flavonoids: Complex diets or dietary supplements? *Adv. Nutr.* **2011**, *2*, 8−14.

(3) Ramos, S. Cancer chemoprevention and chemotherapy: Dietary polyphenols and signalling pathways. *Mol. Nutr. Food Res.* **2008**, *52*, 507−526.

(4) Jagtap, S.; Meganathan, K.; Wagh, V.; Winkler, J.; Hescheler, J.; Sachinidis, A. Chemoprotective mechanism of the natural compounds, epigallocatechin-3-O-gallate, quercetin and curcumin against cancer and cardiovascular diseases. *Curr. Med. Chem.* **2009**, *16*, 1451−1462.

(5) Williams, R. J.; Spencer, J. P. E. Flavonoids, cognition, and dementia: Actions, mechanisms, and potential therapeutic utility for Alzheimer disease. *Free Radical Biol. Med.* **2012**, *52*, 35−45.

(6) Selvaraj, S.; Krishnaswamy, S.; Devashya, V.; Sethuraman, S.; Krishnan, U. M. Flavonoid−metal ion complexes: A novel class of therapeutic agents. *Med. Res. Rev.* **2014**, *34*, 677−702.

(7) Na, H.-K.; Surh, Y.-J. Modulation of Nrf2-mediated antioxidant and detoxifying enzyme induction by the green tea polyphenol EGCG. *Food Chem. Toxicol.* **2008**, *46*, 1271−1278.

(8) Williams, R. J.; Spencer, J. P. E.; Rice-Evans, C. Flavonoids: Antioxidants or signalling molecules? *Free Radical Biol. Med.* **2004**, *36*, 838−849.

(9) Ramos, S. Effects of dietary flavonoids on apoptotic pathways related to cancer chemoprevention. *J. Nutr. Biochem.* **2007**, *18*, 427−442.

(10) Le Nest, G.; Caille, O.; Woudstra, M.; Roche, S.; Burlat, B.; Belle, V.; Guigliarelli, B.; Lexa, D. Zn−polyphenol chelation: Complexes with quercetin, (+)-catechin, and derivatives: II Electrochemical and EPR studies. *Inorg. Chim. Acta* **2004**, *357*, 2027−2037.

(11) Prasad, A. S. Discovery of human zinc deficiency: 50 years later. *J. Trace Elem. Med. Biol.* **2012**, *26*, 66−69.

(12) Maret, W. Zinc biochemistry: From a single zinc enzyme to a key element of life. *Adv. Nutr.* **2013**, *4*, 82−91.

(13) Fukada, T.; Yamasaki, S.; Nishida, K.; Murakami, M.; Hirano, T. Zinc homeostasis and signaling in health and diseases. *J. Biol. Inorg. Chem.* **2011**, *16*, 1123−1134.

(14) Maret, W. Metals on the move: Zinc ions in cellular regulation and in the coordination dynamics of zinc proteins. *Biometals* **2011**, *24*, 411−418.

(15) Wilson, M.; Hogstrand, C.; Maret, W. Picomolar concentrations of free zinc(II) ions regulate receptor protein-tyrosine phosphatase β activity. *J. Biol. Chem.* **2012**, *287*, 9322−9326.

(16) Maret, W. Inhibitory zinc sites in enzymes. *Biometals* **2013**, *26*, 197−204.

(17) Cousins, R. J.; Liuzzi, J. P.; Lichten, L. A. Mammalian zinc transport, trafficking, and signals. *J. Biol. Chem.* **2006**, *281*, 24085−24089.
(18) Krężel, A.; Maret, W. Thionein/metallothionein control Zn(II) availability and the activity of enzymes. *J. Biol. Inorg. Chem.* **2008**, *13*, 401−409.
(19) Laity, J. H.; Andrews, G. K. Understanding the mechanisms of zinc-sensing by metal-response element binding transcription factor-1 (MTF-1). *Arch. Biochem. Biophys.* **2007**, *463*, 201−210.
(20) Truong-Tran, A. Q.; Ho, L. H.; Chai, F.; Zalewski, P. D. Cellular zinc fluxes and the regulation of apoptosis/gene-directed cell death. *J. Nutr.* **2000**, *130*, 1459S−1466S.
(21) Little, P. J.; Bhattacharya, R.; Moreyra, A. E.; Korichneva, I. L. Zinc and cardiovascular disease. *Nutrition* **2010**, *26*, 1050−1057.
(22) Myers, S. A.; Nield, A.; Myers, M. Zinc transporters, mechanisms of action and therapeutic utility: Implications for type 2 diabetes mellitus. *J. Nutr. Metab.* **2012**, *2012*, 173712.
(23) Watt, N. T.; Whitehouse, I. J.; Hooper, N. M. The role of zinc in Alzheimer's disease. *Int. J. Alzheimer's Dis.* **2010**, *2011*, 971021.
(24) Hogstrand, C.; Kille, P.; Nicholson, R. I.; Taylor, K. M. Zinc transporters and cancer: A potential role for ZIP7 as a hub for tyrosine kinase activation. *Trends Mol. Med.* **2009**, *15*, 101−111.
(25) Gao, Z.; Xu, H.; Chen, X.; Chen, H. Antioxidant status and mineral contents in tissues of rutin and baicalin fed rats. *Life Sci.* **2003**, *73*, 1599−1607.
(26) Baiges, I.; Palmfeldt, J.; Blade, C.; Gregersen, N.; Arola, L. Lipogenesis is decreased by grape seed proanthocyanidins according to liver proteomics of rats fed a high fat diet. *Mol. Cell Proteomics* **2010**, *9*, 1499−513.
(27) Kuo, S.-M.; Leavitt, P. S. Genistein increases metallothionein expression in human intestinal cells, Caco-2. *Biochem. Cell Biol.* **1999**, *77*, 79−88.
(28) Kuo, S.-M.; Huang, C.-T.; Blum, P.; Chang, C. Quercetin cumulatively enhances copper induction of metallothionein in intestinal cells. *Biol. Trace Elem. Res.* **2001**, *84*, 1−10.
(29) Sreenivasulu, K.; Raghu, P.; Nair, K. M. Polyphenol-rich beverages enhance zinc uptake and metallothionein expression in Caco-2 cells. *J. Food Sci.* **2010**, *75*, H123−H128.
(30) Kim, E.-Y.; Pai, T.-K.; Han, O. Effect of bioactive dietary polyphenols on zinc transport across the intestinal Caco-2 cell monolayers. *J. Agric. Food Chem.* **2011**, *59*, 3606−3612.
(31) Yang, J.; Yu, H.; Sun, S.; Zhang, L.; Das, U.; Ruan, H.; He, G.; Shen, S. Mechanism of free Zn(2+) enhancing inhibitory effects of EGCG on the growth of PC-3 cells: Interactions with mitochondria. *Biol. Trace Elem. Res.* **2009**, *131*, 298−310.
(32) Chung, M. J.; Kang, A.-Y.; Lee, K. M.; Oh, E.; Jun, H.-J.; Kim, S.-Y.; Auh, J. H.; Moon, T.-W.; Lee, S.-J.; Park, K.-H. Water-soluble genistin glycoside isoflavones Up-regulate antioxidant metallothionein expression and scavenge free radicals. *J. Agric. Food Chem.* **2006**, *54*, 3819−3826.
(33) Zhang, J. J.; Wu, M.; Schoene, N. W.; Cheng, W.-H.; Wang, T. T. Y.; Alshatwi, A. A.; Alsaif, M.; Lei, K. Y. Effect of resveratrol and zinc on intracellular zinc status in normal human prostate epithelial cells. *Am. J. Physiol.: Cell Physiol.* **2009**, *297*, C632−C644.
(34) Uchiyama, S.; Yamaguchi, M. Genistein and zinc synergistically stimulate apoptotic cell death and suppress RANKL signaling-related gene expression in osteoclastic cells. *J. Cell. Biochem.* **2007**, *101*, 529−542.
(35) Kagaya, N.; Kawase, M.; Maeda, H.; Tagawa, Y.-i.; Nagashima, H.; Ohmori, H.; Yagi, K. Enhancing effect of zinc on hepatoprotectivity of epigallocatechin gallate in isolated rat hepatocytes. *Biol. Pharm. Bull.* **2002**, *25*, 1156−1160.
(36) Sun, S.-l.; He, G.-q.; Yu, H.-n.; Yang, J.-g.; Borthakur, D.; Zhang, L.-c.; Shen, S.-r.; Das, U. N. Free Zn^{2+} enhances inhibitory effects of EGCG on the growth of PC-3 cells. *Mol. Nutr. Food Res.* **2008**, *52*, 465−471.
(37) Yang, J. G.; Yu, H. N.; Sun, S. L.; Zhang, L. C.; He, G. Q.; Das, U. N.; Ruan, H.; Shen, S. R. Epigallocatechin-3-gallate affects the growth of LNCaP cells via membrane fluidity and distribution of cellular zinc. *J. Zhejiang Univ., Sci., B* **2009**, *10*, 411−21.
(38) Quesada, I. M.; Bustos, M.; Blay, M.; Pujadas, G.; Ardèvol, A.; Salvadó, M. J.; Bladé, C.; Arola, L.; Fernández-Larrea, J. Dietary catechins and procyanidins modulate zinc homeostasis in human HepG2 cells. *J. Nutr. Biochem.* **2011**, *22*, 153−163.
(39) Hider, R. C.; Liu, Z. D.; Khodr, H. H. Metal chelation of polyphenols. In *Methods in Enzymology*; Lester, P., Ed.; Academic Press: Waltham, MA, 2001; Vol. *335*, pp 190−203.
(40) Ding, W.-Q.; Liu, B.; Vaught, J. L.; Yamauchi, H.; Lind, S. E. Anticancer activity of the antibiotic clioquinol. *Cancer Res.* **2005**, *65*, 3389−3395.
(41) Li, Y.-M.; Shi, J.; Wu, X.; Luo, Z.-F.; Wang, F.-L.; Guo, Q.-X. Tracing of intracellular zinc(II) fluorescence flux to monitor cell apoptosis by using FluoZin-3AM. *Cell Biochem. Funct.* **2009**, *27*, 417−423.
(42) Yu, H.; Zhou, Y.; Lind, S. E.; Ding, W.-Q. Clioquinol targets zinc to lysosomes in human cancer cells. *Biochem. J.* **2009**, *417*, 133−139.
(43) Collins, T. J. ImageJ for microscopy. *Biotechniques* **2007**, *43*, 25−30.
(44) Genç, R. k.; Ortiz, M.; O'Sullivan, C. K. Curvature-tuned preparation of nanoliposomes. *Langmuir* **2009**, *25*, 12604−12613.
(45) Ding, W.-Q.; Yu, H.-J.; Lind, S. E. Zinc-binding compounds induce cancer cell death via distinct modes of action. *Cancer Lett.* **2008**, *271*, 251−259.
(46) Wise, A. Phytate and zinc bioavailability. *Int. J. Food Sci. Nutr.* **1995**, *46*, 53−63.
(47) Li, B.; Tan, Y.; Sun, W.; Fu, Y.; Miao, L.; Cai, L. The role of zinc in the prevention of diabetic cardiomyopathy and nephropathy. *Toxicol. Mech. Methods* **2013**, *23*, 27−33.
(48) Bareggi, S. R.; Cornelli, U. Clioquinol: Review of its mechanisms of action and clinical uses in neurodegenerative disorders. *CNS Neurosci. Ther.* **2012**, *18*, 41−46.
(49) Muylle, F. R.; Adriaensen, D.; Coen, W.; Timmermans, J.-P.; Blust, R. Tracing of labile zinc in live fish hepatocytes using FluoZin-3. *Biometals* **2006**, *19*, 437−450.
(50) Wessels, I.; Haase, H.; Engelhardt, G.; Rink, L.; Uciechowski, P. Zinc deficiency induces production of the proinflammatory cytokines IL-1β and TNFα in promyeloid cells via epigenetic and redox-dependent mechanisms. *J. Nutr. Biochem.* **2013**, *24*, 289−297.
(51) Kim, J.-H.; Jeon, J.; Shin, M.; Won, Y.; Lee, M.; Kwak, J.-S.; Lee, G.; Rhee, J.; Ryu, J.-H.; Chun, C.-H.; Chun, J.-S. Regulation of the catabolic cascade in osteoarthritis by the zinc-ZIP8-MTF1 axis. *Cell* **2014**, *156*, 730−743.
(52) Chevallet, M.; Jarvis, L.; Harel, A.; Luche, S.; Degot, S.; Chapuis, V.; Boulay, G.; Rabilloud, T.; Bouron, A. Functional consequences of the over-expression of TRPC6 channels in HEK cells: Impact on the homeostasis of zinc. *Metallomics* **2014**, *6*, 1269−1276.
(53) Landero Figueroa, J. A.; Subramanian Vignesh, K.; Deepe, G. S., Jr.; Caruso, J. Selectivity and specificity of small molecule fluorescent dyes/probes used for the detection of Zn^{2+} and Ca^{2+} in cells. *Metallomics* **2014**, *6*, 301−315.
(54) Efimova, S. S.; Ostroumova, O. S. Effect of dipole modifiers on the magnitude of the dipole potential of sterol-containing bilayers. *Langmuir* **2012**, *28*, 9908−9914.
(55) Sankaram, M. B.; Shastri, B. P.; Easwaran, K. R. K. Interaction of carrier ionophores with phospholipid vesicles. *Biochemistry* **1987**, *26*, 4936−4941.
(56) Lee, M.-J.; Maliakal, P.; Chen, L.; Meng, X.; Bondoc, F. Y.; Prabhu, S.; Lambert, G.; Mohr, S.; Yang, C. S. Pharmacokinetics of tea catechins after ingestion of green tea and (−)-epigallocatechin-3-gallate by humans: Formation of different metabolites and individual variability. *Cancer Epidemiol., Biomarkers Prev.* **2002**, *11*, 1025−1032.
(57) Schroeter, H.; Bahia, P.; Spencer, J. P. E.; Sheppard, O.; Rattray, M.; Cadenas, E.; Rice-Evans, C.; Williams, R. J. (−)Epicatechin stimulates ERK-dependent cyclic AMP response element activity and up-regulates GluR2 in cortical neurons. *J. Neurochem.* **2007**, *101*, 1596−1606.

(58) Vauzour, D.; Vafeiadou, K.; Rice-Evans, C.; Williams, R. J.; Spencer, J. P. E. Activation of pro-survival Akt and ERK1/2 signalling pathways underlie the anti-apoptotic effects of flavanones in cortical neurons. *J. Neurochem.* **2007**, *103*, 1355−1367.
(59) Oyama, T. M.; Ishida, S.; Okano, Y.; Seo, H.; Oyama, Y. Clioquinol-induced increase and decrease in the intracellular Zn^{2+} level in rat thymocytes. *Life Sci.* **2012**, *91*, 1216−1220.

Reviewer's comments

Zinc Ionophore Activity of Quercetin and Epigallocatechin-gallate: From Hepa 1-6 Cells to a Liposome Model

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The manuscript by Dabbagh-Bazarbachi et al. describes novel results regarding the zinc ionophore activity of Polyphenols and the cancer drug Clioquinol. The authors tested their hypothesis in 2 models, i.e. cell culture and liposomes using the zinc indicator FluoZin-3. As many long-standing concepts trying to explain the effects of Polyphenols on human health and disease are currently discarded, the idea of Polyphenols acting as metal ion ionophores clearly is of scientific interest. However, prior publication the authors need to take some time to address the following questions:

***1**- From a physiological and nutritional point of view, before reaching the bloodstream and further target tissues (i.e. Hepa 1-6 cells), polyphenols in general and quercetin and EGCG in particular, are metabolized by phase II enzymes in enterocytes or hepatocytes or reach the colon where these compounds are substrate of bacterial microbiota and converted to phenolic acids, before absorption. Thus, the active metabolite developing the biological action at hepatic level being different from native polyphenol ingested. Authors need to clarify this issue, and indicate if it is possible that these polyphenols would reach Hepa 1-6 cells in the tested concentrations of this study or mainly their metabolites and in which physiological concentration, and thus develop the reported activity. Another question raised about this is if the metabolites of these polyphenols still retain the ionophore activity.*

We find the proposed discussion addressed to a future prospective of the biological *in vivo* activity, since it differs from the proof-of-concept reported herein. In our work we have demonstrated the zinc ionophore effect of the pure form of quercetin and EGCG under specific experimental conditions optimized for the "*in vitro"* set up. Even though Lee et al. showed that a minor part of the EGCG found in plasma conserved its native form (Lee et al. 2002), additional research is necessary to assess and understand if the metabolized forms of the polyphenols still exert the ionophore effect. In addition, it has been already reported that those metabolites still maintain their ability to chelate and form complexes with metal ions (Kim et al. 2011). Furthermore, physiological concentrations should also be tested in order to evaluate if at those concentrations the ionophore-like effect is maintained. However, taking into account that nanomolar concentrations of polyphenols are able to modulate some metabolic pathways (Williams & Spencer 2012), as well as labile zinc within concentrations ranged from pico- to nanomolar does (Maret 2011), we

believe that very small variations in both concentrations may produce important biological changes. As a concluding remark, we consider that this, together with the use of other cell lines, should be part of a deeper work in a future study.

***2**- Why these concentrations of Zn and polyphenols were chosen? (Nutritional expected values?). It must be clearly indicated.*

The aim of our study was to evaluate the capacity of two of the most abundant polyphenols, quercetin and EGCG, to act as zinc ionophores, and therefore modulate the levels of zinc within the cells. Previous studies in our group demonstrated that specific doses of polyphenols and zinc, 100 μM and 50 μM respectively, were able to act modulating some of the central genes involved in zinc homeostasis (MT and ZnT1 genes), as well as increase the amount of cytoplasmic labile zinc. These effects were observed both in vitro, using Hepa1-6 (mouse cell line) and HepG2 (human cell line), and *in vivo* using C57BL/6J mice. In the present work, we have chosen these concentrations of polyphenols and zinc supported by our past studies as well as by most of the published literature, in where between 10 to 100 μM EGCG is used to elucidate its mechanism of action in vitro (Lee et al. 2002; Chow et al. 2001).

***3**- Considering that 100 µM PFs are far beyond in vivo concentrations, the authors should comment on the biological relevance of their results; especially in light of their comment that "natural flavonoids can be added to an arsenal of drugs that may be used to modulate zinc homeostasis and regulate zinc-dependent biological pathways". How and where do the authors expect this interaction/synergistic effects to occur? Possibly the intestine, but also other organs? Please, explain.*

In our proof-of-concept work, we have shown the zinc ionophore effect of the pure form of quercetin and EGCG under specific experimental conditions optimized for the in vitro set up. However, in vivo after an oral ingestion of polyphenols, the main part found in plasma and in different tissues or organs is not the pure compound, but in its metabolized and/or conjugated form. Therefore there is a need to study, first if those metabolites, such as phenolic acids, glucuronidated, sulfated or methylated forms are able to keep their

ionophore-like effect, and second, if this effect can be maintained at physiological concentrations of both compounds (nanomolar concentrations). In addition, it was reported that several pathways can be activated with only a few nanomolar concentrations of polyphenols or their metabolites (van Praag et al. 2007; Vauzour et al. 2010), therefore, supporting that even very few concentration of polyphenols could exert a biological effect. We expect that the effect does not occur only in the intestine, but also in different tissues and organs of the body, as even certain polyphenols were able to enter the brain across the blood-brain barrier (BBB) (Williams & Spencer 2012).

***4**- Recently, considerable doubt regarding the specificity of labile metal ion fluorescent indicators (including FluoZin-3) has been raised (e.g. Figueroa et al. Metallomics 2014, 6). This aspect ought to be discussed by the authors.*

Although Fluozin-3 has been widely accepted as a fluorophore that specifically interacts with zinc (Wessels et al. 2013; Muylle et al. 2006; Chevallet et al. 2014), a recent report has indicated that this marker may in fact suffer from a lack of specificity (Figueroa et al. 2014). However, this has no impact on the proof-of-concept study reported here as zinc is the only ion present and studied, and thus no interfering effect from other ions will occur.

***5**- Furthermore, additional information on the binding affinities of tested PFs, Clioquinol and FluoZin-3 are needed in order to better understand why/how PF or Clioquinol-chelated zinc can be detected by FluoZin-3.*

In order to quantify the relative capacity of QCT, EGCG and CQ to bind zinc cations in aqueous solutions at physiological pH, we measured their capacity to retrieve zinc cations from FluoZin-3-zinc complexes (K_d = 15 nM), by monitoring the quenching of zinc-dependent fluorescence emitted by FluoZin-3-zinc complexes upon addition of the polyphenols to the solution. We found that QCT, EGCG and CQ reduced the zinc-dependent FluoZin-3 maximum fluorescence for each zinc concentration (Figure 1). These results imply that our compounds bind zinc cations from FluoZin-3-zinc complexes.

***6**- It appears from the literature that the ratio of chelator and zinc matters for the observable biological effects. Thus, it would be interesting to know how the tested chelator to zinc ratio of 2:1 has been selected and whether it has influenced the obtained results.*

This paper is a proof-of-concept of the ionophore activity. Our research group previously hypothesised that the ratio is important in order to obtain this effect, because only some of the several complexes formed between PFs and zinc will cross the membrane, the other ones will just act as zinc sequestrants, chelating the metal ions and making them unavailable to the cell (Personal communication, Isabel M. Quesada 2010, URV, Effects of Dietary Catechins and Proanthocyanidins on Zinc Homeostasis in Hepatic Cells). The ratio 2:1 as well as the concentration, have been selected following the results obtained in other studies done *in vitro* and *in vivo* under the same conditions, where we observed a modulation of the expression of several zinc transporter and homeostasis genes, and also an increase of the intracellular labile zinc. (Personal communication, Mario Bustos 2011, URV, Efecto de Flavonoides Sobre la Homeostasis de Zinc en Células Hepáticas). It would be interesting to continue this experiment with other ratios, and study how this changes could affect the results not only in a liposome model, but also in a cellular model or in an *in vivo* study in order to understand better the mechanisms of action of our compounds.

***7**- More information on the Clioquinol mode of action regarding to zinc uptake and intracellular zinc levels is needed (see e.g. Oyama et al. Life Sci 2012, 91); this information should also be considered in the discussion.*

Although the ionophore effect of Clioquinol is extensively reported (Bareggi & Cornelli 2012), under specific conditions it can act as a zinc sequestrant. In the report published by Oyama et al., they suggest the dual effect of CQ depending on the extracellular zinc concentration (Oyama et al. 2012). The known ionophore effect of CQ only appeared when extracellular zinc was available, and when zinc was not available in the extracellular environment, CQ may cross the membrane and chelate the intracellular zinc ions. Future work should be addressed to elucidate if this behaviour could be happening with the phenolic compounds.

***8**- How good do the cell culture experiments conducted at 37-Celsius degrees compared to the liposome studies done at 25-Celsius degrees?*

The liposome studies were carried out to demonstrate the ionophore activity of the polyphenols in a simple membrane system. In this case, we wanted to ensure that the membrane stability of the liposomes was not affected due to working with temperatures close to the main lipid transition temperature. The transition temperature of DPPC lipid is approximately 41-Celsius degrees, and is around that temperature where the lipid changes its physical state from an ordered gel phase to a disordered liquid crystalline phase, increasing the membrane permeabilization and leakage. In addition of working at 25-Celsius degrees, we have also included the presence of cholesterol in the liposome formulation to enhance the membrane stability, to be, therefore, completely confident when confirming that zinc molecules are within the liposomes only due to their complexation with quercetin, EGCG or Clioquinol. On the other hand, we have also confirmed in previous control experiments that the transport mechanism also occurred at physiological temperature in the liposomal set up system. We have incubated the liposomes together with the polyphenols, with and without zinc, for 30 minutes at 37-Celsius degrees and measured the fluorescence (end point). The results were following the same trends as at 25-Celsius degrees confirming the zinc-ionophore activity of quercetin, EGCG and CQ at same optimum cellular temperature. However, the results from the simple liposomal system cannot be comparable to what is happening in a complex cellular system. Our objective of using liposomes was to demonstrate the ionophore-like effect and not to compare the transport kinetics between both systems.

REFERENCES

Bareggi, S.R. & Cornelli, U., 2012. Clioquinol: review of its mechanisms of action and clinical uses in neurodegenerative disorders. *CNS neuroscience & therapeutics*, 18(1), pp.41–6.

Chevallet, M. et al., 2014. Functional consequences of the over-expression of TRPC6 channels in HEK cells: impact on the homeostasis of zinc. *Metallomics : integrated biometal science*, 6(7), pp.1269–76.

Chow, H.H. et al., 2001. Phase I pharmacokinetic study of tea polyphenols following single-dose administration of epigallocatechin gallate and polyphenon E. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*, 10(1), pp.53–8.

Figueroa, J.A.L. et al., 2014. Selectivity and specificity of small molecule fluorescent dyes/probes used for the detection of Zn2+ and Ca2+ in cells. *Metallomics : integrated biometal science*, 6(2), pp.301–15.

Kim, E.-Y., Pai, T.-K. & Han, O., 2011. Effect of bioactive dietary polyphenols on zinc transport across the intestinal Caco-2 cell monolayers. *Journal of agricultural and food chemistry*, 59(8), pp.3606–12.

Lee, M.-J. et al., 2002. Pharmacokinetics of Tea Catechins after Ingestion of Green Tea and (-)-Epigallocatechin-3-gallate by Humans: Formation of Different Metabolites and Individual Variability. *Cancer Epidemiol. Biomarkers Prev.*, 11(10), pp.1025–1032.

Maret, W., 2011. Metals on the move: zinc ions in cellular regulation and in the coordination dynamics of zinc proteins. *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine*, 24(3), pp.411–8.

Muylle, F.A.R. et al., 2006. Tracing of labile zinc in live fish hepatocytes using FluoZin-3. *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine*, 19(4), pp.437–50.

Oyama, T.M. et al., 2012. Clioquinol-induced increase and decrease in the intracellular Zn2+ level in rat thymocytes. *Life sciences*, 91(23-24), pp.1216–20.

Van Praag, H. et al., 2007. Plant-derived flavanol (-)epicatechin enhances angiogenesis and retention of spatial memory in mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 27(22), pp.5869–78.

Vauzour, D. et al., 2010. Polyphenols and human health: prevention of disease and mechanisms of action. *Nutrients*, 2(11), pp.1106–31.

Wessels, I. et al., 2013. Zinc deficiency induces production of the proinflammatory cytokines IL-1β and TNFα in promyeloid cells via epigenetic and redox-dependent mechanisms. *The Journal of nutritional biochemistry*, 24(1), pp.289–97.

Williams, R.J. & Spencer, J.P.E., 2012. Flavonoids, cognition, and dementia: actions, mechanisms, and potential therapeutic utility for Alzheimer disease. *Free radical biology & medicine*, 52(1), pp.35–45.

4. A simple liposomal assay for the screening of zinc ionophore activity of polyphenols

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Manuscript submitted for publication

Once the zinc ionophore effect of QCT and EGCG was confirmed in a liposome model, this study seek to determine the possible ionophore effect of 14 different dietary polyphenols, and compare them to Clioquinol and Pyrithione, two different well-described zinc ionophores, as well as TPEN, a commercial zinc sequestrant. We also tested their zinc chelation strength in order to correlate those two properties with the structure of our compounds.

Although displaying quantitative and structural differences, all the 14 dietary polyphenols tested, including EGCG and QCT, behave qualitatively as the synthetic pharmacological zinc chelators TPEN, pyrithione and clioquinol, forming complexes with zinc cations and crossing the lipid bilayers.

A simple liposomal assay for the screening of zinc ionophore activity of polyphenols

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List of abbreviations:

Caffeic acid (CAF), catechin hydrate (CAT HYD), catechol (CAT), clioquinol (CQ), epigallocatechin-3-gallate (EGCG), gallic acid (GAL), genistein (GEN), luteolin (LUT), naringenin (NAR), phloretin (PHLO), pyrithione (PYR), quercetin (QCT), resveratrol (RSV), rutin (RUT), tannic acid (TAN), taxifolin (TAX), N,N,N',N'-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN).

Keywords: Ionophore, liposome, polyphenol, zinc

ABSTRACT

An efficient liposomal system for screening the zinc ionophore activity of a selected library consisting of the most relevant dietary polyphenols is presented. The zinc ionophore activity was demonstrated by exploring the use of zinc-specific fluorophore FluoZin-3 loaded liposomes as simple membrane tools that mimic the cell membrane. The zinc ionophore activity was demonstrated as the capacity of polyphenols to transport zinc cations across the liposome membrane and increase the zinc-specific fluorescence of the encapsulated fluorophore FluoZin-3. In addition, the zinc chelation strength of the polyphenols was also tested in a competition assay based on the fluorescence quenching of zinc-dependent fluorescence emitted by zinc-FluoZin-3 complex. Finally, the correlation between the chelation capacity and ionophore activity is demonstrated, thus underlining the sequestering or ionophoric activity that the phenolic compounds can display, thus, providing better knowledge of the importance of the structural conformation versus their biological activity. Furthermore, the developed assays can be used as tools for rapid, high-throughput screening of families of polyphenols.

1. INTRODUCTION

Zinc ions have long been known to mimic the actions of hormones, growth factors, neurotransmitters and cytokines, and it is believed that zinc may act on intracellular signalling molecules [1-3]. In fact, zinc is a known inhibitor of protein tyrosine phosphatases [4] with a constant of inhibition in the nanomolar range [5]. In addition, zinc affects the regulation of transcription factors, and can induce the expression of some genes, including those coding for molecules involved in zinc homeostasis, such as zinc transporters and metallothioneins [6]. The gene expression of metallothioneins by zinc is regulated by metal response element–binding transcription factor-1 [7]. The chemical properties of zinc that differentiate it from other transition metals, such as copper and iron, which display several different oxidation states in biological systems, is that zinc exists as a redox inert Zn^{2+} cation, which does not undergo redox reactions at physiological redox potentials [8,9]. Additionally, zinc can induce the expression and maintain the levels of potentially radical scavenging proteins such as metallothionein (MT), the major zinc binding protein associated with zinc homeostasis [10], DNA protection, oxidative stress,

and apoptosis [11,12]. Furthermore, it can act through stabilization of cell membranes [13] or as a structural component of anti-oxidant enzymes [14].

On the other hand, recent studies have focused on dietary phenolic compounds as natural improvers of health and more than 8000 dietary polyphenols have been identified [15]. The growing interest in these compounds resides in the accumulating evidence regarding their ability to trigger several cellular pathways leading to the prevention and/or amelioration of pathological conditions, acting as anti-oxidants [16], anti-carcinogenics [17,18], anti-inflammatories [19], neuroprotectors [20], anti-lipidemic and vaso-relaxing agents [15].

In the last decades, it has been demonstrated and understood that phenolic compounds interact with different metals, including zinc, and because of their distinctive chemical structure, they can easily form complexes through metal ion chelation [21] in a manner similar to that of other well-known metal chelators such as the drug clioquinol (CQ) and also exerts a ionophore activity comparable to pyrithione (PYR) [22,23]. The first evidence of polyphenol-metal complexes was reported in 1962 between aluminium ions and flavonoids. Since then, more than 40 metal-flavonoid complexes have been investigated [24].

One of the mechanisms by which flavonoids exert their anti-oxidant activity is via the chelation of redox-active transition metals [25], which are known to catalyze many biological processes leading to the production of free radicals [26]. The essential sites for metal chelation are hydroxyl groups, and the most suitable cations for chelation are Fe(II), Fe(III), Cu(II) and Zn(II) as they high charge density, stimulating the interaction with the phenoxide groups that have a high negative charge density [21]. The structure of the formed complexes depends on the type of flavonoid and metal ion involved, which in turn can influence its' biological interactions that may be different from the native flavonoid [27-31]. Depending on the polyphenol and its' potential binding sites, different structures could be formed with different stoichiometries, thus affecting the biological function of the complex [32]. Experimental data has indicated that the chelated compounds are more effective free radical scavengers than flavonoids alone, suggesting that the Zn-Polyphenol complexes not only exert singular biological properties, but can also enhance the effects of both compounds individually [33]. Further studies have revealed that polyphenols not only interact with metal ions, but also deeply modulate expression of MTs, cellular zinc transporters, extracellular zinc carriers, and intracellular zinc accumulation which are key

factors in zinc homeostasis [10]. Zinquin is a fluorescent zinc-specific indicator and an increase in Zinquin-detectable cytoplasmic levels of zinc in a HepG2 cell line has been monitored when treated with phenolic compounds [10]. This increment in intracellular zinc levels have been reported to induce apoptosis of tumour cells [34,35], suggesting that zinc ionophores may serve as anticancer agents [36].

Although the ionophore activity of natural occurring compounds has not been well established, there is strong evidence of their interaction and complex formation with zinc ions [33], suggesting that they could be potential candidates as zinc ionophore molecules. The interaction of quercetin (QCT) and epigallocatechin-3-gallate (EGCG) with zinc, as well as their ionophore activity has been confirmed in a liposome model using the specific zinc indicator FluoZin-3 [37]. Luteolin (LUT) and naringenin (NAR) interact with zinc ions, forming complexes and exerting a biological function acting as strong radical scavengers [38,39]. The ability of genistein (GEN) to bind zinc ions has not been well elucidated, although its' ability to bind iron is well known and these complexes exert a strong anti-oxidant role, and this suggests that it could have a similar action with other metals such as zinc [40]. There is also evidence of the ability of catechin hydrate (CAT HYD), which is the one of the main bioactive components in green tea, to interact and form complexes with zinc ions [41,42], exerting an anti-oxidant activity, but also having an essential role in treatment of different cancers, such as prostate cancer [43]. Several reports have confirmed that rutin (RUT) forms complexes with zinc [44], also acting as a free radical scavenger in a much more effective way than the free flavonoid [45]. The anti-inflammatory activity of this bioflavonoid is also enhanced when complexed with zinc [28]. Taxifolin (TAX) is also able to interact and form complexes with zinc ions, being an effective radical scavenger too [46]. Most phenolic acids are good metal chelators, due to their structure with several catechol and/or galloyil moieties [47]. To our knowledge, there are no reports to date on interactions and complex formation with zinc ions with phloretin (PHLO) or the stilbene resveratrol (RSV), although for RSV there are some evidences on complex formation with copper, suggesting that maybe similar structures can be formed with other metal ions [48]. Catechol (CAT) is one of the simplest naturally occurring polyphenols, and also one of the most important moieties in a high variety of polyphenols, responsible for the interaction with metal ions. CAT forms complexes with Ruthenium, a rare transition metal, suggesting that it could have the same behaviour with other transition metals like zinc [49]. Thus, a high proportion of polyphenols present some kind of interaction with zinc or other

metal ions, although for the majority of polyphenols the ionophore activity is still undescribed.

The aim of this work was to evaluate the capacity of fourteen different phenolic compounds to bind and chelate zinc ions in solution. We focused on fourteen phenolic compounds grouped according to their chemical structure, including the flavonoids quercetin (QCT), epigallocatechin-3-gallate (EGCG), luteolin (LUT), naringenin (NAR), phloretin (PHLO), genistein (GEN), catechin hydrate (CAT HYD), rutin (RUT) and dihydroquercetin or taxifolin (TAX); the phenolic acids gallic acid (GAL), tannic acid (TAN) and caffeic acid (CAF); the stilbene resveratrol (RSV); and other polyphenols such as catechol (CAT). Two different zinc ionophore agents, clioquinol and pyrithione, were used to compare the ionophore activity of the selected polyphenols, as well as the zinc sequestrant molecule, TPEN (N,N,N',N'-tetrakis (2-pyridylmethyl) ethylenediamine).

Binding/chelation of the zinc ions by the polyphenols was evaluated using a competition assay based on the fluorescence quenching of zinc-dependent fluorescence emitted by FluoZin-3. In this competition assay the zinc chelation strength of each phenolic compound was correlated with the decrease in the fluorescence signal due to the dissociation of the zinc-FluoZin-3 complex as zinc cations are sequestered from the fluorophore complex by the polyphenol. In addition, we present a simple and rapid liposome assay for demonstrating the ionophore activity of common polyphenols and compared them to strong, well-established ionophores, such as clioquinol and pyrithione. The correlation between the chelation capacity and ionophore activity underlines the different behaviours the phenolic compounds can display and the developed assays can be used as tools for rapid, high-throughput screening of families of polyphenols.

2. MATERIALS AND METHODS

2.1. Materials

All the phenolic compounds, pyrithione (PYR), quercetin (QCT), epigallocatechin-3-gallate (EGCG), genistein (GEN), taxifolin (TAX), luteolin (LUT), phloretin (PHLO), catechol (CAT), naringenin (NAR), rutin (RUT), catechin (CAT HYD), caffeic acid (CAF), tannic acid (TAN), gallic acid (GAL), resveratrol (RSV) and clioquinol (CQ) were purchased from Sigma-Aldrich, as well as the lipid 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC),

cholesterol, dimethyl sulfoxide (DMSO), ethanol, zinc chloride ($ZnCl_2$), N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) and the phosphate buffered saline (0.01M PBS, pH 7.4). Cell impermeant FluoZin™-3 tetrapotassium salt was obtained from Molecular Probes. A Simplicity 185 Millipore-Water System was used to obtain Milli-Q water (18.2 $m\Omega.cm^{-1}$) for the preparation of buffers and liposomes. The compounds PYR, QCT, EGCG, GEN, TAX, LUT, PHLO, CAT, NAR, RUT, CAT HYD, CAF, TAN, GAL, RSV and CQ were dissolved as 100 μM solutions in 100 % DMSO and aliquoted of at -20 ºC. $ZnCl_2$ was stored as 1 M solution in ethanol/PBS (50/50 % v/v). FluoZin-3 zinc indicator was used at 10 μM in 100 % DMSO.

2.2. Measurement of the interaction of the polyphenols with zinc cations in solution

A competition assay was carried out to test the ability of the polyphenols PYR, QCT, EGCG, GEN, TAX, LUT, PHLO, CAT, NAR, RUT, CAT HYD, CAF, TAN, GAL, RSV and CQ to chelate zinc cations in solution, thus reverting their binding with the zinc specific fluorophore FluoZin-3. TPEN was also included as a positive control, to compare with a well-recognized zinc chelator [50]. FluoZin-3 tetrapotassium salt was used to demonstrate the capacity of the flavonoids and of clioquinol to form complexes with zinc. First, a fluorescence titration curve was constructed using a range of zinc concentrations (from 0 to 1.25 µM) to establish where the fluorescence of 3 µM FluoZin-3 is not saturated by zinc cations. The relative capacity of the polyphenols and clioquinol to quench the zinc-dependent fluorescence of FluoZin-3 at 1 µM zinc concentration was then monitored. The final concentration of test substances in the competition reaction with FluoZin-3 was 10 µM.

Briefly, 3 µM FluoZin-3 was mixed with 1 µM zinc in PBS (0.01 M, pH 7.4) and incubated for 15 minutes at room temperature to facilitate formation of the zinc-complex. Subsequently, 10 µM (final concentration) of the test substances (PYR, QCT, EGCG, GEN, TAX, LUT, PHLO, CAT, NAR, RUT, CAT HYD, CAF, TAN, GAL, RSV, CQ or TPEN), were added, mixed vigorously and incubated at 37 °C for 30 minutes under shaking conditions, protected from light. All fluorescence measurements were performed in an Eclipse fluorescence spectrophotometer from Varian coupled with a Cary temperature controller at 25 °C using quartz cuvettes with 1 cm path length and with a maximum volume of 150 μL. The excitation and emission wavelengths used were 494 nm and 516 nm with slits of 5 nm.

2.3. Preparation of FluoZin-3 loaded liposomes

Liposomes were prepared using the curvature-tuned method previously reported [51]. Briefly, FluoZin-3 (final concentration 3 µM) was mixed with 2 mL of PBS (0.01 M, pH 7.4) in a glass reactor protected from light-induced degradation, under stirring conditions and bubbling argon gas. After 15 minutes, a previously homogenized mixture of DPPC and cholesterol (9:1 molar ratio) in 3 mL PBS was added and maintained under stirring conditions and argon at 25 °C for another 15 minutes. The homogeneous mixture was then subjected to a rapid pH jump from pH 7.4 to pH 11, and then back to pH 7.4 within a 3 seconds frame, followed by an equilibration step of 25 minutes where lipid clusters curl into liposomes entrapping the buffer containing the FluoZin-3 molecules. The resulting FluoZin-3-loaded liposomes were purified to remove any unencapsulated material by size exclusion chromatography (SEC) using a Sephadex G-100 column and the size and charge of the formed liposomes was determined using dynamic light scattering (DLS) and zeta-potential. Prepared liposomes were used immediately.

2.4. Liposome assay to assess zinc ionophore activity

The zinc ionophore activity of the different polyphenols and clioquinol was demonstrated by the increase in zinc-dependent fluorescence of FluoZin-3 loaded liposomes. Freshly prepared liposomes loaded with FluoZin-3 were placed in separate vials and their fluorescence measured. Subsequently, $ZnCl_2$ was added to each solution to a final concentration of 10 µM, the solution was softly vortexed, incubated at 25 ºC for 30 minutes and the fluorescence was measured again. Finally, 50 µM of PYR, QCT, EGCG, GEN, TAX, LUT, PHLO, CAT, NAR, RUT, CAT HYD, CAF, TAN, GAL, RSV or CQ was added to each vial respectively, softly vortexed and allowed to incubate at 25 ºC for 30 minutes under shaking conditions before measuring their fluorescence. The evaluation of the ionophore behavior of each polyphenol was tested in a time-dependent assay to further understand the velocity of the zinc transport. The kinetic experiment was carried out by continuously measuring the fluorescence of the FluoZin-3 loaded liposomes over a period of time of 70 minutes, with the addition of $ZnCl_2$ (final 10 µM) after 5 minutes and the addition of the test substances (50 µM) to each cuvette respectively after 10 minutes. All fluorescence measurements were performed in an Eclipse fluorescence spectrophotometer from Varian coupled with Cary temperature controller at 25 °C using quartz cuvettes with a 1 cm path length and with a maximum volume of 150 μL. The

excitation and emission wavelengths used were 494 nm and 516 nm with slits of 5 nm. Control experiments were performed by adding to the cuvette FluoZin-3 loaded liposomes with 10 μM $ZnCl_2$ and the solvent used to dissolve the ionophores (final DMSO concentration 0.1 % v/v) after 10 minutes.

3. RESULTS AND DISCUSSION

3.1 Zinc chelation strength of polyphenols in solution

Several polyphenols have been widely reported to chelate metals through their deprotonated hydroxyl groups, in which the oxygen possesses a high charge density offering a strong ligand for metal-binding. As expected, the chelation strength depends on the number of hydroxyl ligands, but also on their proximity, thus bi- or poly-dentate ligands are stronger scavengers than mono-dentate ligands. A detailed structure of the identified chelating groups of each of the polyphenols tested in this work, as well as their classification and food source, is presented in Table 1.

In order to quantify the relative capacity of the phenolic compounds tested in the work reported here (QCT, EGCG, GEN, TAX, LUT, PHLO, CAT, NAR, RUT, CAT HYD, CAF, TAN, GAL and RSV), in addition to the established ionophore (CQ and PYR) and sequestrant (TPEN) agents, to bind zinc cations in aqueous solutions at physiological pH, their capacity to retrieve zinc cations from FluoZin-3-zinc complexes was measured, by monitoring the decrease in zinc-dependent fluorescence emitted by the FluoZin-3-zinc complexes upon addition of the polyphenols to the solution.

Firstly, an assay was carried out showing that the fluorescence of FluoZin-3 (3 μM) increments linearly with increasing amounts of zinc cations up to 1.25 μM Zn^{2+}, where a plateau is reached (Figure 1 inset). Therefore, a zinc concentration of 1 μM was selected as optimal to demonstrate the decrease in fluorescence of 3 μM FluoZin-3-Zn complexes upon addition of the phenolic compounds. As can be seen in Figure 1, all 14 polyphenols, together with clioquinol, pyrithione and TPEN, at 10 μM concentrations, resulted in a decrease in the zinc-dependent FluoZin-3 fluorescence to some extent, due to sequestering of the zinc ions. The highest zinc-chelating strength was observed to be produced by TPEN, followed by CQ, with an almost complete decrease of the FluoZin-3 fluorescent signal. Similarly, PYR caused an almost 80% reduction of the fluorescence signal. These observations are anticipated, as they are well-known powerful zinc chelators.

Regarding the phenolic compounds, TAN surprisingly also quenched 100 % of the fluorescence signal, which can be explained by its' complex molecular structure having 25 hydroxyl groups, most of them positioned in a powerful bi-dentate configuration (Table 1), ready to bind and sequester zinc cations from the fluorescent zinc-FluoZin-3 complex. Regarding the rest of the polyphenols, the fluorescence quenching capacity was smaller and similar, within a range from 30 to 15%, and in agreement with the number and position metal-binding sites of present on each compound. For example, the flavonoids having -OH groups positioned together offering a bi-dentate ligand (QCT, EGCG, LUT, GAL, TAX) instead of having the -OH groups positioned on different sides of the molecule (NAR, PHLO, GEN), were observed to have a higher chelating strength. However, containing more hydroxyl groups alone does not result in improved chelation. As it was observed there are other important factors affecting the chelating strength, including the three-dimensional conformation of the potential binding groups, as well as the formed stoichiometry between the polyphenols and the metal. Therefore, the structure of the studied polyphenols does not always correlate with their chelating efficiency. The very low capacity of some of the studied polyphenols to quench the FluoZin-3 fluorescence despite containing several -OH groups, as well as some compounds being observed to have a high ability to chelate zinc ions, whilst only possessing few hydroxyl groups can thus be attributed to their 3-D conformation and stoichiometry.

3.2. Zinc ionophore activity of polyphenols

Polyphenols are known to interact with lipid bilayers and actively modify their membrane fluidity [52-54]. It is believed that the fluidization of the bilayer is due to the intercalation of the lipophilic domains of the molecules within the ordered structure of the lipid membranes. This interaction strongly depends on several characteristics of the polyphenolic molecule, such as its' degree of hydroxylation and their stereochemistry, the polarity and the 3-D structural features. However, the transport phenomena of molecules across lipid membranes is still not fully understood. The permeabilization of the membrane to low-molecular-weight molecules by ionophore molecules has been suggested to also be dependent on the concentration of monovalent ions thus creating a gradient of ions and modifying the membrane potential, thus inducing its' depolarization [55,56]. In addition, other several factors may play an important role and modulate the ionophore strength, such as the type of ion-ionophore complex formed, the different ratios, the kinetic reaction

of complexation/decomplexation, the ion-ionophore membrane interaction as well as its' transmembrane diffusion constant [57]. For example, Yang *et. al.* reported that the behavior of EGCG was modified due to the formation of zinc-EGCG complexes, resulting in an enhancement of the incorporation of EGCG into the liposome membrane, which could cause the formation of ion passages[58].

A wide number of publications have reported the use of liposomes as simple membrane systems for the demonstration of ion transport across lipid bilayers [59-61]. However, there is only one previous report of the use of a liposomal system to study the ionophore properties of phenolic compounds to demonstrate the zinc ionophore activity of QCT and EGCG [37], where they also investigated that the flavonoid compounds did not induce the release of the liposome load, as was also reported by Ollila *et al* [62].

A simple liposome system with zinc-dependent fluorophore FluoZin-3 encapsulated in the inner cavity was used to determine if the polyphenols can transport zinc across the cell membrane. Using the liposome also limited the transport pathway to be solely due to transmembrane transport as no other transduction mechanisms normally present in cells would contribute to the transport of the zinc cations. The zinc ionophore effect was extrapolated as a function of the increase in fluorescence due to the capacity of the polyphenols to carry zinc cations across the liposome membrane to interact with the encapsulated FluoZin-3. Liposomes with a mean size of 1.3 ± 0.2 μm and a net charge of 0.8 ± 0.2 mV loaded with FluoZin-3 before and after the addition of 10 µM $ZnCl_2$ showed a negligible fluorescence signal due to the impermeability of the DPPC:Cholesterol liposome membrane at 25 ºC to zinc cations. The subsequent addition of the phenolic compounds, caused an increase in the fluorescence signal due to the zinc complexation, transport and consequent interaction with FluoZin-3. The well-reported CQ and PYR zinc ionophores, resulted in a marked increase in the fluorescence signal, as expected, whilst each of the other phenolic compounds studied presented very different ionophore properties (Figure 2). In the case of EGCG and QCT, both presented a notable zinc ionophore activity, EGCG > QCT, as previously reported [37]. In agreement with the results obtained in the chelation assay, the fluorescence increment (Δ) observed, can be clearly correlated with their chelation strength towards zinc. The polyphenols analyzed can be classified into three groups: strong, soft and no zinc ionophore activity. The polyphenols exhibiting a strong ionophore activity include EGCG (36-fold Δ), QCT (18-fold Δ), LUT (12-fold Δ), TAN (12-fold Δ) and GAL (8-fold Δ); those displaying a soft zinc ionophore activity were RUT (4-fold

Δ), TAX (4-fold Δ), CAF (3-fold Δ), CAT HYD (2-fold Δ), CAT (2-fold Δ) and GEN (2-fold Δ), whilst those resulting in no fluorescence were RSV, PHLO and NAR, indicative of a complete lack of zinc ionophore activity. In addition, control experiments demonstrated that the addition of a final concentration of 0.1 % DMSO, as present in the tested samples, did not affect or destabilize the liposomes, thus keeping the FluoZin-3 within the liposomes and not resulting in an increase in fluorescence. Furthermore, TPEN was also tested and displayed a soft zinc ionophore activity as it caused a 10-fold increase in the fluorescent signal (data not shown).

An evaluation of the kinetics of the ionophore behavior of each polyphenol was tested in a time-dependent assay to further understand the zinc transport strength (Figure 3). The results showed that the increase of the zinc-FluoZin-3 fluorescence produced by PYR until reaching the maximum fluorescence was instantaneous. CQ also produced a very high increase in the fluorescence and rapidly achieved the maximum fluorescence reaching equilibrium in less than 15 minutes. Regarding the kinetics of the polyphenols studied, almost none of the ionophore active compounds showed a markedly time-dependent increase of the fluorescence. Only in the case of EGCG and QCT a plateau was reached after ca. 40 and 20 minutes respectively, exhibiting a slower, but efficient, chelation and transport kinetics. In addition, control experiments carried out by adding the different compounds to the FluoZin-3 loaded liposomes in the absence of $ZnCl_2$ did not show any increase in the fluorescence signal (data not shown).

In order to confirm the stability of all the liposomes immediately after the fluorescent experiments, DLS and Zeta potential analysis of the vesicles were carried out and the results are presented in Table 2. The DLS results confirmed the presence of stable liposomes that had not leaked the fluorophore following exposure to the polyphenols, maintaining roughly the same size as compared with the starting FluoZin-3 loaded liposomes (1.3 ± 0.2 μm) or the control sample (FluoZin-3 loaded liposomes with 10 μM $ZnCl_2$ at final 0.1 % v/v DMSO) (1.1 ± 0.2 μm) in the absence of the tested compounds. The surface charge of the liposomes following exposure to the polyphenols also demonstrated that the main net charge of the FluoZin-3 loaded liposomes (0.8 ± 2.2 mV) was not significantly affected by the zinc-complexes as all measurements indicated approximately a zero charge. Both size and charge results confirmed that the liposome vesicles were maintained intact and the fluorescence signal was solely due to the transport of the zinc-ionophore complex through the lipid bilayer.

The compounds analyzed have been demonstrated to interact to different extents with zinc cations in solution, as well sequestering zinc from fluorescent zinc-FluoZin-3 complexes, forming metal-chelation complexes. In addition, polyphenols were also tested as zinc-carriers across a liposome membrane, and not all compounds were observed to be zinc ionophores. The results from the comparison of both chelating strength capacity and ionophore activity are presented in figure 4, highlighting some interesting observations. Generally, the compounds with a higher chelating capacity, also presented a high ionophore activity. However, some of the polyphenols did not follow this general trend. It is important to note that all the ionophore compounds could chelate the molecule, but not all the chelators have the ability to act as ionophores. The physicochemical properties of each of the polyphenols will directly have an effect on its' ionophore activity, consequently defining the compound as metal sequestering agent or ionophore agent. However a deeper study is still needed to fully understand their mechanism of action.

4. CONCLUDING REMARKS

It is confirmed that most of the natural occurring phenolic compounds used in this study have the ability to directly interact and form new structures (complexes) bound with zinc. In many cases these complexes act in a similar way to the control ionophores. We have reported a liposome assay that can be used as a tool the for rapid, high-throughput screening of families of polyphenols. In addition, this liposome system can be used to screen the ionophore activity towards other ions such as Ca, Fe or Mg among others. Zinc-specific FluZin-3 loaded liposomes were used to screen the zinc-ionophore activity of a selected library consisting of the most relevant dietary polyphenols, classified according to their zinc-ionophore strength capacity and their chelation efficiency, giving us a better knowledge of the importance of the structural conformation versus biological activity. Synthetic ionophore molecules are currently being used as potential drugs against several chronic diseases including Alzheimer’s and different types of cancer, and as demonstrated, one of the mechanisms by which polyphenols exert their beneficial activity is by acting as zinc ionophores. Polyphenol-zinc ion complexes are yet to be investigated and more extensive studies are needed in order to elucidate their possible clinical potential.

AUTHOR CONTRIBUTIONS:

G. C. and H. D.-B. equally contributed to this work.

REFERENCES

[1] Colvin R.A., Fontaine C.P., Laskowski M., Thomas D., Zn2+ transporters and Zn2+ homeostasis in neurons. *Eur J Pharmacol* 2003, *479* (1-3), 171-85.

[2] Frederickson C., Imaging zinc: old and new tools. *Sci STKE* 2003, *2003* (182), 18.

[3] Beyersmann D., Haase H., Functions of zinc in signaling, proliferation and differentiation of mammalian cells. *Biometals* 2001, *14* (3-4), 331-41.

[4] Brautigan D.L., Bornstein P., Gallis B., Phosphotyrosyl-protein phosphatase. Specific inhibition by Zn. *J Biol Chem* 1981, *256* (13), 6519-22.

[5] Maret W., Jacob C., Vallee B.L., Fischer E.H., Inhibitory sites in enzymes: zinc removal and reactivation by thionein. *Proc Natl Acad Sci USA* 1999, *96* (5), 1936-40.

[6] Palmiter R.D., Huang L., Efflux and compartmentalization of zinc by members of the SLC30 family of solute carriers. *Pflugers Arch* 2004, *447* (5), 744-51.

[7] Lichtlen P., Schaffner W., Putting its fingers on stressful situations: the heavy metal-regulatory transcription factor MTF-1. *Bioessays* 2001, *23* (11), 1010-7.

[8] Eide D.J., The oxidative stress of zinc deficiency. *Metallomics* 2011, *3* (11), 1124-9.

[9] Laitaoja M., Valjakka J., Jänis J., Zinc coordination spheres in protein structures. *Inorg Chem* 2013, *52* (19), 10983-91.

[10] Quesada I.M., Bustos M., Blay M., et al., Dietary catechins and procyanidins modulate zinc homeostasis in human HepG2 cells. *J Nutr Biochem* 2011, *22* (2), 153-63.

[11] Higashimoto M., Isoyama N., Ishibashi S., et al., Tissue-dependent preventive effect of metallothionein against DNA damage in dyslipidemic mice under repeated stresses of fasting or restraint. *Life Sci* 2009, *84* (17-18), 569-75.

[12] Tapiero H., Tew K.D., Trace elements in human physiology and pathology: zinc and metallothioneins. *Biomed Pharmacother* 2003, *57* (9), 399-411.

[13] Powell S.R., The antioxidant properties of zinc. *J Nutr* 2000, *130* (5S Suppl), 1447S-54S.

[14] Klotz L.-O., Kröncke K.-D., Buchczyk D.P., Sies H., Role of copper, zinc, selenium and tellurium in the cellular defense against oxidative and nitrosative stress. *J Nutr* 2003, *133* (5 Suppl 1), 1448S-51S.

[15] Araújo J.R., Gonçalves P., Martel F., Chemopreventive effect of dietary polyphenols in colorectal cancer cell lines. *Nutr Res* 2011, *31* (2), 77-87.

[16] Leopoldini M., Russo N., Toscano M., The molecular basis of working mechanism of natural polyphenolic antioxidants. *Food Chem* 2011, *125* (2), 288-306.

[17] Čipák L., Rauko P., Miadoková E., Čipáková I., Novotný L., Effects of flavonoids on cisplatin-induced apoptosis of HL-60 and L1210 leukemia cells. *Leuk Res* 2003, *27* (1), 65-72.

[18] Ramos S., Cancer chemoprevention and chemotherapy: dietary polyphenols and signalling pathways. *Mol Nutr Food Res* 2008, *52* (5), 507-26.

[19] Bravo L., Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutr Rev* 1998, *56* (11), 317-33.

[20] Russo M., Spagnuolo C., Tedesco I., Bilotto S., Russo G.L., The flavonoid quercetin in disease prevention and therapy: facts and fancies. *Biochem Pharmacol* 2012, *83* (1), 6-15.

[21] Hider R.C., Liu Z.D., Khodr H.H., Metal chelation of polyphenols. *Methods Enzymol* 2001, *335*, 190-203.

[22] Cao B., Li J., Zhu J., et al., The antiparasitic clioquinol induces apoptosis in leukemia and myeloma cells by inhibiting histone deacetylase activity. *J Biol Chem* 2013, *288* (47), 34181-9.

[23] Cao B., Li J., Zhou X., et al., Clioquinol induces pro-death autophagy in leukemia and myeloma cells by disrupting the mTOR signaling pathway. *Sci Rep* 2014, *4*, 5749.

[24] Grazul M., Budzisz E., Biological activity of metal ions complexes of chromones, coumarins and flavones. *Coord Chem Rev* 2009, *253* (21), 2588-2598.

[25] Thompson M., Williams C.R., Elliot G.E., Stability of flavonoid complexes of copper(II) and flavonoid antioxidant activity. *Anal Chim Acta* 1976, *85* (2), 375-81.

[26] Mladenka P., Zatloukalová L., Filipský T., Hrdina R., Cardiovascular effects of flavonoids are not caused only by direct antioxidant activity. *Free Radical Bio Med* 2010, *49* (6), 963-75.

[27] Aherne S.A., O'Brien N.M., Mechanism of protection by the flavonoids, quercetin and rutin, against tert-butylhydroperoxide- and menadione-induced DNA single strand breaks in Caco-2 cells. *Free Radical Bio Med* 2000, *29* (6), 507-14.

[28] Afanas'eva I.B., Ostrakhovitch E.A., Mikhal'chik E.V., Ibragimova G.A., Korkina L.G., Enhancement of antioxidant and anti-inflammatory activities of bioflavonoid rutin by complexation with transition metals. *Biochem Pharmacol* 2001, *61* (6), 677-684.

[29] Fernandez M.T., Mira M.L., Florêncio M.H., Jennings K.R., Iron and copper chelation by flavonoids: an electrospray mass spectrometry study. *J Inorg Biochem* 2002, *92* (2), 105-11.

[30] Mira L., Fernandez M.T., Santos M., et al., Interactions of flavonoids with iron and copper ions: a mechanism for their antioxidant activity. *Free Radical Res* 2002, *36* (11), 1199-208.

[31] Payán-Gómez S.A., Flores-Holguín N., Pérez-Hernández A., Piñón-Miramontes M., Glossman-Mitnik D., Computational molecular characterization of the flavonoid Morin and its Pt(II), Pd(II) and Zn(II) complexes. *J Mol Model* 2011, *17* (5), 979-85.

[32] Wei Y., Guo M., Zinc-Binding Sites on Selected Flavonoids. *Biol Trace Elem Res* 2014, *161* (2), 223-30.

[33] Selvaraj S., Krishnaswamy S., Devashya V., Sethuraman S., Krishnan U.M., Flavonoid-Metal Ion Complexes: A Novel Class of Therapeutic Agents. *Med Res Rev* 2013, *34* (4), 677-702.

[34] Ding W.-Q., Liu B., Vaught J.L., Yamauchi H., Lind S.E., Anticancer activity of the antibiotic clioquinol. *Cancer Res* 2005, *65* (8), 3389-95.

[35] Feng P., Li T., Guan Z., Franklin R.B., Costello L.C., The involvement of Bax in zinc-induced mitochondrial apoptogenesis in malignant prostate cells. *Mol Cancer* 2008, *7* (1), 25.

[36] Liang J.Y., Liu Y.Y., Zou J., et al., Inhibitory effect of zinc on human prostatic carcinoma cell growth. *Prostate* 1999, *40* (3), 200-7.

[37] Dabbagh-Bazarbachi H., Clergeaud G., Quesada I.M., et al., Zinc Ionophore Activity of Quercetin and Epigallocatechin-gallate: From Hepa 1-6 Cells to a Liposome Model. *J Agr Food Chem* 2014, *62* (32), 8085-8093

[38] Chen L., Wu C., Li J., Study on Scavenging Activities of Free Radical by Luteolin-Zn (II) Complex. *Chem Adhes* 2009.

[39] Wang H.-L., Yang Z.-Y., Wang B., Synthesis, Characterization and the Antioxidative Activity of Copper(II), Zinc(II) and Nickel(II) Complexes with Naringenin. *Transit Met Chem* 2006, *31* (4), 470-474.

[40] Harper A., Kerr D.J., Gescher A., Chipman J.K., Antioxidant effects of isoflavonoids and lignans, and protection against DNA oxidation. *Free Radical Res* 1999, *31* (2), 149-160.

[41] Bodini M.E., del Valle M.A., Tapia R., Leighton F., Berrios P., Zinc catechin complexes in aprotic medium. Redox chemistry and interaction with superoxide radical anion. *Polyhedron* 2001, *20* (9-10), 1005-1009.

[42] Le Nest G., Caille O., Woudstra M., et al. Zn–polyphenol chelation: complexes with quercetin, (+)-catechin, and derivatives: II Electrochemical and EPR studies. *Inorganica Chim Acta* 2004, *357* (7), 2027-2037.

[43] Yu H.-N., Shen S.-R., Yin J.-J., Effects of metal ions, catechins, and their interactions on prostate cancer. *Crit Rev Food Sci Nutr* 2007, *47* (8), 711–9.

[44] Bai Y., Song F., Chen M., et al., Characterization of the rutin-metal complex by electrospray ionization tandem mass spectrometry. *Anal Sci* 2004, *20* (8), 1147-51.

[45] De Souza R.F., De Giovani W.F., Antioxidant properties of complexes of flavonoids with metal ions. *Redox Rep* 2004, *9* (2), 97-104.

[46] Donracheva L.G., Mel'nikova N.B., Pegova I.A., et al., Complexes of dihydroquercetin with chromium (III) and zinc (II) asparaginates in water and their effects on the state of lecithin membranes. *Pharm Chem J* 2009, *42* (10), 564-570.

[47] Andjelkovic M., Vancamp J., Demeulenaer B., et al., Iron-chelation properties of phenolic acids bearing catechol and galloyl groups. *Food Chem* 2006, *98* (1), 23-31.

[48] Chiavarino B., Crestoni M.E., Fornarini S, et al., Infrared spectroscopy of copper-resveratrol complexes: a joint experimental and theoretical study. *J Chem Phys* 2012, *137* (2), 024307.

[49] Almeida W.L.C., Vítor D.N., Pereira M.R.G., et al., Redox properties of Ruthenium complex with catechol are involved in toxicity to glial cells. *J Chil Chem Soc* 2007, *52* (3), 1240-1243.

[50] Blindauer C.A., Razi M.T., Parsons S., Sadler P.J., Metal complexes of N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN): Variable coordination numbers and geometries. *Polyhedron* 2006, *25* (2), 513-520.

[51] Genç R., Ortiz M., O'Sullivan C.K., Curvature-Tuned Preparation of Nanoliposomes. *Langmuir* 2009, *25* (21), 12604-12613.

[52] Arora A., Byrem T.M., Nair M.G., Strasburg G.M., Modulation of Liposomal Membrane Fluidity by Flavonoids and Isoflavonoids. *Arch Biochem Biophys* 2000, *373* (1), 102-109.

[53] Saija A., Scalese M., Lanza M., et al., Flavonoids as antioxidant agents: Importance of their interaction with biomembranes. *Free Radical Bio Med* 1995, *19* (4), 481-486.

[54] Yu X., Chu S., Hagerman A.E., Lorigan G.A., Probing the Interaction of Polyphenols with Lipid Bilayers by Solid-State NMR Spectroscopy. *J Agr Food Chem* 2011, *59* (12), 6783-6789.

[55] Alonso M.A., Carrasco L., Action of Membrane-Active Compounds on Mammalian Cells. *Eur J Biochem* 1980, *109* (2), 535-540.

[56] Alonso M.A., Carrasco L., Molecular basis of the permeabilization of mammalian cells by ionophores. *Eur J Biochem* 1982, *127* (3), 567-9.

[57] Erdahl W.L., Chapman C.J., Wang E., Taylor R.W., Pfeiffer D.R., Ionophore 4-BrA23187 Transports Zn2+ and Mn2+ with High Selectivity Over Ca2+. *Biochemistry* 1996, *35* (43), 13817-13825.

[58] Yang J.G., Yu H.N., Sun S.L., et al., Epigallocatechin-3-gallate affects the growth of LNCaP cells via membrane fluidity and distribution of cellular zinc. *J Zhejiang Univ-Sc B* 2009, *10* (6), 411-21.

[59] Weissmann G., Anderson P., Serhan C., Samuelsson E., Goodman E., A general method, employing arsenazo III in liposomes, for study of calcium ionophores: results with A23187 and prostaglandins. *P Natl Acad Sci USA* 1980, *77* (3), 1506-1510.

[60] Kolber M.A., Haynes D.H., Fluorescence Study of the Divalent Cation-Transport Mechanism of Ionophore A23187 in Phospholipid-Membranes. *Biophys J* 1981, *36* (2), 369-391.

[61] Mathew M.K., Nagaraj R., Balaram P., Ionophore-mediated transmembrane movement of divalent cations in small unilamellar liposomes: An evaluation of the chlortetracycline fluorescence technique and correlations with black lipid membrane studies. *J Membrane Biol* 1982, *65* (1-2), 13-17.

[62] Ollila F., Halling K., Vuorela P., Vuorela H., Slotte J.P., Characterization of Flavonoid-Biomembrane Interactions. *Arch Biochem Biophys* 2002, *399* (1), 103-108.

Table 1. Summary of the phenolic compounds used within this work and divided according to class, food source, chemical structure with the proposed chelating groups highlighted and the number of hydroxyl groups present.

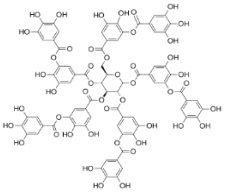
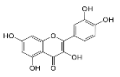
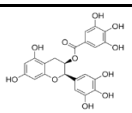
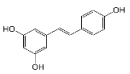
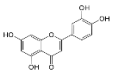
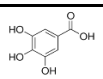
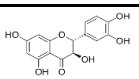
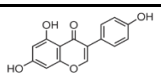
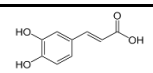
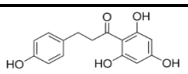
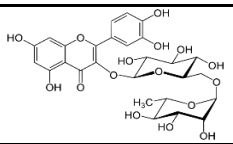
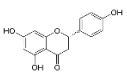
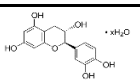
Compound	Class	Dietary source		Structure	# Hydroxyl groups
Tannic acid	Hydroxybenzoic acids	-Grape -Persimmon -Black berry -Myrobalan	-Walnut -Maple -Sumac -Tarragon		25
Quercetin	Flavonols	-Cocoa -Onion -Elderberry	-Wine -Black plum -Kale		5
(–)-Epigallocatechin gallate (EGCG)	Flavanols (Flavan-3-ols)	-Green tea -Black tea -Apple	-Blackberry -Hazelnut -Pecan nut		8
Resveratrol	Stilbenes	-Muscadine grape -Lingonberry	-Cranberry -Red wine		3
Luteolin	Flavones	-Olive -Mexican oregano	-Globe artichoke -Green pepper		4
Gallic acid	Hydroxybenzoic acids	-Chestnut -Black tea	-Blackberry -Green chicory		4
Dihydroquercetin (Taxifolin)	Dihydroflavonols	-Mexican oregano	-Acai berry		5
Catechol	Other polyphenols	-Argan oil -Coffee beverage	-Cocoa		2
Genistein	Isoflavonoids	-Soy	-Red clover		3
Caffeic acid	Hydroxycinnamic acids	-Coffee -Black chokeberry	-Plum		3
Phloretin	Dihydrochalcones	-Apple	-Apricot		4
Rutin	Flavonol	-Buckwheat -Black olive -Black tea	-Plum -Capers -Tomato		11
Naringenin	Flavanones	-Mexican oregano -Grapefruit	-Orange -Tomato		3
Catechin hydrate	Flavonol	-Cocoa -Strawberry	-Grape -Broad bean		5

Table 2. Dynamic light scattering and Zeta-potential measurements of the liposomes loaded with FluoZin-3, as well as the fluorescence increment (Δ) caused by each compound, after the treatment with 10 μM $ZnCl_2$ and polyphenols, clioquinol, pyrithione and TPEN at 50 μM (0.1 % DMSO final concentration). Standard deviations were calculated from the mean data of a series of experiments (n ≥ 3).

Compound	Δ Fluorescence[a]	Liposome parameters	
		Size (μm)	Charge (mV)
Clioquinol (CQ)	57.2	1.3 ± 0.2	2.4 ± 2.9
Pyrithione (PYR)	53.6	1.4 ± 0.1	0.8 ± 1.1
Epigallocatechin-gallate (EGCG)	35.5	1.3 ± 0.1	1.6 ± 2.5
Quercetin (QCT)	18.1	1.3 ± 0.1	2.1 ± 1.8
Luteolin (LUT)	12.2	1.4 ± 0.3	1.1 ± 0.8
Tannic Ac. (TAN)	12.2	1.2 ± 0.1	-2.1 ± 3.6
TPEN	10.4	1.1 ± 0.2	0.0 ± 2.6
Gallic Ac. (GAL)	7.5	1.3 ± 0.3	-3.5 ± 2.8
Rutin (RUT)	4.3	1.6 ± 0.3	-2.0 ± 3.3
Taxifolin (TAX)	4.3	1.0 ± 0.3	3.6 ± 3.0
Caffeic Ac. (CAF)	2.5	1.4 ± 0.4	3.6 ± 1.6
Catechin (CAT Hyd)	2.4	1.2 ± 0.2	3.3 ± 0.6
Catechol (CAT)	1.9	1.4 ± 0.3	2.1 ± 1.3
Genistein (GEN)	1.8	1.4 ± 0.4	-2.1 ± 2.2
Phloretin (PHLO)	1.3	1.4 ± 0.2	2.6 ± 1.1
Resveratrol (RSV)	1.1	1.3 ± 0.2	-1.5 ± 4.1
Naringenin (NAR)	1.1	1.3 ± 0.2	4.6 ± 1.2
Control[b]	0.9	1.1 ± 0.2	-2.1 ± 3.1

[a] Increment of fluorescence is calculated by the signal obtained from the FluoZin-3 loaded liposomes in the presence of 10 μM $ZnCl_2$ and the respective compound at 50 μM divided by the signal obtained from the FluoZin-3 loaded liposomes with 10 μM $ZnCl_2$.
[b] Control contains the FluoZin-3 loaded liposomes with $ZnCl_2$ (10 μM) in the solvent vehicle (0.1 % DMSO)

Figures

A simple liposome assay for the screening of zinc ionophore activity of polyphenols

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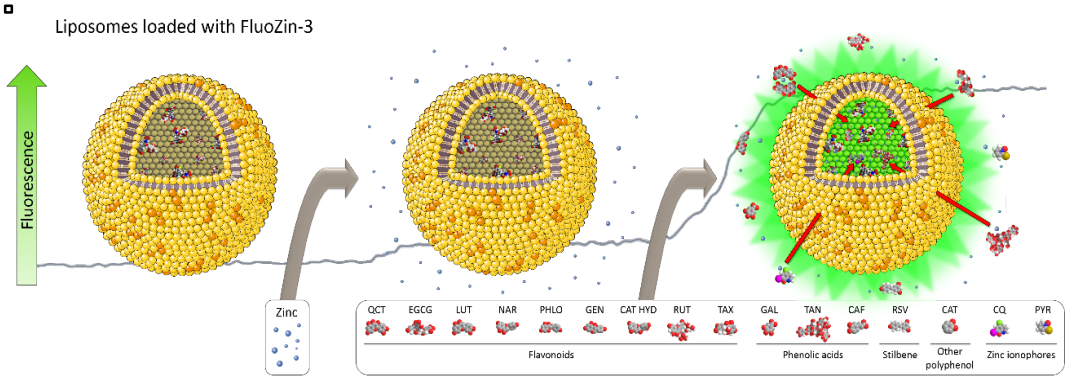


Figure 1. Chelation strength of zinc cations by the polyphenols, clioquinol, pyrithione and TPEN in solution. The quenching of the zinc-dependent fluorescence of FluoZin-3 indicates the capacity of the compounds at 10 µM to retrieve zinc cations from the zinc-FluoZin-3 complex formed between 3 µM FluoZin-3 and 1 µM $ZnCl_2$. Inset 3 µM FluoZin-3 calibration plot. λ_{ex} 494nm / λ_{em} 516nm. All values are means ± SD of three independent experiments.

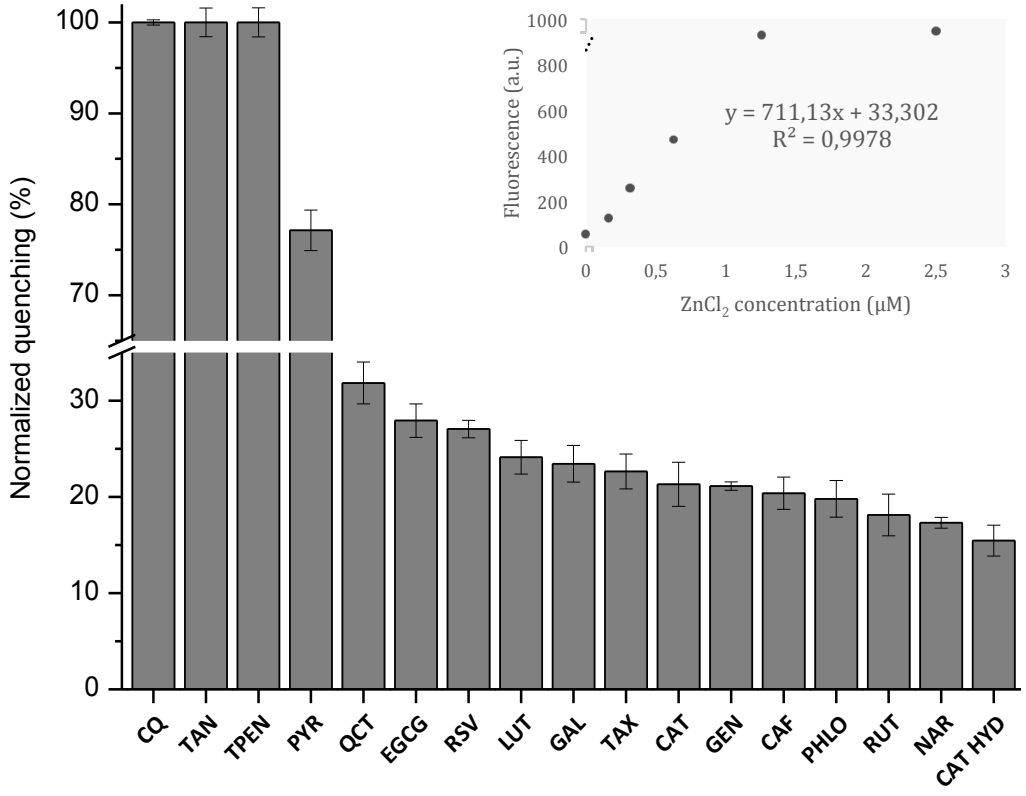


Figure 2. Liposome assay for the determination of zinc ionophore activity of polyphenols, clioquinol and pyrithione. The increase in the zinc-dependent fluorescence of FluoZin-3 indicates the capacity of the compounds at 50 µM to interact with 10 µM zinc cations, transport them across the liposome membrane and present them to 3 µM FluoZin-3 in the liposome inner cavity. Control are FluoZin-3 loaded liposomes in the presence of 10 µM zinc cations with 0.1% DMSO. FluoZin-3 loaded liposomes in the absence of zinc (Lipo-FZ3) and in the presence of 10 µM $ZnCl_2$ (Zn10). λ_{ex} 494nm / λ_{em} 516nm. All values are means ± SD of three independent experiments.

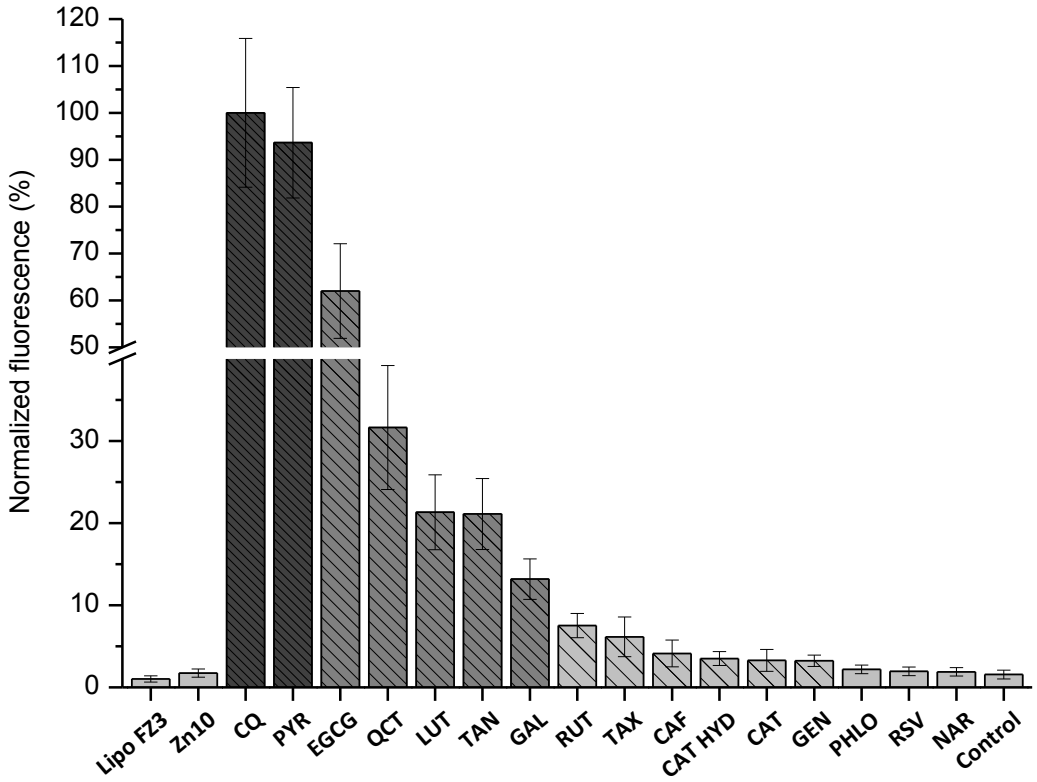


Figure 3. Time-dependent fluorescence emission of FluoZin-3 loaded liposomes before (minute 0) and after (minute 5) addition of 10 μM $ZnCl_2$. The fluorescence increased upon the addition of the compounds (50 μM) at minute 10, and the fluorescence was monitored over the period of an hour. λ_{ex} 494nm / λ_{em} 516nm. Results are representative of at least three experiments. In the control sample, only the solvent used to dissolve the ionophores (0.1 % DMSO) was added at minute 10 without showing any fluorescent increase.

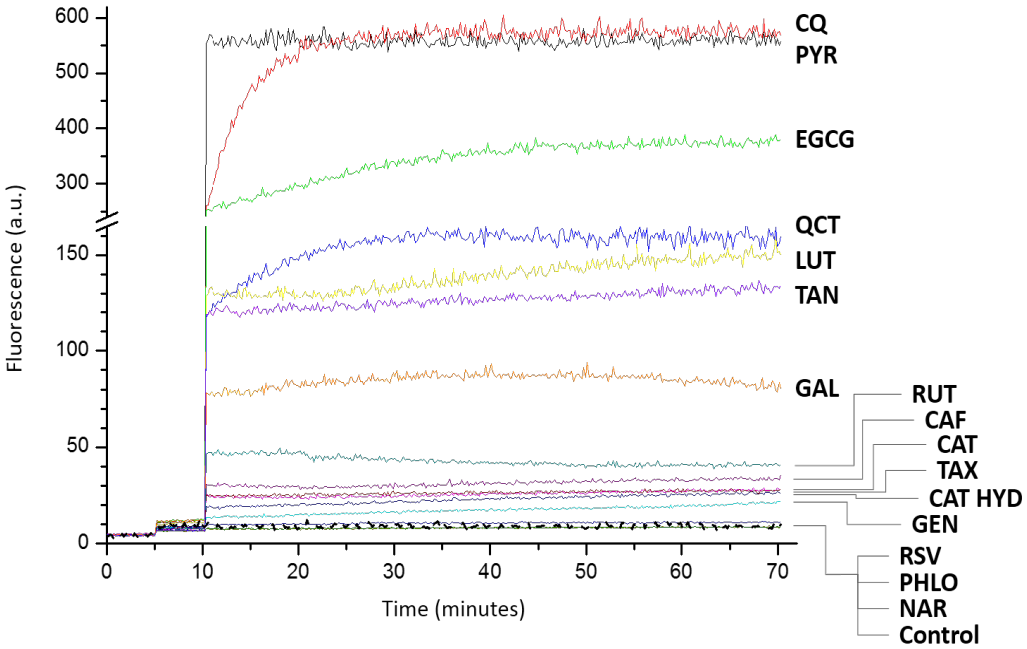
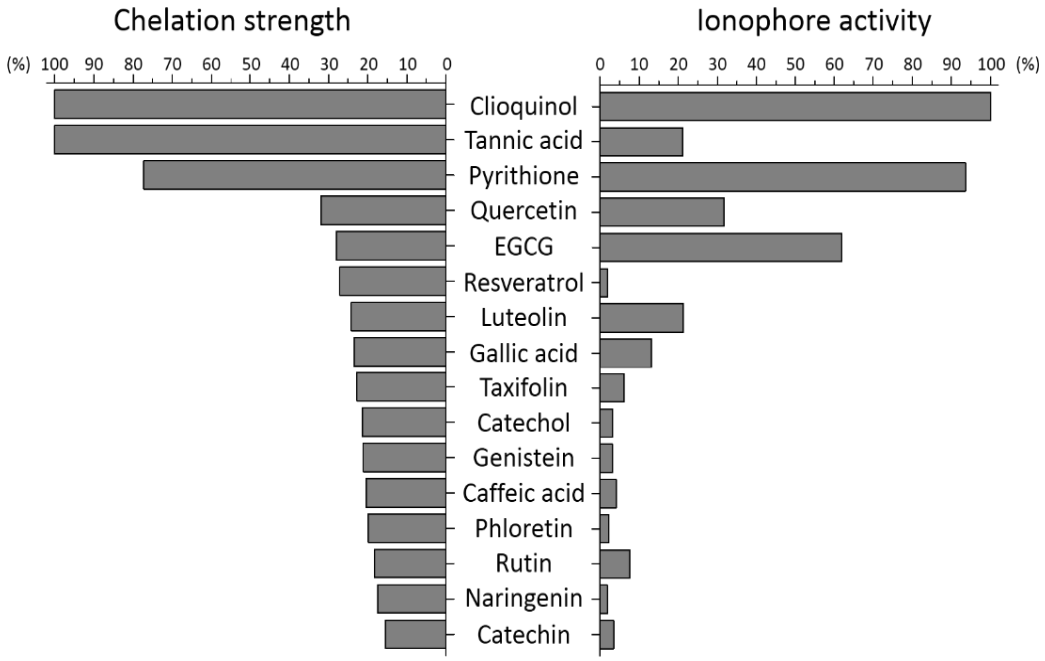


Figure 4. Schematic comparison between zinc chelating strength and ionophore activity.



5. Zinc-dependent pro-apoptotic activity of Quercetin in hepatocarcinoma Hepa 1-6 cells

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Manuscript in preparation

Our previous work confirmed that the administration of quercetin together with additional zinc to hepatoma cells boost the gene expression of the zinc-store protein metallothionein and the zinc-export transporter ZnT1. Moreover, total intracellular zinc content and cytoplasmic labile zinc are increased. Western-blot analyses show that quercetin, greatly enhance the zinc-induced phosphorylation of Akt. QCT also was confirmed to act as a zinc ionophore, increasing rapidly the intracellular concentrations of labile cytoplasmic zinc.

The aim ofthis work is to evaluate the effects of combined quercetin-zinc administration on proliferation and apoptosis the hepatoma cell line model Hepa 1-6. MTT viability assays were carried, as well as the avaluation of the nuclar morphology in order to check our hypothesis. Additionally, DNA fragmentation was evaluated by electroforesis analisys.

The results confirmed that Appropriate combinations of zinc and quercetin result in morphological changes characteristics of apoptotic processes, as revealed by propidium iodide staining. The electroforesis assay confirmed those results, exhibiting disctitive apoptotic ladder patters. Polyphenol-zinc complexes may be useful for controlling multiple cellular processes such as cancer.

Zinc-dependent pro-apoptotic activity of Quercetin in hepatocarcinoma Hepa 1-6 cells

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List of Abreviations:

Zinc (Zn), metallothionein (MT), Pyrithione (Pyr), N,N,N',N'-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN), Clioquinol (CQ), quercetin (QCT), Zinc chloride ($ZnCl_2$) dimethyl sulfoxide (DMSO), propidium iodide (PI), phosphate buffered saline (PBS).

Keywords: Quercetin, zinc, apoptosis, ionophores, polyphenols

ABSTRACT

Zinc is the second most imporant transition metal after iron, found in organs, tissues, bones, fluids, and cells. It's appropriate intracellular homeostasis is essential for the correct function and cellular viability. Metal-binding compounds have been shown to exert anticancer action and are being evaluated clinically as anticancer agents. The modulation of intracellular zinc concentrations through metal sequestrants or metal ionophores are known to kill cells via apoptosis. Quercetin, a metal chelator and zinc ionophore, exert differential effects on cell viability when combined with zinc. Morphological changes suggest that this combination might be inducing apoptosis to the mouse hepatoma Hepa 1-6 cell line. DNA fragmentation has been confirmed by electrophoresis analysis. The results suggest that zinc-quercetin complex could be used together as a new potential anticancer agent.

1. INTRODUCTION

Apoptosis is an active, energy dependent process of cellular self-destruction characterized by a distinct set of morphological and biochemical changes, including apoptotic body formation, nuclear condensation, and chromosomal DNA fragmentation [1]. It is a controlled biological mechanism required for the removal and deletion of unessential or damaged cells in response to a harmful agent. Contrasting the cellular "homicide" which occurs during necrotic cell death, apoptosis It could be described as a cellular "suicide" [2]. It can be activated through an intracellular mechanism with a genetically defined developmental program, or by extracellular stimuli thru proteins, cytokines, hormones, or drugs.

Zinc (Zn), one of the most important trace elements in the organism, is involved in the stabilization and catalysis of over 300 enzymes and a higher number of metalloproteins [3,4]. Advances in analytical methods for isolating proteins and measuring metals in biological material, led to the discovery of other zinc proteins whose importance emerged more recently including proteins for zinc signaling, transport, buffering, and storage. As the molecular mechanisms of cellular zinc homeostasis are just beginning to be elucidated, the number of these proteins and thus the size of zinc proteomes is likely to be larger than

what is currently realized [5,6]. Four main groups described for zinc coordination environments in proteins: catalytic, cocatalytic, structural and protein interface [7]. The chemical properties of zinc different from other transition metals, such as copper and iron which display several different oxidation states in biological systems, is that zinc exists as a redox inert Zn^{2+} cation, which does not undergo redox reactions at physiological redox potentials [8,9]. Additionally, zinc can induce the expression and maintaining the levels of potentially radical scavenging proteins such as metallothionein (MT), the major zinc binding protein associated with zinc homeostasis [10], DNA protection, oxidative stress, and apoptosis [11,12]. It can act through stabilization of cell membranes [13] or as a structural component of antioxidant enzymes [14]. Although it has been studied for many decades, the molecular bases are still not so clear.

Zinc dyshomeostasis can induce cell death. However, the mechanisms involved have not been fully elucidated. Studies in prostate cancer cells (PCa) have revealed that an excess of zinc mediated by a zinc ionophore agent such as Pyrithione (Pyr) stimulated cell death at low micromolar concentrations. Then same effects were observed after using the zinc sequestrant N,N,N',N'-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) [15]. Other studies have reported that both zinc deficiency and zinc overloading trigger cell death, nevertheless acting through different signaling mechanisms and resulting in different patterns of cellular death. Zinc chelation resulted in apoptotic cell death, while zinc overload led to a mixed death pattern, comprised of both necrosis and apoptosis. [16]

Further reports have suggested that zinc-binding molecules may have anticancer effects inducing apoptosis acting via different mechanisms [17]. The first and more common, occurs when cells are deprived of zinc, principally under the action of ROS excess; this mechanism involve the formation of a complex with a zinc sequestrant, such as TPEN, making zinc ions non available to the cell [16]. Another mechanism includes zinc transportation into cells through specific Ionophores [18]. Zinc becomes cytotoxic if its extracellular concentration exceeds the capacity of the Zn homeostatic system. Elevated extracellular Zn concentrations lead to the breakdown of the Zn transporting system of the plasma membrane. The resulting enhanced intracellular Zn concentration activates the apoptotic machinery [19] One of the most important ionophore agents being studied due to its anticancer properties is the drug Clioquinol (CQ) [20].

Diet is a lifestyle factor that plays a major role in the primary and secondary prevention of several chronic diseases. There is an abundance of evidence that regular consumption of fruits and vegetables is associated with a reduced risk of chronic and degenerative pathologies. In the last decades significant number of studies have focused in dietary phenolic compounds as natural improvers of health. More than 8000 dietary polyphenols have been identified [21]. This growing interest for this compounds resides in the accumulating evidence for their ability to trigger several cellular pathways leading to the prevention and/or amelioration of pathological conditions acting as Antioxidants [22], anticarcinogenics [23,24], anti-inflammatories [25], neuroprotectors [26], antilipidemic and vasorelaxing among others [21]. There is an emerging view that flavonoids and their *in vivo* metabolites do not act only as conventional antioxidants but may also exert modulatory actions on cellular system through direct action on various signalling pathways [27].

In the last decades, phenolic compounds were known to interact with different metals, and because of their distinctive chemical structure, they can easily form complexes through metal ion chelation [28] in a comparable way of other well-known metal chelators such as the anticancer drug Clioquinol [29–31]. Further studies have revealed that polyphenols not only interact with metal ions, but they deeply modulate expression of MTs, cellular zinc transporters, extracellular zinc carriers, and intracellular zinc accumulation which are key factors in zinc homeostasis [10]. In addition, an increase of Zinquin (fluorescent specific zinc indicator)-detectable cytoplasmic levels of zinc in HepG2 cell line was monitored when treated with phenolic compounds [10]. This increment in intracellular zinc levels have been reported to induce apoptosis of different tumoral cells [30,32]. Quercetin (QCT), a water-insoluble flavonoid present in onions, nuts, and many other vegetables is one of the most abundant polyphenols distinctive of the Mediterranean Diet. Previous reports presented how QCT profoundly alter zinc homeostasis in cultured human and mouse hepatoma cells, enhancing total zinc accumulation as well as MT and ZnT1 expression (M. Bustos, personal communication, 2011, Universitat Rovira i Virgili). Lately, QCT has been classified as a zinc ionophore molecule, increasing the intracellular labile zinc quantities in a comparable way of other zinc ionophores such as Pyr and CQ [33].

In the present study, the combined effect of QCT and zinc on the viability and apoptosis induction in Hepa 1-6 cells has been investigated. MTT was used to determine cellular

viability, whereas cell death was detected and identified using morphologic studies, propidium iodide staining and DNA electrophoresis analyses.

2. MATERIALS AND METHODS

2.1. Materials

Zinc chloride ($ZnCl_2$), quercetin (QCT), TPEN [N,N,N′,N′-tetrakis(2-phridylmethyl) ethylenediamine], dimethyl sulfoxide (DMSO), MTT (Thiazolyl Blue Tetrazolium Bromide), ethanol, 2-propanol, propidium iodide (PI) and phosphate buffered saline (0.01M PBS, pH 7.4) were purchased from Sigma-Aldrich. RNase A (10mg/mL) and Proteinase K (recombinant) were purchased from Thermo Scientific. A Simplicity 185 Millipore-Water System was used to obtain Milli-Q water (18.2 mΩ.cm-1) for the preparation of buffers. QCT was dissolved as 100 μM solutions in 100% DMSO and aliquoted of at -20ºC. $ZnCl_2$ was stored as 0,5 M solution in ethanol/Milli-Q water (50%/50% v/v).

2.2. Cell cultures and treatments

The mouse hepatoma cell line Hepa 1-6 was obtained from the European Collection of Cell Cultures (BW7756 ECACC) and propagated in Dulbecco's Modified Eagle medium (DMEM; BioWittaker) supplemented with 10% fetal bovine serum (BioWittaker), 2 mM glutamine in 0.85% NaCl, Penicillin/Streptomycin 1000 U/mL and 1.25 M HEPES. Cells were cultured at 37 °C in a humidified, 5% CO_2-enriched atmosphere and routinely split every 3-4 days at a 1:5 ratio upon reaching approximately 80% confluence. For treatments, cells at 80% confluence were detached with Accutase© (Sigma-Aldrich) and resuspended at a density of 5×10^5 cells/mL; 1000 μL of this cell suspension was then seeded per well in 12-well plates (Orange Scientific) and 100 μL in 96-well plates (for MTT assay). Twenty-four hours after plating, medium was removed and cells were treated by adding 1000 µL of fresh medium containing either TPEN, $ZnCl_2$, QCT or the combination of $ZnCl_2$ with QCT for 24 hours. As a control treatment, cells were incubated just with medium (final 0.1% DMSO and 0.05% ethanol).

2.3. Cell viability assay

Cell viability was analyzed with a modified tetrazolium assay using MTT reagent following the manufacturer's protocol. This method is based on the conversion of MTT to formazan

by certain mitochondrial enzymes [34]. In brief, after 24 hours treatment, the media was removed and 250 μL of fresh media containing MTT was added to all treated cells. Then, Hepa 1-6 cells were incubated at 37ºC under dark conditions for 4 hours. After that, the MTT containing media was removed and replaced by 225 μL of DMSO-glycine buffer. Finally, the plate was shaken vigorously and absorbance at 570 nm and 690 nm was detected.

2.4. Propidium iodide staining

PI binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4-5 base pairs of DNA. PI also binds to RNA, requiring treatment with nucleases to distinguish between RNA and DNA staining. When bound to nucleic acids, the absorption maximum for PI is 535 nm and the fluorescence emission maximum is 617 nm. After the treatments, media was removed and cells were washed twice with 1 mL per well of PBS. Cells were then fixed for 1 hour at room temperature and washed again with PBS. Finally, 1 mL per well of a PBS solution containing PI and RNase A was added to the cells and incubated for 30 min minutes at 37ºC. Cells were visualized under a Nikon Eclipse TE 2000-S fluorescent microscope.

2.5. DNA laddering assay

Internucleosomal DNA fragmentation was analyzed by agarose gel electrophoresis. Media containing treatments was collected in different 15 mL falcon tubes. Cells were detached with 200 μL Accutase per well and added over treated media. Falcon tubes were centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and the pellet resuspended with 1 mL lysis (TE/triton) buffer and incubated for 30 minutes on ice. After that, pellets were centrifuged again at 14000 G for 5 minutes under cold conditions (4ºC). The supernatant was resuspended with 20 μL RNase A and 10 μL Proteinase K and incubated at 50ºC for 30 minutes, pellet was discarded. 500 μL of Isopropanol and 150 μL of NaCl 5M were added and let DNA precipitation overnight at -20ºC. The mixture was then centrifuged at 14000 G for 15 minutes and the supernatant discarded. Finally, 100 μL of TE buffer were added. After the determination and quantification of DNA, 5μL of loading buffer (0.25% bromophenol blue, 30% glycerol) was added to the samples and loaded on a 2% agarose gel and run at 80V (Constant V) for about 4 hours. DNA was detected by ethidium bromide under UV light.

3. RESULTS AND DISCUSSION

3.1. MTT cell viability assay

In order to test the viability of Hepa 1-6 cells treated with our compounds, an MTT assay was performed. Cells were treated for 24 hours with QCT 50, 100, 200, 400 and 500 μM (Fig.1A). It was observed how the viability decreased rapidly for QCT concentrations over 50 μM. For QCT 100 μM it was observed a viability nearby 80%, which means that this treatment begins to become harmful for the cells, however is not much aggressive if compared to the subsequent treatments which have viability values between 50-60%, suggesting that those concentrations exert a significant toxicity to the cells. For $ZnCl_2$ treatments, the concentrations were the same as for QCT (50-500 μM). Conversely, no significant changes in viability were observed till 400 μM, where the viability fell around a 20%. For the last treatment (Zn 500μM), the viability was lower than 50%, and it was considered a toxic amount of zinc under our conditions after 24 hours treatment (Fig.1B). Once knowing that QCT can interact with zinc ions, we wanted to test if those interactions affect somehow the cell viability. We selected the first value of zinc presenting a minor reduction in viability ($ZnCl_2$ 400 μM), and combined it with different amounts of QCT (Fig.1C). It was observed how the viability drastically fell down with all the combinations to levels around 30-40%, which means that in all cases the new treatments were lethal for Hepa-16 cells. Non-toxic concentrations of QCT (50 and 100 μM) when combined with a non-toxic amount of zinc (400 μM), presented a very significant decrease in cell viability, suggesting a combined, maybe synergistic effect on cell viability, different from single treatments. In all cases after combination, cell viability decreased to values around 10-20% after 48 hours treatment (data not shown), denoting a cell death induction. Subsequently, we can confirm that there is a differential effect on cell viability with the interaction of our compounds, inducing cell death, however we cannot confirm if that effect is due an induction of apoptosis, a regulated and programmed cell death, or by necrosis, an unordered and accidental form of cellular dying.

3.2. Propidium iodide nuclear staining

Since its introduction, the propidium iodide assay has been widely used for the evaluation of apoptosis in different cellular models. It is based on the principle that apoptotic cells are characterized by DNA fragmentation and, consequently, loss of nuclear DNA content. Use

of a fluorophore, such as PI, with the ability to bind and label DNA, makes it a rapid and accurate method for the identification of characteristic apoptotic features "*in vitro*" [35]. In our study, Hepa 1-6 cells were treated with QCT 100 μM, $ZnCl_2$ 400 μM and both in combination for 24 hours. For our control reference, non-treated cells containing only culture media with the same final concentration of DMSO and Ethanol were used (Fig.2A). No significant alterations in nuclear morphology were observed after 24 hours of QCT 100 μM treatment, reflecting similar results obtained in 3.1, and denoting that under this conditions quercetin does not trigger the apoptotic machinery in Hepa 1-6 cell line (Fig.2B). Notwithstanding, when cells were treated with zinc 400 μM (Fig.2C), partial nuclei condensation and fragmentation were observed in various nucleus, however most of cells didn't show nuclear changes if compared to the control vehicle, which could represent a beginning phase of an apoptotic process. When both compounds were administered in combination, the obtained results were completely different of the single compounds individually (Fig.2D). Several apoptotic conformations (nuclear condensation, giant cell formation or fragmentation) were distinguished. This phenomenon is completely consistent with the results obtained in the MTT cell viability assay, where cells under a combined treatment with zinc and quercetin appear to be extremely damaged, now with evidences that the cellular death is owing to an apoptotic process.

3.3. DNA fragmentation

Biochemically, apoptosis is characterized by double-stranded cleavage at the linker regions between nucleosomes, resulting in the formation of multiple DNA fragments that are the sizes of integer multiples of a nucleosome length (180-200 bp) [36,37]. Because of their characteristic patterns revealed by agarose gel electrophoresis, these nucleosome DNA ladders are widely used as biochemical markers of apoptosis. In order to confirm the previous results, which suggest but not confirm an apoptotic mechanism for cellular death, a DNA fragmentation was analyzed through agarose gel electrophoresis (Fig.3). Our non-treated cells did not exhibit any ladder pattern as expected for a non-fragmented DNA. It is well reported that zinc dishomeostasis lead to an apoptotic mediated cell death. Therefore, zinc sequestrants could induce apoptosis in many cellular lines [15]. However, zinc ionophores, a new group of potential anticancer agents that target zinc, may also induce a lysosome-mediated apoptosis [38]. In this experiment, TPEN, a specific zinc sequestrant, was used as a positive control treatment in order to confirm the DNA ladder fragments. At

low concentrations of TPEN (2 μM), DNA fragments were not observed, indicating that cells can afford the effect of a weak zinc sequestrant without undergoing apoptosis. On the other hand, after a 24 hour treatment of TPEN 5 μM, cells underwent apoptosis, displaying the typical fragments or ladders of 180 bp as expected. For the QCT 100 μM treatment, very weak, almost negligible fragments appeared; suggesting that the decrease in cell viability, in some cases to very low values, lead to a cellular death probably mediated through an apoptotic process. Since this dose (100 μM) only decreased the viability of Hepa 1-6 cells a roughly 20%, the DNA laddering assay seems to be consistent with those results. Zinc 400 μM exhibited an evident DNA fragmentation pattern, confirming that the cellular decrease of viability and the morphological changes observed by the nuclear staining assay were due to apoptosis, discarding other cellular death mechanisms. Again, when zinc and quercetin were combined, the laddering was enhanced and the DNA fragmentation was more prominent than the single treatments independently. Thus confirm us the interaction of Zn and QCT, but also a differential molecular mechanism in cell signaling, contributing to maximize the effects on cell death mediated through apoptosis.

4. CONCLUDING REMARKS

Over the last years, metal-binding compounds have played a significant role in the development of new therapies against chronic disease such as cancer. The results obtained in our studies demonstrate that, in combination, QCT and zinc enhance the proapoptotic effects once compared to the single molecules, obtaining a distinctive effect, which could be used as a new potential tool in the study of cancer progression.

Even though metal-binding compounds have been considered to be promising anticancer agents [39], the cellular mechanisms of their anticancer action remain completely uncharacterized. Based on their physical and chemical properties [39], its reasonable to assume that individual metal-binding compounds might act through distinctive mechanisms. Chelation of zinc by a sequestrant such as TPEN leads to cellular zinc deficiency and subsequent apoptotic cell death in a variety of cell studies [40–43]. On the other hand, the activity of zinc ionophores, increases intracellular zinc concentration resulting in cancer cell death [30]. Therefore, zinc ionophores are becoming an attractive strategy to consider for selectively killing cancer cells. Taking into account that several

phenolic compounds act as zinc chelators, forming complexes with zinc ions, they could become new potential candidates as anticancer agents.

The complexation of metal ions to flavonoids gives a specific spatial orientation, and this could be the responsible for the pharmacological activity. These metal ion complexes therefore can display similar characteristics as their parent flavonoids and also present unique features distinct from their parent flavonoid due to their structural characteristics [44]. Several recent reports have indicated that the flavonoid-metal ion complexes possess more potent biological activities than the parent. Many pharmacological effects have been identified for flavonoid-metal ion complexes [44].

Quercetin, a zinc ionophore [33], has been confirmed to induce, or maximize the apoptotic effect when combined with zinc. Other dietary polyphenols with similar ionophore effects such as epigallocatechin-3-gallate, should be taken in consider for future studies. These results suggest that metal-binding compounds need to be characterized individually to fully understand their mechanisms of action during the course of anticancer drug development.

Additional experiments are required in order to elucidate the exact molecular mechanism and identify the specific metabolic pathways involved in apoptosis and to fully realize the potential of combining flavonoids with zinc. Clarifying the molecular basis about the apoptotic process will be essential for understanding and applying the basic knowledge of programmed cell death to clinical and therapeutic fields. Additionally, accelerated research is required to achieve a clear understanding of other definitive bioassays including protein expression and documentation of specific molecular markers to establish the exact mechanism for zinc-quercetin interactions on the induction of apoptosis.

REFERENCES

1. Chimienti, F., Seve, M., Richard, S., Mathieu, J. & Favier, A. Role of cellular zinc in programmed cell death: temporal relationship between zinc depletion, activation of caspases, and cleavage of Sp family transcription factors. *Biochem. Pharmacol.* 62, 51–62 (2001).

2. Nath, R. Metallothioneins, oxidative stress and the cardiovascular system. *Toxicology* 155, 17–26 (2000).

3. Parkin, G. Synthetic analogues relevant to the structure and function of zinc enzymes. *Chem. Rev.* 104, 699–767 (2004).

4. Vallee, B. L. & Falchuk, K. H. The biochemical basis of zinc physiology. *Physiol. Rev.* 73, 79–118 (1993).

5. Maret, W. Zinc biochemistry: from a single zinc enzyme to a key element of life. *Adv. Nutr.* 4, 82–91 (2013).

6. Eide, D. J. Homeostatic and adaptive responses to zinc deficiency in Saccharomyces cerevisiae. *J. Biol. Chem.* 284, 18565–9 (2009).

7. Auld, D. S. Zinc coordination sphere in biochemical zinc sites. *Biometals* 14, 271–313 (2001).

8. Eide, D. J. The oxidative stress of zinc deficiency. *Metallomics* 3, 1124–9 (2011).

9. Laitaoja, M., Valjakka, J. & Jänis, J. Zinc coordination spheres in protein structures. *Inorg. Chem.* 52, 10983–91 (2013).

10. Quesada, I. M. *et al.* Dietary catechins and procyanidins modulate zinc homeostasis in human HepG2 cells. *J. Nutr. Biochem.* 22, 153–63 (2011).

11. Higashimoto, M. *et al.* Tissue-dependent preventive effect of metallothionein against DNA damage in dyslipidemic mice under repeated stresses of fasting or restraint. *Life Sci.* 84, 569–75 (2009).

12. Tapiero, H. & Tew, K. D. Trace elements in human physiology and pathology: zinc and metallothioneins. *Biomed. Pharmacother.* 57, 399–411 (2003).

13. Powell, S. R. The antioxidant properties of zinc. *J. Nutr.* 130, 1447S–54S (2000).

14. Klotz, L.-O., Kröncke, K.-D., Buchczyk, D. P. & Sies, H. Role of copper, zinc, selenium and tellurium in the cellular defense against oxidative and nitrosative stress. *J. Nutr.* 133, 1448S–51S (2003).

15. Carraway, R. E. & Dobner, P. R. Zinc pyrithione induces ERK- and PKC-dependent necrosis distinct from TPEN-induced apoptosis in prostate cancer cells. *Biochim. Biophys. Acta* 1823, 544–57 (2012).

16. Ding, W.-Q., Yu, H.-J. & Lind, S. E. Zinc-binding compounds induce cancer cell death via distinct modes of action. *Cancer Lett.* 271, 251–9 (2008).

17. Orlova, M. & Orlov, A. Role of zinc in an organism and its influence on processes leading to apoptosis. *Br. J. Med. Med.* (2011).

18. Magda, D. *et al.* Synthesis and anticancer properties of water-soluble zinc ionophores. *Cancer Res.* 68, 5318–25 (2008).

19. Beyersmann, D. Homeostasis and Cellular Functions of Zinc. *Materwiss. Werksttech.* 33, 764–769 (2002).

20. Yu, H., Zhou, Y., Lind, S. E. & Ding, W.-Q. Clioquinol targets zinc to lysosomes in human cancer cells. *Biochem. J.* 417, 133–9 (2009).

21. Araújo, J. R., Gonçalves, P. & Martel, F. Chemopreventive effect of dietary polyphenols in colorectal cancer cell lines. *Nutr. Res.* 31, 77–87 (2011).

22. Leopoldini, M., Russo, N. & Toscano, M. The molecular basis of working mechanism of natural polyphenolic antioxidants. *Food Chem.* 125, 288–306 (2011).

23. Čipák, L., Rauko, P., Miadoková, E., Čipáková, I. & Novotný, L. Effects of flavonoids on cisplatin-induced apoptosis of HL-60 and L1210 leukemia cells. *Leuk. Res.* 27, 65–72 (2003).

24. Ramos, S. Cancer chemoprevention and chemotherapy: dietary polyphenols and signalling pathways. *Mol. Nutr. Food Res.* 52, 507–26 (2008).

25. Bravo, L. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev.* 56, 317–33 (1998).

26. Russo, M., Spagnuolo, C., Tedesco, I., Bilotto, S. & Russo, G. L. The flavonoid quercetin in disease prevention and therapy: facts and fancies. *Biochem. Pharmacol.* 83, 6–15 (2012).

27. Mansuri, M. L., Parihar, P., Solanki, I. & Parihar, M. S. Flavonoids in modulation of cell survival signalling pathways. *Genes Nutr.* 9, 400 (2014).

28. Hider, R. C., Liu, Z. D. & Khodr, H. H. Metal chelation of polyphenols. *Methods Enzymol.* 335, 190–203 (2001).

29. Ding, W.-Q. & Lind, S. E. Metal ionophores - an emerging class of anticancer drugs. *IUBMB Life* 61, 1013–8 (2009).

30. Ding, W.-Q., Liu, B., Vaught, J. L., Yamauchi, H. & Lind, S. E. Anticancer activity of the antibiotic clioquinol. *Cancer Res.* 65, 3389–95 (2005).

31. Schmitt, S. M., Frezza, M. & Dou, Q. P. New applications of old metal-binding drugs in the treatment of human cancer. *Front. Biosci. (Schol. Ed).* 4, 375–91 (2012).

32. Feng, P., Li, T., Guan, Z., Franklin, R. B. & Costello, L. C. The involvement of Bax in zinc-induced mitochondrial apoptogenesis in malignant prostate cells. *Mol. Cancer* 7, 25 (2008).

33. Dabbagh-Bazarbachi, H. *et al.* Zinc Ionophore Activity of Quercetin and Epigallocatechin-gallate: From Hepa 1-6 Cells to a Liposome Model. *J. Agric. Food Chem.* (2014).

34. Duarte, I. D., Nakamura, M. & Ferreira, S. H. Participation of the sympathetic system in acetic acid-induced writhing in mice. *Braz. J. Med. Biol. Res.* 21, 341–3 (1988).

35. Riccardi, C. & Nicoletti, I. Analysis of apoptosis by propidium iodide staining and flow cytometry. *Nat. Protoc.* 1, 1458–61 (2006).

36. Chiarugi, V., Magnelli, L., Cinelli, M. & Basi, G. Apoptosis and the cell cycle. *Cell. Mol. Biol. Res.* 40, 603–12 (1994).

37. Huang, P., Ballal, K. & Plunkett, W. Biochemical Characterization of the Protein Activity Responsible for High Molecular Weight DNA Fragmentation during Drug-induced Apoptosis. *Cancer Res.* 57, 3407–3414 (1997).

38. Xue, J. *et al.* Chloroquine is a zinc ionophore. *PLoS One* 9, e109180 (2014).

39. Huang, R., Wallqvist, A. & Covell, D. G. Anticancer metal compounds in NCI's tumor-screening database: putative mode of action. *Biochem. Pharmacol.* 69, 1009–39 (2005).

40. Canzoniero, L. M. T., Manzerra, P., Sheline, C. T. & Choi, D. W. Membrane-permeant chelators can attenuate Zn2+-induced cortical neuronal death. *Neuropharmacology* 45, 420–428 (2003).

41. Adler, M., Shafer, H., Hamilton, T. & Petrali, J. P. Cytotoxic actions of the heavy metal chelator TPEN on NG108-15 neuroblastoma-glioma cells. *Neurotoxicology* 20, 571–82 (1999).

42. Hashemi, M., Ghavami, S., Eshraghi, M., Booy, E. P. & Los, M. Cytotoxic effects of intra and extracellular zinc chelation on human breast cancer cells. *Eur. J. Pharmacol.* 557, 9–19 (2007).

43. Sakabe, I., Paul, S., Dansithong, W. & Shinozawa, T. Induction of apoptosis in Neuro-2A cells by Zn2+ chelating. *Cell Struct. Funct.* 23, 95–9 (1998).

44. Selvaraj, S., Krishnaswamy, S., Devashya, V., Sethuraman, S. & Krishnan, U. M. Flavonoid-Metal Ion Complexes: A Novel Class of Therapeutic Agents. *Med. Res. Rev.* (2013).

Figures

Zinc-dependent pro-apoptotic activity of Quercetin in hepatocarcinoma Hepa 1-6 cells

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Figure 1. MTT viability assay in Hepa 1-6 cells treated with QCT (A), $ZnCl_2$ (B) and a combination of $ZnCl_2$ 400 μM with QCT (C). First bar in A and B represents the viability of the vehicle control. In figure C, first bar indicates the viability of cells treated with a single dose of $ZnCl_2$ 400 μM before the combined treatment. For statistical analysis t-test and one-way ANOVA were performed using SPSS software. All data are the result of at least 3 independent experiments. Differences were considered significant for P values 0.05.

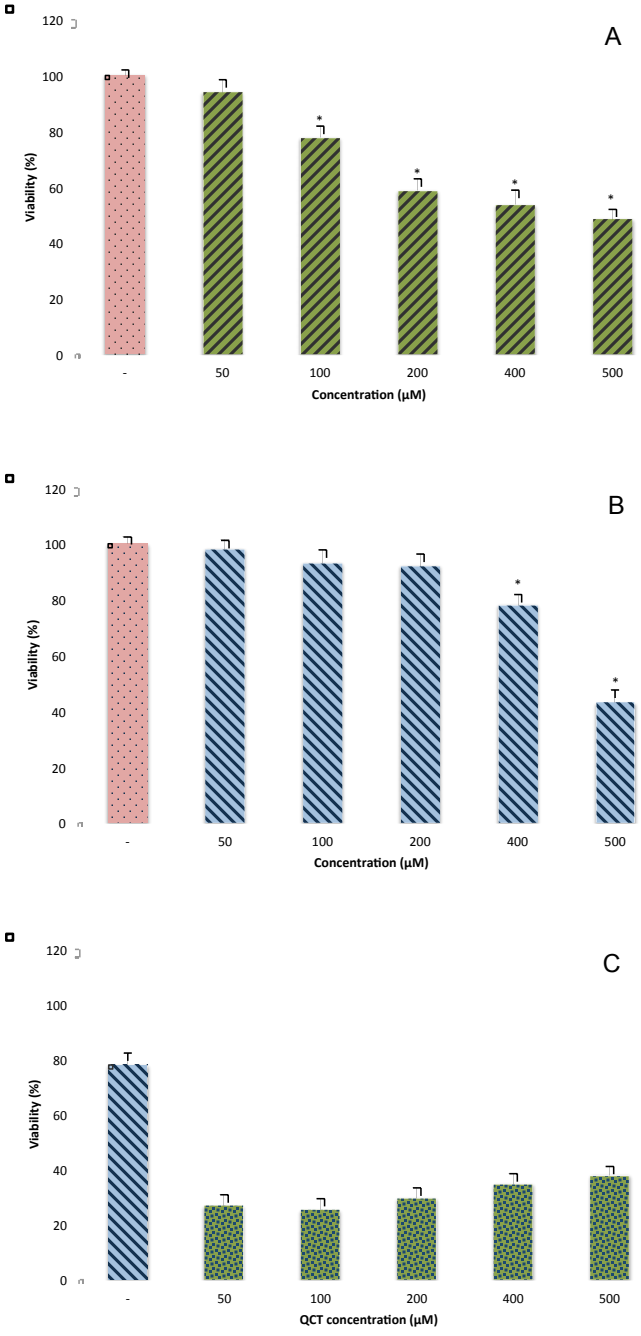


Figure 2. Confocal fluorescence microscope images of IP stained Hepa 1-6 cells after 24 hours treatment. (A) Vehicle control and (B) QCT 100 μM treated cells show a normal nuclear pattern. (C) $ZnCl_2$ 400 μM treatment shows condensed and partial fragmentation nuclei. (D) QCT 100 μM combined with $ZnCl_2$ 400 μM present significant evidences of nuclear condensation and apoptotic bodies, giant cell formation and fragmentation. Scale bars are 50 μm.

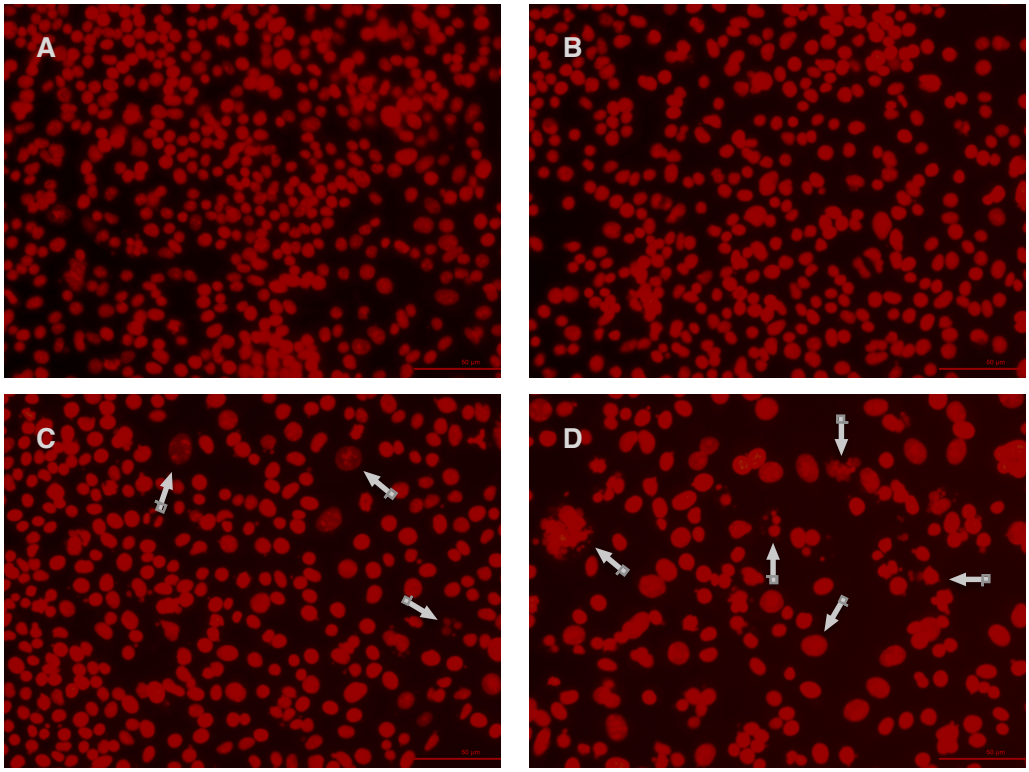
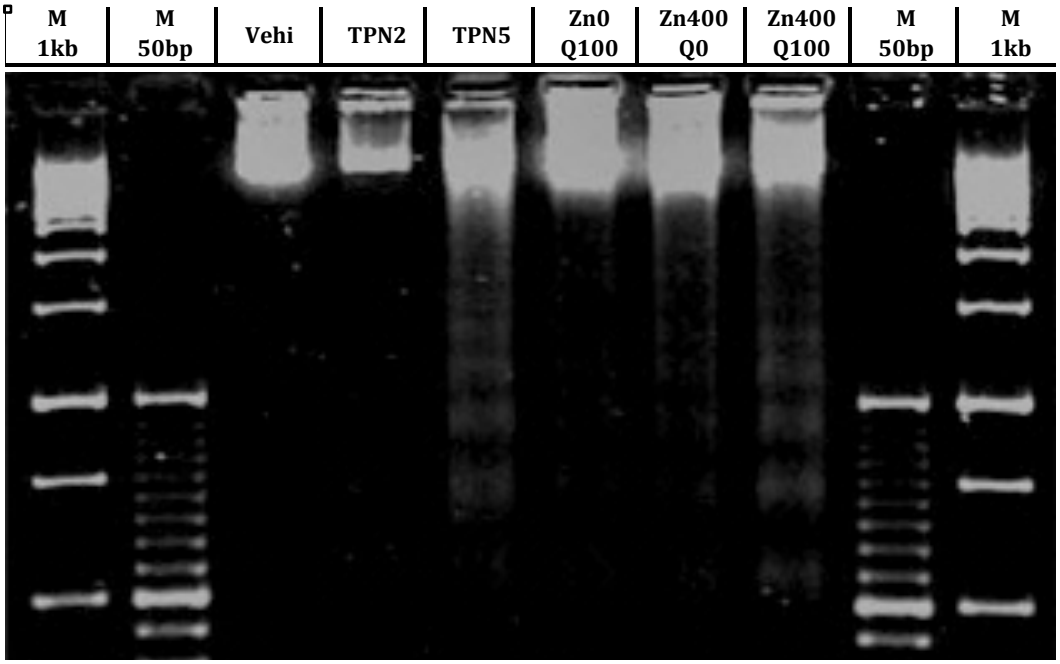


Figure 3. Effect of Vehicle control (Vehi), TPEN 2 μM (TPN2), TPEN 5 μM (TPN5), QCT 100 μM (Zn0, Q100), Zn 400 μM (Zn400, Q100) and both Zn and QCT in combination (Zn400, Q100) on DNA fragmentation detected by agarose gel electrophoresis. Analysis corresponds to 24 hours treatment. Two different molecular-weight size markers (M) were used in order to identify the approximate size of the fragments. Three independent experiments were carried out with similar results. A representative experiment is shown.



IV. DISCUSSION

Discussion

The results of studies outlined in this thesis provide a current understanding on the biological effects of polyphenol-zinc ion complexes and their relevance to human health. It is clear, from the literature that many potentially useful biological activities of wide variety of polyphenol-zinc ion complexes are yet to be investigated. From the scan of literature, it is observed that the greater pharmacological activities of polyphenol-metal ion complexes are mainly due to the type of polyphenols forming the complex. Therefore the binding affinity of the polyphenol with the metal ion is extremely important in determining its stability in the biological systems. This study also adds accumulating observations underlying the importance of zinc as an intracellular signaling molecule. Given the fundamental functions of zinc in cell biology, it comes as no surprise that perturbations of the homeostatic mechanisms that regulate intracellular zinc amounts are involved in many diseases, including degenerative diseases, such neurodegeneration (Alzheimer disease, stroke), diabetes, cancer, and wound healing. The World Health Organization considers zinc deficiency as the 5th leading cause of morbidity and mortality in developing countries (11th worldwide).

The control of a fluctuating intracellular pool of zinc at remarkably low concentrations and with the participation of many proteins provides a new perspective on the molecular functions of zinc in biology in general and for the impact of zinc on human health in particular (Rink 2011). Roles of zinc ions in biological phosphorylations and in redox signaling are already well documented and are part of the spectrum of actions of zinc in cellular proliferation, differentiation, and cell death. Zinc is not just required for the function of proteins; it participates in the control of cellular metabolism and paracrine and intracrine signaling (Maret 2011). Not only the availability of zinc itself, but the limited cellular zinc buffering capacity and the many mutations that affect the functions of proteins involved in the cellular control of zinc have major implications for the balance between health and disease. The concentration range at which zinc ions occur is critical for explaining the global functions of zinc. Zinc is generally considered to be an antioxidant. However, it is redox inert and thus can serve such a function only indirectly. The term pro-antioxidant is more appropriate (Maret 2008). Whether zinc elicits antioxidant, anti-inflammatory, anti-

diabetic, or anti-apoptotic effects depends on its concentration. Outside the physiological or pharmacological range, under conditions of both zinc overload and zinc deficiency, zinc ions have the opposite effect: they become pro-oxidants with pro-inflammatory and proapoptotic properties. This intricate balance at a relatively narrow and tightly controlled range of concentrations needs to be considered when the physiological significance of results from experiments performed with zinc are evaluated and in nutrition when supplementation with zinc is considered (Maret & Sandstead 2006). It is fair to conclude that the impact of zinc for health and disease will be at least as far-reaching as that of iron.

Polyphenols have been demonstrated to interact and directly bind zinc ions (Grazul & Budzisz 2009; Scalbert et al. 1999; Wei & Guo 2014; Tarahovsky et al. 2014), thus modulating the intracellular pool of zinc acting as zinc sequestrants or zinc ionophores . As explained, controlling the fluctuations of zinc provides several important biological outcomes. Since several phenolic compounds have been demonstrated to act as zinc ionophores, a new investigation field needs to be explored. Polyphenol rich diets provide significant protection against the development and progression of many chronic pathological conditions including cancer, diabetes, cardio-vascular problems, neurodegenerative diseases or aging. Although several biological effects based on epidemiological studies can be scientifically explained, the mechanism of action of some effects of polyphenols is not fully understood. A better knowledge of some variables of polyphenol bioavailability, such as the kinetics of absorption, accumulation and elimination, will facilitate the design of such studies.

The role of polyphenols in human health is still a fertile area of research. Moreover, the interaction between dietary polyphenols and several metal ions such as zinc ought to be investigated in order to increase our understanding of many novel aspects of its physiological and pathophysiological functions. Furthermore, it is noteworthy that several polyphenol rich food sources also contain significant amounts of zinc, such as olive oil (La Pera et al. 2002; Angioni et al. 2006), red wine (BanoVIć et al. 2009), chocolate (cocoa) (da Silva et al. 2006), green tea (Tsushida & Takeo 1977), black grapes, berries, tomatoes, nuts and oilseeds (Jorhem & Sundström 1993; Leblanc et al. 2005), providing a new understand on their correlation and biological role.

Taken together, the results of this Thesis elucidate the chronology of different phases on the biological importance of the intracellular zinc modulation. Although it has been reported the interaction between metal ions, such as zinc, and several phenolic compounds (Leopoldini et al. 2011; Georgiades et al. 2011), herein we confirmed the interaction of different dietary polyphenols with zinc ions under physiological conditions; We used different zinc fluorophores, in order to test and quantify the relative ability of our polyphenols to bind zinc cations in aqueous solutions. Their capacity to retrieve zinc cations from the fluorophore-zinc complexes was measured by monitoring the decrease in zinc-dependent fluorescence emitted by the fluorophore-zinc complexes upon addition of the polyphenols to the solution, confirming that dietary polyphenols are zinc chelators.

Initially, our studies demonstrated that GSPE, as well as a some pure phenolic compounds were able to modulate some of the most important genes controlling zinc homeostasis such as Metallothionein (MT) and Zinc transporters (ZnT1) in the human carcinoma cell line HepG2 (Quesada et al. 2011). Herein, we have elucidated how the water-insoluble flavonoid QCT, and EGCG a water-soluble flavonoid affect those genes in different ways, therefore modulating the zinc homeostasis system in the hepatic carcinoma Hepa 1-6 cell line, reconfirming and giving a better understand of previous reports of our research group.

Dietary polyphenols have been known to modify the activity of various receptor tyrosine kinases and particular pathways of signal transduction, thereby altering the expression of genes involved in protein synthesis, immunity, cell survival, proliferation, glucose metabolism, cardiovascular homeostasis and neuroprotection. Various inhibitory or stimulatory actions of polyphenols on these pathways greatly affect cellular functions by altering the phosphorylation state of targeted molecules and also modulate various gene expressions through activation of various transcription factors. Moreover, dietary polyphenols can interfere at the initiation, development and progression of various cancers through the modulation of different cellular processes, showing certain common signaling events, such as the arrest of cell cycle, induction of apoptosis, activation of caspases, inhibition of survival/proliferation signals such as Akt, MAPK and NF-kB or inflammation signals such as COX-2, and TNF secretion, as well as suppression of key proteins involved in angiogenesis and metastasis (Orlikova & Diederich 2012). It is worth mentioning that the cancer chemopreventive effect of dietary phenolic compounds seems

to be specific, as carcinogenic cells demonstrate higher sensitivity than normal cells when incubated with the phenolic compounds. In addition, differences on protein modulation and regulatory events over time have been observed, which also suggests a specific and differential manner of gene expression regulation by dietary polyphenols (Granado-Serrano et al. 2010). On the other hand, zinc is an essential micronutrient involved in cell proliferation and differentiation, remarkably for the regulation of DNA synthesis, genomic stability and mitosis (Sharif et al. 2012). On the molecular level, zinc is known to be related to activation of more than 300 enzymes and 1000 transcription factors (Wang et al. 2013). In many cases, zinc act through similar metabolic pathways of polyphenols, exerting a signaling activity. (Fig.1) However, it is remarkably important to understand these shared modulation of several metabolic pathways by zinc and dietary polyphenols, which seems to be enhanced when both act together mainly due to the modulation of intracellular labile zinc, which could trigger the activation of those pathways, and in many cases seems to be indispensable since several enzymes are directly dependent on zinc.

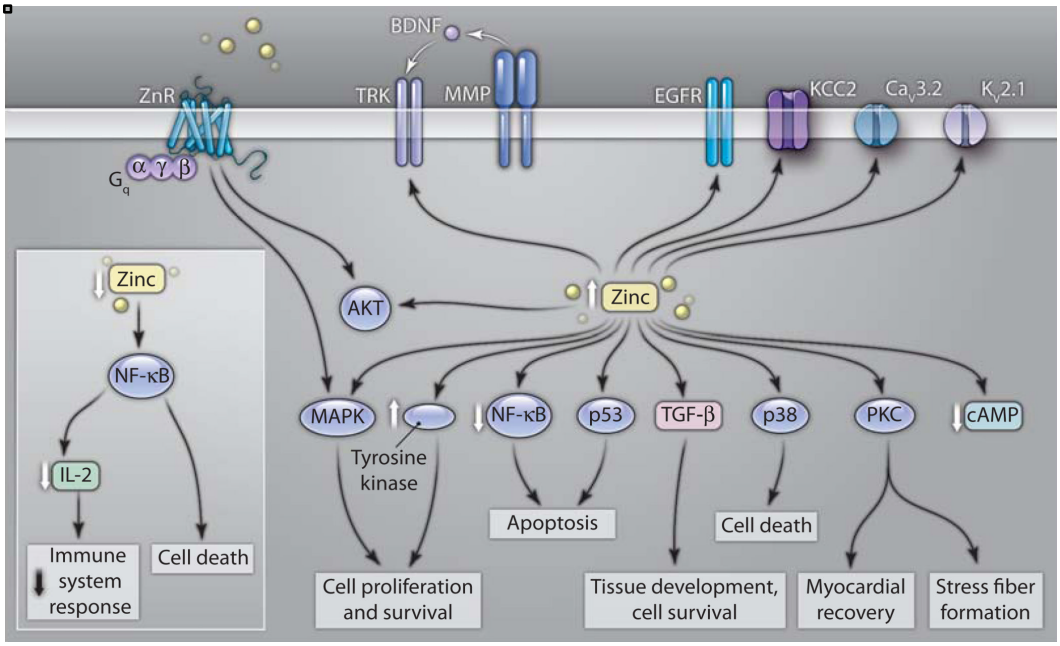


Figure 1. Major signaling pathways regulated by intracellular and extracellular zinc. The physiological roles of these pathways are also presented. BDNF, brain-derived neurotrophic factor. All the presented pathways are also regulated by dietary polyphenols. (Hershfinkel et al. 2010)

After observing that QCT and EGCG rapidly increase intracellular labile zinc in Hepa 1-6 cells, we have here elucidated that they function as ionophores for zinc in a liposomal system. Thus, natural flavonoids can be considered novel agents that may be used to modulate zinc homeostasis and regulate zinc-dependent biological pathways (Dabbagh-Bazarbachi et al. 2014). Furthermore, it has been confirmed that most of the natural occurring phenolic compounds have the ability to directly interact and form new structures (complexes) bound with zinc, and for the first time, have been classified according to their zinc-ionophore strength capacity and their chelation efficiency, giving us a better knowledge of the importance of the structural conformation versus biological activity.

The activity of zinc ionophores, increasing intracellular zinc concentration result in several cancers cell death (Ding et al. 2005). Therefore, zinc ionophores are becoming an attractive strategy to consider for selectively killing cancer cells. Taking into account that several phenolic compounds act as zinc chelators, forming complexes with zinc ions, they could become new potential anticancer candidates. Quercetin, which interacts with zinc ions, modulates Akt phosphorylation and acts as a zinc ionophore has been confirmed to induce, or maximize the apoptotic effect when combined with zinc, becoming a good candidate to act as an anticancer agent.

In this Thesis we have successfully demonstrated the proof of concept of how dietary polyphenols display zinc ionophore activity and modulate zinc signaling in hepatocarcinoma cells. Further experiments are needed in order to characterize the specific mechanisms of action. This requires more in-depth *in vitro* studies with different cell lines, primary hepatic cell cultures and *in vivo* experiments. In addition, the liposome system designed herein could be used to screen the ionophore activity towards other metal ions such as Ca, Fe, Cu or Mg.

There remains a pressing need to develop an absolute understanding of the underlying biology of zinc trafficking in terms of its dynamic and quantitative processing in specific organelles (Jobe et al. 2011). Thus progress does not come from the flurry of activities synthesizing yet another probe but from furthering an understanding of the biological issues that are interrogated with specifically designed probes. What is needed are experiments, in which zinc ion transients are induced specifically in biologically meaningful

ways, progress in the inorganic chemical cell biology of zinc and an understanding of how cellular and subcellular zinc ion fluxes are controlled by zinc transporters and metallothioneins, which chemical species of zinc are involved in cellular regulation, and insights into the molecules and mechanisms that buffer and muffle the zinc ion (Maret 2014).

To sum up briefly, zinc can be considered an almost omnipresent metal ion, not only as a vital element in various physiological processes but also a dietary drug in the prevention and management of many diseases. The importance of this element has taken much time for recognition, with just a few years since it has been accorded its due status. However, zinc is now being extensively investigated for utilization in a wide area ranging from therapeutic activity, drug and gene delivery, to nanotechnology with promising outcomes. It can be concluded that the role of zinc will be an important research area in the future and, with the significant discoveries at hand, well justifies the tag “metal of life” (Kaur et al. 2014).

References

Angioni, A. et al., 2006. Influence of olive cultivars and period of harvest on the contents of Cu, Cd, Pb, and Zn in virgin olive oils. *Food Chemistry*, 99(3), pp.525–529.

BanoVIć, M., IrIn, J.K. & KoVače, K., 2009. Influence of Vintage on Cu, Fe, Zn and Pb Content in Some Croatian Red Wines.

Dabbagh-Bazarbachi, H. et al., 2014. Zinc Ionophore Activity of Quercetin and Epigallocatechin-gallate: From Hepa 1-6 Cells to a Liposome Model. *Journal of agricultural and food chemistry*.

Ding, W.-Q. et al., 2005. Anticancer activity of the antibiotic clioquinol. *Cancer research*, 65(8), pp.3389–95.

Georgiades, S.N. et al., 2011. Identification of a potent activator of Akt phosphorylation from a novel series of phenolic, picolinic, pyridino, and hydroxamic zinc(II) complexes. *Journal of biological inorganic chemistry : JBIC : a publication of the Society of Biological Inorganic Chemistry*, 16(2), pp.195–208.

Granado-Serrano, A.B. et al., 2010. Quercetin modulates NF-kappa B and AP-1/JNK pathways to induce cell death in human hepatoma cells. *Nutrition and cancer*, 62(3), pp.390–401.

Grazul, M. & Budzisz, E., 2009. Biological activity of metal ions complexes of chromones, coumarins and flavones. *Coordination Chemistry Reviews*, 253(21), pp.2588–2598.

Hershfinkel, M. et al., 2010. Zinc bells rang in Jerusalem! *Science signaling*, 3(129), p.mr2.

Jobe, K. et al., 2011. Modular “click” sensors for zinc and their application in vivo. *Chemical communications (Cambridge, England)*, 47(21), pp.6036–8.

Jorhem, L. & Sundström, B., 1993. Levels of Lead, Cadmium, Zinc, Copper, Nickel, Chromium, Manganese, and Cobalt in Foods on the Swedish Market, 1983–1990. *Journal of Food Composition and Analysis*, 6(3), pp.223–241.

Kaur, K. et al., 2014. Zinc: The Metal of Life. *Comprehensive Reviews in Food Science and Food Safety*, 13(4), pp.358–376.

Leblanc, J.-C. et al., 2005. Dietary exposure estimates of 18 elements from the 1st French Total Diet Study. *Food additives and contaminants*, 22(7), pp.624–41.

Leopoldini, M., Russo, N. & Toscano, M., 2011. The molecular basis of working mechanism of natural polyphenolic antioxidants. *Food Chemistry*, 125(2), pp.288–306.

Maret, W., 2014. Analyzing free zinc(ii) ion concentrations in cell biology with fluorescent chelating molecules. *Metallomics : integrated biometal science*.

Maret, W., 2011. Metals on the move: zinc ions in cellular regulation and in the coordination dynamics of zinc proteins. *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine*, 24(3), pp.411–8.

Maret, W., 2008. Metallothionein redox biology in the cytoprotective and cytotoxic functions of zinc. *Experimental gerontology*, 43(5), pp.363–9.

Maret, W. & Sandstead, H.H., 2006. Zinc requirements and the risks and benefits of zinc supplementation. *Journal of trace elements in medicine and biology : organ of the Society for Minerals and Trace Elements (GMS)*, 20(1), pp.3–18.

Orlikova, B. & Diederich, M., 2012. Power from the garden: plant compounds as inhibitors of the hallmarks of cancer. *Current medicinal chemistry*, 19(14), pp.2061–87.

La Pera, L. et al., 2002. Derivative Potentiometric Stripping Analysis (dPSA) Used for the Determination of Cadmium, Copper, Lead, and Zinc in Sicilian Olive Oils. *Journal of Agricultural and Food Chemistry*, 50(11), pp.3090–3093.

Quesada, I.M. et al., 2011. Dietary catechins and procyanidins modulate zinc homeostasis in human HepG2 cells. *The Journal of nutritional biochemistry*, 22(2), pp.153–63.

Rink, L., 2011. Zinc in human health.

Scalbert, A. et al., 1999. Polyphenols, metal ion complexation and biological consequences. *Basic life sciences*, 66, pp.545–54.

Sharif, R. et al., 2012. The role of zinc in genomic stability. *Mutation research*, 733(1-2), pp.111–21.

Da Silva, E.G.P. et al., 2006. Determination of manganese and zinc in powdered chocolate samples by slurry sampling using sequential multi-element flame atomic absorption spectrometry. *Microchemical Journal*, 82(2), pp.159–162.

Tarahovsky, Y.S. et al., 2014. Flavonoid-membrane interactions: involvement of flavonoid-metal complexes in raft signaling. *Biochimica et biophysica acta*.

Tsushida, T. & Takeo, T., 1977. Zinc, copper, lead and cadmium contents in green tea. *Journal of the science of food and agriculture*, 28(3), pp.255–8.

Wang, Y. et al., 2013. Effects of exogenous zinc on cell cycle, apoptosis and viability of MDAMB231, HepG2 and 293 T cells. *Biological trace element research*, 154(3), pp.418–26.

Wei, Y. & Guo, M., 2014. Zinc-Binding Sites on Selected Flavonoids. *Biological trace element research*.

V. CONCLUSIONS

V. CONCLUSIONS

1. Quercetin interacts with zinc cations to exert a signaling effect on Akt.

- Quercetin chelates zinc in solution with strength comparable with TPEN and Clioquinol, at physiological pH and near physiological concentrations.
- When applied to the mouse hepatic carcinoma Hepa 1-6 cell line:
  - Toxic amounts of QCT counteract the toxic effects of excess zinc. The other way around, toxic amounts of zinc neutralize the toxicity of excess QCT.
  - QCT increases the amount of total intracellular zinc, and does so with intensity directly proportional to added zinc. *
  - QCT increases the expression of cytoplasmic zinc-chelating proteins MT-1 and MT2 and of plasma membrane zinc-exporter ZnT1 with intensity directly proportional to added zinc. *
  - QCT increases the amount of Zinquin-detectable labile zinc in a zinc-dependent manner, proportionately to the quantity of added zinc, and with less strength than Clioquinol.
  - QCT increases Akt phosphorylation in a zinc-dependent way, i.e., it does so only when applied together with certain amounts of zinc. It is inferred that QCT affect Akt signaling in a zinc-dependent manner.
  - Zinc alone displays a small effect on Akt phosphorylation in the conditions tested.

2. Epigallocatechin-gallate interacts with zinc cations to affect Akt phosphorylation.

- EGCG chelates zinc in solution with strength comparable with TPEN and Clioquinol, at physiological pH and near physiological concentrations.
- When applied to the mouse hepatic carcinoma Hepa 1-6 cell line:
  - Toxic concentrations of EGCG counteract the toxic effects of excess zinc. *Vice versa*, toxic amounts of zinc neutralize the noxious effect of excess EGCG.
  - EGCG decreases the amount of total intracellular zinc. *
  - EGCG decreases the expression of cytoplasmic zinc-chelating proteins MT-1 and MT2 and of plasma membrane zinc-exporter ZnT, with intensity dependent on added zinc. *

- EGCG increases the amount of Zinquin-detectable labile zinc in a zinc-dependent manner, proportionately to the quantity of added zinc, and with less strength that Clioquinol.
- EGCG increases Akt phosphorylation in a zinc-dependent way, i.e., it does so only when applied together with certain amounts of zinc. It is inferred that EGCG affect Akt signaling in a zinc-dependent manner.

3. Epigallocatechin-gallate and Quercetin behave as true zinc ionophores in a liposomal system.

- EGCG and QCT enhance the FluoZin-detectable labile zinc when applied to Hepa 1-6 cells, in a way dependent on extracellular zinc concentration.
- The entry of zinc cations inside protein-free liposomal vesicles is dependent on the addition of EGCG or QCT.
- It could be deduced that EGCG-zinc complexes and QCT-zinc complexes are membrane permeable and go across the lipid bilayer, without the intervention of any protein, i.e., by concentration-driven passive transport.
- It may be concluded that, one inside the liposome, zinc cations bound to EGCG or to QCT dissociate and may bound by FluoZin.
- The ionophoric action of QCT and EGCG *in a liposomal system* provides an explanation for the increment of cytoplasmic labile zinc in Hepa 1-6 cells treated with QCT and zinc or with EGCG and zinc.
- Although not tested directly in this work, the zinc ionophoric behaviour of QCT and EGCG implies that QCT and EGCG also enter the liposomal cavity as zinc-polyphenol complexes, and provides a mechanism for the entrance of this polyphenols to cells, i.e., EGCG and QCT may cross lipid bilayers as metal ion complexes.

4. Polyphenols, in general, act as zinc chelators and as zinc ionophores.

- Although displaying quantitative differences, and in spite of structural differences, all 14 dietary polyphenols tested, including EGCG and QCT, behave qualitatively as the synthetic pharmacological zinc chelators TPEN, Pyrithione and Clioquinol,

in that of all them form complexes with zinc cations that may cross through lipid bilayers.

- It may be inferred that, *vice versa*, polyphenols may cross lipid bilayers when they form complexes with metal cations.

5. Non-toxic amounts of Quercetin induce apoptosis in Hepa1-6 cells when combined with under-toxic amounts of zinc.

- The combination of QCT with zinc, at low concentrations that individually by themselves have no effect on cell viability, result in a drastic decrement of cellular viability.
- The decrease in cell viability is due to an apoptotic process, as manifested by nuclear fragmentation and DNA laddering.
- Triggering of apoptotic process is accompanied by an increment in FluoZin3-detectable cytoplasmic labile zinc, although the intervention of labile zinc in the apoptotic process remains to be elucidated.

***** *These conclusions have already been presented in a previous PhD Thesis of the Nutrigenomics Research Group, and are included here because they are part of the publications presented in this Thesis.*

General conclusions:

- Chelation of zinc by polyphenols entails both a sequestering and an ionophoric action on zinc, exerting a dual effect on zinc bioavailability, metabolism and signalling.

- Zinc sequestration by QCT and EGC is concluded to determine their protective effect against excess zinc.

- The ionophoric action of QCT is concluded to underlie the triggering of apoptosis in liver-carcinoma cells; whether the direct cause is the increment of intracellular Quercetin, the increment of labile zinc, of both, remains to be investigated.

- Polyphenols can both act as donor and as a receptor of zinc cations, i. e., may retrieve zinc bound to enzymes and *vice versa*. Thus, polyphenols affect the function of metal-binding proteins. Zinc-binding proteins are targets of polyphenol action.

- The effect of polyphenols on Akt phosphorylation, and hence Akt signaling, are modulated by zinc, i.e., by the complexation of polyphenols with zinc, and is concluded to depend on the amount and structure of polyphenol-zinc complexes.