The impact of polypharmacology on chemical biology

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també pot ser màgica.

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"Phantasie ist wichtiger als Wissen, denn Wissen ist begrenzt."

"La imaginació és més important que el coneixement, perquè el coneixement és limitat"

Albert Einstein

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Abstract

There is now ample evidence that drugs have biologically relevant interactions with more than one protein, a behavior that is commonly referred to as polypharmacology. This finding is starting to have a true impact on the drug discovery process, transforming it into a more holistic endeavor. In contrast, chemical biology continues to be a reductionist discipline, still regarding chemical probes as highly selective small molecules that enable the modulation and study of one specific target. In an effort to bring a more comprehensive perspective to the practice of chemical biology, this Thesis aims at demonstrating that chemical probes, like drugs, tent to bind to more than one protein, a behavior that may have confounded many of the biological insights gathered using these tool compounds. Accordingly, in this Thesis we use the Poly(ADP-ribose) polymerase (PARP) enzyme superfamily to illustrate the consequences that chemical probe polypharmacology have for the practice of chemical biology and follow-on drug discovery. Next, we extend this analysis to a collection of chemical probes to demonstrate the ubiquity of polypharmacology and we provide guidelines to derisk the practice of chemical biology using potentially promiscuous tool compounds. Chemical biology cannot continue to overlook the existence of polypharmacology and the results presented in this Thesis urge it to become a more holistic discipline that looks at the use of tool compounds from a systems perspective.

Resum

Avui dia un gran nombre de proves demostren que els fàrmacs exerceixen interaccions biològicament rellevants amb més d'una proteïna, un comportament generalment anomenat polifarmacologia. Aquest descobriment

està començant a influir en el procés de descobriment de fàrmacs, transformant-lo en una empresa més holística. Per contra, la biologia química continua essent una disciplina reduccionista que encara considera les sondes químiques com molècules molt selectives que permeten la modulació i l'estudi d'una diana específica. En un esforç per portar una visió més àmplia a la pràctica de la biologia química, aquesta Tesi té l'objectiu de demostrar que les sondes químiques, com els fàrmacs, tendeixen a unir-se amb més d'una diana, un comportament que podria haver confós moltes de les conclusions biològiques derivades del seu ús. En conseqüència, en aquesta Tesi utilitzem la superfamília de les proteïnes Poly(ADP-ribose) polymerasa (PARPs) per il·lustrar les conseqüències de la polifarmacologia de les sondes químiques en la pràctica de la biologia química i el conseqüent descobriment de fàrmacs. Seguidament, ampliem aquest anàlisi a una col·lecció de sondes químiques per demostrar la prevalença de la polifarmacologia i aportem recomanacions per a convertir l'ús de sondes químiques potencialment promiscues en biologia química en una pràctica menys arriscada. La biologia química no pot seguir obviant l'existència de la polifarmacologia i els resultats presentats en aquesta Tesi demostren que la biologia química ha d'esdevenir una disciplina més holística que consideri l'ús de sondes químiques des d'una perspectiva de sistemes.

Preface

Our understanding of drug action has evolved with time, from the early conception of drugs as 'magic bullets' modulating specifically one single target to the discovery that the vast majority of drugs bind to more than one target, a promiscuous behavior now commonly known as polypharmacology. Accordingly, we are witnessing an early impact of polypharmacology on drug discovery with new business models like drug repurposing already exploiting drug promiscuity and with ongoing academic efforts to rationally design multi-target drugs. Drug discovery is, slowly, becoming a more holistic endeavor.

Unfortunately, this holistic perspective is not permeating to other disciplines. Since the 1990s, the field of chemical biology has emerged to use chemical tools to study basic biology. However, a much reductionist view of these chemical probes to study biology still prevails. Chemical probes are considered to selectively modulate one single target and, therefore, the biological insights gathered using these probes are linked to their known primary target regardless of the concentration being used. These chemical probes and the biological insights derived from their use are not only fundamental to the advancement of biology but also a knowledge-base for follow-on drug discovery campaigns. Accordingly, this reductionist view in chemical biology is putting at risk many biological conclusions and drug discovery programs.

The main aim of this Thesis is precisely to bridge these two disciplines by exploring the existence of polypharmacology among chemical probes and their impact in the practice of chemical biology by using poly(ADP-ribose)polymerases (PARPs) as a proof-of-concept target family. To accomplish this goal, this Thesis has been divided in five parts. In the first part a historical perspective of drug discovery, chemical biology and the PARP enzyme superfamily is presented. After this introductory section, the main objectives of this Thesis are listed. Next, the results of the Thesis are presented and discussed

in the form of four research articles and two book chapters. Then, a discussion section globally addresses the impact of polypharmacology on chemical biology and the lessons learned during this Thesis to derisk the practice of chemical biology. Finally, the main conclusions derived from this Thesis are outlined and the main bibliographic citations are listed.

List of publications from this Thesis

Articles

- Antolín, A. A.; Jalencas, X.; Yélamos, J.; Mestres, J. Identification of Pim Kinases as Novel Targets for PJ34 with Confounding Effects in PARP Biology. *ACS Chem. Biol.* **2012**, 7, 1962–1967.
  Journal Impact Factor: 6.446; Journal Ranking: Q1 Biochemistry & Molecular Biology; Citations: 16.

- Antolín, A. A.; Mestres, J. Linking off-target kinase pharmacology to the differential cellular effects observed among PARP inhibitors. *Oncotarget*. **2014**, 5, 3023-28. Journal Impact Factor: 6.636; Journal Ranking: Q1 Oncology.

- Antolín, A. A.; Mestres, J. Distant polypharmacology among MLP chemical probes, *ACS Chem Biol*. **2014**, *in revision*.

- Antolín, A.A.; Carotti, A.; Nuti, R.; Hakkaya, A.; Camaioni, E.; Mestres, J.; Pellicciari, R.; Macchiarulo, A. Exploring the Effect of PARP-1 Flexibility in Docking Studies. *J Mol Graph Model.* **2013**, 45C:192-201.
  Journal Impact Factor: 2.325; Journal Ranking: Q1 Computer Science, Interdisciplinary Applications; Citations: 1.

Book chapters

- Antolin, A. A.; Mestres, J.; Knowledge Base for Nuclear Receptor Drug Discovery. In "Therapeutic Targets: Modulation, Inhibition, and Activation" Edited by Luis M. Botana and Mabel Loza, Wiley, New Jersey. **2012**, 309-26. Citations: 1.

- Antolín, A. A.; Mestres, J. The impact of distant polypharmacology in the chemical biology of PARPs. In "Concepts and Case Studies in Chemical Biology" Edited by Petra Janning and Herbert Waldmann, Wiley-VCH, Weinheim. **2014**.

Oral communications:

- Antolín, A.A.; Mestres, J. Towards a Complete Probing Chemome for the PARP Superfamily. Oral communication presented at the 6th Summer School on Drug Design. **2011** Sep 11-16. Vienna, Austria.

- Antolín, A.A.; Mestres, J. De-risking Chemical Biology: Identification of Novel Confounding Targets for PJ34 Warns on its Use to Probe the Biological Role of PARPs. Oral communication presented at the 3rd European Chemical Biology Symposium ECBS2012. **2012** July 1-3. Vienna, Austria.

Poster communications:

- Antolín, A.A.; Mestres, J. Using Cross-Pharmacology Networks to Identify Novel Targets for Chemical Probes: The Case of PARPs. Poster presented at the Quantissue Meeting: Computational Approaches to Networks, Cells and Tissues. **2013** Apr 10-11. Barcelona, Spain.

- Antolín, A.A.; Mestres, J. De-risking Chemical Biology: A Critical View on the Polypharmacology of Chemical Probes. Poster presented at the Gordon Research Conference in High Throughput Chemistry & Chemical Biology. **2013** Jun 2-7. New London (NH), USA.

Part I: Introduction

The use of **natural products** as **therapeutics** has an uncertain origin. The first univocal evidence on the use of plants for healing dates back to our earliest written records,[1] so probably started much further back.[2] Supporting this idea, recent evidence of widespread animal self-medication suggests that the use of natural products as remedial substances likely predates the human race.[3] Despite this uncertainty, our knowledge of the medical effects of natural products has increased continuously since the earliest Sumerian writings.[4,5] The mechanisms underlying drug action, however, remained elusive for millennia. In fact, it was not until the mid-19th century that truly scientific studies of drug action began,[6] and we had to wait until the 20th century before therapeutics started being developed in a scientific manner.[6] In this last century, the discovery and synthesis of new drugs has contributed to the progress of medicine more than any other factor.[7] This notwithstanding, we are still far from having a complete understanding of drug action.

I.1 Polypharmacology in Drug Discovery

The foundation of pharmacology

The history of pharmacology really began when humans started to show an interest to investigate how natural products exert their beneficial actions. It is therefore not surprising that the etymological meaning of the word pharmacology is 'the science of drugs' (Greek ***pharmacos***, medicine or drug; and ***logos***, study).[6] Today, the word pharmacology refers more specifically to the science of studying the interactions between chemicals and living beings with a focus on preventing, ameliorating or curing the deleterious consequences of a disease.[8] The first scientists who tried to understand the effects of natural products were François Magendie and Claude Bernard, physicians who studied the effect of the arrow poison curare using animals in the 19th Century.[6] The

first University Chair of pharmacology was established in **1847** when Rudolf Buchheim was appointed Professor at the University of Dorpat in today's Estonia, a date now considered as the origin of pharmacology as a separate discipline from medicine.[9] However, it was **Oswald Schmiedeberg**, a student of Buchheim and the author of the classic text *Outline of Pharmacology*, who is considered the true founder of modern pharmacology.[6] One of the key theories that originated during the late 19th century was the **side-chain theory** of immunity proposed by **Paul Ehrlich.** Fascinated by the different affinity of different dyes for certain biological tissues, Ehrlich connected chemistry with biology for the first time to explain the action of bacterial toxins.[10] In the side chain theory, Ehrlich proposed that cells contained certain side-chains that would bind **selectively** to certain toxins (Figure 1).[11] It was the beginning of man's quest to explain the mechanism of action of drugs.

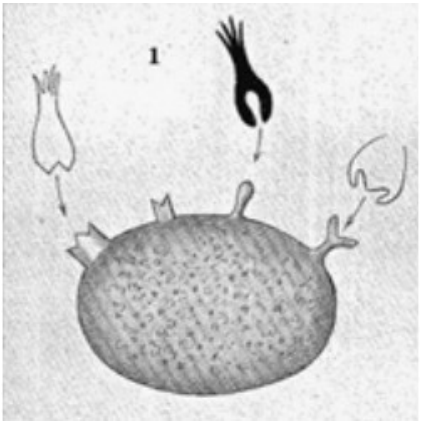


Figure 1. Ehrlich side-chain theory of immunity to explain toxin binding in 1901.[11]

While natural products would continue to be used and studied, humans were close to a historical change in the way novel therapeutics would be sought and discovered, evolving the early anecdotic evidence of the therapeutic benefit of plants into a scientific process.

The initial drug discovery process

Following the establishment of pharmacology as a separate science, the interdisciplinary scientific process with an industrial base that we refer today as **drug discovery** was born about a century ago.[7] At that time chemistry was already a well-established discipline, with Avogadro's atomic hypothesis confirmed and a periodic table of elements settled. There was also a theory on the structure of aromatic molecules that had already boosted research in an incipient **coal-tar derivatives** industry, initially focused in dyes. Synthetic organic chemistry had already begun in 1829 with the synthesis of urea from inorganic substances. And analytical chemistry had already enabled the isolation of **natural products** from plant extracts, like the isolation of morphine in 1815.[7] These technologies yield an early drug discovery process that consisted in screening small numbers of compounds synthesized from available coal-tar derivatives. At that time, the screening process was merely observational and performed directly on living organisms due to the limited understanding of the underlying biology.[12] Therefore, initially drug discovery was largely driven by the **creativity** of **medicinal chemists**, often getting inspiration from natural products,[13] and the molecular mechanism-of-action of drugs remained elusive.[7] In these last years of the 19th century, new companies started to emerge from pharmacies or as divisions of chemistry or dye companies. It was the beginning of a new way of finding, characterizing and developing medicines and it was originating a new industry.[7]

As a consequence of that early drug discovery process, for the first time in history the biological effect of non-natural molecules obtained by synthetic organic chemistry was being obtained. Today, we refer to these compounds as **bioactive small molecules**. Drugs represent the most studied and privileged minute portion of these bioactive small molecules.

The work of **Paul Ehrlich** during that period beautifully illustrates that early drug discovery process. Paul Ehrlich had already noticed that the binding of certain dyes to certain tissues could have therapeutic applications, like the effect of methylene blue on malaria parasites.[14] Next, Paul Ehrlich turned his efforts to try to combat syphilis, a major public health problem in the beginning of the 20th century.[15] To this aim, Ehrlich synthesized and screened derivatives of an arsenic dye. This process of synthesis and screening yield finally the compound **606**, later called **salvarsan**, the first antibacterial drug against syphilis (Figure 2).[14] Salvarsan was rapidly licensed to the pharmaceutical company Hoechst, today part of Sanofi, showing the early connection between academic institutions and pharmaceutical companies[14]. Interestingly, at that time the awareness of side-effects was already present, and the wide use of salvarsan unrevealed toxicities that were addressed by Paul Ehrlich leading to a new drug named neosalvarsan (Figure 2). [14] Also from that time, the relationship between the chemical structure of a compound and its pharmacological action (**SAR**) started being studied systematically,[16] despite the underlying mechanisms by which drugs exerted their effects were still unknown. To overcome this limitation, a breakthrough theory to explain drug action was about to be postulated.

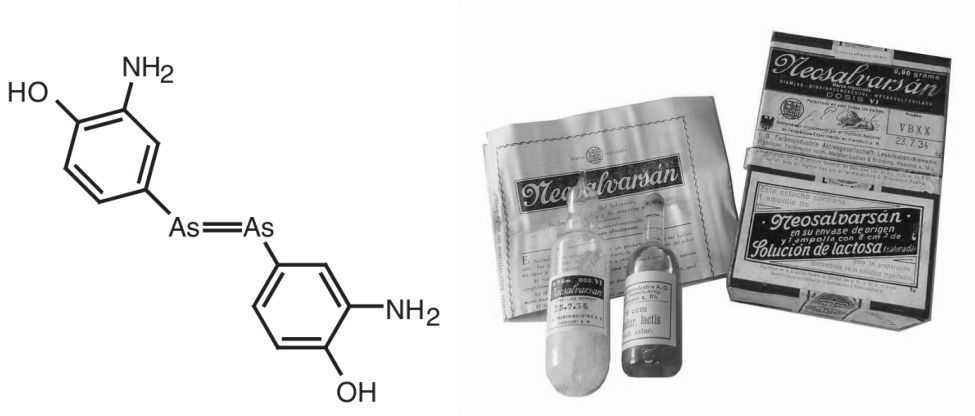


Figure 2. Salvarsan chemical structure (left).[17] Neosalvarsán marketed by I.G. Farbenindustrie Aktiengesellschaft Leverkunsen, Germany (right).[18]

The formulation of the drug receptor theory

The **drug receptor theory** originated during those first years of the 20th century. Following the side-chain theory of toxin binding from Paul Ehrlich (Figure 1), **John Newport Langly** postulated that drugs interacted with a "receptive substance" in biological tissues, inspired also by the concept of chemical reactivity.[16] Ehrlich accepted Langley's point shortly afterwards and evolved the concept of side-chains to **chemoreceptors**, proposing that drugs were '**magic bullets**' that should go straight to their intended cell-structural targets.[16] The idea of Ehrlich, coming from the treatment of infectious diseases, was that molecules should bind to parasites with the highest possible affinity and display the lowest affinity for the human host.[15] However, following generations of pharmacologists would interpret the concept of 'magic bullet' as a compound that targets a single receptor in an exclusive, highly specific fashion.[10] In any case, the drug receptor theory wouldn't be directly accepted. Conversely, it would meet considerable criticism from the academic community and alternative theories of drug action would delay the acceptance and practical application of the drug receptor theory.[16]

Natural products as drugs

Despite the drug discovery process was focused on synthetic organic molecules obtained from coal-tar derivatives, the search for natural product drugs was not abandoned. Conversely, natural products not only inspired medicinal chemists in the design of new synthetic drugs but became drugs themselves. A breakthrough example came from **Microbiology** and the discovery of **penicillin** by **Alexander Flemming** in 1929.[7] Thanks to its efficacy and lack of toxicity, penicillin opened a new era with the incorporation of Microbiology and Fermentation departments in pharmaceutical industry. Those new departments enabled the discovery many other microorganism metabolites that

ultimately became drugs, mainly antibiotics but also immunosuppressants and cholesterol-lowering agents.[7]

The lock and key analogy

Next influencing discipline was **Biochemistry** that introduced the concept of **enzymes**.[7] Emil fisher had already made the famous **lock and key** analogy to explain the specificity of enzyme action as early as 1894.[19] However, he also recognized that this idea couldn't be demonstrated until enzymes were isolated. This happened in 1926, when J. B. Sumner **isolated urease**,[20] a discovery followed closely by the isolation of carbonic anhydrase in 1933.[7] The fortuitous discovery that the active drug metabolite sulphanilamide inhibited carbonic anhydrase helped to create better carbonic anhydrase inhibitors that turned better diuretics.[7]

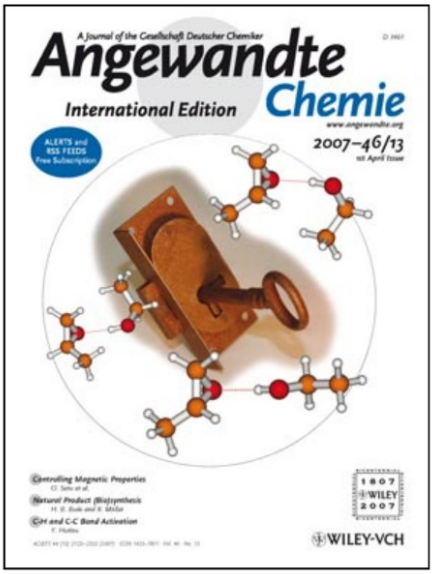


Figure 3. Cover of *Angewandte Chemie International Edition* of March 2007 showing how the lock-and-key concept is applied to the molecular recognition between propylene oxide and ethanol.[21]

These discoveries expanded Fisher's idea of molecular recognition from enzyme substrates to drugs.[22] Drug receptors started to be considered proteins, although the nature of the majority of drug receptors was still unknown. Since then, the metaphor of the lock-and-key has provided successive generations of scientists with a mental picture of molecular recognition processes (Figure 3)[19]. Provably, this lock-and-key concept also helped to settle the idea of **drugs** as **keys** interacting specifically with only one molecular target.[23] Interestingly, Emil Fisher evolved a little further his metaphor to account for the promiscuity of some yeasts at fermenting different sugars.[19] The idea of '**special keys**' opening more than one lock, however, has passed largely unnoticed.

The acceptance of the drug receptor theory

Also by the early 1930s, the first evidence supporting the drug receptor theory came from the first **quantitative analysis of drug action**.[16] **Alfred Joseph Clark** was not satisfied with available qualitative descriptions and analyzed mathematically a large amount of pharmacological data. In his publication *the mode of action of drugs on cells*, Clark showed that for many drugs the relationship between the drug concentration and the biological effect corresponded to a hyperbolic curve.[9] He also related the hyperbolic shape of the **dose-response curve** to the equilibrium binding equation proposed by Hill and later by Langmuir to explain the adsorption of a gas onto a metal surface (Law of Mass Action).[9] Moreover, we also have to thank A. J. Clark for the iconic representation of the dose-response curve in a logarithmic scale (Figure 4). But most importantly, from his calculations of molecular size and cell surface area, Clark concluded that at the low concentrations needed for their biological effects drugs were likely to "exert their action uniting with certain specific receptors of cells".[16] This way, Clark gave an impulse to establish the drug receptor theory by discarding other coexisting theories to explain drug action.[9]

Unfortunately, the Second World War would delay the practical application of the drug receptor theory.[24] After the war, a further step forward towards the acceptance of the drug receptor theory was made by **R. P. Ahlquist** in **1948**.[7] In his seminal paper about adrenergic receptors (or adrenoceptors), Ahlquist concluded that there existed two types of receptors, which he named **alpha** and **beta**.[24] His further observation that the increase of heart rate corresponded solely to the β-adrenoceptor would probe key for the design of the first target-based drug.[24]

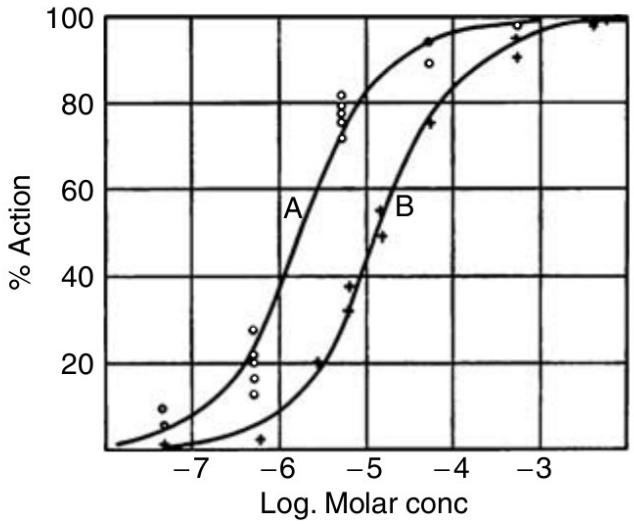


Figure 4. Figure included in the original paper '*The mode of action of drugs on cells'* by AJ Clark that demonstrates the hyperbolic shape of the dose-response curve represented in a logarithmic scale. The concentration-effect curves are for acetylcholine on (A) frog heart and (B) frog muscle.[9]

In 1950s, Clark's occupancy theory was modified to differentiate between **affinity** (the attraction between the drug and the receptor) and **efficacy** (the ability of the drug-receptor complex to induce an effect after binding).[16] These concepts would settle the base for the subdiscipline of pharmacology that we refer today as **pharmacodynamics**.[24] The drug receptor theory was nearly

accepted but it still needed a technological breakthrough and the proof-of-concept development of the first target-based drug.

The first target-based drug

The last breakthrough that strengthened the confidence on the drug receptor theory was the first **direct measure of drug binding**.[9] Although from an analogy to enzymes it was believed that receptors should be proteins, this had not been demonstrated yet. The first evidence of direct measurement of drug binding to receptors came from autoradiographic studies of curare marked with ^{14}C binding to mouse diaphragm performed in the 1960s.[9] These experiments showed a localized binding of curare in the endplate region of the diaphragm (Figure 5). Closely afterwards, quantitative drug binging experiments thanks to tritium labelling and liquid scintillation counters became available (Figure 5).[9] Few years later these ligands were used to isolate and purify their receptors, that turned to be proteins.[9]

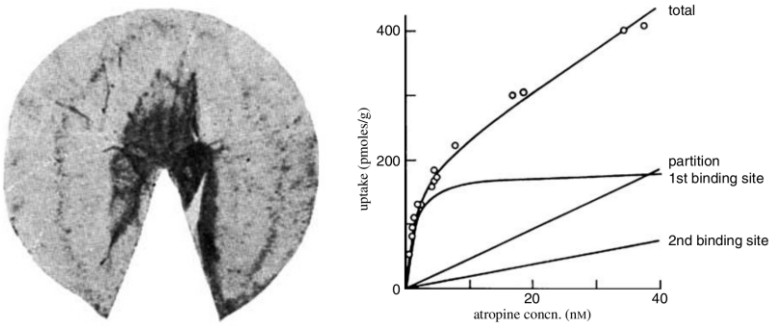


Figure 5. Binding of curare to the diaphragm by autoradiography (left) and quantitative tritium-labelled atropine binding measured by scintillation counters (right).[9]

Finally, the last event in the establishment and acceptance of the concept of receptor was the successful development of a **selective inhibitor** of β-

adrenoceptors.[24] **James Black** was the first to translate the dual receptor theory of Ahlquist from an academic to an industrial setting while working at ICI, now part of Akzo Nobel.[25] The introduction in **1965** of **propranolol** as the first **selective beta-blocker** was a breakthrough in the treatment of anginas and arrhythmias.[25] Propranolol soon became a best-selling drug, still used today for the original and several other indications. For this discovery, James Black was awarded the Nobel Prize in 1988.[24] This way, the high affinity of propranolol for β-adrenoceptors and his lack of affinity for α-adrenoceptors boosted the acceptance of the 'magic bullet' concept in drug discovery. Under this principle, best drugs were totally selective against one target and propranolol was a fantastic example of how this selectivity could be engineered, what James Black called "**rational drug design**". However, it's interesting to acknowledge that propranolol binds in reality a high number of other receptors with high affinity (Figure 6).[26] The initial screening of propranolol only against alpha and beta adrenoceptors highly biased the perception of its selectivity.

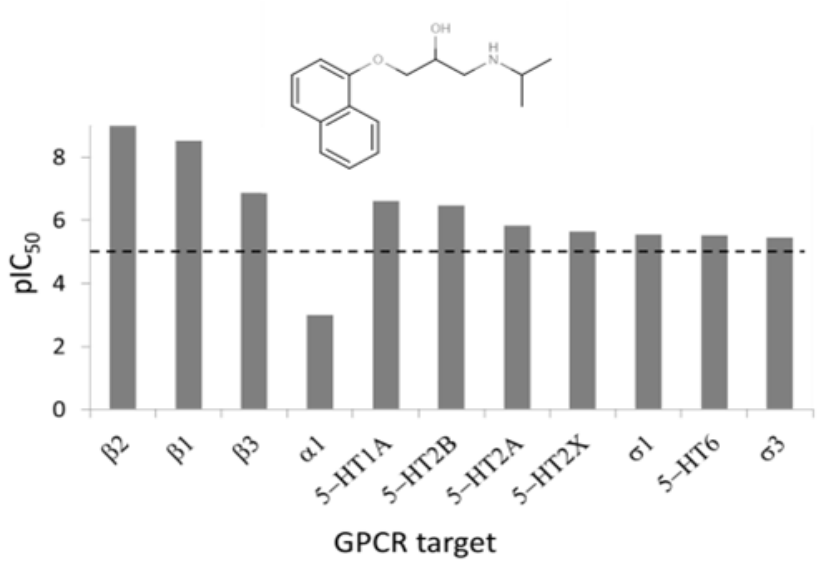


Figure 6. GPCR target profile of propranolol as extracted from ChEMBL 17 database.[27]

Therefore, the full acceptance of drug receptors in cells as the mechanism of drug action is a concept not yet 50 years old. However, the necessary technological advances to fully exploit the drug receptor theory in drug discovery were yet to come.

Paradigm change: Target-based drug discovery

In the mid-1980s the full acceptance of the drug receptor theory and key technological advances enabled a paradigm change in the drug discovery process. Drug discovery changed from a merely **observational** process to a **hypothesis-driven** endeavor. Drugs stopped being tested directly in whole organisms through phenotypic tests. Conversely, a hypothesis about the **single** biomolecule (**target**) responsible of the phenotypic effect would be the first step in that new drug discovery process, changing from reverse to **forward pharmacology**.[28] This way, drugs would no longer be the result solely of the imagination of chemists, but the result of a team work that included chemists, biologists, pharmacologists and several other scientists.[12] However, as the case of propranolol illustrates (Figure 6), the information on the selectivity of drugs across the entire proteome was very limited at that time. The bet that was done in pharmaceutical industry towards the selection of single targets for following drug discovery campaigns was a risky and highly speculative step forward.

The first and most relevant technological advance that enabled that paradigm change was the rise of **molecular biology**.[7] The elucidation of the **double helical structure** of **DNA** had already been performed by Whatson and Crick in 1953. And since the 1960s scientists started to characterize, isolate and manipulate the molecular components of cells. However, the discovery of the **polymerase chain reaction** (**PCR**) in 1983 would be the catalysts to widely start cloning, expressing and purifying genes that encode therapeutically useful proteins.[7]

Another advance towards target-based drug discovery came from the wide adoption of **structural biology in** drug discovery. X-ray crystallography was already a mature technique since the first crystallization of protein-ligand complexes in the 1960s.[29] However, its application to drug discovery remained challenging, with few protein structures solved until the molecular biology revolution enabled a wide access to proteins in sufficient quantity and purity to facilitate crystallization. The last step forward to use structural biology in drug discovery was made thanks to advances in **robotics**, enabling a much more feasible exploration of crystallization conditions.[30] Since then, the availability of crystal structures of protein-ligand complexes has increased dramatically enabling the understanding of the interactions between drugs and their biomolecular targets at the molecular level, which constitutes the basis of **structure-based drug design (SBDD).** Interestingly, SBDD first used physical models (Figure 7) that were soon replaced by computational ones (Figure 8)[31] profiting from the advancement of computer science and its successful application in drug discovery.

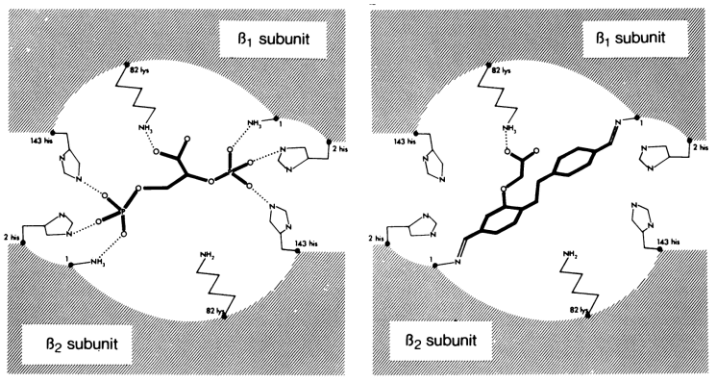


Figure 7. First structure-based design on hemoglobin ligands using physical Kendrew wireframe models published in 1976.[32] On the left side structure of the natural ligand. On the right side, structure of a ligand successfully designed to fit in the protein cavity.

Molecular biology was increasing the number of targets available and, therefore, pushing other disciplines forward. Chemistry and biochemistry also exploited robotics to be able to cope with the increasing demand to identify molecules that interacted with all the targets being cloned, expressed and purified.

From the 1960s to the 1980s, radiochemistry became increasingly popular and biological testing of drugs started to shift from being directly analyzed in cells or whole organisms to being analyzed in simplified biochemical systems, despite the still limited availability of purified proteins. Immunoassays, that used antibodies and radiochemistry to test the effects of drugs directly on their target(s), became one of the methods of choice.[33] The last impulse to the technique would be given thanks to the discovery of ELISA in 1971, that suppressed the need to use radioactivity.[34,35] However, biochemical testing, also referred to as *in vitro* screening, was a relatively slow process until the 1980s that needed also high volumes and high amounts of products.[36] Thanks to robotics, screening down-sized and speeded up leading to **high throughput screening (HTS)**. HTS changed from using single-tube 1 mM assays to using at least 96-well plates and 50-100 μl assay volumes. This miniaturization increased dramatically the capacity to test compounds, changing from 50 compounds per week to thousands.[36]

Chemical synthesis was also pushed forward to keep with the increasing testing capacity. **Combinatorial chemistry** was born by implementing solid-phase synthesis, originally developed by Bruce Merrifield to synthesize peptides, in small molecule synthesis.[37] As a consequence, compound libraries were generated instead of single compounds, benefiting also from smaller quantity requirements for HTS and the number of different compounds available increased dramatically. The creation of large compound databases and its screening substituted the imagination of the medicinal chemist that had

dominated until that moment,[12] often getting its ideas from naturally occurring hormones and substrates.[13]

Target-based drug discovery became increasingly popular until practically becoming the only paradigm supporting drug discovery after the 1990s. The technological advances that supported this paradigm change pushed the drug discovery process towards the exploration of more targets and the screening of more compounds, a brute-force approach that unfortunately was not used to screen each compound against a high number targets. Selectivity would only be evaluated on few and closely related targets despite the increasing evidence of the complex interactions between drugs and the human body.

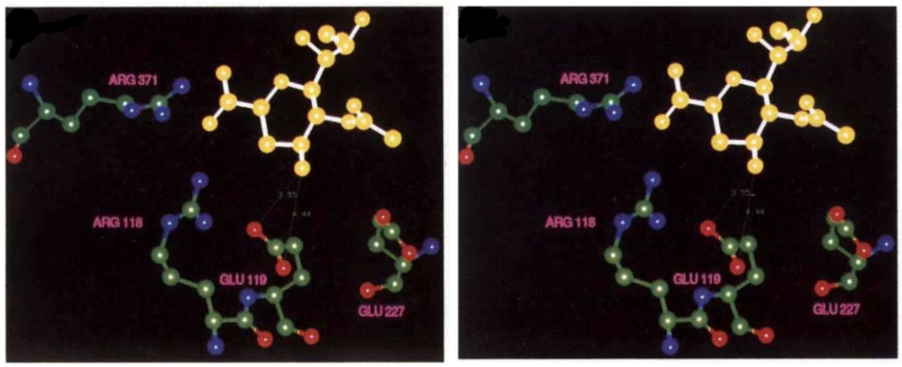


Figure 8. Computational representation of a co-crystal structure of the influenza drug Zanamivir complexed with the protein salanidase (left) as published in Nature in 1993.[38] In the right side, computational calculations of the binding mode of the drug in the receptor performed in the study already show good performance.

Pharmacokinetics and targets of drug metabolism

Pharmacokinetics originated at the same time as pharmacology, with the evidence of the transformation of ingested molecules that occurred inside the human body and the first studies of the concentration of drugs over time performed around the 1850s.[39,40] Today, we can define pharmacokinetics as the

branch of pharmacology that studies the temporary evolution of a drug and its metabolites inside the body, including **Absorption**, **Distribution**, **Metabolism**, and **Excretion** (**ADME**).[41] During many years, pharmacokinetics only dealt with the investigation of the rate at which different drugs were absorbed, transformed and excreted *in vivo* and deriving mathematical models that reproduced them.[39] In the late 1940s it started to be apparent that some of these effects were mediated, at least in part, through **protein-drug binding**. The first evidences came from the discovery that drugs were binding **plasma proteins** in order to get distributed though the body.[42] Shortly afterwards it was also discovered that drug-metabolizing enzymes located in liver cells were responsible for many other pharmacokinetic processes. The final discovery of **P450s cytochromes** (CYP450s) in the mid-1960s changed completely the understanding of drug metabolism.[43] However, despite the discovery of all these new interactions between drugs and biomolecules, drugs continued to be considered selective for their primary target. Targets of drug metabolism were considered to be only responsible for the ADME functions without interfering with the disease phenotype.

The increasing identification and characterization of biomolecules mediating ADME functions, including **drug-efflux pumps** like **ABCB1** and **conjugating enzymes**, made apparent that many drugs were binding to the same biomolecules.[44] Especially relevant was the discovery of the cytochrome P450 **CYP3A4** as a central player in the metabolism of many drugs, leading to drug-drug interactions becoming a concern during the 1970s.[39,45] This discovery also uncovered the broad specificity of these targets, as they were capable of binding totally different drugs.[45] This lack of selectivity, however, was understood as an evolution characteristic exclusive of ADME targets, not applicable to other target families.

This way, it started to be recognized that, in order to successfully perform their function, drugs needed to bind a plethora of other biomolecules to be absorbed, distributed, metabolized an excreted. Moreover, these targets of drug metabolism where recognized to have a promiscuous behavior, capable of binding totally different drugs. However, this knowledge did not change the view of drugs as 'magic bullets' selective for their primary target. In addition, the lack of selectivity of ADME targets was considered an exclusive characteristic for the need of these targets to sense a broad spectrum of compounds. Despite this fact, increasing evidence was starting to link targets of drug metabolism to different disease phenotypes.[46,47] In addition, the identification of many **nuclear receptors regulating drug metabolism** targets and showing also high promiscuity at binding many different ligands increased the number of targets showing broad specificity.[48] A much more complex interaction between drugs and biomolecules was slowly starting to emerge (Figure 9).

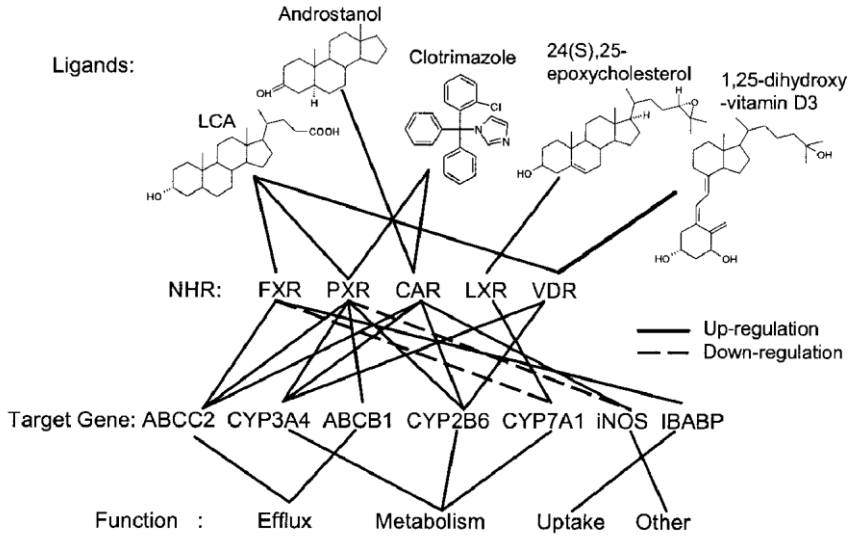


Figure 9. Representation of the promiscuity of different nuclear receptor targets that regulate the expression of ADME genes by means of the recognition of similar ligands. This figure also represents the emerging complexity of the regulation of ADME genes by nuclear receptors.[49]

From toxicology to safety pharmacology and off-target identification

The knowledge of the toxic properties of some plants and minerals has accompanied mankind for millennia.[50] When modern drug discovery started in the early 20th century, it became soon clear that drug candidates could also be toxic and physicians started to study **dose-response** relationships in order to achieve maximum efficacy without toxicity.[51] However, despite common awareness, pre-clinical **toxicology** wasn't initially taken too much into consideration during the drug discovery process nor required by governmental regulation. In 1938, the U.S. Food, Drug, and Cosmetic Act subjected new drugs to pre-market safety evaluation for the first time, despite the tests required for approval were not specified.[52] Although **animal testing** of **drug safety** started in the 1950s, a worldwide disaster was needed to move safety forward.[53] **Thalidomide**, a very popular sedative drug developed in the 1950s, was found to produce severe congenital malformations that affected 10,000 infants worldwide and led to the withdrawal of the drug in the 1960s.[54] This prompted a change in the law of different countries including UK and USA and the evaluation of **reproductive toxicity testing** (especially teratogenicity) was included as part of the standard non-clinical test battery.[53] However, as it happened in the beginning of drug discovery, toxicology was initially evaluated directly on animals as a detailed understanding of the molecular mechanisms of toxicity was still missing.[55]

During the 1980s a fundamental change started to occur. Scientists and doctors started to recognize that drugs could also interfere with organ functions different from the intended mechanism-of-action of the drug.[56] These effects on organ function, that passed undetected to available toxicological tests, were more frequent than serious toxicity and a leading cause of **side-effects** (also referred to as **Adverse Drug Reactions**, **ADRs**).[56] It was the beginning of **safety pharmacology**, later defined as the "studies that investigate the

potential undesirable pharmacodynamic effects of a substance on physiological functions in relationship to exposure in the therapeutic range and above".[56] Despite organ function testing became popular in drug discovery research, governmental regulations provided only general references to evaluations of drug effects on organ system functions that were regarded as quite unimportant when compared to drug efficacy.[56] Another sounded withdrawal was needed in order to advance in the regulation of drug safety.

In the late 1980s it became apparent that the H1-antihistamine drug **terfenadine** was causing **torsade de pointes** (**TdP**), a potentially life-threatening ventricular tachyarrhythmia, in a reduced number of patients.[57] At that time, no regulatory guidance to the pharmaceutical industry existed for studying non-cardiac drugs that could produce this side-effect. Between 1990 and 2001, eight non-cardiovascular pharmaceuticals including terfenadine were removed from the market for this same reason.[57] These drugs were found to prolong the QT interval by inhibiting IKr (**hERG**), the rapid component of the delayed rectifier potassium current. Therefore, this channel present in the heart and the brain was found also to have a broad specificity at being able to bind a wide diversity of drugs (Figure 10), leading to this severe side-effect.[58]

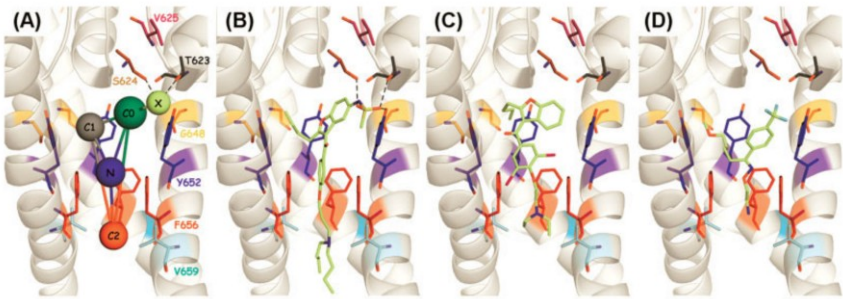


Figure 10. Homology model of the 3D structure of the hERG protein with (A) a pharmacophore model of the moieties governing the binding of drugs to the hERG channel and with the binding models of the drugs (B) dronedarone, (C) amiodarone and (D) fluvoxamine.[59]

The situation changed in the year 2000. The **International Conference on Harmonization** (**ICH**), involving both regulators and research-based industry from the US, EU and Japan, finally achieved its goal of harmonizing critical guidelines on drug quality, safety and efficacy worldwide, including guidelines on safety pharmacology called ICH S7A.[56] These ICH S7A guidelines were mainly *in vivo* and organ function assays, but they included for the first time an *in vitro* pharmacology assay on hERG. This way, hERG testing became the first target-based ADR mechanisms to be required for drug approval in many countries over the world.[60]

Since the inclusion of hERG *in vitro* pharmacology assay in drug regulation, many other toxicity issues and ADRs have been linked to the binding of a drug to another biomolecule different from the drug intended target.[61] The terms **on-target** and **off-target** were coined to distinguish between the target that the drug was designed to bind (also called **primary target**) and the binding to other, generally unknown targets (also called **secondary targets**).[62] This way, on-target toxicity refers to exaggerated and adverse pharmacologic effects at the target of interest in the test system and off-target refers to adverse effects as a result of modulation of other targets, either related biologically or totally unrelated to the target of interest.[62] However, safety pharmacology regulation has been reluctant to include *in vitro* assays on other off-targets apart from hERG.[53,60] Despite the limited regulation supporting *in vitro* safety pharmacology, since the early 1990s pharmaceutical companies started to screen drugs *in vitro* against a predefined panel of targets before starting clinical trials.[61] However, the number and nature of targets varied largely across organizations and these assays were increasingly performed through a growing number of contract research organizations (CROs).[60,61]

In summary, the increasing knowledge on the mechanisms of drug-induced toxicity made apparent that some drugs, despite being designed as selective

inhibitors of their primary target, were in reality binding a plethora of other secondary targets that in many cases were responsible for the side-effects. Moreover, it was also recognized that some of these off-targets, such as hERG, had a low selectivity that conferred them the capacity of binding very different drugs. However, off-target binding was interpreted as an undesirable effect that could be corrected through an improved rational drug design process. The search for selective 'magic bullets' continued to be the dominating paradigm in drug discovery and the identification of secondary targets of drugs became increasingly considered one of the main challenges in the development of new drugs.[63]

One century of drug discovery

In the late-20th early- 21st Century, drug discovery had evolved substantially from the rational process started 100 years before.[64,12] Small companies that emerged from pharmacies or dye companies had maturated into large organizations commonly referred to as **Big Pharma,** with worldwide annual market sales around US **$300 billion** and investing large amounts of money in R&D.[65,66] **New technologies** and **disciplines** developed during the last decades had been integrated in the drug discovery process, from HTS to advances in organic synthesis, informatics, crystallography, proteomics and genomics,[64,12] accomplishing the turn towards target-based drug discovery that started in the 1980s. The established **one drug-one target paradigm** in drug discovery considered that "good drugs should be so **potent** and **specific** that they inhibit their target so completely and specifically as if the target was absent" (Figure 11).[63] Under this paradigm, secondary targets were considered always undesirable and one of the major reasons of the recent increase in costly late-stage drug failures.[64,63] This business model was also making Big Pharma

highly dependent on the approval of many drugs per year to sustain the increasing costs of R&D, patents and regulation.

However, this evolved drug discovery process had become a **complex, long, costly and risky** endeavor, with the average cost and time of bringing a drug into the market being around US $802 million during 15 years.[67] This process, inspired by manufacturing industry, had also been discretized in several steps, each of them having different time and technology requirements (Figure 11).

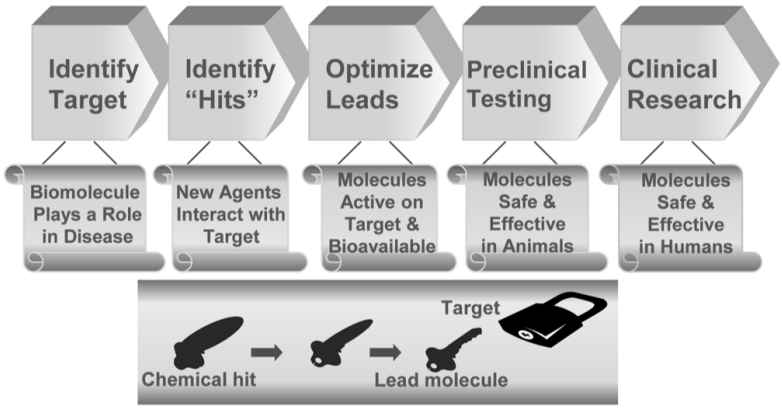


Figure 11. Scheme of the drug discovery process highlighting the prevalence of the one drug-one target paradigm in drug discovery illustrated by the lock-and-key analogy.[23]

The first step consisted in the selection and validation of a single molecular target believed to be essential for modulating a disease.[63] **Target identification** could arise from many different sources of knowledge including biomedical data, genetics and phenotypic screens and was the only step that could be performed outside industry.[68,69] Moreover, the recent publication of the draft sequence of the **human genome** would help to increase the number of possible targets. However, the selection of an appropriate target for a subsequent industrial drug discovery campaign also depended on many non-scientific factors, including strategic level of research policy (research area of interest for the company, budget), managing of research portfolio (number and

scope of projects per therapeutic area) and risk management.[70] **Target validation**, essential to cope with the increasing number of targets identified in academia,[71] was performed using different biological approaches including genetically modified animals and siRNA but these biological methods could not access the protein's amenability to functional modulation by a small molecule. **Chemical biology** was increasingly necessary in target validation as used small-molecule tool compounds (later called chemical probes) to validate the target druggability.[72]

After target validation, the second **hit identification** step consisted in obtaining active compounds (hits) as defined by fixed criteria in a screening assay on the isolated target.[73] Therefore, this step included also thorough efforts on **assay development**. Many different approaches to hit identification could be pursued. One of the most popular strategies was high-throughput screening (**HTS**) against the large compound collections available inside pharmaceutical companies. However, more **focused** or knowledge-based screenings of a portion of this internal database were also common. **Fragment** screening also emerged as an alternative to these methods using smaller compounds and biophysical methods to detect these low-affinity hits. Moreover, computational methods including **virtual screening** and **structure-based drug design** could also be used instead.[73] This process identified generally dozens to hundreds of hits. These hits were then validated during a follow-up process to account for possible false-positives of the assays. Once validated, the hits were clustered into **chemical series** and prioritized. After confirmation in dose-response experiments and evaluation of initial SAR data, usually a small hit optimization phase occurred with synthesis of few derivatives and evaluation of initial *in vitro* ADME (Absorption, Distribution, Metabolism and Excretion), physicochemical, pharmacokinetic (PK) and selectivity properties. In the end, one or several hits emerged from this second step with potencies between 100

nM – 5 μM and a follow-on chemistry program would start to further optimize each of the selected hit series.

Next, the **hit-to-lead** process started with the aim of refining each hit series to produce compounds more potent, selective and with better PK properties to examine their efficacy in the chosen *in vivo* animal model.[73] This process was characterized by intensive synthesis and systematic investigation of structure-activity relationships (SAR) among analogues of the identified hits. Generally, structure-based drug design was used to guide this optimization with structural information (X-ray crystallography, NMR, …) developing the SAR faster and in a more focused way thanks to the available understanding on the molecular interactions driving drug binding.[74] Generally, at this point a **screening cascade** was introduced, accounting for the biological tests and thresholds that would define the ideal candidate for that specific drug discovery project (Figure 12). In general, first the **HTS** on the isolated human target was maintained followed by similar assays on targets where **selectivity** might be known, or expected to be, an issue. Generally targets screened for selectivity were phylogenetically related to the primary target of the project. When a compound met initial criteria it was escalated to further assays such as functional investigations in **cell line** models and the investigation of the affinity of the compound in **orthologs** of the species that would be later used as animal models (generally mice, rat or dog). Also, in this phase a more detailed profiling of **physicochemical** and **ADME properties** was carried out in parallel, like solubility, microsomal stability of CYP450 inhibition. When these criteria started being met, compounds followed-on to first **PK** studies in animal models to ensure absorption and a sufficient half-life *in vivo*.[73] Finally, initial assessment of few anti-targets widely-known to cause serious side-effects would be evaluated such as **hERG** binding.[60] Overall, at the end of this step a few leads from different series would emerge as meeting the majority of the aforementioned thresholds on the different biological assays of the screening

cascade and also intellectual property assessment would be considered (Figure 12). However, the evaluation of selectivity and promiscuity of the compounds was performed on a reduced number of targets as compared to the 20,000 genes present in the human genome, highly biasing the perception of the selectivity of leads.

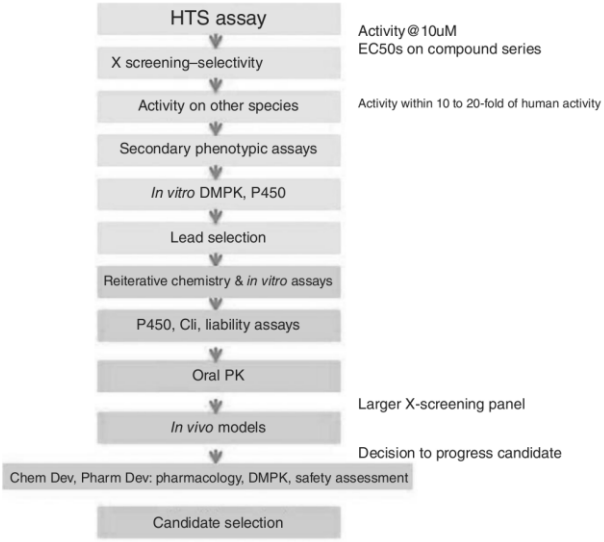


Figure 12. Example of a general screening cascade.[73] Note that the selectivity screening and the screening of possible liabilities is done in a reduced number of targets as compared to all possible off-target interactions on the human genome.

Next, the **lead optimization** phase tried to improve on deficiencies of the lead while maintaining favorable properties.[73] If these deficiencies were finally addressed, the final characterization before being declared as pre-clinical candidate occurred, while medicinal chemistry efforts continued in order to produce back-up molecules to anticipate further failures. The final lead characterization process differed largely among organizations but generally included examination in models of **genotoxicity** (Ames test), *in vivo* models of **general behavior** (Irwin's test), **high-dose PK/PD** studies, dose linearity and repeat dosing PK looking for drug-induced metabolism and metabolic

profiling.[73] Generally in this phase a broader *in vitro* profiling of the lead also occurred but even the largest profiles performed in pharmaceutical industry or through Contract Research Organizations (CROs) were capable of covering less than 2% of the human proteome.[60] After these tests, the lead was ready to enter pre-clinical development.

The **preclinical testing** phase usually covered further efficacy experiments in animal models and all the non-clinical safety pharmacology and preclinical toxicology required by the **International Conference on Harmonization** (**ICH**) such as ICH S7A and ICH S7B that were mainly *in vivo* experiments.[60] This stage, if successful, ended with the filling of the **Investigational New Drug** (**IND**) application to the FDA and regulatory agencies from other countries, that included all the preclinical results and a plan for the clinical trials and had to be reviewed and accepted by regulatory agencies and hospitals for the clinical trials to begin.

Clinical trials were generally discretized in three phases, despite ongoing research to speed up the clinical development of compounds has recently introduced the concept of **Phase 0**.[75] In general, **Phase 1** clinical trials tested the drug in humans for the first time under close-monitoring conditions and in a low number of healthy volunteers (20 - 100). The main goal was to discover if the drug was safe and evaluate the pharmacokinetics and pharmacodynamics. **Phase 2** clinical trials aimed to evaluate the drug efficacy in a slightly larger number of patients (100 - 500) suffering from the disease. Researchers also evaluated the dose and the schedules for dosing. Finally, **Phase 3** clinical trials aimed to extensively investigate for safety and efficacy in a much larger number of patients (1000 – 5000) to generate statistically significant data. After clinical trials, drug companies submitted a **New Drug Application** (**NDA**) to the FDA or corresponding regulatory agency and waited for approval. Finally, all **manufacturing** had to meet all the guidelines and **Phase 4** clinical trials would

continue after approval to ensure no side-effect has passed undetected to previous clinical trials.

In the first Century of drug discovery, **approximately 1200 small-molecule drugs** and **~150 biologics** were developed, targeting **324 biomolecules** out of the approximately 20,000 genes in the human genome, a low proportion even if we consider that the number of druggable genes could be much lower.[76,77] More than half of the small-molecules developed during those last 100 years targeted one of the four main target families, namely Class I G-protein-coupled receptors (**GPCRs**), nuclear receptors (**NRs**), ligand-gated **ion channels** and voltage-gated ion channels and had an average potency of 20 nM (Figure 13).[76] Therefore, there was an historical **bias** in our knowledge towards these major target families in drug discovery.[78,79] Despite the majority of these drugs were developed following the initial drug discovery process and therefore usually inspired in natural products,[80] the paradigm change in the drug discovery process occurring since the 1980s was starting to give the first target-based drugs.[81] In summary, in the 20th Century the introduction of many successful drugs greatly improved patients' lives, contributing to a dramatic increase in life expectancy.[82] Despite this success, many drugs continued to lack a well-defined mechanism of action,[83,84] many targets remained undrugged and many diseases representing global health burdens remained untreatable.[85]

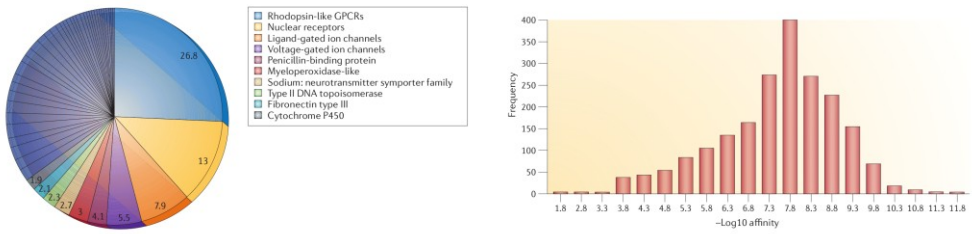


Figure 13. Gene-family distribution of drugs in 2006 (left) and frequency distribution of the potencies of the same small-molecule drugs.[76]

Big Pharma: a business model in crisis

Since the 1980s, the hype with new technologies that were unable to meet with the expectations started being recognized. The benefits of large numbers promised by **combinatorial chemistry** lead to the selection of compounds from mixture-based libraries that usually were inactive or had inappropriate properties to become drugs, ultimately failing in clinical trials. Therefore, combinatorial chemistry was abandoned in favor of more reasonable parallel synthesis of discrete project-focused libraries.[86] From this experience, 'drug-like' and ADME properties became a concern in earlier phases of the drug discovery process to prevent the failures of drug candidates with inappropriate properties. **High-throughput screening** (HTS) also received much criticism, accused of reducing creativity and problems with false positives and false negatives, among others.[87] However, the quality of chemical libraries increased, the problems with frequent hitters were identified and addressed[88] and the quality of HTS data also increased leading to the maintenance of an improved HTS as a hit identification method in drug discovery. The lack of correlation between animal models and human diseases was also increasingly highlighted as a problem in drug discovery.[89] But it was at the beginning of the 21st century when it became apparent that, above punctual problems, the business model followed by Big Pharma during the last decades was unsustainable.[23] The **costs were rising** exponentially (Figure 14), with R&D being about 25% higher in 2003 than in 1998, total industry spending in R&D being around $50 billion and marketing and administrative expenses also rising.[90] However, this increase in R&D spending was not translated to an increased number of approved drugs, with the **number of FDA approved drugs** per year remaining constant at around 20 new drugs per year since 1950 (Figure 14). This problem was coined the **innovation gap**.[91]

Even more worrisome was the overall **success rate** of the drug discovery process, remaining very low (**11%**) despite the efforts to increase it. The solution of pharmacokinetics problems that dominated clinical failure of drugs in the 1990s just lead to lack of efficacy and safety being the major reasons of failure in the 2000s.[92] Moreover, **sounded drug withdrawals** due to side-effects like erivastatin or rofecoxib exacerbated even more the decline on the efficiency of the drug discovery process.[93] This withdrawals also increased the prudence of regulatory bodies, leading to new safety requirements and **regulatory pressure** increasing since the 1980s.[91]

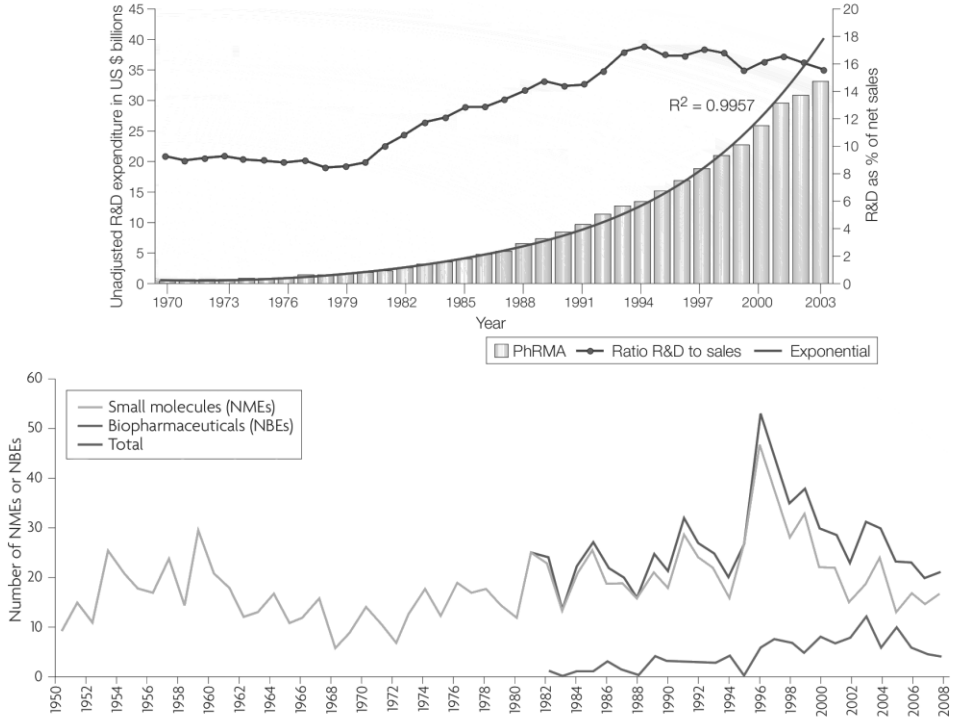


Figure 14. Exponential evolution of the overall R&D costs and their relation to the sales of pharmaceutical companies between 1970 and 2003 (top).[94] Evolution of the number of drugs approved by the FDA between 1950 and 2008 showing the number of drugs approved was basically constant despite the measures introduced in the drug discovery process (bottom).[91]

Moreover, a **decline in innovation** was also starting to be acknowledged, with pharmaceutical companies usually competing to drug exactly the same targets. Despite the incremental innovation that may correspond to the so called "**me-too's**" (second-in-class drugs also referred to as 'me-betters') and the fact that the collective therapeutic advantage of a whole drug class may be higher than the first drug being approved (first-in-class),[94] in the 1990s only 6% of approved drugs targeted a previously undrugged domain.[76] Overall, many R&D resources where allocated on the same targets, with the corresponding reduction on innovation.

Even worse, **key patent expirations** and **increasing prize competition from generics** was also starting to affect the profits of Big Pharma. In 1984 the Food, Drug and Cosmetic Act (also called the **Hatch–Waxman Act**) had created a process by which generics companies could challenge patents to branded or 'innovator' pharmaceutical products.[95] However, during the first decade few generics companies took advantage of this process called '**Paragraph IV**'. But from 1995 on, an increasing number of generics companies started to use the financial incentives provided by the Hatch–Waxman Act to bring generics faster to the market, highly reducing the price of these drugs.[95] Despite these practices already started to reduce the benefits of Big Pharmaceutical companies, patents on a big number of blockbusters ending around 2010 and the reduced pipeline to cover these patents became a growing concern during the 2000s, a situation commonly referred to as the **patent cliff**.

Finally, despite legislators started accusing pharmaceutical industry of questionable practices in the 1950s and critical articles appeared in the literature from the 1980s, it was in the 2000s where the criticism started to level in the sector. In fifty years pharmaceutical industry went from being one of the most admired to one of the most unpopular.[96] Unethical marketing practices, making diseases become chronic instead of curing them and high drug prices that

increased international price controls **tarnished the image** of pharmaceutical companies, culminating their overall crisis.

Many causes were deemed as the reason for the unsustainability of Big Pharma business model. The lower rate of success could be accounted for, at least, the greater complexity of current diseases as compared to easier early diseases (called the low-hanging fruit theory), the competition of drugs with higher standards of care in many diseases and the increasing regulation.[23] Also the persistence on the **blockbuster model**, with Big Pharma focusing on drugs which annual sales could exceed US $1 billion despite the low probability of a drug to become a blockbuster.[91]

Regardless of the causes, since the 2000s Big Pharma started a series of harsh measures to reverse the aforementioned problems of their business model and please shareholders. First of all, Big Pharma started to use their available cash to **acquire** smaller pharmaceutical or biotech companies to fill in their pipelines. Also, some Big Pharma companies started to **merge**. This process would increasingly reduce the number of Big Pharmaceutical companies. Moreover, Big Pharma started also to **downsize** and **cut the number of research projects** adopting measures like **portfolio management** to focus in a reduced number of **therapeutic areas**. Finally, Big Pharma started also to **outsource** many functions, externalizing in Contract Research Organizations (CROs) processes previously done internally and opening sites in cheaper countries like China and India. Overall, these processes yield a painful **loss of jobs** in pharmaceutical industry, many of them attaining R&D scientists.[23]

Overall, the R&D model that enabled the drug discovery successes of the 20th Century was showing signs of fatigue, and increasing voices claimed a redesign of Big Pharma business model.[91] These claims co-occurred with the serendipitous discovery that some drugs had a much more complex mechanism-of-action than previously anticipated.

Imatinib and the unanticipated benefits of kinase promiscuity

Initial anticancer drug discovery consisted in developing toxic chemotherapeutic agents against DNA and the cell cycle, but the discovery of **oncogenes** and the rise of target-based drug discovery transformed anticancer research in the 1980s.[12] **Imatinib** (Gleevec/Glivec) was the first anticancer **target-based drug** developed by Ciba-Geigy (now Novartis) scientists. They selected **BCR-ABL** as a target due to the knowledge that this genetic translocation produced a protein with elevated kinase activity that was the single alteration driving Chronic Myelogenous Leukaemia (**CML**).[97] After evolving a lead from a screening against protein kinase C (PKC), they found a drug candidate devoid of PKC activity and with high affinity for BCR-ABL (IC_{50} = 0,25 μM) and good pharmacokinetic properties that would be finally approved by the FDA in 2001.[97]

However, Imatinib was not only inhibiting BCR-ABL. Initial **kinase profiling** performed by Novartis scientists already found PDGF receptor as an off-target (IC_{50} = 0,1 μM). But when these initial screens widened, **c-KIT** also appeared as a potent imatinib off-target (IC_{50} = 0,1 μM).[98] Interestingly, c-KIT was known to have a key role in the pathogenesis of rare Gastrointestinal Stromal Tumors (**GIST**). In collaboration with scientists of the Dana Farber Cancer Institute, Novartis scientists demonstrated that Imatinib was inhibiting c-KIT driven GIST cells. Both groups patented the new indication of the drug (US6958335)[99] that would be finally approved in 2002.[97]

During the 2000s, larger kinase panels for target profiling and the development of chemo-proteomics continued to increase the targets of imatinib and other kinase drugs, uncovering the wide promiscuity of the kinase family (Figure 15).[100,101] New kinase targets lead to the expansion of the uses of other kinase drugs in cancer, like the off-target affinity of crizotinib in ALK being pivotal to their approval in ALK-positive lung cancer.[102] Therefore, the identification of

new kinase targets among kinase drugs started to provide cases showing the benefits of unanticipated off-targets that didn't cause relevant side-effects but expanded the uses of these drugs. The value of **drug selectivity** started to be challenged and, despite major limitations,[103,104] some kinase drugs like sorafenib (Nevaxar) started being developed to inhibit several kinases (Figure 15).[105] However, there was still unclear to which extent multi-target inhibition contributed to the overall efficacy of these promiscuous drugs.[105] For better or worse, many off-target kinases remained to be identified and fully exploited in cancer treatment and the advantages of **drug promiscuity** remained largely considered valuable only in the kinase field.

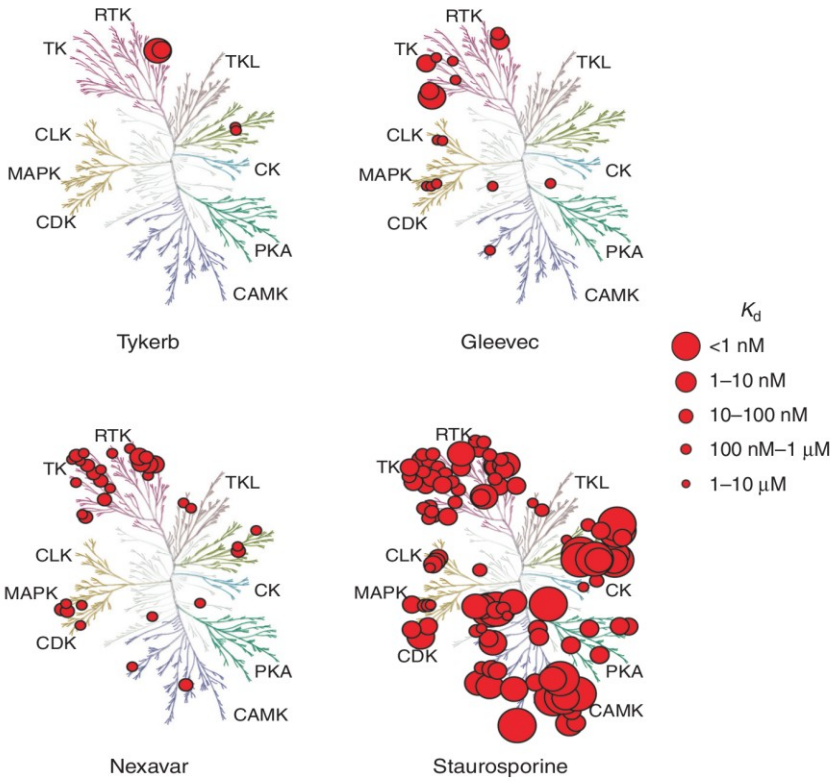


Figure 15. Profiles of three approved kinase drugs (including imatinib or gleevec and sorafenib or nevaxar) and one kinase inhibitor (staurosporine) across 113 kinases. As it can be observed none of the approved drugs is totally selective. Sorafenib (nevaxar), in particular, is shows the highest promiscuity at binding to many kinases with similar affinity.[106]

Drug polypharmacology: The downfall of the one drug-one target paradigm

By mid-2000s, it became apparent that target-based drug discovery was being unsuccessful at delivering drugs for certain diseases, like the many failed attempts to develop selective GPCR drugs to treat central nervous system (CNS) disorders. The efforts to understand the mechanism of action of old pre-target-based drugs that proved to be effective in these diseases started to uncover a much more complex picture of drug pharmacology as first acknowledged by Dr. **Hugo Kubinyi**.[86] Shortly afterwards, a group of scientists lead by Dr. **Bryan L. Roth** not only acknowledged the pleiotropic actions of most clinically effective CNS drugs at binding several GPCRs, but also linked this promiscuity with efficacy, refuting the established conception that promiscuity was solely the cause of side-effects (Figure 16).[107] Since advances in genetics where also showing that many CNS disorders were **polygenic**, the concept of '**magic shotgun**' was proposed to exemplify the benefits of non-selective drugs as opposed to Ehrlich's 'magic bullet' concept. These observations co-occurred with the raise of **systems** and **network biology**, both questioning the reductionist approaches that dominated the study of biology in the 20th Century in favor of more holistic and integrated approaches.[108,109] The one drug-one target paradigm that had dominated drug discovery since the 1980s was receiving its first criticism.

Soon, more concerns with the established drug discovery model started to rise. Some linked the increase of drug failures due to lack of efficacy with the over-simplification of experimental systems used in target-based drug discovery and proposed a **return to phenotypic screening**.[110,111] Others acknowledged the case of **imatinib** (Gleevec) and other kinase drugs to propose the utility of multi-target agents in cancer, another highly complex disease.[112] Interestingly, the case of imatinib also showed that not only drugs developed before target-

based drug discovery were promiscuous. Imatinib was developed as a target-based drug for BCR-ABL. However, due to the limited time and resources, imatinib's **selectivity was initially evaluated only against a few number of other targets** believed to be possible off-targets. When the number of targets was increased it was shown that imatinib was also binding c-kit and other kinases (Figure 15). Therefore, target based drugs could also be promiscuous when their selectivity was further investigated against a larger number of targets, showing the **incompleteness of current knowledge on drug selectivity**. Overall, the one drug-one target paradigm was being increasingly criticized in favor of '**dirty drugs**'.[113] However, drug promiscuity was initially conceived as lack of selectivity against **targets of the same family**. A more global picture of drug selectivity was still missing.

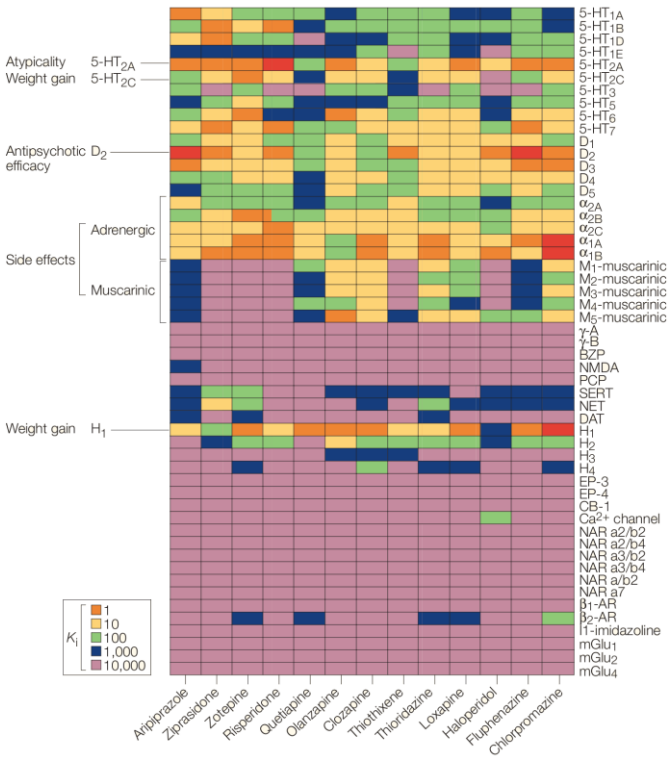


Figure 16. Matrix of the interaction between 13 antipsychotic drugs and 53 receptors colored by Ki values (nM).[107]

The **global mapping of pharmacological space** lead by Dr. **Andrew L. Hopkins** and the publication of the first **drug-target network** lead by Dr. **Marc Vidal** represented a step forward in our global understanding of drug selectivity.

In a pioneering effort to integrate private databases of protein-ligand data, Dr. Hopkins and collaborators constructed a network composed by 700 human proteins (nodes) and 12,119 interactions (edges) showing that 35% of compounds in the integrated database hit more than one target.[114] The concept of linking proteins sharing a high number of ligands would be later coined target **cross-pharmacology**.[115] Dr. Hopkins also popularized the term polypharmacology to refer to the lack of drug selectivity. The word **polypharmacology** had been coined in the 1970s as a synonym of polypharmacy, that is, the use of multiple drugs in a single prescription (*polypharmakos* + *logos*: the study of multiple drugs).[116] However, in 1997 scientists from Pfizer including Professor **Julian Blagg** used the term polypharmacology for the first time to refer to the **lack of selectivity** of a GPCR drug (*poly* + *pharmakologos*: the multiple studies of drugs).[117] Today polypharmacology is the most common word used to refer to the binding of a small molecule to multiple targets.[116] The global mapping of pharmacological space also showed that the majority of promiscuity occurred between targets of the same family, despite significant interactions between different gene families was also observed, with 25% of all promiscuous compounds hitting targets from different gene families. This concept would be later referred to as **distant polypharmacology**.[118] Target promiscuity analysis highlighted targets from GPCRs, CYP P450s and kinases as the most promiscuous, although the incompleteness of the data matrix was already acknowledged and an initial attempt to predict drug polypharmacology was also presented.

Closely afterwards, a group of scientists lead by Dr. Marc Vidal constructed the first **drug-target network** by using data from **Drugbank**, a recently created public database containing drug-target information (Figure 17).[84] They reported an average of **1.8 targets per drug** and observed that the drug-target network was highly interlinked due to drug polypharmacology, with local clustering of similar drugs regarding their indications. By analyzing the topology of the network they also observed an overabundance of "follow-on" drugs highlighting the historical focus on a reduced number of targets.[84] Overall, available data suggested that polypharmacology was a common phenomenon among drugs.

However, the initial drug-target network was highly **incomplete**. As showed by Dr. **Mestres**, available information was **highly biased** towards certain areas of interest.[119] This bias had been created because, due to limited time and resources, drugs had not been systematically screened against a large panel of targets to acquire knowledge about their complete pharmacological profile but solely to the few targets of interest for the particular project at work. These targets had been usually selected on the basis of safety concerns and phylogenetic relationships to the primary target, leading to that historical misconception of drug selectivity.[120] In addition, the biased perception of drug selectivity was aggravated by the fact that, from all the data generated, only a portion was ultimately published and even that was found scattered over numerous bibliographic sources.[121] When a larger drug-target database recovering a higher proportion of scattered data was integrated with Drugbank in the analysis, the topology of the network changed dramatically, questioning some of the conclusion achieved by previous analysis (Figure 17). Moreover, the importance of ***in silico*** **methods for target profiling** to complete the existing gaps in current understanding of drug polypharmacology was also illustrated. Using these methods, the average **number of targets per drug** was

found to increase up to **6.3**.[119] An unexpectedly complex picture of drug–target interactions had begun to emerge.[121]

During next years, new targets for drugs continued to be discovered specially thanks to the creation of many contract research organizations (**CROs**) such as Cerep that offered the possibility of screening compounds across an increasing number of targets.[61] Moreover, initial resources and attempts to settle the bases for rationally designing multi-target drugs also started being reported. [122–124] The emerging complexity of robustness of biological networks suggested that modulating several proteins simultaneously would be often required to modify a given phenotype, supporting also network pharmacology approaches.[125]

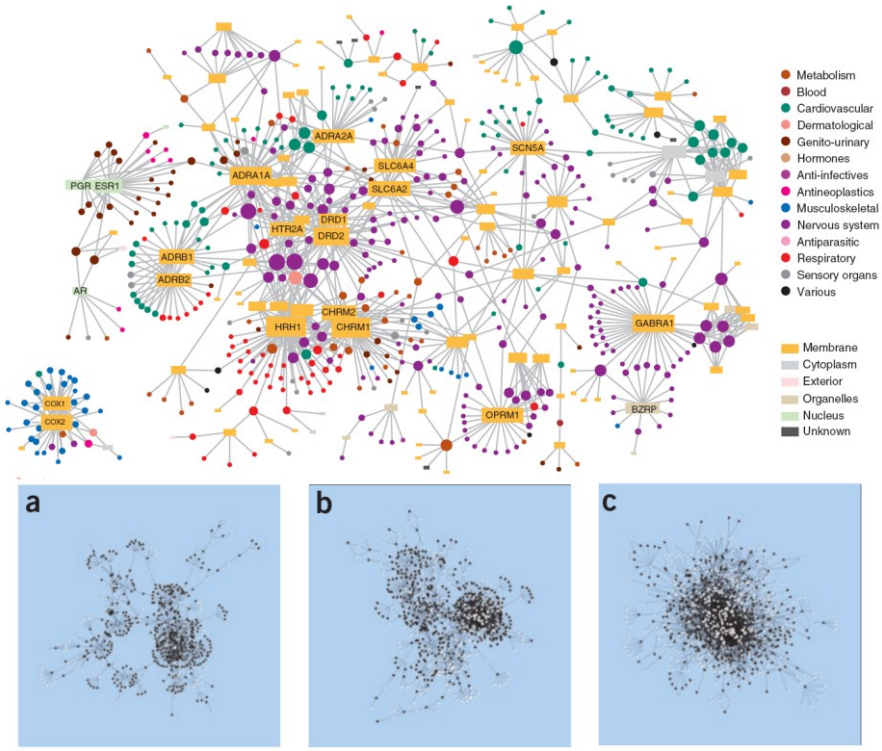


Figure 17. Drug-target network generated by known associations between FDA-approved drugs (circles) colored by their ATC classification and their target proteins (rectangles) colored according to cellular localization as available in Drugbank (top).[84] In the bottom the same drug-target network is reproduced from Drugbank (a), from Drugbank and the drug-target interaction database Wombat (b) and Drugbank + Wombat + *In silico* predictions (c).[119]

However, the 'magic bullet' concept would continue to be considered as a less-risky approach, remaining as a dominating paradigm in industrial drug discovery. Nevertheless, it would become increasingly clear that most drugs exhibited some degree of polypharmacology and drug discovery would slowly embrace more holistic approaches.[126]

Predicting drug polypharmacology: *in silico* target profiling

Informatics is probably the technology that has most widely transformed drug discovery since its introduction, permeating today to all aspects of the process including target profiling.[127] **Computational chemistry** calculations started in the late 1920s**,** rooted in the theoretical developments of quantum mechanics. However, first electronic computers wouldn't appear until the 1940s and the first paper on the application of computer technology in chemistry wouldn't be published until 1946.[128] Second generation computers would follow shortly afterwards and high level computer language would substitute machine code and punched cards. In the 1960s, third generation computers supporting FORTRAN language started to become available at major research laboratories, leading to an explosion in the use of computers. First **chemical database** retrieval systems started in the 1960s, like the Cambridge Structural Database (**CSD**) and the Chemical Abstract Service (**CAS)**.[128] Also in the 1960s first computational files describing the 2D structure of chemical molecules and algorithms to compare molecules among them appeared, such as the first substructure-matching algorithm.[128] The 1960s saw the dramatic expansion of two other research areas of **chemoinformatics**. First, **molecular descriptors** based on the graph theory were proposed as numerical representations of chemical structures, marking the beginning of systematic studies on molecular descriptors.[129] Second, early work led by Professor **Corwin Hansch** demonstrated the need to account for different molecular properties and

describe these properties **quantitatively** in order to rationalize a series of structure-activity data of herbicides.[130] The first **quantitative structure-activity relationship** (**QSAR**) was published in **1962** and the construction of mathematical models relating a molecular structure to a chemical property or biological effect using statistical techniques would become an essential component of pharmacological research ever since.[131] After this discovery, many pharmaceutical companies launched research programs on QSAR.[128] Informatics had become an integral part of the drug discovery process.

In the 1970s major software companies today, like Microsoft and Apple, were born together with the **UNIX** operating system and the programming language C. In 1971 the Protein Data Bank (**PDB**) was established as a database containing experimental crystallographic data (**3D structures**) of macromolecules, software to generate 3D structures from 2D drawings was also published and 3D structure searching systems were also developed.[128]

In the 1980s the first personal computers (**PCs**) were introduced, leading to the wide popularization of informatics.[128] In 1988 **SMILES** (Simplified Molecular Input Line Entry System) was first described and, as a natural extension of the topological representation of a molecule, the geometrical aspects of molecular structures were taken into account since the mid-1980s, leading to the development of 3D-QSAR.[129] During the 1980s also **computer-aided molecular design** became increasingly possible, with theoretical chemistry calculations being implemented on fast computers yielding accurate predictions of thermodynamic and kinetic properties.[132] First protein-ligand **docking** paper was published in 1982 and thanks to the rise on protein structures in the PDB it became increasingly popular in pharmaceutical research, making structure-based approaches an alternative to ligand-based methods.[31] Finally, **molecular dynamics** simulations also became available during the 1980s.[133]

In the 1990s the **World Wide Web** was born and the widespread adoption of graphics-based Web browsers led to the **Internet** revolution.[128] Internet would change the way information was accessed leading to online journals becoming the major source of scientific information today. During the 1990s some of the current standard file formats to represent chemical structures using connection table blocks were introduced, such as the MOLfile or the **SDfile**. Also during the 1990s the first pharmacophore-mapping system was introduced, profiting from the early-developed concept of the **pharmacophore,** a 3D arrangement of molecular features necessary for bioactivity.[131] In the 1990s the **virtual screening** concept was also introduced as an extension of QSAR along the chemical dimension to rank molecules in large chemical libraries according to their likelihood of having affinity for a certain target, thus representing an alternative to HTS.[131] As QSAR methods were performed on series of congeneric molecules, their extension to a larger proportion of the chemical space required of further methods and concepts to generalize local models. **Structure-based methods** using structural information of the target such as docking where developed together with **ligand-based methods** relying on the **central-similarity property principle** that states that similar molecules should state similar properties defined by molecular descriptors, and thus **chemical similarity** calculations lie at the core of these methods.[131] Finally, during the 1990s the Human Genome Project led to a revolution on genomics and the wide adoption and popularization of **bioinformatics**. Bioinformatics profited from the co-occurrence of the Internet and, as opposed to chemical databases that had been largely private, they embraced an open model with resources freely available online.[134]

During the 2000s, chemistry started to slowly embrace the openness of bioinformatics with the launch of several free databases integrating information on the biological activities of small molecules.[128] **PubChem** was launched in 2004 and **ChemBank** and **DrugBank** would follow shortly afterwards in an

early effort that culminated with the final launch of **ChEMBL** in 2010, a manually-curated database offering high quality data.[135] The existence of all these databases would make the classification and annotation of data at the interface of chemistry and biology an increasingly important issue. The use of both unified nomenclatures (**ontologies**) and appropriate classification schemes started being developed to allow an integrative and information-rich knowledge generation.[136] As an example, during the 2000s the IUPAC (International Union of Pure and Applied Chemistry) released the first version of its International Chemical Identifier, the **InChI** code, widely used today as a non-proprietary unique chemical identifier.[128] With respect to the functional annotation of proteins, several classification schemes coexisted, like the Enzyme Commission (EC) code for enzymes or the one developed by the Nuclear Receptors Nomenclature Committee. However, an universal protein code coined **UniProt** had been also recently proposed.[137]

At the turn of the new century, computers and computer technology were an integrated part of our lives and had permeated to all aspects of the drug discovery process.[127] Despite their limitations, computational methods had been successfully used in compound selection, library screening, de-novo design, ADME, biological properties and drug-likeness prediction, protein-ligand binding prediction with advanced approaches, data visualization and network analysis.[127,138] However, the recognition of drug polypharmacology demanded taking *in silico* pharmacology a step further.

By mid-2000s, first ***in silico* target profiling** methods were developed giving to virtual screening a further biological dimension.[131] Early methods for target profiling could be divided into ligand-based and target-based methods. **Ligand-based** methods relied on increasingly abundant annotated chemical libraries that connected small molecules with target proteins to create ligand-based protein models. Several strategies to develop these models could be pursued,

from Bayesian statistics, neural networks or machine learning approaches such as the PASS computer system to the use of pharmacophore models or chemical similarity. To calculate chemical similarity metrics a molecular descriptor was necessary to produce a numerical representation of each chemical structure. The most commonly used descriptors for *in silico* target profiling were **topological fingerprints** encoding the presence of substructural fragments in molecules in a binary fingerprint, like MDL MACCS or Daylight.[139] These fingerprints could be precalculated and compared, usually using Tanimoto distances, in a fast and efficient manner. However, it was proposed that the use of pharmacophoric features could be more relevant for *in silico* target profiling than topology or substructures.[140] Therefore, descriptors based on **topological atom-centered feature-based distributions** such as CATS or SHED, developed in our laboratory, were also implemented and successfully used for *in silico* target profiling.[131,141,142] Although extremely computationally demanding compared with ligand-based methods, applications of **target-based virtual** profiling were also developed, mainly relying on docking.[131]

Despite the successful development of all these methods, there was a lack of confidence on the ability of computational tools to identify new targets of drugs.[143] In 2009, two large-scale discoveries ultimately brought awareness to the use of *in silico* target profiling to identify new targets for old drugs.[144] First, a work led by Dr. **Peer Bork** used phenotypic side-effect similarity to identify new targets for known drugs. By analyzing 746 marketed drugs, 13 new drug-target relations were identified.[145] Second, a group of scientists led by Dr. **Brian K. Shoiket** and Dr. **Brian L. Roth** used the SEA (Similarity Ensemble Approach) method of statistical similarity computed using two fingerprints as topological descriptors to identify new targets of known drugs.[146] By analyzing a panel of 3665 drugs and pharmaceutical compounds, the study validated 23 new drug–target associations, five of which highly potent, that enabled to

explain the efficacy or side-effects observed among famous drugs such as Prozac (Figure 18).

These publications demonstrated that it was possible to predict polypharmacology and the increasing availability of data in the public domain would boost the developments of other methods for predicting drug promiscuity. *In silico* predictions for target profiling had come of age and were ready to impact the drug discovery process.[143]

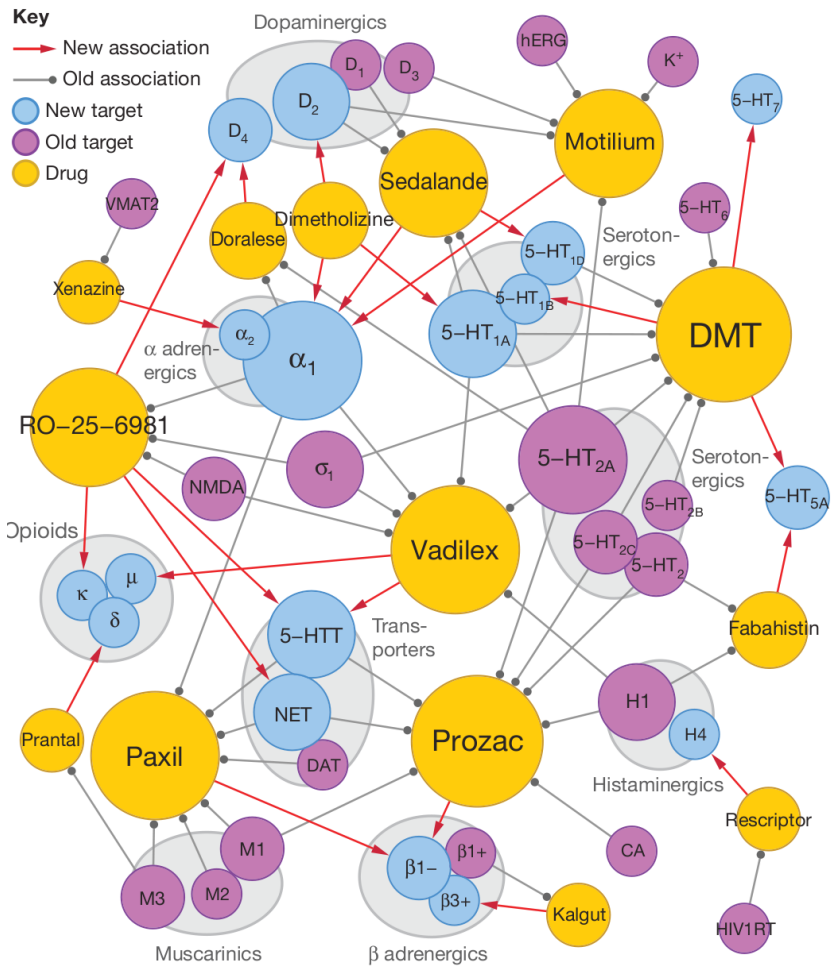


Figure 18. Bipartite network where drugs (yellow) are linked by grey edges to their known targets (violet) and by red arrows to their discovered off-targets (blue).[146]

The impact of polypharmacology on drug discovery

Today, the one drug-one target-one disease model still remains as the established paradigm in drug discovery and Big Pharma maintains its business strategy.[147] However, the harsh measures adopted by Big Pharma during the past 15 years have catalyzed alternative drug discovery models relying, at least in part, in drug polypharmacology. Moreover, the polypharmacological nature of drugs is becoming increasingly accepted by the broad scientific community, contributing to the embracement of more holistic approaches to pharmacology.[148] We are, slowly, turning towards systems drug discovery.

Since the 2000s, Big Pharma has received an increasing amount of critics and criticism until becoming one of the most unpopular professions today.[96] The initiatives implemented to overcome the hurdles of the 2000s have been painful and produced new problems without reversing the stagnant R&D productivity.[91] Fifteen years of mergers and acquisitions have shown that this strategy does not increase the output of drugs approved.[149] Conversely, a **decrease on the rate of progress of compounds** through the pipeline has been reported. Also, **massive job firings**, **research cuts** and **closure of research sites** have produced a devastating impact on the internal innovation of resulting organizations.[149] As a result of these measures, **the number of pharmaceutical companies** with the financial capacity to bring a new drug to the market has been dramatically reduced, contributing to the stagnant number of new drug approvals that cannot cope with a steepening patent cliff.[91,150]

However, these measures have not been totally unsuccessful. Drug industry continues to earn good money in a growing market of global prescription sales reaching **$875 billion** in 2013 and the valuation of Big Pharma companies has resisted past hurdles, in part thanks to the reduction on R&D expenses.[151,152] There are also recent signs of increased value of new drug approvals.[153] But without increasing the number of drugs being approved the pharmaceutical

business model will become, at some point, unsustainable.[152] Despite the recent proposal of mega-merger between Pfizer and AstraZeneca showing that Big Pharma continues to pursue old strategies,[154] some alternative business models and strategies have also flourished, giving hope to the future of drug industry.

First, **Chemical Biology** departments are opening in Big Pharma.[155–157] On the one hand, these departments focus on **target validation**, as targets coming from academia have been blamed of having poor reproducibility and target selection is considered a major responsible for the high number of drug failures due to lack of efficacy.[69,152,155] One the other hand, these Chemical Biology departments are responsible for **phenotypic screening** projects as an strategy to recover some of the success of the initial drug discovery process that is gaining increasing popularity also in academia.[158,159] To avoid the development of drugs without knowing their mechanism of action, one of the limitations of the early drug discovery process,[28] **target identification** (or target fishing) strategies are gaining popularity. Experimental methods such as chemoproteomics have been successfully used but also ***in silico*** **target profiling** has demonstrated to be a useful method for target identification, highlighting the impact that these methods are already having in drug discovery.[158,160,161] This return to phenotypic screening could indirectly foster the development of multi-target drugs as no prior single-target hypothesis is being imposed.[162]

Second, we are also seeing new alternative business models in pharmaceutical industry some of them relying, at least in part, in drug polypharmacology. Besides the change from blockbusters to '**niche busters'** following the success of some biotech companies in rare diseases,[163] drug industry is increasingly interested in **drug repurposing** (or repositioning), that is, the use of a drug in another indication.[164,165] This strategy is not new and has always been used in pharmaceutical industry (like the case of viagra illustrates) but it is increasingly

pursued also by biotech companies and academia.[166,167] Despite the identification of a new target is not mandatory to repurpose a drug (the same target can be involved in different pathways and tissues) and despite repositioning a drug goes far beyond finding a new target, some drug reprofiling strategies are already exploiting drug polypharmacology.[165]

Third, the crisis of the Big Pharma business model is forcing industry to realign resources away from the early R&D,[149] making **small start-up companies**,[91,168] **academia**[169–171] and **non-profit organizations** increasingly important players in drug discovery.[172,173] This is accompanied by a considerable embrace of more **openness** and **collaboration**, including joint development of drugs, public-private partnerships to develop chemical probes, the successful release of proprietary compounds and patents from pharmaceutical industry to public databases, prizes, competitions, innovation networks, consortia and initiatives like the Medicines for Malaria Venture (MMV) of the Bill & Melinda Gates foundation to advance antimalarials.[91,174–178]

Another interesting move is the one lead by cancer drug discovery, betting for a more **personalized** and **precision medicine** with **biomarker** identification and the use of companion diagnostics as a means to a more informed use of drugs in a selected patient population.[179,180] The rationale behind this approach is that current clinical trial failures are due to the persistence of Big Pharma in one-size-fits-all blockbuster drugs not targeted to the specific molecular characteristics of individual patients. This old approach leads to expensive clinical trials that account today of more than 60% of overall costs of developing a drug. By developing smarter, smaller and shorter clinical trials and moving proof-of-concept studies (POC) to earlier clinical phases the cost of drug development could be substantially reduced.[152] Due to the big number of kinase drugs among anticancer therapeutics and their known promiscuity, off-targets are increasingly considered as therapeutically meaningful.[102,181] However,

personalized medicine strategies generally consider drugs as 'magic bullets', aiming to develop fully selective drugs targeting specific genetic defects to which tumors are addicted and rationally combine them to overcome drug resistance. Despite recent evidences pointing towards a pivotal role of polypharmacology in drug synergism,[182] drug promiscuity remains to be fully exploited in personalized cancer medicine.

These examples show how polypharmacology is already having a meaningful impact on drug discovery despite Big Pharma continues to largely rely on the selective drug paradigm. In recent years, drug polypharmacology has been increasingly recognized as a property of the majority of drugs, a promiscuous behavior that might recall that of endogenous hormones and metabolites and that has been proposed to lie at the heart of protein evolution.[116,183] One of the main reasons for this gain of acceptance of drug polypharmacology is the increasing availability and integration of **public data** as we move towards a **Semantic Web** in life sciences.[184,185] Public databases of ligand-target interactions continue to exponentially grow in size and ontologies and integration are also being developed in pharmacology. Despite this daunting task if far from complete and this increasing amount of information creates new challenges, big efforts including European projects such as Open PHACTS are enabling a much wider access to this data for knowledge generation and pharmaceutical R&D.[186–190] By analyzing these increasingly publicly available databases, several authors have **linked polypharmacology to molecular properties and fragment composition** of small-molecules, as the proposal of a relationship between the presence of positively charged fragments and promiscuity or the observation that most promiscuous drugs tend to be highly hydrophobic.[116,191,192]

Computational methods have greatly contributed to the identification of polypharmacology, with more than 249 new drug-target interactions identified

between 2008 and 2013 using *in silico* approaches, representing a 7% increase in known drug-target interactions.[116] The majority of the new targets identified correspond to the same family of the primary target, but sounded cases of distant pharmacology have also been identified, like the recent identification of nanomolar GPCR affinity in the kinase drug sorafenib.[193] New computational methods to detect polypharmacology have also flourished during these last years, including those relying in binding site similarity with the introduction of the concept **chemoisosterism** as the property that relates different protein environments interacting with the same chemical fragments.[22,116,194] These computational methods are becoming increasingly sophisticated, sensitive and specific and thanks to the increasing availability of public ligand-target interaction data cover today around 5000 targets, a large proportion of the human genome. However, polypharmacology also continues to be serendipitously identified in large-scale drug profiling experiments, like the recent and sounded identification of nanomolar bromodomain affinity among several clinical kinase inhibitors or the identification of MTH1 as an off-target of the kinase inhibitor crizotinib.[195,196] Both computational and serendipitous identification of polypharmacology are contributing to expand a drug-target network that is becoming increasingly complex, illustrating nicely the intricate inter-family cross-pharmacologies observed in current drugs (Figure 19).[197]

The majority of new targets of drugs identified have an affinity around 10 μM, potent enough to be relevant at high drug doses (overdoses) and therefore relevant for **adverse drug reactions**.[116] Accordingly, Big Pharma is becoming increasingly interested in predicting polypharmacology as a means to identify off-targets that could cause unwanted **side-effects** on their drug candidates. Their approach is to identify these off-targets as early as possible in the drug discovery process and use rational drug design to eliminate any off-target affinity, maintaining the selectivity of their drug candidates.[198] In this same direction, large-scale consortia of Big Pharma, biotech companies and academia

are also trying to integrate internal data from Big Pharma and develop better models to predict toxicology and side-effects early in the drug discovery process, like the European projects ARITMO, EU-ADR and eTOX.[199–201] Unfortunately, drug regulation is not keeping pace with current understanding of drug polypharmacology. Despite *in vitro* target profiling of drug candidates against pre-defined panels of targets known to be relevant for side-effects is a common practice in pharmaceutical industry, only *in vitro* hERG affinity is required for New Drug Applications.[60] This lack of regulation might be a lost opportunity to speed current understanding of drug polypharmacology and its role in many side-effects.

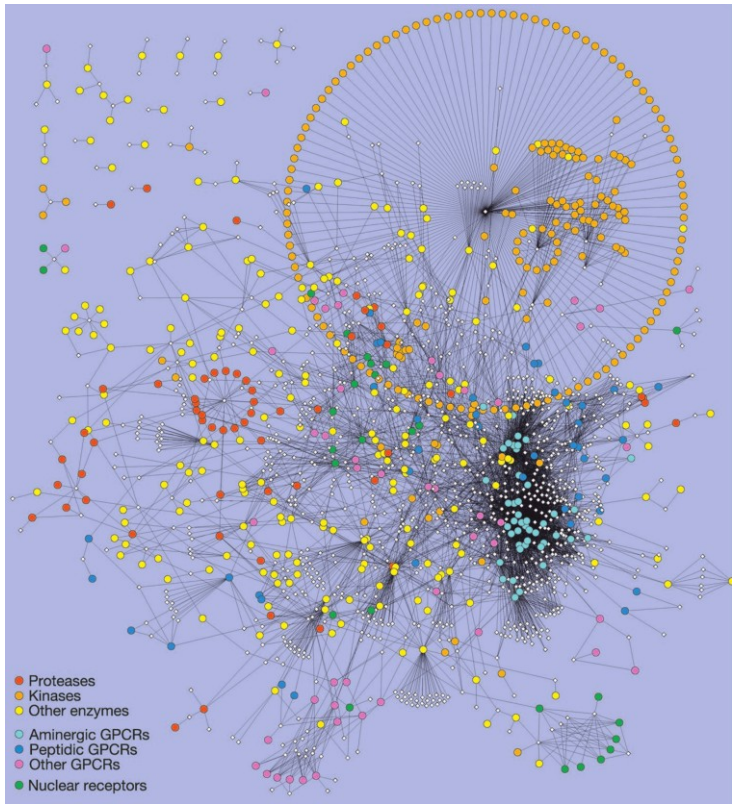


Figure 19. Drug-target network. Drugs (small white circles) are linked to targets if their affinity is better than 1 μM.[197]

Despite the reluctance of Big Pharma, during the last years several academic initiatives are also trying to settle the basis for rationally designing multi-target drugs, a strategy also referred to as **targeted polypharmacology**.[202] These initiatives are mainly coming from the two protein families where polypharmacology was first acknowledged: kinases and GPCRs. In the kinase field, Dr. **Kevan M. Shokat** lead the first efforts to design dual inhibitors of tyrosine and phosphoinositide kinases and a recent approach using directly flies as an animal model while Dr. **Philip J. Hajduk** lead first conceptual bases to navigate the polypharmacological space for multi-kinase drug discovery.[104,203–205] In the GPCR field, Dr. **Andrew L. Hopkins** and Dr. **Bryan L. Roth** led an outstanding computational approach that demonstrated the feasibility of designing molecules towards a concrete polypharmacological profile.[206] These and novel initiatives to target polypharmacology continue their way in academia, showing how pharmacology is slowly becoming a more holistic discipline.

In the last decade, the word **systems pharmacology** was coined as an emerging concept to give a more holistic perspective to the study of drug action.[207] Systems pharmacology is broadly defined as the approach to translational medicine that combines computational and experimental methods to elucidate, validate and apply new pharmacological concepts to the development and use of drugs and the determination of the mechanisms of action of new and existing drugs in preclinical animal models and in patients.[208,209] Accordingly, new systems pharmacology groups, centers, education programs, journals, concepts and (omics) technologies have been generated, giving a broader view of the actions of drugs in biological systems and pathways, from its subcellular distribution to its genome-wide localization.[147,208–215] Accordingly with this less reductionist approach, drugs are increasingly placed in the context of the proteins with which they can potentially interact, their metabolites, the organs and tissues they can reach and

the polymorphisms of the person that takes the drug (Figure 20). As in systems biology, **computer science and quantitation** have a principal role in this new discipline, sometimes also called quantitative and systems pharmacology, with special focus in multi-scale and network modelling and requiring the integration of different types of data.[138,208]

This turn towards systems pharmacology and the embracement of more holistic approaches is increasingly uncovering the lagoons existing in current understanding of drug action, especially thanks to the embracement of omics technologies. The reduction of sequencing costs is enabling to **sequence cancer cell lines and patients after drug exposure**, showing that some drugs are unexpectedly effective against specific cancer alterations.[216–218] Other types of omics data such as gene expression and siRNA screening are also uncovering previously unanticipated effects of drugs.[219,220]

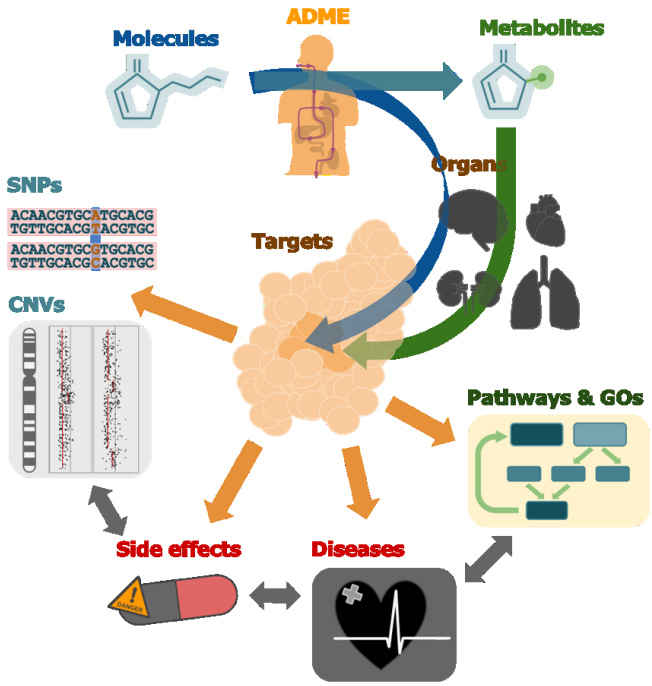


Figure 20. Scheme showing the many different actions and levels of interaction that a drug can have inside the human body (courtesy of Nikita Remez, Chemotargets SL).[221]

An increasing evidence of our limited understanding of drug action is also coming from specialized target areas, such as the discovery that some GPCR drugs affect only one of the downstream signaling pathways of their targets, a behavior called **functional selectivity**.[222,223] Interestingly, the first target-based drug, propranolol, is among these functionally selective drugs, illustrating how the understanding of drug action evolves with time.[224] New advances in pharmacology have also come from the kinase field where ATP-competitive kinase drugs have been found to differentially affect the recycling of their target by the **HSP-90 chaperone system**.[225] These are just two of the more recent examples showing that a more detailed understanding of the exact mechanism-of-action of many drugs is still missing. Even worst, many drugs lack any validated mechanism-of-action,[83] with the mechanism of some old widely-used drugs like paracetamol having been clarified only recently.[226]

Today, after 150 years of pharmacology and 100 years of drug discovery, our understanding of drug action has evolved substantially and mankind has succeeded in developing new drugs to treat many life-threatening diseases.[227] However, we still die of diseases and the drug discovery process continues to be a very difficult endeavor with high failure rate mainly due to our limited understanding of biology and disease but also of drug action.[228] Much is still left to be learned about the connection between drugs' mechanism of action, associated side-effects, and interaction with multiple protein targets.[197] Fortunately, science and knowledge grow at an accelerated rate and we are more conscious of the need to get a more holistic approach to biology, medicine and pharmacology in order to succeed. Today, the quest to understand, exploit and discover drugs continues more alive than ever.

Chemistry and biology originated very closely in time. First, **modern chemistry** was established in the 18th Century after the quantitative analysis of Antoine Laurent Lavoisier and the support of the scientific method from Robert Boyle transformed ancient alchemy into a science.[229] Second, the foundation of **modern biology** as a coherent field, rooted on the ancient developments of natural history and medicine, arose during the 19th Century.[230] Initially, both sciences evolved tightly interconnected as scientists were usually familiar with both chemistry and biology. Unfortunately, with time and increasing knowledge, both sciences developed unique training and languages that torn them apart.[231] Accordingly, small chemical molecules have been underrepresented in major biological advances occurring in the 20th Century, such as the central dogma of molecular biology.[232] The development of molecular biology tools also led to an underutilization of chemical tools to study biology. Despite the success of drug discovery at bringing these two and other disciplines together, both sciences continued to be largely separate worlds. However, at the end of the 20st Century, an effort to bring Chemistry and Biology together gave birth to a new discipline.[231]

I.2 Chemical Biology

The origins of chemical biology

First experiments using chemistry to advance biology and *vice versa* co-occurred with the masterworks founding both chemistry and biology.[17] **Joseph Priestley** experiments exposing animals to gases performed on the 18th century are provably the first attempts to get insights into pure chemistry using biology without a clear interest in medicine. Next, the experiments of the effects of nitrous oxide performed by Sir **Humpfrey Davy** on himself would open the door to increase our understanding of biology, with the role of nitric oxide in

cell signaling finally receiving the Nobel Prize of medicine in 1998.[233] This way the origins of chemical biology, despite modest, are rooted more than two centuries ago.

In 1828, Friedrich Wöhler discovered by accident that he could obtain the organic molecule urea starting from inorganic substances, giving birth to **organic chemistry**.[17] As the new discipline developed a unique language, organic chemists became increasingly interested in synthesizing and confirming the structures of molecules isolated from biological samples without a deeper interest in their biological effects.[234] In 1847 **pharmacology** was born as the science of studying the interactions between chemicals and living beings but with a specific focus on preventing, ameliorating or curing the deleterious consequences of a disease, not with the aim of undestanding basic biolgy.[8] At the beginning of the 20th Century **biochemistry** was also founded as a new discipline.[235] Initially focused on enzymes and in the chemical basis of life processes, the discipline would turn slowly into a biological discipline with little chemistry.[236] During the 20th Century, the separation between chemistry and biology continued despite the drug discovery process successfully joined both disciplines creating new fields like **medicinal chemistry**.[12,237] However, this union didn't transcend to the basic research in biology and chemistry. During that period, the discovery and development of new chemical reactions led to the synthesis of many biological molecules such as the development of the carbodiimide reaction by Goblind Khorana that led to the synthesis of ATP.[234] A turning point occurred in 1955, when Eugene P. Kennedy used the carbodiimide reaction to synthesize and test CDP-choline in biological experiments even before this small molecule was isolated from nature.[234] These experiments showed to biochemists how useful could chemical tools be for their studies of the chemical reactions of life, fostering a renewed interest in chemistry.[234] Next, **Bioorganic chemistry** was born with the aim of applying synthetic and physical organic chemistry to biological questions and would be

the precursor of chemical biology.[238] However, this interest wouldn't last long. The **molecular biology** revolution in the 1980s gave outstanding new tools to biologists and biochemists to manipulate directly the DNA. This powerful technology produced an abandonment of chemical tools in many laboratories, despite some scientists specially in **cell biology** would maintain an interest in using small molecule tools. Chemistry and biology had developed so different languages and tools that they were a barrier for the communication between both sciences.[239]

Chemical Biology, the emergence of a new discipline

During the 1990s, an increasing number of **organic chemists became interested in biology**, provably due to the boost in biological research.[240] New journals promoting interdisciplinary science and a common language between chemistry and biology also started popping up.[231,240] It was the beginning of a new discipline.

Today, **chemical biology** can be broadly defined as the use of chemistry to advance a molecular understanding of biology and the harnessing of biology to advance chemistry.[241] However, corresponding to a dynamic and rapidly growing area of research, the term chemical biology still means slightly different things to different scientists.[242,243] Despite its clear **interdisciplinary** nature, provably the application of chemistry methods and techniques to study biological systems has been more fruitful than the application of biological insights to the advancement of chemistry.[244] Many examples of the first are available, including the development of **chemical probes** to previously considered undruggable targets, **protein tags** to trace proteins inside cells, the development and use of **unnatural aminoacids** and **optogenetics** while **foldamers** and **enzyme engineering** to create new reactions are among the few examples from the latter.[243,245] Following the emergence of chemical

biology, a new wave of research centers and journals settled this new discipline since the mid-2000s, with the opening of new departments in universities, the recruitment of faculty members and the development of specific training programs.[244,246,247]

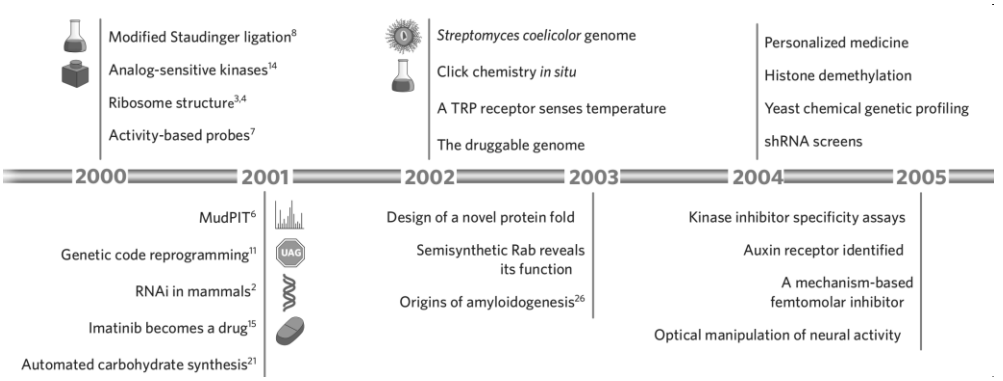
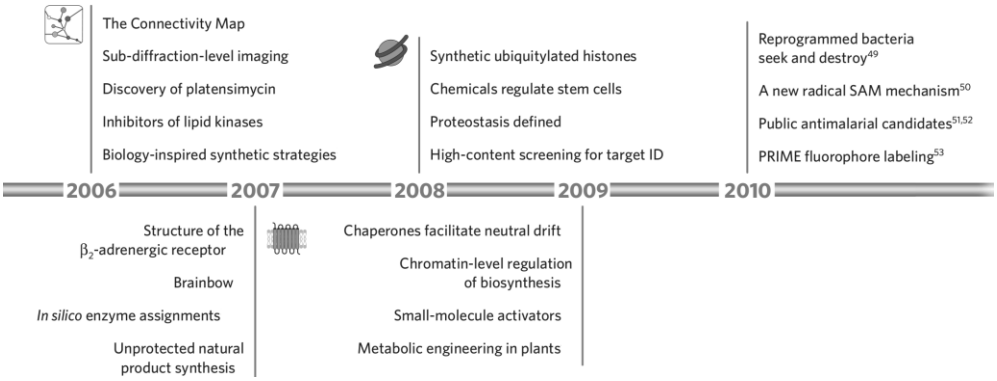


Figure 21. Timeline showing key chemical biology discoveries over the 2000-2010 decade.[238]

In 2010, after two decades of chemical biology, the discipline had maturated to embrace a changing scientific culture and could be considered as fully established. The initial interest of organic chemists in developing tool compounds to study biology had evolved thanks to a new wave of chemical biologists that were also interested in applying new tools and methods to make profound discoveries in biology. Therefore, it was no longer solely the interest of organic chemists in biology what was driving chemical biology. Accordingly, major contributions of chemical biology during the 2000-2010 decade, from *in situ* **click chemistry** as a foundation to **bioorthogonal chemistry** to a catalogue of **genomic signatures after drug exposure**, were being widely acknowledged by the broad scientific community (Figure 21).[238] Also the demonstration that chemical molecules could affect biological systems in unsuspected new ways opened new fields of research, like the discovery that small-molecules could regulate stem cells.[248] The emergence of 'big science' and

systems biology was also encouraging to embrace the complexity of biological systems in an increasingly collaborative manner. And the increasing availability of public compound libraries and a wider access to HTS methods in academia fostered research at the chemical-biology interface.



Chemical probes as tools to study biological systems

One of the most logic, straightforward, and earlier uses of chemistry to study biology was through the use of **bioactive chemical molecules**. Despite it was soon acknowledged that synthetic chemicals could produce biological effects,[17] the mechanism was initially unknown and, therefore, chemical molecules could not be used to gain a molecular understanding of biological processes. However, during the 1960s it became apparent that drugs, natural products and synthetic small-molecules were exerting their biological effects through **directly binding biomolecules**.[16,24] Since then, small molecules had been used as a non-invasive means to perturb and study the function of the macromolecules they target in biological systems. Initially, the small molecules used to study biology were mainly **natural products** or **drugs**.[249,250] Accordingly, the 'magic bullet' concept of drug action was translated to small molecules tools used to

study biology, believed to be fully selective for their primary target regardless of the concentration used. As an example, the natural hormones norepinephrine, epinephrine and isoproterenol were pivotal to distinguish between alpha- and beta-adrenoceptors before their genes or protein structures were known, and associate only to beta-adrenoceptors a pivotal function at increasing heart rate.[24,183] Also, colchicine was used to investigate cell division more than 50 years ago.[251] However, the rise of **target-based drug discovery** and **HTS** in the 1980s would facilitate the interrogation of the chemical space in a more systematic manner directly on isolated and purified proteins, identifying more **synthetic small-molecules** that would later serve as **chemical tool compounds**, also referred to as **standard inhibitors**.[252] However, initially, these HTS approaches were largely restricted to industrial settings.[249] The rise of **molecular biology** and the discovery of the PCR gave new powerful tools to study biological systems and the later development of RNA interference (**RNAi**) technology became widely used to understand the biological function of protein targets.[238] Despite the reduced use of small molecules, chemical tools maintained an important role in annotating the human genome and validating new molecular targets due to their high complementarity to RNAi and genetic approaches.[253] The capacity of small-molecules to inhibit the function of proteins instead of eliminating the target protein from the system is highly valuable as it avoids multiple functions or scaffolding effects. Moreover, small molecules also offer immediate inhibition and a greater control of the extent and kinetics of the inhibition, as compared to RNAi and genomics techniques.[249,251]

Accordingly, paired with the rise of chemical biology in the 2000s, the National Institutes of Health (**NIH**) **Molecular Libraries Program** (**MLP**) began in 2004 with the aim of expanding the availability, flexibility, and use of small-molecule chemical probes for basic research, helping to popularize the term '**chemical probe**'.[254] This big project brought HTS technology and compound

libraries from pharmaceutical industry to academic settings and facilitated the access of all the information generated through the development of PubChem. The MLP would also pave the way for European replicas developed afterwards.[255] However, a lot of discussion accompanied the MLP program since its inception, with much concern about the high amount of funding involved.[256,257] Interestingly, the program also fostered much debate around the **definition of a chemical probe**, a concept that had been previously unclear and not subjected to regulation or general guidelines.[257] The first chemical probes were envisioned to have "adequate potency and solubility to be useful for *in vitro* cell-based experimentation" and much freedom was given to each screening center to decide the exact thesholds.[254] But the lack of specification created controversy and the criteria to define a chemical probe were evolved further until defining that chemical probes should have an **affinity below 100 nM** for the primary target and **at least ten-fold selectivity** against related targets.[258] However, as it occurred with drugs, the selectivity of chemical probes was generally evaluated across few phylogenetically related off-targets and, in some cases, also against predefined panels of a few dozens of targets, covering a reduced proportion of the human proteome. In 2009, an initial evaluation of the program by a group of experts found problems with 25% of the chemical probes developed and the lack of characterization and availability of chemical probes was also criticized.[258,259] To address this issue, **Stephen V. Frye** proposed a series of principles to guide chemical probe qualification, including sufficient *in vitro* potency and selectivity data to confidently associate its *in vitro* profile to its cellular or *in vivo* profile.[260,261] However, it was later argued by Professor Dr. **Paul Workman** and collaborators that too strict rules could foster innovation, with fitness factors proposed as opposed to strict rules.[249] Finally, a more context-dependent definition of chemical probes prevailed, defining chemical probes as compounds that represent an improvement over existing art.[262] The MLP project ended in 2011 surrounded by much discussion

about their continuation with almost two hundred chemical probes identified, one of them in Phase I clinical trials.[263] Today, the discovery of chemical probes continues in academic settings with an increasing number of publications reporting the identification of these useful tool compounds.[260]

Chemical systems biology and the limits of reductionism

The rise of **systems biology** made it soon apparent that a systematic understanding of how small molecules were affecting biological systems was missing, stressing the need to develop **cheminformatics** tools to **integrate** and interpret the huge amounts of data from large-scale experimental approaches that were being made publicly available, such as the MLP program.[264,265] The identification of drug **polypharmacology** also uncovered that many small molecules perturbed multiple targets in a cellular system, a property that could be used to modulate biochemical pathways in robust biological systems that would be missed with selective compounds or RNAi.[265] Therefore, it was argued that chemical biology should lessen their **reductionist one compound-one target view** and avoid the over-simplification of describing small molecules as single-target inhibitors, such as glycogen synthase kinase 3β inhibitors. Conversely, it was proposed that chemical biology should embrace a **systems approach** defining the biological activity of small molecules using their **chemical genomics profile** and using more **phenotypic** and ***in vivo*** screens.[266] Moreover, an analysis found that **few drugs qualified as chemical probes** due to the high selectivity criteria being imposed to probes, creating concerns on the capacity of chemical probes to be appropriate starting points for drug discovery campaigns.[267] Accordingly, the concept of **multiple probes** was proposed to account for compounds able to modulate more than one target at the same time (Figure 22). This idea was evolved further to propose the systematic probing of multiple targets, an ambitious goal coined the

'**probing chemome**' (Figure 22) aiming to identify a complete set of small molecules that cover all possible profile combinations arising from a given set of targets.[267] This approach would enable a true systems approach to interrogate biology.

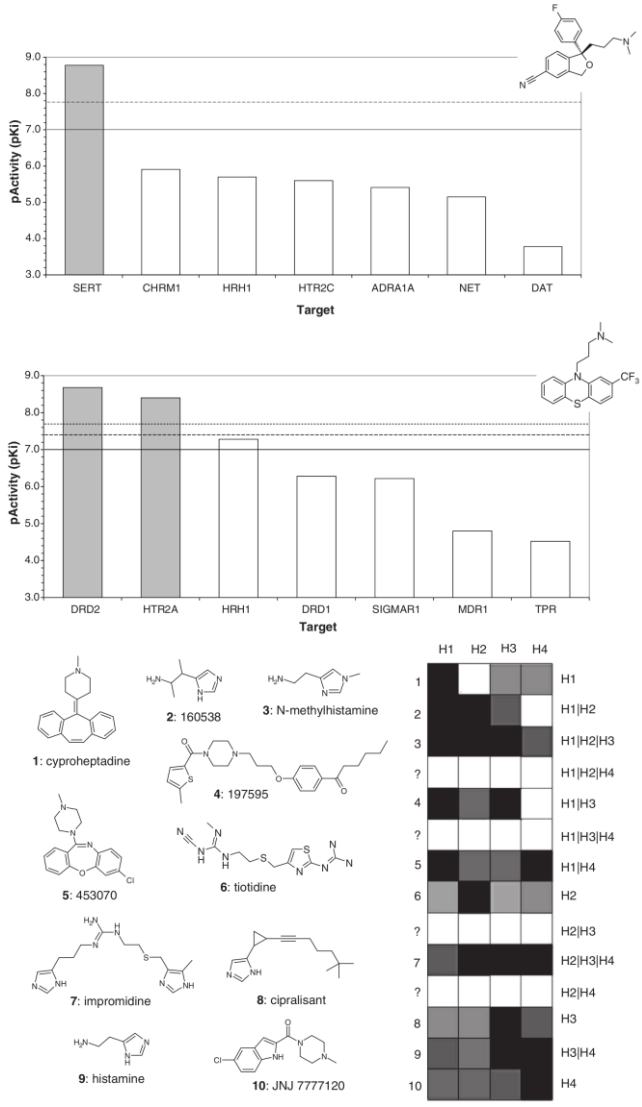


Figure 22. Escitalopram (top) as an example of a drug designated as a selective chemical probe of the sodium-dependent serotonin (SERT) transporter. Trifluprom azine (mid) as an example of a drug designated as a level 2 multiple probe of the target profile defined by the dopamine D2 (DRD2) and serotonin 5-HT2A (HTR2A) receptors. Current level of coverage (bottom) for a complete probing chemome of the family of histamine receptors. Adapted from ref. 267.

During the past years, several **phenotypic screening probes** have been developed, already showing some embracement of a more holistic view of chemical biology.[261] However, the view of probes as selective for their primary target continues to be the dominating paradigm in chemical biology. A 2013 editorial in *Nature Chemical Biology* stressed that "**chemical probes should be held to a higher standard than drugs**" especially regarding their **selectivity**.[268] Also one of the key goals of chemical biology continues to be the identification of a small molecule to modulate specifically each gene on the human genome, showing how the **reductionist** view still imperates in chemical biology.[267] Despite more elaborated guidelines for target validation using chemical probes have been proposed, including the use of siRNA, the use of several active and inactive analogs to rule out off-target effects and the use of **target engagement** to validate the observed effect,[155,269] the lack of characterization of the target profile of chemical probes and the use of high concentrations could compromise some of the conclusion achieved using small molecule tool compounds being made available.[270] Today, chemical probes continue to be strongly sought to functionally annotate the undrugged human genome and advance towards smarter therapeutics[175,249] but a much broader view of chemical probes and the full embracement of a systems approach to chemical biology are still missing.

Until now, we have reviewed the impact of polypharmacology on drug discovery and the history of chemical biology separately. However, today both disciplines are tightly interconnected. Chemical probes are necessary to study the biological function of proteins as the pharmacological modulation is highly complementary to other methods used to study protein function, like siRNA and knock-out models. This chemo-biological information is generally used in follow-on drug discovery campaigns once the target is validated for a certain disease. Therefore, chemical probes serve as an inspiration for medicinal chemists in the design of new drug candidates and the biological insights gathered using chemical probes shape the therapeutic strategy during drug development. In this final chapter of the introduction, we will review the case of the poly(ADP-ribose)polymerase (PARP) enzyme superfamily. In this PhD Thesis, PARPs serve as a proof-of-concept target family to illustrate the connection between chemical biology and drug discovery and to study how polypharmacology affects them both.

I.3 PARPs: from Chemical Biology to Drug Discovery

The discovery of poly(ADP-ribose)

The first evidence of the existence of an acid-insoluble product in chicken nuclei came from the lab of Dr. **Paul Mandel** in **1963**.[271] Mandel's group in Strasbourg and two groups from Japan independently characterized the product shortly afterwards and by 1966 it became clear that the product was a **polymer of adenosine diphosphoribose** generated from NAD^+.[272] After the demonstration that the polymer could be obtained from enzymatic extracts of nuclei, a race started in order to isolate and characterize the enzyme responsible

for the synthesis of this new biopolymer.[273] As it has usually happened in biology, a reductionist approach was dominating research at that moment and no-one conceived that there could be more than one enzyme catalyzing the formation of the biopolymer. The enzyme, initially called **poly(A)DPR polymerase**, was purified from many sources between 1971 and 1977,[274] demonstrating also the ubiquity of this enzyme across different organs and organisms. Also, the branched structure of poly(ADP-ribose) was characterized during the late 1970s.[275] However, the function of the biopolymer was being more difficult to characterize, as scientists believed that the function had to be unique and evidences were pointing towards totally different directions. The principal biological functions for the polymer **poly(ADP-ribose)** (PAR) that started being suggested were the modulation of gene replication, DNA repair or expression and the maintenance of chromatin architecture.[274] However, the key discovery that finally boosted the interest on poly(A)DPR polymerase was about to happen.

A chemical tool to study poly(ADP-ribose) biology

During the 1970s, in parallel with the biological investigations of poly(A)DPR polymerase function, several groups started to search for inhibitors of this enzyme to gain further insights into its function. Initially, the search focused on naturally-occurring small molecules such as **nicotinamide** but in 1975 it was described that **benzamide**, a close analog of nicotinamide, was also an inhibitor of the enzyme, which name had evolved to **poly(ADP-ribose) polymerase**.[276] By 1980, four inhibitors were known (Figure 23): nicotinamide, component of the natural substrate NAD^+, the natural products **thymidine** and **methylxanthines**, and benzamide.[277] However, these natural products had other known functions and were therefore blamed to lack "physiological specificity", contributing to ongoing difficulties at identifying the function of

poly(ADP-ribose).[276] Benzamide, in contrast, was too insoluble to have a practical application *in vivo*. To overcome these limitations, **Michael R. Purnell** and **William J. D. Whish** developed **3-aminobenzamide (3-AB)**, a "physiologically specific" and cell-permeable inhibitor of poly(ADP-ribose) polymerase (Figure 23).[276] 3-AB was demonstrated to have a K_i of 4.4 μM in permeabilized L1210 mouse cells and to be selective over NAD glycohydrolase.[277] Despite all these molecules were modest poly(ADP-ribose) polymerase inhibitors, their availability was essential for the proof-of-concept study demonstrating that poly(ADP-ribose) participated in **cellular recovery from DNA damage**.[278] In this study published in *Nature* in 1980, Dr. **Sydney Shall** and colleagues demonstrated also that the inhibition of poly(ADP-ribose) polymerase using milimolar concentrations of 3-AB and other inhibitors **prevented re-joining of DNA** and was thus **synergistic with DNA-damaging chemotherapeutics**. From this study, **3-AB emerged as the most useful chemical tool** to study poly(ADP-ribose) polymerase biology and it is still used today in some publications.

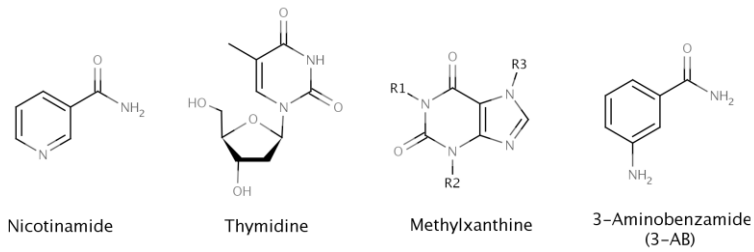


Figure 23. Structures of first chemical tools used to study poly(ADP-ribose) polymerase.

Second generation of poly(ADP-ribose) polymerase inhibitors: chemical probes and drug candidates

The development of a chemical tool and its use to demonstrate a key role of poly(ADP-ribose) in DNA repair boosted the capacity and interest to study this

enzyme. During the 1980s, **3-AB** was used in more than 150 publications to get further insights into poly(ADP-ribose) polymerase biology and therapeutic potential, many of them in animal models. Moreover, 3-AB was pivotal to **purifying the enzyme** in sufficient homogeneity to enable the complete characterization of their molecular properties and their modular structure by using affinity chromatography.[279] Initial SAR studies on benzamides were also performed.[280] However, the appearance of **molecular biology** would also boost the use of biological techniques to study poly(ADP-ribose)polymerase biology.[281] Cloning their cDNA and gene uncovered that poly(ADP-ribose)polymerase was a zinc-finger nuclear protein of 116 kDa coded by a gene on chromosome 1 that bind to nicks of DNA triggering its activation.[282] It was also shown that the enzyme poly(ADP-ribosyl)ated several nuclear proteins, including histones and poly(ADP-ribose)polymerase itself, playing a key role in recruiting the cellular machinery that triggered DNA repair (Figure 24).[282,283] Moreover, at the late 1980s, Poly(ADP-ribose) polymerase would start being abbreviated as **PARP**.

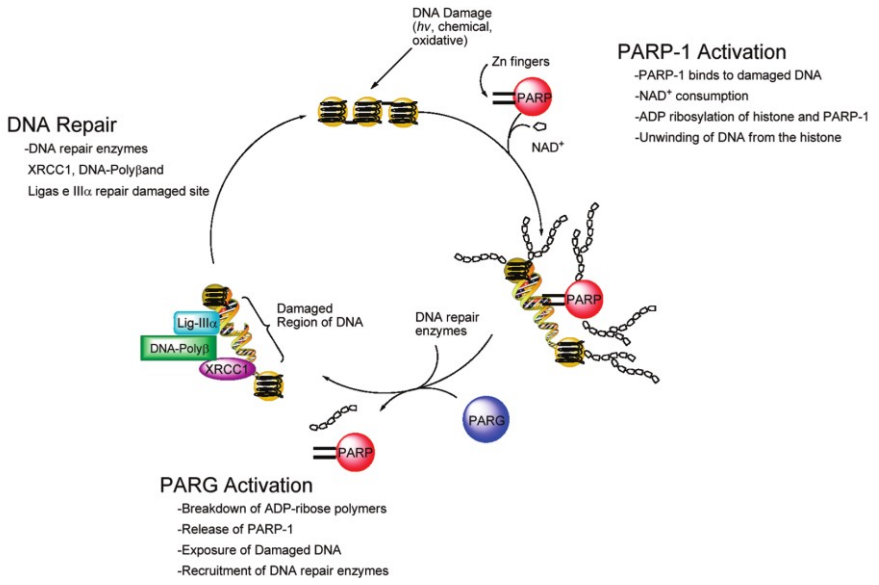


Figure 24. Role of poly(ADP-ribose) polymerase (PARP-1) in DNA repair.[278]

Further insights on the exact catalytic mechanisms by which poly(ADP-ribose) was formed were clarified during the 1990s thanks to the first crystal structure of the chicken PARP catalytic domain being published in 1996 (Figure 25).[284,285]

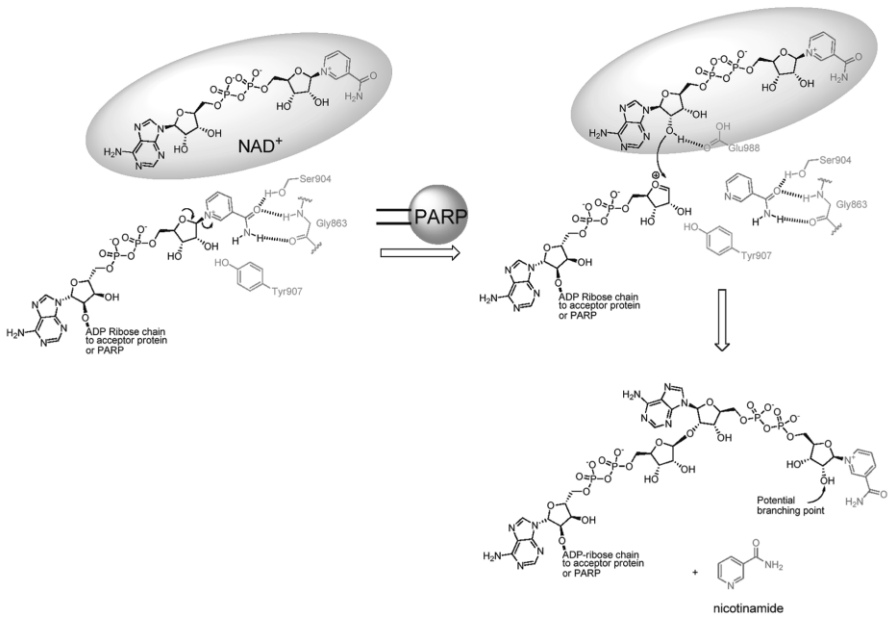


Figure 25. Poly(ADP-ribosyl)ation mechanism catalyzed by PARP.[278]

Also during the 1990s, a key work would boost finally the race to discover current PARP drug candidates and chemical probes. In an attempt to improve the potency of first chemical probes to study PARP biology and avoid some off-target effects already acknowledged even for 3-AB,[280] **Ueda** and **Banasik** from Kyoto University screened over 100 compounds from several structural classes and discovered that several bicyclic and tricyclic lactams were submicromolar inhibitors of PARP, demonstrating that constraining the arilamide of 3-AB into a ring was beneficial for PARP potency (Figure 27).[278] This work, together with the first crystal structure of PARP and earlier docking studies helped to refine the benzamide PARP pharmacophore (Figure 26).[278,286]

Some of the bicyclic lactams identified became widely-used chemical probes due to their improved potency over 3-AB, like 4-Amino-1,8-naphthalimide (4-ANI), 1,5-dihydroxyisoquinoline (ISQ), and phenanthridinone (PHE), among others (Figure 27).[280] These lactams also became the basis for medicinal chemistry programs aimed to finding PARP drugs, with the pioneering work from the Universities of Bath and Newcastle and several Big Pharma companies entering the race in the late 1990s, either independently like BASF or Merck, or acquiring the programs initiated in Universities or small biotech companies, such as Pfizer, Sanofi, Eisai or AstraZeneca (Figure 27).[278] Many of the structures of the drug candidates coming from these programs were undisclosed for a long time but some failed drugs like **PJ34** or NU1025 that never entered clinical trials were published, becoming among the most used PARP chemical probes for their improved potency and solubility (Figure 27).[287]

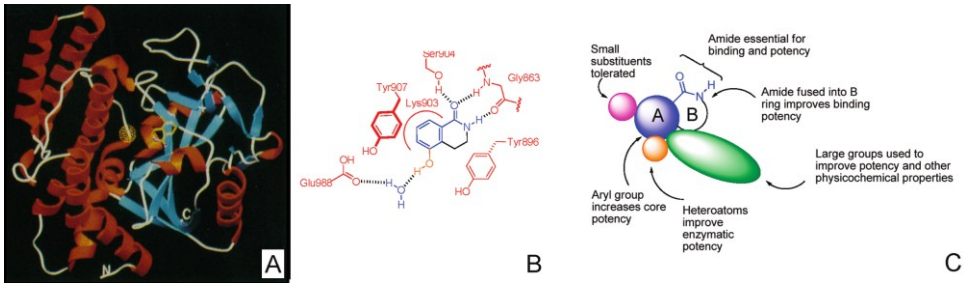


Figure 26. (A) First crystal structure of the catalytic domain of chicken PARP.[285] (B) Binding mode of the benzamide moiety of PARP inhibitors as exemplified by ISQ cocrystal structure.[278] (C) Pharmacophore of PARP inhibitors.[278]

The rationale for developing PARP inhibitors during the 1990s was to potentiate alkylating chemotherapeutics or radiation.[280] But since the mid-1990s the evidence that PARP was playing a significant role in ischemic damage of cells prompted the investigation of PARP inhibitors also in ischemia.[278] Later, the structures of drug candidates that today are under clinical development

would be made available, being rucaparib, olaparib and veliparib the more advanced ones (Figure 27). According to the dominating target-based paradigm in drug discovery all PARP drugs were considered to be PARP-selective.

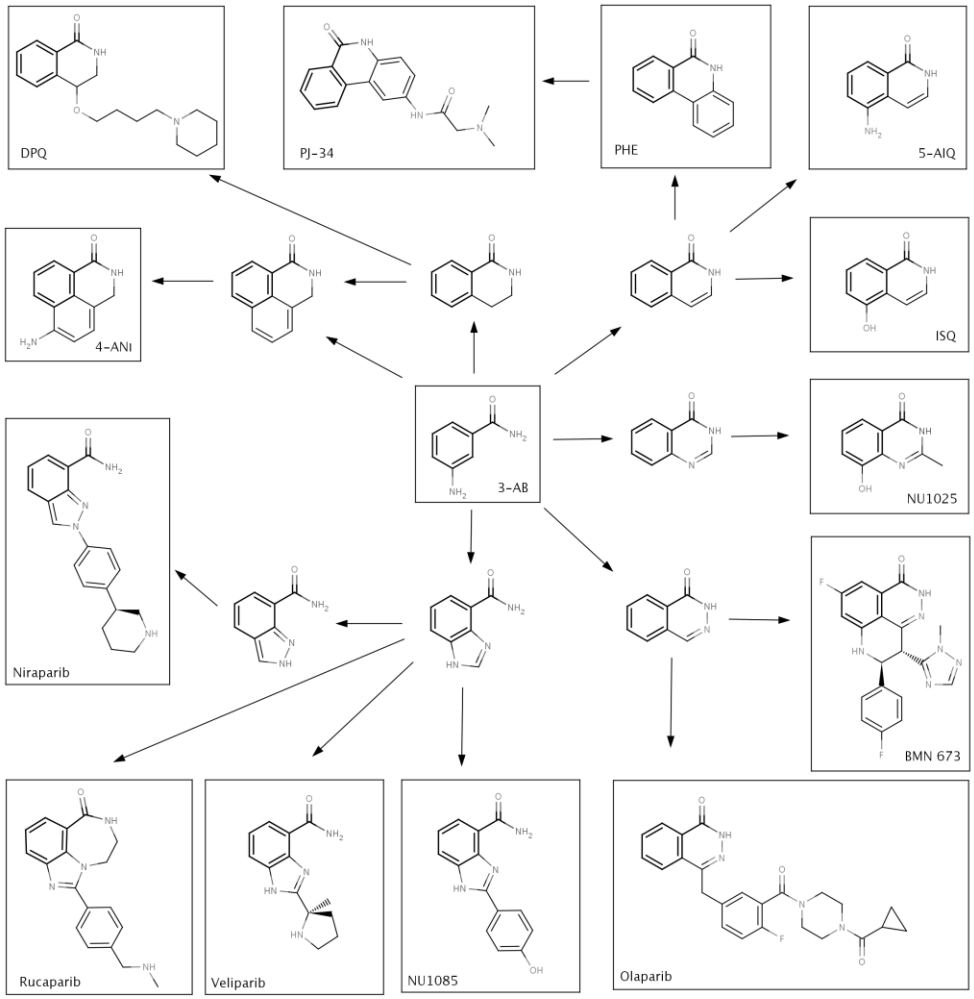


Figure 27. Evolution of the structures of main PARP drug candidates and chemical probes (in boxes) from the classical benzamide pharmacophore of 3-AB (in the center), highlighted in bold.

During the 1990s it also became evident that, besides their prominent function in DNA repair, PARP was having a more complex role than previously

anticipated. A large number of studies showed that PARP was a multifunctional enzyme involved in a wide range of biological processes, including cellular differentiation and chromatin organization.[280,288] PARP-deficient mouse models had a predominant role in uncovering new functions, such as the role of PARP in cell death after ischemia-reperfusion and its role in various inflammation processes.[289] Moreover, the residual PARP activity found in PARP-deficient cells was pivotal to identifying a new DNA damage-dependent poly ADP-ribose polymerase (62 kDa; PARP-2), confirming earlier evidences suggesting the existence of other PARP isoforms.[289] Once more, biology was more complex than previously anticipated.

The PARP superfamily: implications for chemical biology and drug discovery

During the 2000s, the increasing availability of bioinformatics tools and databases and the sequence of the human genome helped to uncover that PARPs were in reality an enzyme family composed by **18 members** (17 PARPs and poly(ADP-ribose) glycohydrolase or PARG) (Figure 28).[290] Accordingly, the enzyme that had been previously characterized and referred to as PARP was renamed to **PARP-1**. Their molecular characterization showed that all 18 members shared a PARP catalytic domain (Figure 28) despite initially only PARP-1 and PARP-2 were known to be catalytically active.[290] All these new members helped to expand the functions of poly(ADP-ribos)ylation to cell proliferation, transcriptional regulation, telomere cohesion, mitotic spindle formation, intracellular trafficking and energy metabolism, with possible therapeutic opportunities also in neurodegenerative and inflammation disorders.[290,291] Genetic tools were key to establish these new functions but PARP chemical tools continued being used as well, like the use of 3-AB to uncover that the PARP pathway was playing a key role in tumor necrosis factor

(TNF)-mediated necroptosis.[292] However, besides PARP-1 only three new PARP members concentrated the majority of the scientific interest. On the one hand, the study of **PARP-2** mainly through knockout mouse models would uncover both shared functions with PARP-1 in DNA repair and unique functions in spermatogenesis, adipogenesis and T cell development.[293] On the other hand, PARP-5 would be subdivided into **tankyrase-1** and **tankyrase-2** with an important role in regulating telomere homeostasis.[291] The molecular characterization of the rest of PARP family members would be substantially delayed.

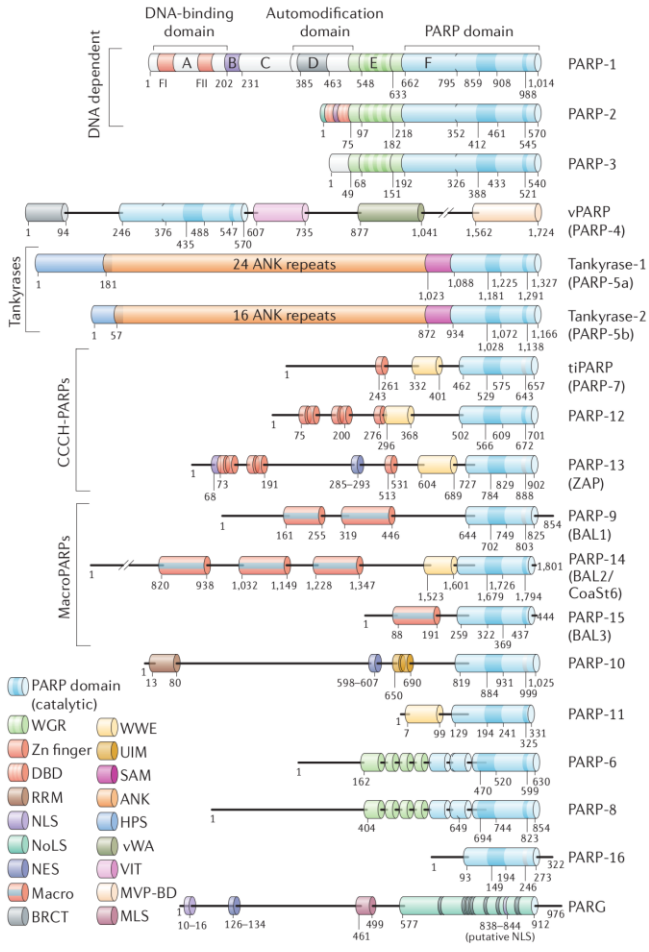


Figure 28. The domain architecture of the 17 members of the poly(ADP-ribose) polymerase (PARP) superfamily and of poly(ADP-ribose) glycohydrolase (PARG).[291]

In 2004, the publication of the crystal structure of the catalytic fragment of murine PARP-2 and their high homology with PARP-1 would raise awareness on the possible lack of selectivity of PARP-1 inhibitors.[294] Moreover, several attempts to design isoform-specific PARP inhibitors would meet a very limited success.[295–297] However, while it would become increasingly clear that all PARP inhibitors were unselective between PARP-1 and PARP-2, their selectivity against the full PARP family would continue to be a big unknown.[298] Surprisingly, the discovery of all these new PARP family members with a highly conserved PARP domain and the increasing evidence of widespread drug polypharmacology[114] wouldn't force the screening of PARP chemical tools and drug candidates against the other members of the PARP family. Conversely, they would continue being used as if they were selective PARP-1 inhibitors and the biological insights extracted from their use would continue being attributed to PARP-1 and in some cases also to PARP-2.

Despite this uncertainty, in 2005 the breakthrough application of **synthetic lethality** in cancer therapy would open another clinical application of PARP inhibitors, speeding their clinical development. In two articles published in *Nature* lead by Dr. **Alan Ashworth** and Dr. **Thomas Helleday**, it was uncovered that PARP inhibitors were selectively killing BRCA-deficient cancer cells without harming normal ones.[299,300] The underlying principle was to exploit the redundancy of the DNA repair mechanisms base excision repair (BER) and homologous recombination (HR). In BRCA-deficient cells, DNA damage was dependent only on BER and therefore blocking PARP-1 would kill only cancer cells, as normal cells could repair their DNA by homologous recombination. (Figure 29).[301] The confirmation of this theory in a clinical trial lead by Dr. **Johan de Bono** in 2009 would boost the clinical development of PARP inhibitors in cancer beneath their role as chemotherapy potentiators. However, some reports started to challenge the exact molecular mechanism by which

PARP inhibitors produced synthetic lethality.[302] Moreover, their true selectivity remained unclear.[301,303]

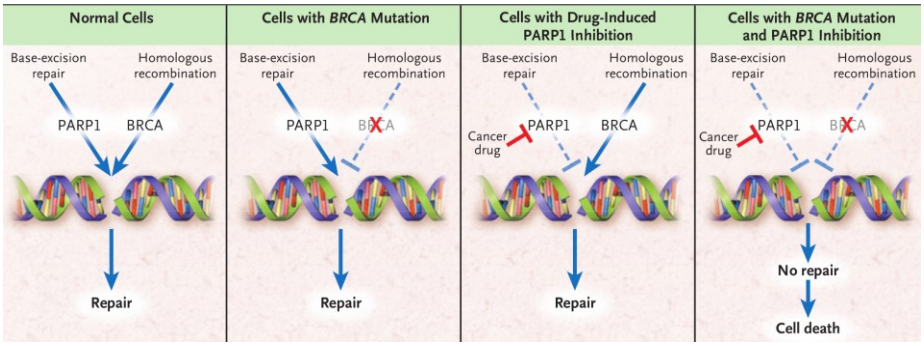


Figure 29. Mechanism of cell death by synthetic lethality as induced by PARP-1 inhibitors.[301]

In 2012, scientists of the Karolinska Institutet and the University of Perugia profiled for the first time 185 PARP inhibitors against 13 of the 17 human PARP family members.[298] This work uncovered that **PARP inhibitors showed a high degree of polypharmacology** against different members of the PARP family. Many of the PARP chemical probes, including TIQ-A, PHE and PJ34, were binding the majority of PARPs (Figure 30). PARP drugs, in contrast, were showing more specificity towards PARP-1-4 except rucaparib that was also binding tankyrases. Overall, PARP inhibitors were highly promiscuous, challenging many of the assumptions in PARP chemical biology.

Even more worryingly, increasing evidence of the different cellular effects of some PARP inhibitors started to accumulate since 2010. PJ34 emerged as the PARP inhibitor with a more divergent effect on cells, with several studies pointing towards their "PARP1-independent effects" on cell cycle arrest, centrosome de-clustering and prevention of *Helicobacter pylori* preneoplasia.[304–306] Some of these differences were ascribed to the different PARP polypharmacology of PJ34 but others were difficult to explain even when their

promiscuity against members of the PARP family was considered. The prominent role of PJ34 at probing PARP biology urged to clarify these "PARP-1 independent effects".

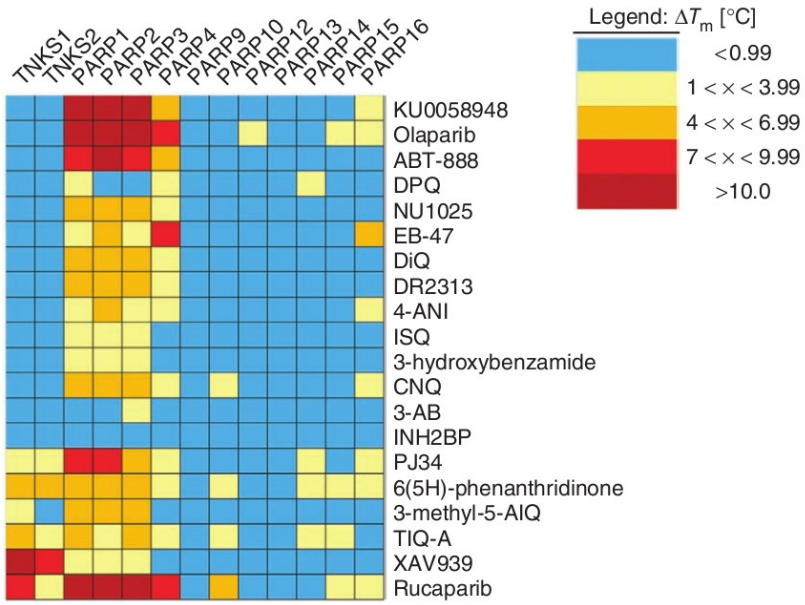


Figure 30. Profiling of most widely used PARP chemical probes and drugs against 13 human PARP catalytic domain using differential scanning fluorimetry.[298]

Today, many functions of PARPs remain unknown while unselective PARP inhibitors continue their clinical development in BRCA-deficient cancers and as chemotherapy potentiators despite the lagoons on the understanding of their exact mechanism-of-action. When chemical probe qualification criteria are considered, current PARP chemical probes lack inter-family selectivity and have never been profiled *in vitro* against a diverse panel of targets to get a wider perspective on their selectivity. Far from being a concern, PARP chemical probes continue to be generally used as if they were PARP-1 selective. Today, a comprehensive understanding of PARP inhibitors effects on cells if far from complete and, with PARP inhibitors in late-stage clinical trials, more necessary than ever.

Part II: Objectives

This PhD Thesis aims at exploring the existence and extent of polypharmacology in chemical probes and their impact in the practice of chemical biology by using poly(ADP-ribose)polymerases (PARPs) as a proof-of-concept target family. Concrete objectives can be summarized as follows:

i) To investigate whether the differential cellular effects observed between PJ34 and other PARP inhibitors can be ascribed to their unknown polypharmacology beyond the PARP superfamily by using ligand- and structure-based approaches to target profiling.

ii) To explore the implications that the differential cellular effects of PJ34 could have for PARP chemical biology.

iii) To assess if PJ34 and follow-on PARP drug candidates currently used in clinical trials have differential polypharmacology.

iv) To analyze whether distant polypharmacology is common among chemical probes and discuss their implications for the practice of chemical biology.

v) To study the importance of considering PARP-1 flexibility in computational studies.

The first objective was accomplished by identifying Pim kinases as novel targets of PJ34 using ligand-based *in silico* target profiling (see Chapter III.2). The second objective was addressed by compiling the concentrations at which PJ34 was used to probe PARP biology and realizing that, at these high concentrations, PJ34 could lead to confounding effects (see Chapter III.2). These results fostered our participation with a book chapter in an educational book aimed at chemical biology students that enabled us to further elaborate on the implications of new targets of PJ34 on PARP chemical biology and to

discuss a recent example showing that one biological function had been wrongly associated to PARP-1 due to the use of a promiscuous tool compound (see Chapter III.3). This contribution has also enabled us to introduce for the first time the concept 'polypharmacology' to master students on chemical biology. The third objective was achieved by identifying that PARP drug candidates have different kinase polypharmacology, contributing to explain their increasingly observed differential effects and alerting on the transfer of pre-clinical and clinical outcomes from one PARP inhibitor to another (see Chapter III.4). The fourth objective was addressed by using *in silico* target profiling to predict and *in vitro* confirm the existence of distantly related off-targets among the Molecular Libraries Program (MLP) collection of chemical probes, alerting on the extent of distant polypharmacology among chemical probes and their implications for the practice of chemical biology (see Chapter III.5). Finally, the last objective was fulfilled by using Replica Exchange Molecular Dynamics (REMD) to demonstrate the importance of considering the dynamic nature of PARP-1 in any structure-based attempt to study the selectivity/polypharmacology of this enzyme (Chapter III.6).

Part III: Results

III.1: Knowledge Base for Nuclear Receptor Drug Discovery.

Antolin, A. A.; Mestres, J.; Knowledge Base for Nuclear Receptor Drug Discovery. In "Therapeutic Targets: Modulation, Inhibition, and Activation" Edited by Luis M. Botana and Mabel Loza, Wiley, New Jersey. 2012, 309-26. DOI: 10.1002/9781118185537.ch8

The Results section of this PhD Thesis begins with this **review** of an **historical target family** that has always received much attention in drug discovery. **Nuclear receptors** (**NRs**) have been the targets of numerous drug discovery campaigns and account for many FDA-approved drugs for several diseases. Accordingly, this target family enables us to nicely illustrate some of the concepts presented in the introduction and get further insights on the impact of drug polypharmacology on this target family that was not historically linked to polypharmacology. First of all, the collection of the **knowledge-base** enables us to realize how **biased** and **incomplete** it is. Moreover, as the amount of public ligand-target data increases, the complex polypharmacology of many nuclear receptor drugs by both inhibiting other nuclear receptors and other targets distantly related by sequence becomes apparent in an increasingly **complex NR drug-target network**. It is also interesting to acknowledge how the early evidence of NR modulating CYP expression has evolved into a full family of **promiscuous NR** modulating xenobiotic metabolism, widely recognized as off-targets due to their lack of substrate specificity. Also, the fact that many nuclear receptors share a big number of ligands enables us to illustrate the concept of **cross-pharmacology**. Finally, the family of orphan NR shows the importance of chemical biology efforts to de-orphanize these targets with *in silico* prediction of polypharmacology playing an increasingly important role at completing our biased knowledge-base.

III.2: Identification of Pim Kinases as Novel Targets for PJ34 with Confounding Effects in PARP Biology

Antolín, A. A.; Jalencas, X.; Yélamos, J.; Mestres, J. Identification of Pim Kinases as Novel Targets for PJ34 with Confounding Effects in PARP Biology. *ACS Chem. Biol.* 2012, 7, 1962–1967. Journal Impact Factor: 6.446; Citations: 16.

After reviewing the impact of polypharmacology in the historical target family of Nuclear Receptors, in this second part of the Results section of this PhD Thesis we shift our interest towards chemical biology. As a proof-of-concept to fulfil the final goal of assessing **the impact of polypharmacology on chemical biology**, we start by studying another protein family that is currently attracting much attention in both chemical biology and drug discovery: The Poly(ADP-ribose)polymerase or **PARP** superfamily. Interestingly, one of the **chemical tool** compounds most used to study PARP-1, **PJ34**, has been recently found to have PARP-1 independent effects. Here we use *in silico* target profiling to investigate if polypharmacology can help to explain the observed differential effects of this tool compound.

An oral communication and a poster were also presented on this topic:

- Antolín, A.A.; Mestres, J. De-risking Chemical Biology: Identification of Novel Confounding Targets for PJ34 Warns on its Use to Probe the Biological Role of PARPs. Oral communication presented at the 3rd European Chemical Biology Symposium ECBS2012. **2012** July 1-3. Vienna, Austria.

- Antolín, A.A.; Mestres, J. De-risking Chemical Biology: A Critical View on the Polypharmacology of Chemical Probes. Poster presented at the Gordon Research Conference in High Throughput Chemistry & Chemical Biology. **2013** Jun 2-7. New London (NH), USA.

Antolín AA, Jalencas X, Yélamos J, Mestres J. Antolín AA, Jalencas X, Yélamos J, Mestres J. Identification of pim kinases as novel targets for PJ34 with confounding effects in PARP biology. ACS Chem Biol. 2012 Dec 21;7(12):1962-7. doi: 10.1021/cb300317y.

III.3: The impact of distant polypharmacology in the chemical biology of PARPs

Antolín, AA.; Mestres, J. The impact of distant polypharmacology in the chemical biology of PARPs. In "Concepts and Case Studies in Chemical Biology". H. Waldmann and P. Janning (Eds). Wiley-VCH, Weinheim 2014.
DOI: 10.1002/9783527687503.ch21

The Pim kinase polypharmacology of PJ34 explained in the previous Chapter was selected as an important example to illustrate the concept of 'polypharmacology' in the sequel of the reference book for the training of Chemical Biology students "Concepts and Case Studies in Chemical Biology", edited by Herbert Waldmann and Petra Janning. This way, **the concept of polypharmacology is introduced to chemical biology students for the first time**. In this chapter, we contextualize the case of PJ34 giving more background on both PARP biology and the methods of *in silico* target profiling used to predict polypharmacology. Moreover, we include the **recent impact of our publication** in the chemical biology of PARPs, explaining how the **incorrect involvement of PARP-1 in one biological function has been revisited**, in part, thanks to our publication. Finally, we give some **lessons** that can be extracted from the case of PJ34 **for the practice of Chemical Biology**.

III.4: Linking off-target kinase pharmacology to the differential cellular effects observed among PARP inhibitors

Linking off-target kinase pharmacology to the differential cellular effects observed among PARP inhibitors, *Oncotarget*. **2014**, 5, 3023-28.

The proof-of-concept study of PJ34 demonstrates the implications of polypharmacology on PARP chemical biology. Before trying to generally address this issue on a collection of chemical probes, here we investigate **how the polypharmacology of a chemical probe translates into drugs** that have been developed inspired on this chemical probe. There are different PARP-1 drug candidates in late stage clinical trials that have recently been found to have also different cellular effects that cannot be explained through their known affinity for members of the PARP family. In this third chapter of the Results section of this PhD Thesis, we investigate **the existence of a link between the different cellular effects of PARP inhibitors in clinical trials and their possible differential kinase polypharmacology** using Pim1 cross-pharmacology.

Antolín AA, Mestres J. Linking off-target kinase pharmacology to the differential cellular effects observed among PARP inhibitors. Oncotarget. 2014 May 30;5(10):3023-8 DOI: 10.18632/oncotarget.1814

III.5: Distant polypharmacology among MLP chemical probes

Antolín, A. A.; Mestres, J.

Distant polypharmacology among MLP chemical probes.

ACS Chem Biol., *in revision*.

After demonstrating that the unknown polypharmacology of a chemical probe strongly influences chemical biology and does not necessarily translate into drugs developed inspired on this chemical probe, it was essential to **access the extent of polypharmacology across a collection of chemical probes**. In this fourth chapter of the Results section of this PhD Thesis, we apply *in silico* target profiling to the NIH Molecular Libraries Program (MLP) chemical probe collection in order to identify cases of polypharmacology to distantly related and difficult-to-anticipate off-targets. We predict and *in vitro* confirm novel targets of chemical probes that question the utility of these chemical probes at probing their primary target(s), demonstrating that **distant polypharmacology is more common among chemical probes than previously anticipated** and we extract conclusions for the practice of chemical biology.

Antolín AA, Mestres J. Distant polypharmacology among MLP chemical probes. ACS Chem Biol. 2015 Feb 20;10(2):395-400. doi: 10.1021/cb500393m.

III.6: Exploring the Effect of PARP-1 Flexibility in Docking Studies

Antolín, A.A.; Carotti, A.; Nuti, R.; Hakkaya, A.; Camaioni, E.; Mestres, J.; Pellicciari, R.; Macchiarulo, A. Exploring the Effect of PARP-1 Flexibility in Docking Studies. *J Mol Graph Model.* 2013, 45C:192-201.
Journal Impact Factor: 2.325; Journal Ranking: Q1 Computer Science, Interdisciplinary Applications

In this last chapter of the results section of this Thesis **the importance of considering PARP-1 flexibility is demonstrated** using Replica Exchange Molecular Dynamics (REMD) and docking studies. This research settles a knowledge-base for the long term objective of using structure-based methods to continue the study of the selectivity/polypharmacology of PARP-1.

Antolin AA, Carotti A, Nuti R, Hakkaya A, Camaioni E, Mestres J, Pellicciari R, Macchiarulo A. Exploring the effect of PARP-1 flexibility in docking studies. J Mol Graph Model. 2013 Sep;45:192-201. doi:10.1016/j.jmgm.2013.08.006

Part IV: Discussion

IV.1 The impact of polypharmacology on chemical biology

In this Thesis, I have pursued the main objective of assessing the impact that polypharmacology could have on the practice of chemical biology. I have demonstrated that polypharmacology (and even distant difficult-to-anticipate polypharmacology) is more common among chemical probes than previously expected, with profound implications for the practice of chemical biology and their translation into drug discovery, as illustrated for the PARP field. Despite not having been the first to warn on the promiscuous nature that tool compounds might have,[266] a recent editorial in Nature Chemical Biology nicely illustrates how a reductionist view still prevails in chemical biology.[268] Chemical probes continue to be seen as single-target 'magic bullets' probing specifically for their primary target. Hopefully, this PhD Thesis will contribute to the transition towards a more holistic understanding of chemical biology as a more solid knowledge-base for follow-on drug discovery.

A critical view on the polypharmacology of chemical probes

We started this Thesis by studying the polypharmacology of the PARP chemical probe PJ34. We used ligand-based *in silico* target profiling to identify that this tool compound also inhibits Pim1 and Pim2 kinases with micromolar affinity. Moreover, we also warned that the high concentrations at with PJ34 was being used could lead to confounding effects due to the shared functions between PARPs and Pim kinases. Nicely, this hypothesis was later confirmed by the identification that PARP-1 had been wrongly associated to the TNF-necroptosis pathway due to the use of a promiscuous tool compound.[307]

Far from being a particular behaviour of PJ34, the polypharmacology of other chemical probes has also been recently uncovered. This year, scientists from

GlaxoSmithKline reported that the widely used PI3-kinase chemical probe LY294002 was inhibiting BET bromodomains.[308] Moreover, we performed a large-scale *in silico* profiling of the NIH Molecular Libraries Program (MLP) chemical probe collection, predicting new distantly related off-targets for 86% of the chemical probes. We next went on confirming *in vitro* half of the predictions from a sample of eight chemical probes. Even more importantly, we were capable of identifying possible confounding effects for all the off-targets identified, in agreement with an emerging view of biology as highly redundant and inter-connected.

Overall, the existence of polypharmacology among chemical probes is becoming increasingly apparent and we have provided examples of how this polypharmacology can lead to confounding effects. These discoveries demand to rethink the current practice of chemical biology and to adopt measures to avoid further confusion. Unfortunately, the consequences of chemical probe polypharmacology expand beyond the practice of chemical biology.

The impact of unknown chemical probe polypharmacology on drug discovery: PARPs and beyond

In this Thesis, the trail of PJ34 polypharmacology led us to unveil that PARP inhibitors have a different kinase polypharmacology between them and between PJ34, one of the most widely used chemical probes to study PARP biology. Accordingly, the unknown polypharmacology of a chemical probe and the fact that it is not equally translated into follow-on drug candidates questions both the selective design of drugs and the information flow from chemical biology to drug discovery. If chemical probes are not selective, drugs designed gaining inspiration from chemical probes are also likely to be promiscuous. Moreover, the validation of some targets for certain indications using chemical probes could be misleading, fostering the investigation of drugs developed for the

primary target in indications where unknown chemical probe off-targets play a significant role. From the results obtained during this Thesis, we can start to explore the impact of chemical probe polypharmacology on PARP drug discovery.

When I started this PhD Thesis, PARP inhibitors were a promising class of anticancer therapeutics in clinical trials as chemotherapy potentiators and in BRCA-defective cancers. However, their wide use in an ample population of Triple-Negative Breast Cancer (TNBC) patients led to poor results that fostered a crisis on the development of this drug class in 2011.[309] Nevertheless, some patients did respond to the treatment. The reanalysis of these data presented at the 2013 ASCO meeting uncovered that BRCA-mutated cancers responded strongly to the treatment.[310] Today, after the recent filling of the New Drug Application, olaparib is awaiting FDA approval as a first-in-class PARP drug for targeting BRCA-deficient cancers with much excitement on the field.[311] However, there were many non-BRCA-mutated patients that did also respond to treatment while some BRCA-mutated didn't. While several other PARP inhibitors continue their clinical development, huge efforts are being devoted to identify all the patients that would respond to PARP inhibitors.[312] All these biomarker identification strategies focus on targets involved in DNA repair similar to BRCA.[313] However, doubts on the exact mechanism by which PARP inhibitors produce synthetic lethality continue to be a matter of much concern and discussion.[302,314,315] In the meantime, evidences pointing towards the different cellular effects of PARP inhibitors continue to accumulate.[316–319] I am afraid that these cycles of much excitement followed by results failing to meet the expectations will continue until the exact mechanism of action of PARP inhibitors is clarified.

What is the impact of the unknown kinase polypharmacology of PJ34 on PARP drug discovery? On the one hand, it is obvious that if the kinase

polypharmacology of PJ34 had been known, medicinal chemistry campaigns aiming to develop PARP drug candidates would have included Pim kinases as off-targets in the screening cascade, and maybe other kinases too. Accordingly, today PARP drug candidates would be most likely devoid of kinase affinity or we would have rational multi-target inhibitors of PARPs and a selected set of kinases. As this did not happen, today's PARP drug candidates were developed unaware of their putative kinase polypharmacology and, therefore, some of them have maintained, and others have lost, their affinity for Pim and other kinases. On the other hand, the influence of unknown chemical probe polypharmacology on the selection of indications for PARP inhibitors where polypharmacology could play a relevant role is more difficult to assess. However, some examples could be already discussed.

A recent report lead by Dr. Yves Pommier and collaborators found that olaparib was more synergistic with temozolomide than veliparib and linked this difference with the recently reported different capacity of PARP inhibitors to trap PARP at the DNA damage site.[317] However, another recent report from Dr. Douglas A. Levine found that the differences between olaparib, veliparib and PJ34 extended to the cell cycle and linked them to different off-target effects, with olaparib and PJ34 having a more similar cell-cycle effect.[319] Regarding the known involvement of several kinases in temozolomide sensitivity,[320] it is likely that kinase polypharmacology plays a relevant role in temozolomide sensitivity. Interestingly, the potentiation of temozolomide with PARP inhibitors was first reported using 2 mM 3-AB in 1996 and later with 50 μM NU1025 and 10 μM NU1085 in 2000.[321,322] These initial reports fostered the acceptance that PARP-1 inhibition synergized with temozolomide and all PARP inhibitors were believed to be equally effective. However, the polypharmacology of these tool compounds at the high concentrations used and their relevance to temozolomide potentiation are still unknown. If some PARP inhibitors are better temozolomide partners due to polypharmacology

we might have lost many valuable resources in costly clinical trials testing less beneficial combinations.

The combination of PARP and kinase inhibitors offers several other examples where the different kinase polypharmacology could play a significant role. We have already warned that the combination of rucaparib and dinaciclib could be more beneficial due to the off-target affinity of rucaparib for CDK1 and that ongoing clinical trials with PARP inhibitors devoid of CDK1 affinity, such as veliparib, could lead to significantly different results (Chapter III.4). Also, significant differences between the combination of PI3K and PARP inhibitors emerged from a recent report with veliparib being surprisingly more effective than olaparib despite ongoing clinical trials combining olaparib and PI3K inhibitors.[323] Overall, with increasing combinatorial clinical trials between PARP and kinase inhibitors, a comprehensive view of which PARP inhibitors offer the best synergistic combinations is increasingly needed in order to avoid persistent scientific distractions and focus the huge economic costs of clinical trials on the most promising PARP-kinase drug combinations.

All the aforementioned results contribute to the mounting evidence that PARP inhibitors, in spite of their structural resemblance, have all an essentially unique pharmacological profile, showing how the reductionist nomenclature of drug classes adds to the confusion. As we have showed, the PARP pharmacophore seems to be compatible with the kinase hinge region, making all PARP inhibitors putative kinase binders (Chapter III.4). I envisage that we will have a more complete picture of the kinase landscape of PARP inhibitors in the near future and, hopefully, this kinome-wide perspective will enable us to advance in the precise use of this promising class of therapeutics in defined patient populations beyond BRCA.

Beyond PARPs, the identification of bromodomain polypharmacology on the chemical probe LY294002 has also contributed to the recent publication that

several clinical kinase inhibitors have nanomolar bromodomain off-targets.[195] Like in the case of PARPs, this discovery might have profound implications for the clinical development of these drugs due to the relevant role that bromodomains have in different types of cancer. Overall, with the increasing evidence of widespread polypharmacology among chemical probes, more examples of their impact on drug discovery will appear, highlighting the need of a more holistic understanding of the action of chemical probes to safely translate these results to a more efficient drug discovery process.

Lessons learned for the prediction of polypharmacology

In this Thesis, several computational methods have been applied to predict polypharmacology with different degrees of success. Interestingly, the use of PARPs as a proof-of-concept target family enabled us to get a deeper understanding of the underlying biology. This understanding was later used to back polypharmacology predictions with biological insights. Therefore, the use of biological information to back polypharmacology emerges as an important asset from this PhD Thesis.

The predictions of Pim1 and Pim2 kinases on PJ34 were originally regarded as low confidence predictions, as only one neighbor with Pim kinase affinity was identified within the pre-defined applicability domains. However, the involvement of Pim kinases in the differential cellular effects observed in PJ34 in cell cycle arrest and centrosome de-clustering increased our confidence in the predictions. Similarly, despite the fact that ligand-based *in silico* target profiling did not predict any kinase off-target of the PARP drug candidates olaparib, veliparib and rucaparib, their increasingly reported differential cellular effects suggested modulation of kinases. Accordingly, we decided to perform a cross-pharmacology analysis on kinases related to Pim1 and we screened these drugs on a panel of 16 kinases. From this analysis, we identified a differential kinase

polypharmacology among PARP inhibitors. Finally, systems pharmacology data was also used to select two additional kinases, one of them being a true micromolar off-target of rucaparib (ALK) and opening the door to the use of genomic biomarkers of drug sensitivity as new putative off-targets. Overall, biological insights emerge as an under-exploited source of information to develop innovative knowledge-based strategies to predict polypharmacology.

Beyond biological information, the identification of improved docking predictions and a side pocket to PARP-1 using Replica Exchange Molecular Dynamics suggests a relevant role of protein flexibility that should be included in any structure-based effort to predict polypharmacology.

Lessons learned for the practice of chemical biology

After witnessing the ubiquity of chemical probe polypharmacology and its impact on PARP chemical biology and follow-on drug discovery several lessons and reflections can be extracted.

1. Single-target vs. multi-target chemical probes

The discovery of many secondary targets of chemical probes, especially the ones with low selectivity over the primary target, poses a difficult question. Should we throw away these probes? I believe that the answer is no. On the one hand, in spite of the fact that we have identified situations in which secondary targets could lead to confounding biology, this is not always the case. Many off-targets will not be involved in the biological pathway under study. Moreover, as far as the target profile of the tool compound is known, the effect of secondary targets can be controlled and distinguished from the effect of the primary target using other tool compounds or siRNA. On the other hand, it is also important to stress that the simultaneous modulation of more than one

target holds great potential to perturb robust biological systems that will often succeed in compensating for the modulation of a single target. The concept of multi-target probes was already proposed to back the design of multi-target drugs.[267] As an example, the reported off-target effects of the S1P3 chemical probe ML006 on mTOR kinase could have synergistic applications in modulating autophagy in cancer.[324] In summary, we need all the armamentarium to better understand how to modulate biology in our path towards more effective therapeutics. However, knowing the target profile is essential to use both single- and multi-target chemical tools without leading to confounding results. As it is currently impossible to know the target profile of small molecules across the complete human proteome, I propose the following recommendations to derisk the practice of chemical biology.

2. Follow state-of-the-art guidelines for target validation (if possible)

State-of-the-art recommendations for target validation using chemical probes have been widely discussed and documented.[155,249,261] Independently of the use of single- or multi-target chemical probes, I believe that the best controls to avoid confounding effects due to unknown polypharmacology are the use of alternative chemical probes, inactive analogs and target engagement. Alternative chemical probes should ideally have a different scaffold but maintain the same target profile, thus minimizing the likelihood of unknown off-targets. Inactive analogs that maintain the same scaffold increase the probability of sharing the same off-targets whereas they are known to be devoid of affinity for the primary target(s). Finally, target engagement is emerging as a key control to ensure that the target(s) of interest are being modulated in the biological system of interest. The use of these three controls may help reducing significantly the chances of confounding effects due to polypharmacology and illustrates the value of developing more than one chemical probe for each target and the need

to publish and sell inactive analogs as important tool compounds. However, from the experience gathered during this PhD Thesis, these high-level controls are not widely used. It is difficult to know the reasons of not using these controls, but I envisage that it might not always be economically feasible to use more than one tool compound or they might not be always available. Several actions could be performed to reduce the impact of polypharmacology in these cases.

3. In silico target profile your chemical probes

One of the main conclusions of this Thesis is that *in silico* target profiling is an efficient and cost-effective method to identify new potentially confounding targets of chemical probes that derisk the practice of chemical biology. Many of these methods are publicly available and I believe that chemical biologists, especially those developing new chemical probes, should use them to explore the target profile of new chemical probes as a complement to *in vitro* profiling against diversity panels of targets. Of course these methods have their limitations: they only cover a portion of the chemome and proteome (the one with known ligands or protein structures) and they are constrained to available metrics to compute similarity and extract essential features for binding, among other limitations. Moreover, I fear that lack of cheminformatics expertise might prevent many experimental groups from using these computational methods. However, I also envisage that they will increasingly contribute to unveil new targets of chemical probes. Beyond these *in silico* approaches, I also propose a control and one simple practice that could aid to reduce confounding effects due to unknown polypharmacology.

4. Use siRNA and the chemical probe as an additional control

From the expertise gathered during this Thesis, the most widely used control to validate observed effects using chemical probes is the use of siRNA. Even though eliminating the target from the system is clearly different from inhibiting the function of the target, when siRNA and the use of a chemical probe have similar effects scientist conclude that the effect of the chemical tool can be attributed to the target. The use of other chemical probes as controls is also common but, from my experience, the use of inactive analogs is less extended. However, the widespread use of siRNA cannot distinguish synergistic from antagonistic effects due to unknown polypharmacology. The majority of the earlier studies that reported PARP1-independent effects of PARP inhibitors used an additional control: they used the siRNA together with the chemical probe. With this simple action, they could compare the effect of siRNA alone with the effect of siRNA and the chemical probe. When the chemical probe had an effect despite their target had been eliminated from the system with siRNA, they could attribute this effect to polypharmacology. Accordingly, I believe that adding this additional control would facilitate the identification of unknown polypharmacology in a very accessible way, as there is no need of secondary chemical probes to be available. The fact that the use of siRNA and the chemical probe as an additional control is a rare practice prompts me to strongly recommend the use of this control. However, this is still another control that can increase the cost of the experiment and it will not always be possible to use it, such in some animal models. Therefore, we also propose a general practice to reduce the likelihood of masked off-target effects.

5. Use the lower concentration possible (or mistrust effects seen only at high concentrations)

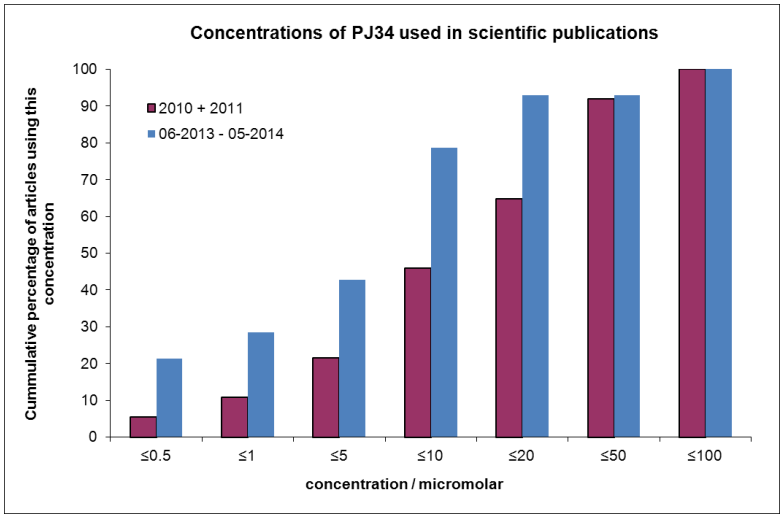
During this Thesis, we have highlighted the use of very high concentrations in chemical biology experiments. Even when there is a biomarker of inhibition in the biological system of interest, like monitorization of PAR polymer formation, usually concentrations far beyond total inhibition of the target are investigated. However, even though effects seen only at high concentrations might be highly interesting, they are more like to be due to polypharmacology. Despite this evidence, chemical biologists seem to be unaware of this fact and usually link effects only seen at high concentrations to the primary target of the chemical probe, fostering the confusion. I fear that chemical biologists are trying to push their experiments until a relevant effect is observed, a practice that assumes that chemical probes retain selectivity at any concentration used and thus entails considerable risks.

Oppositely, I recommend using the minimum concentration possible of chemical probes. Like we have shown for PJ34, the higher the concentration the higher the number of targets that the chemical probe can be modulating. Therefore, especially when there is a biomarker of target engagement *in vivo*, the lower concentration at which the target is fully inhibited should be used. When biological effects are seen only at high concentrations, the involvement of polypharmacology should be carefully controlled. Recently, a report by Gupta and collaborators uncovered that the synergistic effect of veliparib with temozolomide in temozolomide-resistant cells in only observed at high concentrations that are clinically unattainable due to toxicity,[325] blaming that higher concentrations than the Cmax should not be used in cellular experiments. I believe that it might be too drastic to ban the use of high concentrations, as some of the effects seen at high concentrations can help us to advance in our understanding of the best therapeutic strategies. However, a

detailed understanding of polypharmacology and the exact targets being modulated at each concentration is essential to prevent confounding effects due to unknown off-target interactions.

Along the same lines, it is interesting to assess the impact that identifying Pim kinases as new targets of PJ34 has had in the use of this tool compound. Have the concentrations of PJ34 been reduced below the 1 μM threshold that we proposed to selectively probe for PARP-1 and PARP-2? Have effects seen only at high concentrations been linked to PJ34 polypharmacology? Have we contributed to a better use of this tool compound? It is nicely apparent that our discovery has already had some impact in the use of PJ34, like the recent report by Fischer JMF and collaborators where they acknowledge our work to include an additional control of PJ34: "Since […] it has been revealed that PJ34 exhibits significant PARP-independent off-target effects, we used the clinically relevant pharmacological PARP inhibitor ABT888 in a second approach…".[326] However, it was interesting to address this issue more generally. To this aim, the publications using 'PJ34' or 'PJ-34' in PARP biology during the last year (June 2013 – May 2014) were searched through PubMed and the concentrations of PJ34 extracted. As it can be observed, a tendency towards using lower concentrations can be observed with 28% of the publications using concentrations of PJ34 equal or below 1 μM as proposed to probe only for PARP-1 and PARP-2 (Graphic 1). In our previous analysis of the years 2010 and 2011 only 11% of publications agreed with this threshold. However, the overwhelming majority of publications (70%) continue to use concentrations above 1 μM, showing how the impact of our message has been limited. Even more worrisome is the fact that the effects observed at high PJ34 concentrations continue to be linked only to PARP-1 and PJ34 continues to be considered a "selective PARP inhibitor".[327,328] Unfortunately, our publication and the uncovering of the family-wide inhibition of PJ34 against other members of the PARP family has not permeated to many scientists working in

the field. How could we improve the information flow from the identification of new targets of chemical probes to the end users of these tool compounds?



Graphic 1. Comparison of the concentrations of the chemical probe PJ34 used in cellular experiments during the periods 2010 and 2011 (magenta) and june-2013 to May-2014 (blue).

6. A centralized repository of chemical probe information backed by the wide chemical biology community

In the PARP field there is not an agreement of which chemical tool should be used to probe for PARP biology. Still today, with more potent second generation chemical probes, many studies continue to use the old 3-AB chemical probe with clearly suboptimal affinity and with recognized side-effects. And from the second generation chemical probes, some studies use PHE, whereas others use TIQ-A or PJ34. A quick look at the websites of chemical compound providers uncovers information on many PARP-1 inhibitors that are being sold as tool compounds. Regarding PJ34, none of these compound providers collects the information on the newly-identified off-target kinases nor our recommendation of using this tool compound at concentrations lower than 1 μM to selectively probe for PARP-1 and PARP-2. Could this situation be improved?

I recognize that self-regulation is essential and that too strict guidelines foster innovation. However, I believe that a centralized repository of chemical probe information reproduced by chemical vendors as guidelines for chemical probe utilization would facilitate the correct selection and use of the best chemical tool compounds to probe for each target(s). This centralized repository would facilitate the task not only to scientists, but also to reviewers. The chemical probe reports elaborated for NIH MLP chemical probes are a comprehensive piece of information, but they are too extensive. I think the wide chemical biology community should embark into the selection of the minimum information that should be available for a chemical probe in a similar exercise as the recently reported MIABE covering the minimum information required for a bioactive small molecule to be deposited in a database.[329] Clarifying the targets that have been screened for chemical probe selectivity and the concentration recommended in a centralized site would be incredibly valuable. However, all this information evolves with time, as this Thesis exemplifies. I envisage that this centralized repository would be difficult to maintain and that the complicity of journals publishing in chemical biology would be essential. The complicity of journals could also enable to keep track of the concentrations being used. With the engagement of the broad scientific community including authors, reviewers, journals and chemical vendors a centralized repository would facilitate a safer and more comprehensive use of chemical probes to study biology. This approach would enable us all to keep track of new targets of tool compounds. Exemplifying how our understanding of chemical probe polypharmacology is always expanding, this year the N-terminal domain of coronavirus nucleocapsid protein has been reported as a new off-target of PJ34, with 10 μM PJ34 significantly inhibiting the interaction of these protein with RNA.[330] Our understanding of chemical probe selectivity is far from completeness and we urge a centralized place to keep track of new off-targets.

IV.2 Future directions of research

I envisage two promising future lines of research. First, to **develop new methodologies** to predict polypharmacology by exploiting new sources of systems pharmacology data. Second, to continue with the **application** of available computational methods to uncover the polypharmacology of chemical probes and drugs with the long-term goals of derisking the practice of chemical biology and improving drug discovery.

Exploiting systems pharmacology data to predict polypharmacology

The embracement of a systems view in pharmacology and the increasing availability of omics technologies are facilitating the public release of large-scale studies that mix pharmacological information with other types of data, from cancer cell line profiling to siRNA screening or mRNA expression profiling. This Big Data remains underexploited and smart and innovative uses of this information for knowledge generation are increasingly needed to take the most out of these costly experimental efforts. In Chapter III.4 we started to explore the use of systems pharmacology data to validate our target profiling predictions. For instance, we compared the mRNA expression levels of a PARP inhibitor and found that this compound modulated gene expression in a similar way as the kinase drug gefitinib, thus validating our predictions that PARP inhibitors inhibit kinases directly. However, understanding which kinases are being modulated from gene expression levels is a big challenge. Despite different systems pharmacology initiatives are trying to address this challenge, I think that our current understanding of biology is still too limited. Accordingly, I think that **comparing systems pharmacology data from drugs belonging to the same class** that are supposed to have the same targets represents a much more feasible strategy today. In Chapter III.4 we compared the cancer

cell line profiling of the three PARP drugs olaparib, veliparib and rucaparib and we found that some kinases like ALK were predicted as sensitivity biomarkers only of one or two of these PARP drugs. Since all three drugs share their main PARP targets, these differences had the potential to illuminate their differential polypharmacology. Accordingly, we demonstrated that ALK is only an off-target of rucaparib and not of olaparib or veliparib. Therefore, comparing systems pharmacology data against drugs assigned to the same class holds great potential to uncover their differential pharmacology. During my post-doc I plan to integrate different sources of systems pharmacology information, from cancer cell line profiling to siRNA screening, in a global systems pharmacology strategy to predict polypharmacology of new drug families.

Applying computational methods to predict drug-target interactions proteome-wide

There is still much left to be learned about the connections between the mechanism-of-action small-molecules and their interaction with multiple protein targets. Accordingly, many opportunities remain to be explored, both to identify new targets of small molecules that derisk their use as tool compounds to study biology as well as to identify new targets for drugs that explain their side-effects or facilitate their full clinical exploitation. I am convinced that many new targets of PARP inhibitors and other drug families will be discovered in the future and I hope to contribute to the completion of a global ligand-protein interaction network that enables a more comprehensive understanding of the action of small molecules in biological systems.

I think that the smart use of available **systems pharmacology** data will uncover new targets of drugs that aid in the identification of new patient populations that respond to these drugs. Under this hypothesis, off-targets could qualify as pharmacodynamic biomarkers like the use of imatinib in c-kit-

driven tumors illustrates. Accordingly, I propose **off-target biomarkers** as a new concept and I hope to use this strategy to identify new targets of drugs that facilitate a more precise use of these drugs in selected patient populations.

Besides using systems pharmacology, other methods for target profiling remain to be fully exploited. Ligand-based methods will certainly improve their performance as databases of ligand-target interactions continue to increase their size. Moreover, one goal of this PhD that was finally not accomplished was to use structure-based methods for target identification. These methods hold great potential but their novelty makes them still not fully optimized for this purpose. I hope to be able to continue exploring the use of structure-based methods, especially binding site similarity methods relying on chemoisosterism,[194] to explore the polypharmacology of drugs and probes. Moreover, since one of the conclusions of this Thesis is that PARP-1 flexibility is important, I would like also to explore the impact of considering flexibility in structure-based methods of target profiling.

Overall, I believe that completing the full matrix of interactions between drugs and protein targets might be an affordable goal for the next 20 years, regarding how the sequence of the human genome has transformed biological research just over the last decade.[331] So far, the human proteome project has confirmed the existence of **30,057 proteins** from 17,294 genes, accounting for the 84% of the total annotated protein-coding genes in humans.[331,332] The release of the **draft sequence of the human proteome** next November will set de bar,[333] but it will be definitely too high to routinely *in vitro* screen small molecules against the human proteome. I envisage that computational methods will play an increasingly important role in this global understanding of proteome-wide drug interactions. We already have known ligands for 2800 human protein targets,[334] around 6000 different human proteins have a known 3D structure,[335] and the

availability of systems pharmacology data is increasing exponentially. Two exciting decades lie ahead.

Part V: Conclusions

The main contributions of this Thesis can be summarized as follows:

i) Pim1 and Pim2 kinases have been predicted to be micromolar off-targets of the PARP chemical probe PJ34 using ligand-based *in silico* target profiling and these predictions have been confirmed experimentally.

ii) The micromolar affinity of PJ34 for Pim1 and Pim2 kinases has been found to have implications for PARP chemical biology at the high micromolar concentrations at with PJ34 is normally used due to the shared biological functions between PARPs and Pim kinases. Concentrations lower than 1 μM have been recommended for this tool compound to probe selectively for PARPs biology.

iii) The newly identified off-targets of PJ34 have already contributed to unmask the wrong association of PARP-1 in TNF-necroptosis illustrating how unknown chemical probe polypharmacology can confound chemical biology.

iv) Differential polypharmacology across a panel of sixteen kinases selected using Pim1 cross-pharmacology has been identified between the PARP drug candidates olaparib, veliparib and rucaparib, with rucaparib inhibiting nine kinases with micromolar *in vitro* affinity, veliparib only two of them and olaparib, none. Moreover, these results also demonstrate that chemical probe polypharmacology can be used to identify new targets of drugs.

v) The different kinase polypharmacology identified among PARP drug candidates has been linked to their reported differential cellular effects. Moreover, due to the high doses of these drugs that are currently administered to humans in ongoing dose-escalation clinical trials, we suggest to consider these off-targets as clinically meaningful, providing new opportunities to expand the patient population that could respond to these

drug candidates and alerting on the transfer of pre-clinical and clinical outcomes from one PARP inhibitor to another.

vi) Cancer cell line profiling data has been used to identify one kinase biomarker of rucaparib response, ALK, as a direct off-target of rucaparib, proposing for the first time a link between genomic biomarkers of drug sensitivity and polypharmacology. Moreover, these results also show how biological insights and systems pharmacology data can be used to validate and extend computational methods that predict polypharmacology.

vii) Based on crystallographic information, a putative capacity of the benzamide pharmacophore common to all PARP inhibitors at binding to the conserved kinase hinge region has been proposed. Accordingly, all PARP inhibitors would share the inherent capacity to bind to kinases and their size and decoration would provide a unique kinase signature to each PARP inhibitor.

viii) The differential Pim1 kinase affinity among olaparib, rucaparib and veliparib also demonstrates that the polypharmacology of chemical probes does not necessarily translate into drug candidates developed inspired of these chemical probes, with profound implications for the translation of chemical biology insights into drug discovery.

ix) The Molecular Libraries Program chemical probe collection has been *in silico* profiled and four new targets of chemical probes have been identified, three of them with low selectivity over the primary targets of the chemical probes and with the potential to confound the biological insights obtained using these tools compounds regardless of the concentration used.

x) Accordingly, it has been demonstrated that distant polypharmacology is more common among chemical probes than previously anticipated and should be always considered, with profound implications for the practice of chemical biology and challenging the current screening of chemical

probes only across pylogenetically related targets and targets known to cause side-effects.

xi) *In silico* target profiling emerges as an efficient and cost-effective derisking strategy in chemical biology to identify new potentially-confounding off-targets of chemical probes that should be added to the toolbox of chemical biologists aiming to discover new chemical probes.

xii) We also have proposed the use of multi-target compounds as chemical probes and a series of guidelines to prevent confounding biology due to unknown off-targets of chemical probes. These guidelines include the embracement of state-of-the-art controls for target validation, the use of the siRNA together with the chemical probe to control polypharmacology, the use of the lower concentration possible and the creation of a central repository of chemical probe information backed by the wide scientific community.

xiii) PARP-1 flexibility has been demonstrated to be relevant for computational studies with the report of an additional site pocket that could explain the better docking results of a panel of PARP-1 ligands in an ensemble PARP-1 conformations obtained using REMD.

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Appendix A

Contributions to other publications not included in this Thesis:

Rubio-Perez, C.*; Tamborero, D.*; Schroeder, M. P.; Antolín, A. A.; Deu-Pons, J.; Perez-Llamas, C.; Mestres, J.; Gonzalez-Perez, A.; Lopez-Bigas N. *In silico* prescription of anti-cancer drugs to cohorts of 28 tumor types reveals novel targeting opportunities. *Cancer Cell*, *in revision*.

The main contribution on this work consisted in providing the conceptual framework to include data and their thresholds from bioactive small-molecules and drugs to the cancer-driver identification methods developed at the group of Dr. Lopez-Bigas in order to perform a global perspective of the drugs and small molecules targeting all known cancer drivers from currently sequenced cancer genomes. Moreover, I provided critical perspective on drug repurposing opportunities and the possibilities of exploiting polypharmacology in cancer therapeutics and contributed in the manuscript preparation.