APPLICATIONS OF FLOW CHEMISTRY METHODS AND COMPUTER-AIDED APPROACHES TO EXPEDITE THE DEVELOPMENT OF HBV INHIBITORS

Justine Raymond

UNIVERSITAT ROVIRA i VIRGILI

ICIQ Institut Català d'Investigació Química

Applications of Flow Chemistry Methods and Computer-Aided Approaches to Expedite the Development of HBV Inhibitors

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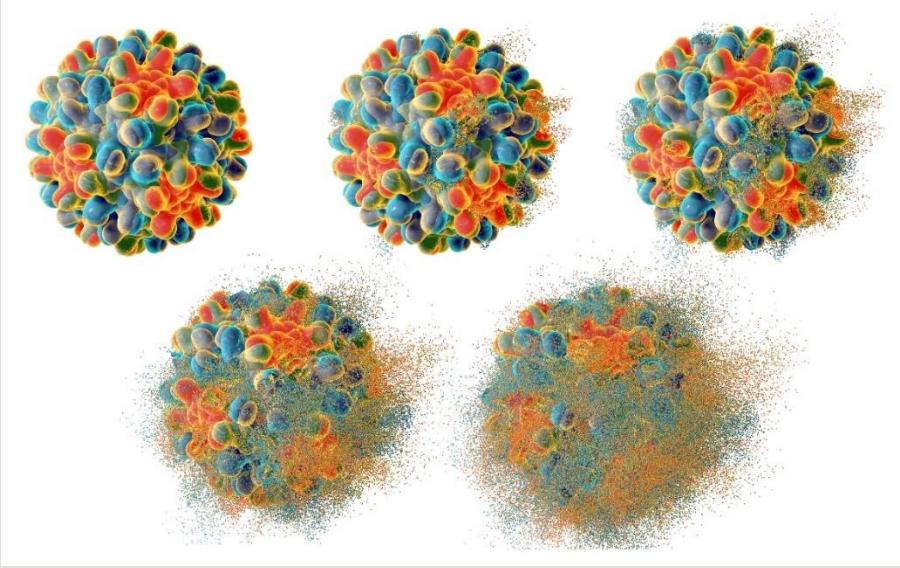
JUSTINE LAURENCE RAYMOND



DOCTORAL THESIS

2021

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Cover illustration: “Destruction of hepatitis B virus”, 3D illustration. Conceptual image for hepatitis B treatment. Stages of viral destruction.

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Abridged version of the thesis due to confidentiality agreement

The present doctoral thesis is the result of VIRO-FLOW Industrial Doctorate Programme. It has been possible thanks to the funding received from the European Union's Horizon 2020 research and innovation program under the Marie Sklodowska-Curie grant agreement No. 766058.

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Viro-Flow

AiCuris Anti-infective Cures

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Applications of flow chemistry methods and computer-aided approaches to expedite the development of HBV inhibitors

Doctoral Thesis by

Justine L. Raymond

Developed under the supervision of:

Prof. Miquel A. Pericàs and Dr. Helmut Buschmann

Departament de Química Analítica i Química Orgànica (URV)

Institut Català d'Investigació Química (ICIQ)

Tarragona

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STATE, that the present Doctoral Thesis entitled: **"Applications of flow chemistry methods and computer-aided approaches to expedite the development of HBV inhibitors"**, presented by Justine L. Raymond to receive the degree of Doctor, has been carried out under our supervision at the Institute of Chemical Research of Catalonia (ICIQ) and at AiCuris Anti-infective Cures GmbH.

Tarragona, 17 May 2021

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ACKNOWLEDGEMENTS

First, I would like to express my gratitude to my supervisors Prof. Miquel A. Pericàs and Dr. Helmut Buschmann for giving me the opportunity to be a part of this research programme, for their advice and useful insights.

Then, I would like to address my sincere gratitude to Esther Alza: for her support on daily basis, especially during my time at ICIQ, for always having an open door to my technical and personal struggles, for her commitment and rigour in revising my work, and for her encouragements. Thanks as well to Anna Maria Banet, Elena Masdeu and Sara Garcia for their support in project coordination, organization and administrative matters.

At AiCuris, my gratitute goes to Thomas Goldner for his unwavering support and useful insights, also for spreading his motivation and zeal to tackle new challenges. I would like to extend my thanks to Dr. Andreas Urban for always being available for fruitful discussions and sharing his expertise. Special thanks to Dr. Alastair Donald for introducing me to the world of computer aided drug discovery with a steady patience and kindness, for providing constructive critiques that allowed me to grow and improve my work.

I want to acknowledge and express my gratitude to Dr. Mauro Fianchini and Elena Detta for their contribution in the fifth chapter of this thesis. Mauro, for supporting our experimental endeavors with rigorous computational insight. I have greatly appreciated our cheerful discussions and your commitment to excellence. Elena, for dedicating your motivation and persistence to completing the experimental work needed to conclude this story.

My gratitude also goes to Prof. Thierry Langer for his positive influence and keenly sharing expertise that was critical to my understanding of pharmacophore modelling. As well, I want to thank Stefan Kohlbacher for clarifying important implications of my work and sharing his expertise on machine learning and QSAR.

To both my fellow VIRO-FLOW researchers, Elena Detta and Tamás Vermes, I am grateful for many moments of joy and sincere companionship along with helping each other to look on the bright side.

I would like to thank as well all my coworkers at ERTFLOW Unit: Alba Camarasa for her steady enthusiasm and encouragements as well as technical support in all lab matters, Dr. Anna Sobolewska for introducing me to the great world of flow chemistry, Dr. Laura Amenòs for always taking the time to answer my interrogations with keenness. As well my

gratitute goes to the labmates that I had the chance to meet in Pericàs group: Marco, Patri, Pedro, Carles, Carla, Santi, Junshan, Parijat, Nicola, Stefania, Mauro, Sándor. I have truly enjoyed conducting my research project in such a positive environment. I would extend my thanks to all my coworkers at ICIQ and the ChromTAE Unit: Simona Curelli, Meritxell Diaz Estirado and Marta Serrano Torne, as well as, Xisco Caldentey at the Cellex-HTE lab. I am also grateful for all my co-workers at AiCuris from and beyond the virology department, for keeping a joyful environment in the lab and out. Especially helpful to me during this time were Jan Hoffmann, Kirsten Geiger, Wiebke Schultze, Ilva Leckebusch and Lucas Grebe who provided me with technical support whenever needed. Special thanks go to Angelica Corcuera for her patience and rigor in teaching me new techniques, while generously sharing constructive insights.

I also want to thank all the beautiful souls that I have encountered during the course of this PhD, with whom I have enjoyed happy distractions, that gave me motivation and strength to pivot or persevere: Giulia, Franziska, Ece, Chiara, Marco, Bradley, Madhura, Arancha, Cagla and more…

J'aimerais aussi remercier mes professeurs et éducateurs de classe préparatoire au Lycée Lavoisier, sans qui j'ai le sentiment que je n'en serais pas arrivé là. Qui en plus de me former de façon rigoureuse et passionnée, ont témoignés d'un soutien inestimable durant la période la plus difficile de ma vie. Merci à mes ami.e.s de France et d'ailleurs, toujours là pour me sortir la tête du guidon et me rappeler aux bonheurs simples. Merci en particulier à Hélène de m'avoir prêté son oreille attentive et son oeil scrupuleux lorsque j'en avais besoin.

Je remercie évidemment toute ma famille : mes soeurs adorées, mon père, mes tantes et oncles, cousins et cousines, neveu et nièces, pour leur présence, leur attention, pour être ma maison où que je sois.

Enfin, mon immense gratitude revient à Ivo, mon partenaire de vie, pour son amour et son soutien inconditionnel, pour toujours me faire voir la lumière au bout du tunnel même dans les moments les plus sombres.

Ce travail de thèse est dédié aux femmes qui m'ont portée : Jeanne Blum, Marie-Joséphine Lomba et Céline Raymond. Mes (grand)-mères qui par leur influence et leur courage m'ont insufflé que oui : "Quand on veut, on peut".

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SUMMARY

Hepatitis B is a serious liver infection which can be either “acute” or “chronic”. It is the primary cause of liver cancer (also known as hepatocellular carcinoma), which is the 2nd leading cause of cancer deaths in the world. Even though an efficient prophylactic vaccine is available, there remains a need for chronic hepatits B patients. Currently, FDA-approved antiviral therapies are limited to type 1 interferons (IFNs) and nucleos(t)ide analogues (NAs) which reduce HBV antigen levels. The HBV core protein (Cp) is an essential component of the virus replication cycle, including capsid assembly, pgRNA packaging and cccDNA maintenance. Thus, the HBV Cp has become an important target for developing direct-acting antivirals. A new class of compounds named capsid assembly modulators (CAMs) have been identified, showing the potential to efficiently eliminate HBV DNA from infected liver cells.

This doctoral thesis aims at proposing novel methodologies whether in continuous flow or computationally driven, that will support the fast discovery of HBV inhibitors.

The first research project described in chapter two, reports the development and the optimization of three chemical reactions in continuous flow: a CDI-mediated amidation, a thermal aminolysis and a Boc-deprotection. The processes that were developed quantitatively facilitated the obtention of relevant building blocks necessary to generate a library of oxalyl-amide-containing scaffolds: a novel chemotype of HBV capsid assembly modulators (CAMs).

The following chapter (chapter three) encompasses the synthesis and biological evaluation of a focused library of oxalyl-amide analogues which lead to the identification of two potential lead compounds. The analysis of structure-activity relationship led to a reasonable perception of the physico-chemical features responsible for the potency within this compound series. In addition, the mode of action of the of several analogues was characterized *in vitro*.

The fourth chapter illustrates a virtual screening workflow aimed at supporting and prioritizing the synthesis of new oxalyl-amide analogues. This workflow combined pharmacophore-based screening and molecular docking, leading to the selection of 90 new compounds with putative high potency. Several compounds from this selection were synthesized and tested where they effectively displayed higher potency than the initial lead.

The next project reports the investigation in continuous flow of the sulfilimine-based synthesis of 1,2,4-triazolo-[1,5-*a*]-pyridine-2-carboxylate. The limitations of the chemical reaction were assessed in continuous flow and a computational modelling approach was conducted, affording an acute mechanistic understanding of the reaction process.

The last project reported in chapter six attempted to identify novel HBV capsid assembly modulators. A combination of molecular dynamics and pharmacophore modelling was used to isolate representative ligand-complex conformations that were then used as queries for a large virtual screening (ca. 65 million compounds). The method predicted novel chemotypes that have the potential to behave as CAMs with the expected mode of action. A total of 30 potential molecules with excellent druglikeness were selected to be developed further experimentally.

ABBREVIATIONS

In this document the abbreviations and acronyms most commonly used in organic chemistry have been used, according to the recommendations of the ACS "Guidelines for authors":

http://pubs.acs.org/paragonplus/submission/joceah/joceah_authguide

aa	amino acid
ACS	American Chemistry Society
ADMET	absorption, distribution, metabolism, excretion, toxicity
aSEC	analytical size exclusion chromatography
AUC	area under the ROC curve
BPR	back pressure regulator
CAA	capsid assembly assay
CADD	computer-aided drug discovery
CAM	capsid assembly modulator
cccDNA	closed circular covalent DNA
CHA	common hits approach
CHB	chronic hepatitis B
Cp	core protein
CTD	C-terminal domain
CYP3A4	cytochrome P450 3A4
DBT	dibenzothiazepinecarboxamide
DD	drug discovery
DFT	density functional theory
DMTA	design, make, test, analyze
DNA	deoxyribonucleic acid
DoE	Design of Experiment
DSF	differential scanning fluorimetry
EC_{50}	half-minimal effective concentration in cell culture
EF	enrichment factor
EM	electron microscopy
EMA	European Medicines Agency
FDA	Food and Drug Administration
FOA	fractional overlap area
FPR	false positive rate
GCI	Green Chemistry Institute
GPA	glyoxamoylpyrroloxamide
HAP	heteroaryldihydropyrimidine
HBA	hydrogen bond acceptor
HBA	hydrogen bond acceptor

HBD	hydrogen bond donor
HBD	hydrogen bond donor
HBeAg	hepatitis B e-antigen
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
hCp149	human HBV core protein
HDX-MS	hydrogen-deuterium exchange mass spectrometry
hERG	human ether-à-go-go-related gene
HPLC	high-pressure liquid chromatography
HTE	high-throughput experimentation
HTL	hit-to-lead
(v)HTS	(virtual) high-throughput screening
ITN	innovative training network
LBDD	ligand-based drug design
LJ	Lennard-Jones
LO	lead optimization
MD	molecular dynamics
MoA	mode of action
MS	mass spectrometry
MW	molecular weight
NCE	new chemical entity
NDA	new drug application
NME	new molecular entities
NME	new molecular entities
NMR	nuclear magnetic resonance
NMS	native mass spectrometry
NTD	N-terminal domain
PAT	process analytical technology
PCA	principal component analysis
pgRNA	pregenomic RNA
PK/PD	pharmacokinetic/pharmacodynamic
PM	pharmacophore model
PME	particle mesh Ewald
PPA	phenylpropenamide
PT	phenylurea
R&D	research and development
rcDNA	relaxed circular DNA
RMSD	root-mean-square deviation
RNA	ribonucleic acid
Ro5	rule of 5
ROC curve	receiver operating characteristic curve
RPM	representative pharmacophore model
RT	reverse transcriptase
SAR	structure-activity relationship

SBA	sulfamoylbenzamide
SBDD	structure-based drug design
SDE	standard deviation ellipse
TPR	true positive rate
TPSA	topological polar surface area
VT	VapourTec®
wCp149	woodchuck HBV core protein
WHO	World Health Organization

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CHAPTER I. GENERAL INTRODUCTION

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I.1 THE DRUG DISCOVERY PARADIGM

According to Segens's Medical dictionary, drug discovery is: „The process of developing a therapeutical active substance for a defined target molecule or pathway".[1] A drug discovery campaign is initiated in response to an unmet clinical need to offer a treatment or a cure.

Historically, new therapeutic leads have been discovered from traditional plant remedies or by serendipity. An emblematic example of the latter being the discovery of penicillin in 1928 by Sir Alexander Fleming: During his summer vacation, one of his staphylococcus culture plates was contaminated and developed a mold that created a bacteria-free circle. At the time, Fleming was working in an old building with considerable dust and contamination was likely to occur. Despite that, Fleming recognized the possible significance of the bacteria-free circle and isolated the mold in pure culture. Eventually he found that it produced a substance that has a powerful destructive effect on many of the common bacteria that infect Man.[2] He named the antibacterial substance liberated into the fluid in which the mold was grown "penicillin," after *Penicillium notatum*, the contaminant of the staphylococcus colony that led to the discovery.[i]

Serendipity aside, Mother Nature stands as a tremendous source of novel chemotypes and pharmacophores.[3] Plants, microorganisms and animals, represent a largely underexploited tank of numerous and diverse therapeutic applications. Until the late 1800s, most drugs were actually based on herbs or extraction of ingredients from botanical sources.[4] Until now, natural products have been the major sources of chemical diversity as starting materials for driving pharmaceutical discovery.[5] Moreover, many natural products and synthetically modified natural product derivatives have been successfully developed for clinical use to treat human diseases in almost all therapeutic areas.[6] A notable example in this field is the acetyl salicylic acid, more commonly known as „Aspirin".

Indeed, salicylated-rich plants such as willow and myrtle had been used to treat fever or rheumatoid arthritis for over 4000 years.[7] The active substance, salicylic acid", was isolated

[i] It should be noted that while Fleming generally receives credit for discovering penicillin, he in fact technically rediscovered the substance. Indeed, 32 years earlier a French medical student named Ernest Duchesne originally discovered the antibiotic properties of Penicillium.[215215]. It is not clear why his initial discovery remained unnoticed but Duchesne was posthumously honored in 1949, 5 years after Alexander Fleming had received the Nobel Prize.

in the first half of the 19th century and its derivative acetyl salicylic acid - our modern version of Aspirin - was synthesized by Felix Hoffman, a German chemist working for Bayer in 1897 in Germany (Figure I.1).

Figure I.1. (**A**) Felix Hoffmann (Portrait of Felix Hoffmann, BAYER, public domain).[7] By acetylating the phenol group of salicylic acid, he obtained acetylsalicylic acid in its purest form. **(B)** Aspirin in its original crystal powder form. (Aspirin flask, BAYER, public domain).[8]

The subsequent development of this molecule into a pharmaceutical drug by Bayer at that time paved the way for what is now known as „modern drug discovery“. In fact, drug discovery really took a turn during the 19th century: advances in chemistry enabled isolation of active substances and *de novo* synthesis of active compounds were successfully applied to market drugs such as Aspirin by Bayer in 1899.[7] Shortly after, chemical modifications to increase potency were elaborated. To go back to the example of the penicillin: after its (re-)discovery in 1928 and its approval in clinical use in the 1940s, series of semisynthetic penicillin derivatives with improved therapeutical properties were introduced over the next 40 years.[9] In parallel, identification of distinct enzymes and cellular receptors by pharmacologists and biochemists were providing the basis for rational drug discovery and development that is still in use today (Figure I.2).[10]

Nowadays, the Food and Drug Administration (FDA) in the United States acts as one of the main regulatory authorities in terms of drug development along with the European Medicines Agency (EMA).

The FDA describes the drug discovery process in five basic stages:

1) Early Drug Discovery
2) Preclinical research
3) Clinical research
4) Review and approval
5) Post-release monitoring

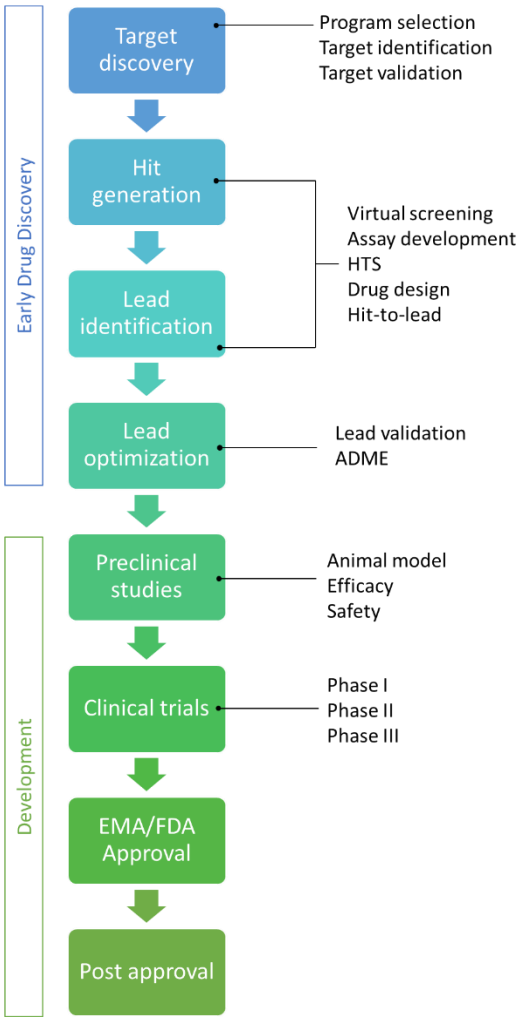

Figure I.2. Drug discovery process.

The „Early Drug Discovery "phase generally lasts three to six years and entails several steps. The target, which can be a protein, a gene or RNA, needs to be efficacious, safe, and "druggable". "Druggable" means that the target is accessible to a putative drug molecule (a small molecule or a larger biological entity). The target must elicit a biological response upon binding with the putative drug and methods must exist to measure that response *in vitro* and *in vivo*. Drugs in development typically fail in clinical phases due to lack of efficacy and/or due to toxicity. For this reason, properly choosing and validating the initial target is of paramount importance. Then, the hit-to-lead phase will assess several hit clusters and identify a few hit series that have the best potential as a drug-like lead. Structure-activity relationship (SAR) will be investigated to confirm appropriate response from the biological target. Additionally, initial assessment of *in vivo* ADMET[ii] properties

[ii] Absorption, distribution, metabolism, elimination and toxicity studies are an assessment of the pharmacokinetic/pharmacodynamic (PK/PD) properties of the compound.

will help selecting the most promising lead for further optimization.

Consecutively, the lead optimization phase characterizes a potential clinical candidate with optimal physico-chemical properties. The structure of the lead will be derivatized and modified in order to obtain a compound with suitable solubility, permeability and pH while maintaining good potency and selectivity for the biological target.[12] This first stage lasts in general between three and five years whereby several thousands of compounds are screened, which directly underlines the need for considerable synthetic capacities at this point.

The „Preclinical research“ stage lasts about a year and is distinctly essential to assess whether the envisioned drug can cause harm to the patient. Preclinical trials test the candidate *in vitro* and *in vivo* for efficacy, toxicity and pharmacokinetics to build a pharmacological profile for the potential candidates.[12] Information is also collected to guide the selection of dosage form, drug delivery method, side effects, effects on gender or ethnicity, interaction with other treatments and effectiveness compared to similar drugs.

The third stage „Clinical trial“ is made of several sub-phases that include dose studies, healthy volunteer study, patient population studies, PK/PD studies in human drug stability. It is considered the most lengthy and costly stage of drug development.[13] Once satisfying results are obtained in clinical trials and are in compliance with the regulatory authority, a New Drug Application (NDA) can be presented and is reviewed and approved in stage four.

The final stage „Post-release monitoring“ involves pharmacovigilance activities and surveillance in case safety alerts are raised about the drug on the long run.[12]

Even though the drug discovery stages have not evolved much over the last century, technological revolutions especially occurring in the late 1980s and 1990s have broad-opened the landscape of possibilities to deliver more effective treatments in a fast and cost-effective manner. A few of these novel disruptive technologies worth mentioning are: 1) the development of quick and reliable analytical techniques such as Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS); 2) the novel separation techniques with High-Pressure Liquid Chromatography (HPLC); 3) the identification of novel protein structures from crystallography techniques; 4) the exponential increase of computer power which, combined with protein structures has set the base of computer-aided drug design.

However, despite numerous technological breakthroughs and broader understanding of biological systems, drug discovery is still a "lengthy, expensive, difficult and inefficient process".[14] In 2012, Scannell *et al.* coins the term „Eroom's law" (in contrast to „Moore's law", a term generally used for technologies that improve exponentially over time) to report a curious trend in pharmaceutical R&D: Over the last 50 years, despite major advances in many of the scientific and technological inputs into drug research and development (R&D), the rate of FDA drugs approved has steadily declined while R&D cost keep increasing (Figure I.3).[14] Overall, this results in the decline of R&D efficiency, measured in terms of the number of new drugs brought to market by the global biotechnology and pharmaceutical industries per billion US dollars of R&D spending.

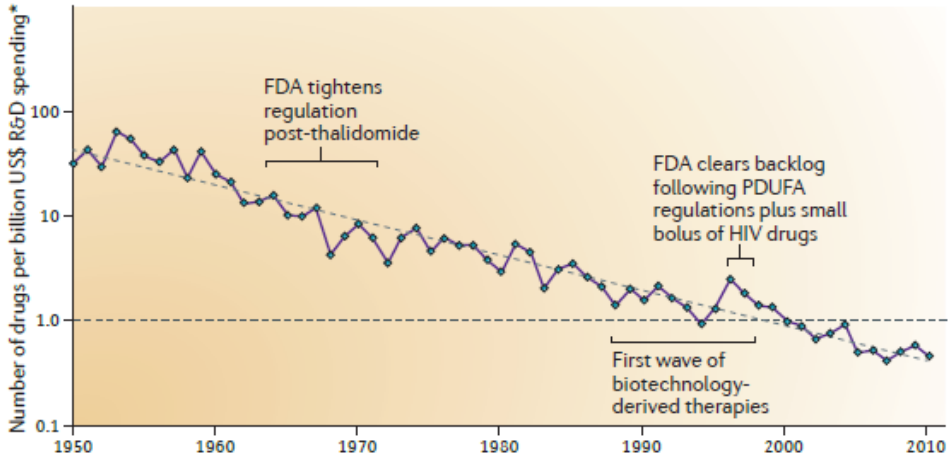

Figure I.3. Eroom's law in pharmaceutical R&D.[14] Overall trend in R&D efficiency (inflation-adjusted). Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nature Reviews Drug Discovery, REF 14, 2012.

The cost of drug development is the full cost of bringing a new drug to market from early drug discovery through clinical trials and approval. In 2020, a study published by the Journal of the American Medical Association estimates the average cost to bring a new drug to market at $985.3 million. The study spans from 2009 to 2018 and accounts for failed clinical trials.[15] Notwithstanding this cost-wise assessment, drug development remains extremely time-consuming, considering that it generally takes from ten to fifteen years to develop a single new drug molecule, from the time it is identified in early drug development until it is available in the market for patients.

As Paul *et al.* wrote: "Without a substantial increase in R&D productivity, the pharmaceutical industry's survival (let alone its continued growth prospects), at least in its current form, is in great jeopardy." The study estimates that without a cost reduction of 50% per new chemical entity (NCE), a viable business model cannot be sustained.[16]

This statement inevitably pushes all actors of the drug discovery process on an individual, institutional, and industrial level, to re-assess their "old ways" and to truly reflect on how they can take part in the change in paradigm.

I.2 HOW TO BOOST DRUG DISCOVERY?

As aforementioned, early drug discovery entails the validation of a biological target, the identification of screening hits, and the subsequent optimization of those hits to increase affinity, selectivity, efficacy, metabolic stability and additional ADME parameters. It is - with the clinical trial phase – often considered a rate-limiting step of the drug discovery process. It requires extensive iterative rounds of screening and optimization and significant synthetic capabilities.

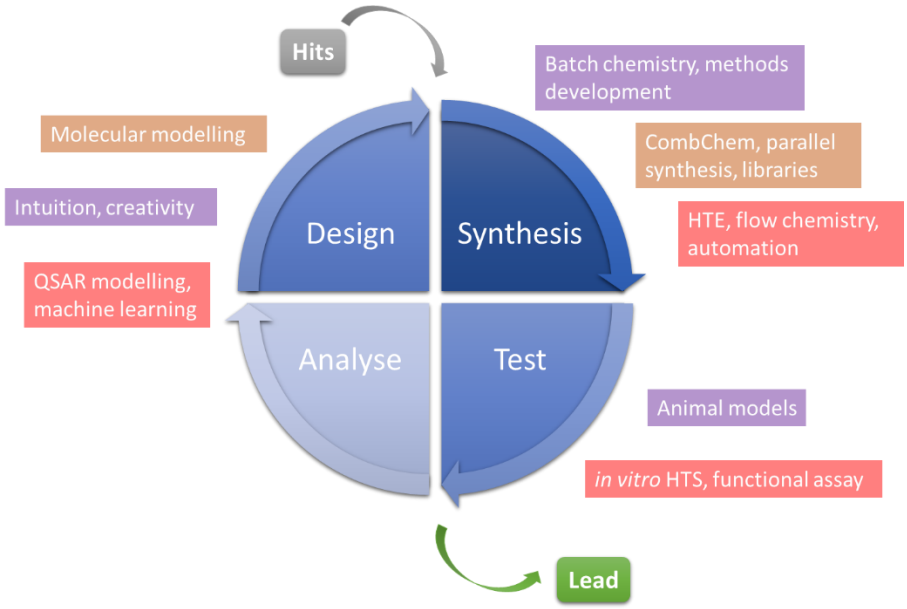

Figure I.4. Iterative learning cycles of medicinal chemistry based on diverse discipline activities with examples of key approaches used before 1980 (purple), up to 2000 (orange), and nowadays (red).[17]

The design-make-test-analyze (DMTA) cycle is the central iterative process in lead optimization (Figure I.4). It involves four steps:

- **Design:** a hypothesis is proposed to improve the profile of the lead molecule.
- **Make:** the compounds designed are synthesized.
- **Test:** compounds with confirmed structure and purity are tested in one or more validated assays.
- **Analyze:** the experimental data are analyzed, and the results used to amend a design hypothesis for the next cycle.

In recent years, novel enabling technologies have emerged to support the drug discovery process, especially in the early drug discovery stage.

The following section will be focusing on two approaches that are key to boosting early drug discovery:

- The development of novel chemical synthesis methods.
- The development of acute predictive models via computational methods.

I.2.1 INTENSIFYING CHEMICAL SYNTHESIS

Reducing costs and accelerating the timelines of drug discovery is key for pharmaceutical companies.[14] Process chemistry departments play a role in meeting this demand by providing robust and efficient manufacturing routes to active pharmaceutical ingredients (API) faster than ever before. Thanks to the growing implementation of automation and parallelization principles in existing synthetic methodologies, robust chemical process intensification takes a new momentum.[18]

I.2.1.1 High-throughput experimentation: miniaturization

High-throughput experimentation (HTE) can be broadly defined as "the workflow of running multiple reactions in parallel".[19] Classically, it is applied in combination with design of experiment[iii] (DoE) techniques and rational design to probe reaction mechanisms, examine the scope of reagents/catalysts or to determine critical parameters for a specific chemical transformation.[19] In the past decade, there has been a large increase in major pharmaceutical companies adopting HTE platforms.[20] Companies realize the benefit of this technology to accelerate the optimization of a synthetic route or for downstream processing while also covering a wider chemical space than the classic one-factor-at-a-time optimization. Recent advances in automation and process analytical technology (PAT) have warranted higher efficiency, allowed more reactions with less material, and lessened the necessity of human intervention.[21,22]

Eli Lilly has reported a large-scale automated drug discovery platform capable of performing > 16,000 reactions on a 100 mg scale per year. A diversity of reaction types

[iii] Design of experiments (DOE) is defined as a branch of applied statistics that deals with planning, conducting, analyzing, and interpreting controlled tests to evaluate the factors that control the value of a parameter or group of parameters.

were carried out, such as: organometallic cross-coupling reactions, alkylations, reductive aminations and multicomponent reactions.[23] The platform also extends to the concept of self-optimized synthesis that was introduced by Buchwald and Jensen in 2016.[24] In that latter case, a DoE-based algorithm was coupled to online HPLC analysis to optimize the yields in a Suzuki-Miyaura reaction (Figure I.5).[24]

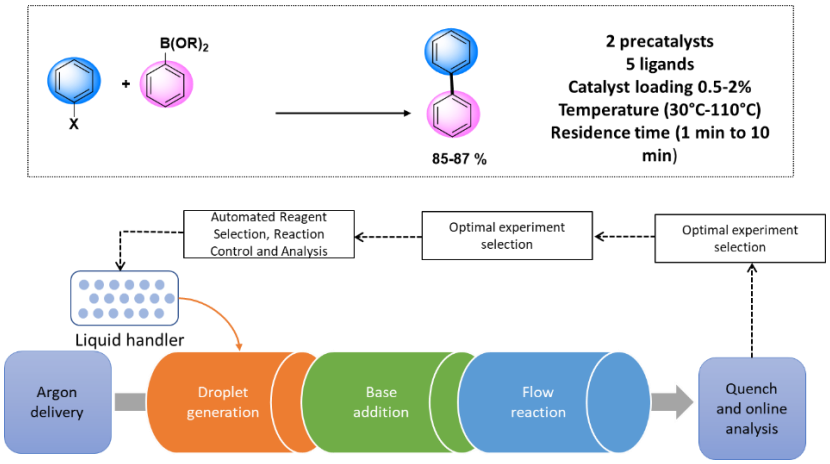

Figure I.5. Automated Suzuki–Miyaura cross-coupling optimization.[24]

In 2017, Cernak and co-workers described the application of micromole-scale high-throughput experimentation in the early drug discovery phase.[22] The aim was the identification and optimization of a diacylglycerol acyltransferase 1 (DGAT1) inhibitor lead. They report the optimization and application of a S_NAr reaction with HTE approach that enabled the synthesis of thousands of analogues. A rich SAR could be mapped and contributed to the progression of a (piperidinyl)pyridinyl-1H- benzimidazole in advanced preclinical studies (Figure I.6).[22] Later on in 2019, a first-time acoustic droplet ejection (ADE) approach was disclosed by AstraZeneca and the University of Groningen.[25] In ADE, short and precise acoustic waves are applied to a liquid, and very small nanodroplets of defined size are ejected and transported to a destination.[26] The platform is an automated ADE platform that was used to scout a novel Ugi-multicomponent approach for isoquinoline derivatives synthesis (Figure I.7). 384 reactions were performed in less than 2 days, including quality evaluation of each reaction by SFC-MS and TLC-UV-MS. Sixty-two substituted isocyanides were combined with seven various benzylamines to evaluate the reaction scope.

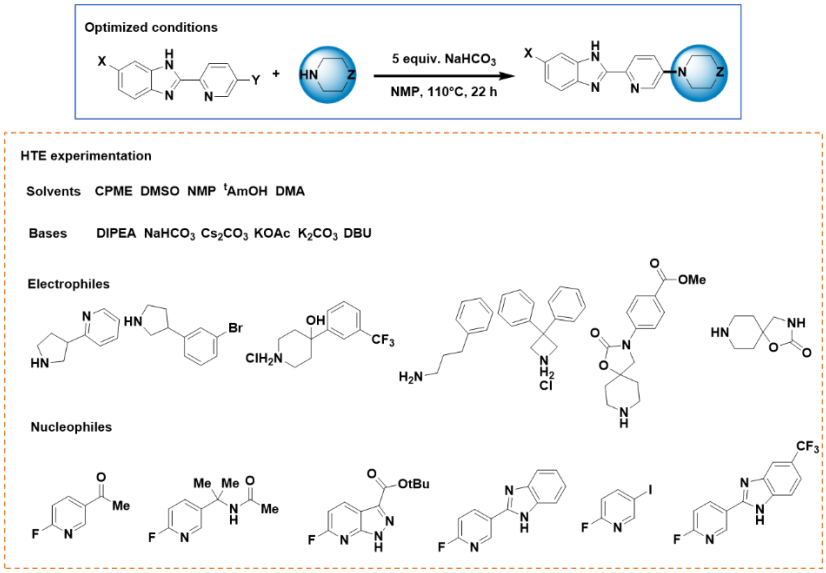

Figure I.6. Micromole-scale parallel-in-parallel reaction optimization. Screening enabled the discovery of robust reaction conditions for library synthesis.

About 80% of the nanoscale reactions revealed the product, and the functional group compatibility was exerted. Twenty-nine examples were successfully reproduced on mmol scale, underlining the scalability of the reaction. This type of technology offers fast and fair assessment of the scope of a new reaction but also very low material consumption. Indeed, the total amount of building block used for the successful synthesis of more than 300 isoquinolines derivatives is less than 50 mg.

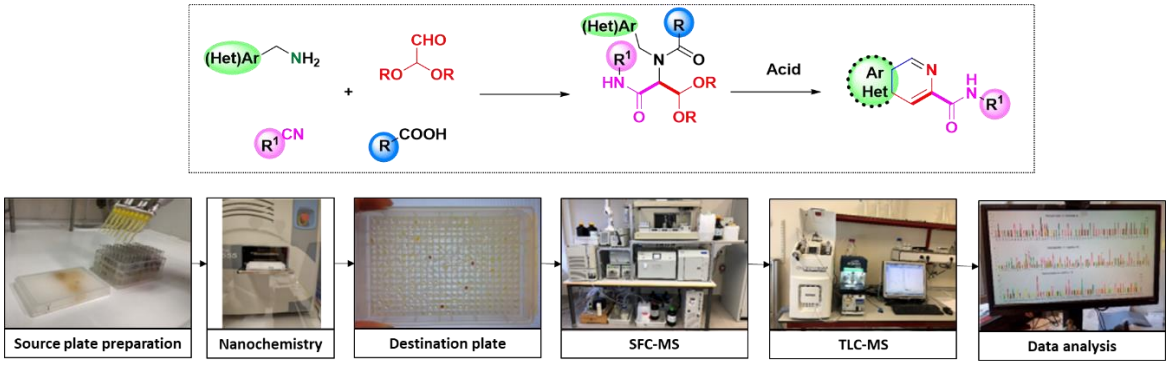

Figure I.7. Experimental workflow of nanochemistry and newly designed reaction synthesis of isoquinolines. Adapted from REF 26 authorized under ACS AuthorChoice agreement.

I.2.1.2 Flow chemistry, meso- and microfluidics

Flow chemistry can be trivially defined as the proceeding of a reaction in a continuous manner.[27–31] According to the channel's size of the reactor used for the continuous process, one can differentiate mesofluidics (millimeter- to centimeter-sized channels) from

microfluidics (micrometer-sized channels). Microfluidic and mesofluidic technologies enable the operation with small volumes in a well-controlled environment, flexible technical setup, and parameter control to an extent that is not available for batch-based methods. Their technical setup typically consists of a pumping unit, a mixer, reactor chamber, detectors, and separating or receiving units (Figure I.8).

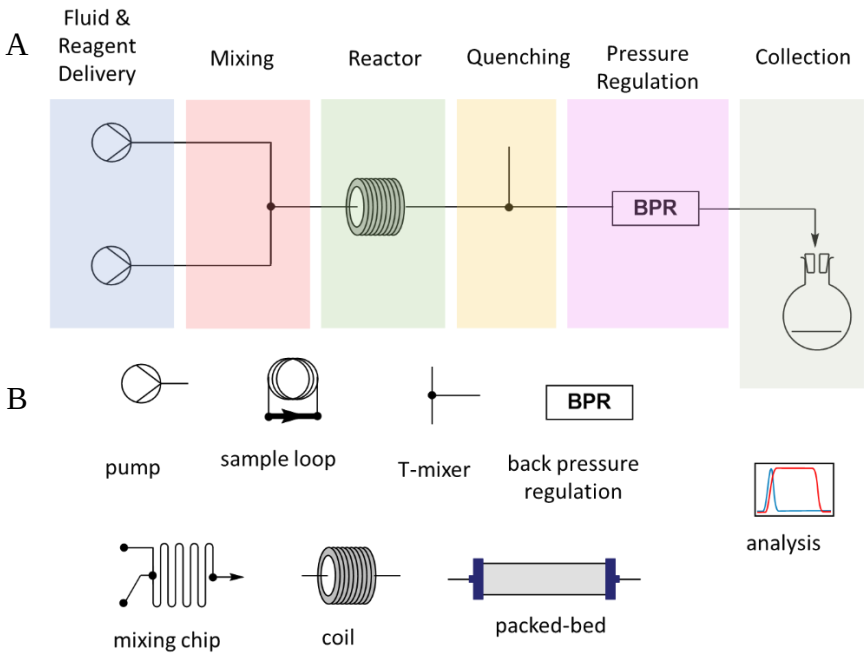

Figure I.8. (**A**) Zones of a standard two-feed continuous flow setup. (**B**) Diagram legend.

Numerous benefits of continuous flow processing reside in the improved mass transfer and heat transfer. This is enabled by the high surface area to volume ratio environment and the channels architectures inherent to flow reactors (Figure I.9).[32,33] The benefits of continuous manufacturing (CM) include: 1) the ability to operate at high temperature in a low-boiling solvent; 2) improved safety for a hazardous reaction; 3) better yields, improved containment; 4) efficient solvent stripping with enhanced performance in terms of product stability; 5) elimination of one isolation and elimination of solids handling in another isolation; 6) increased quality assurance and process understanding provided by online PAT and process automation. The continuous feature is suitable for the design of multistep processes and promises an easy scale up by duplicating the number of reactors in parallel.

Altogether, flow chemistry also addresses sustainable chemistry values by potentially leading to cleaner, more efficient, less consumptive, and safer chemical processes, while also being a tool to develop entirely novel chemical transformations.[35]

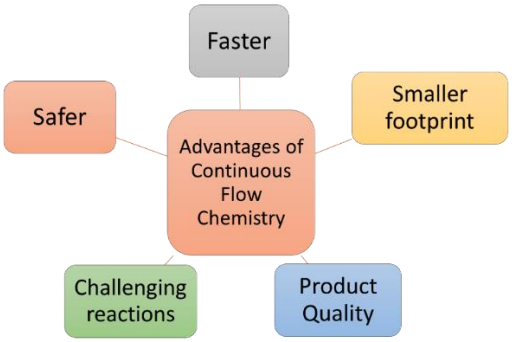

Figure I.9. Strategic drivers for the adoption of continuous flow approaches for the synthesis of chemicals.[43]

It should be noted that until the 90s, flow chemistry was solely employed by heavy chemistry industries like petrochemicals and piloted by chemical process engineers. It was just about 20 years ago that flow chemistry started to invade fine organic chemistry laboratories, enforcing the idea that continuous processing can renew the discipline. In parallel, continuous flow technologies such as micro- and mesofluidics made an entrance in pharmaceutical industries at different stages of the drug discovery process. In 2019, IUPAC identified „ten chemical innovations that will change our world“ and cited flow chemistry as an emerging technology with the potential to make our planet more sustainable.[36] The article explains that flow chemistry perfectly tackles one of the United Nations´s 2030 Agenda for Sustainable Development: responsible consumption and production, as a critical technology that increased productivity while lowering the environmental impact.[36]

In 2007, Novartis AG decided to invest $65 million to Massachusetts Institute of Technology (MIT) over 10 years to create the Novartis-MIT Center for Continuous Manufacturing, a research center dedicated to transforming pharmaceutical production.[37] The Center developed new technologies to replace the pharmaceutical's industry conventional batch-based system with a continuous flow manufacturing process. More pharmaceutical companies have followed that lead: GlaxoSmithKline committed a $50 million investment in a Singapore plant for continuous manufacturing and expanded the facilities with an additional $95 million in 2019;[38] J&J is collaborating with Rutgers University School of Engineering with a $6 million investment;[37] Eli Lilly has funded a continuous flow manufacturing facility in Ireland with an initial $40 million investment[39] and finally; Vertex Pharmaceuticals had their FDA-approved cystic fibrosis drug Symdeko produced by continuous manufacturing.[40]

In 2015 Janet Woodcock, the Director of the Center for Drug Evaluation and Research (CDER) called on the Commissioner of the FDA to „award grants to institutions of higher education and nonprofit organizations for the purpose of studying and recommending improvements to the process of continuous manufacturing of drugs and biological products and similar innovative monitoring and control techniques".[41] This so-called „mindset momentum" will encourage pharmaceutical companies to take a step towards innovative continuous manufacturing process. Just over the past few years, dozens of reviews have been praising flow chemistry as the next technology that will revolutionize the medicinal chemistry field.[17,30,42–47]

Flow chemistry has proven useful to strengthen and reliably perform some of the most commonly used reactions in early drug discovery: 1) amide formation (including peptide synthesis);[48,49] 2) Suzuki–Miyaura cross-coupling;[50–52] 3) aromatic nucleophilic substitution (SNAr);[53–55] 4) reductive amination;[56–59] or 5) Boc protection/deprotection.[60,61] When transferred to continuous flow, all these useful transformations exerted a high versatility and a productivity that superseded their batch version.[44] More interestingly, flow chemistry also represents a tool to access novel chemical space, particularly in the fields of photochemistry, electrochemistry and when unstable intermediates are involved. Controlling reactive intermediates is an essential asset to selectively divert the outcome of a reaction. Starting from oxadiazolines, Ley and co-workers generated unstable diazo compounds *in situ* to conduct the $C(sp^2)$–$C(sp^3)$ cross-coupling reaction of arylboronic acids.[62] Similarly, reactions involving reactive organometallic reagents formed *in situ* were greatly enabled in continuous flow.[63–65] As well, the use of continuous flow reactors in photochemical transformations provides a more efficient and homogeneous irradiation of the reaction mixture, which will generally result in decreased reaction times and increased selectivity.[66–70] In a collaboration between Janssen Pharmaceutica and the University of Eindhoven, the trifluoromethylation of heterocycles using iridium catalysts and CF_3SO_2Na as trifluoromethylating agent was conducted as an entirely innovative methodology.[71,72]

From the perspective of drug development, an important aspect is the issue of scaling up: from few milligrams during primary testing, the production of API quickly scales to tens of grams for early preclinical studies, then hundreds of grams for toxicology, kilograms for clinical trials and finally hundreds of tons. Having this in mind, scaling up is generally easier for a continuous process than for a batch process: by numbering up flow devices or scaling up the reactor volumes, the reaction throughput can be increased while maintaining the performance of the reactor (smart dimensioning).[73,74] In this application, flow chemistry

has been effectively introduced in key steps for active pharmaceutical ingredients (API) manufacture.[43] In a recent example, Merck reports a one-step diazotization synthesis of 2-fluoroadenine using Olah's reagents under continuous flow.[75] Starting from commercially available 2,6-diaminopurine, the process was critically improved by acute control of the temperature and residence time. The product could be isolated in 98% purity by recrystallization.[75] Similarly, Schuster and co-workers took advantage of the tight temperature control enabled by a flow setup to optimize a Matteson reaction, a key step in the synthesis of β-lactamase inhibitor Vaborbactam (Figure I.10).[76] The Matteson reaction is typically performed at -95°C to -100°C as it involves the formation of an unstable intermediate: (dichloromethyl)lithium, a labile species prone to carbene formation.[77] The continuous process was successfully scaled up from gram to 100-kilogram scale with high productivity, energy efficiency and reduced waste.[76] The process was later adapted by Novartis.[78]

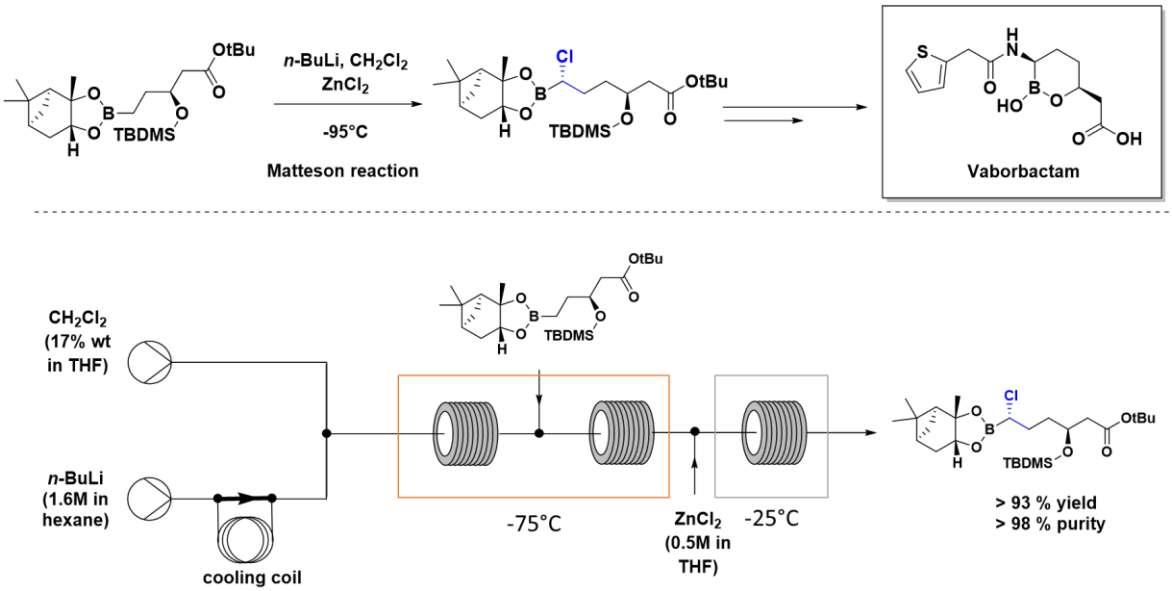

Figure I.10. Matteson Reaction Used in the Synthesis of Vaborbactam API in continuous flow.

Moreover, multistep synthesis and end-to-end production of active pharmaceutical ingredients is an attractive application for flow chemistry in the pharmaceutical industry, particularly since such flow processes have a lower space-time demand. A pioneering example is from Eli Lilly with their kilogram-scale manufacture of Prexasertib – an

anticancer drug – in a continuous flow system (Figure I.11).[34] The continuous flow process afforded 3 kg per day of cGMP[iv] material in a standard laboratory fume hoods.

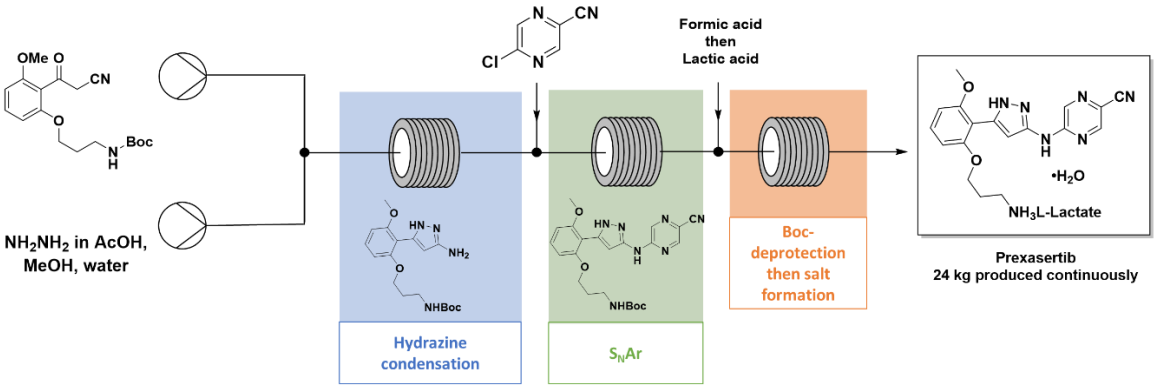

Figure I.11. Continuous manufacturing production route for Prexasertib monolactate monohydrate.

I.2.1.3 Automation of the DMTA cycle

Over the last decade, extensive efforts were made across industry to automate and integrate the routine aspects of the design-make-test-analyze (DMTA) cycle.[79] One of the most ambitious visions is to integrate machine-learning algorithms in the design of novel compounds, their chemical synthesis and subsequent testing in biological assay. Those algorithms would ultimately be able to interpret SAR data to iteratively command the synthesis of next rounds of compounds with potentially improved activity.

Several companies such as Abbott, Abbvie, Eli Lilly and Cyclofluidic have tackled the challenge with relative success. In 2017, Djuric and colleagues at Abbvie disclosed a fully automated and integrated platform for synthesis, purification, quantitation, dissolution and bioassay testing of small molecules. In comparison, preparation of identical libraries of compounds through conventional approaches, consisting of autonomous synthesis, purification, and testing was conducted in the span of several days, reflecting industry standards. The correlation of bioassay data between both approaches was excellent.[80] The platform enables batch-supported compound synthesis of up to 48-member libraries with purification and quantitation of compounds. The platform was validated with the effective preparation of 22-member amide library and 33-member aromatic amine library in 15 h and 30 h, respectively.[80]

[iv] cGMP: current Good Manufacturing Practice

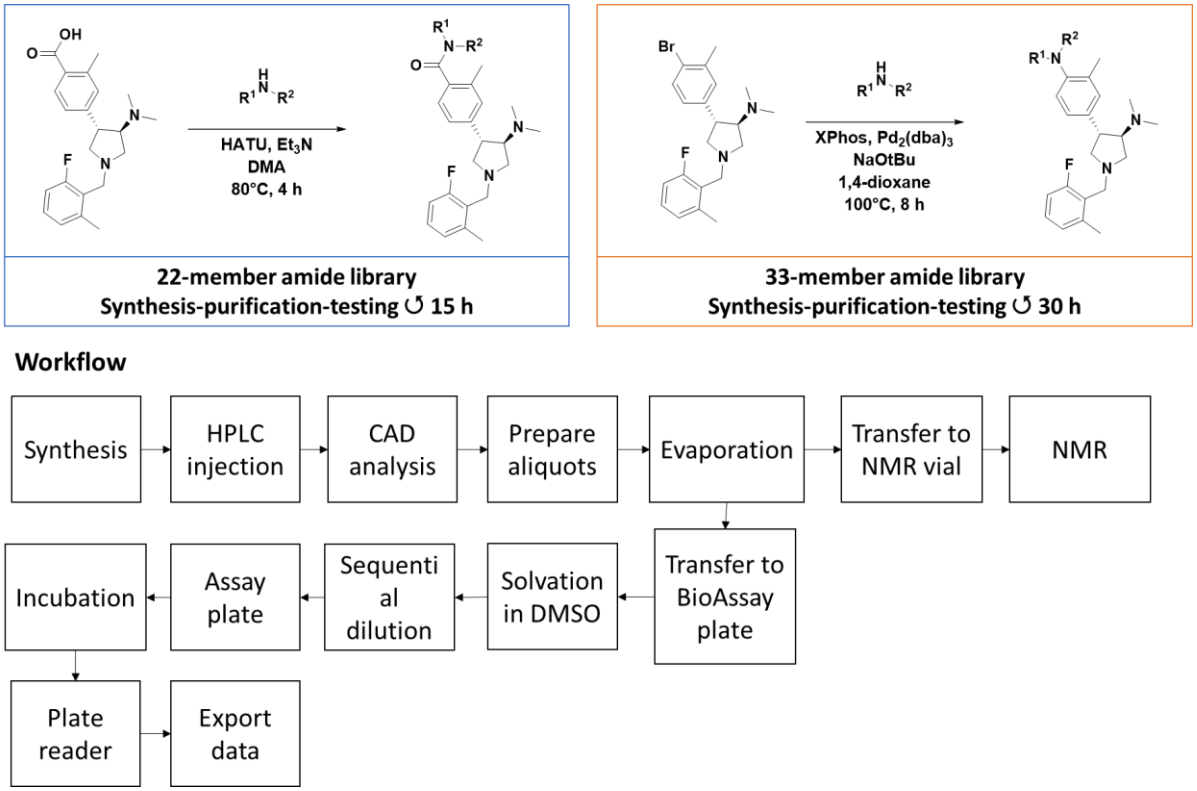

Figure I.12. Schematic representation of integrated synthesis-purification-bioassay platform developed by Abbvie.

In 2019, Cyclofluidic Ltd narrated their great epic effort towards a fully integrated closed loop design, synthesis, and screening platform.[81] With the platform named "CyclOps" (Figure I.12), the team achieved significant technical progress in demonstrating the potential for rapid SAR generation utilizing a fully automated design make and test process.[81] Notably, CyclOps was successfully utilized in the lead optimization of ABL kinases inhibitors.[82] The platform ran in fully automated mode over a long weekend, completing 72 cycles without any intervention. New compounds were synthesized, and valuable SAR was uncovered in a fully automated way and in record time.[81]

Overall, full integration of all aspects of compound design synthesis, testing and automated iteration throughout the molecular design cycle has not yet been productively applied on a broader scale but has already demonstrated very promising outcomes.

I.2.2 DEEPEN PREDICTIVE CAPABILITIES

It was during the 1970s that computational modelling of macromolecules as a mean of understanding and predicting chemical and biological processes was first introduced. Notably, Martin Karplus, Michael Levitt and Arieh Warshel shared the Nobel Chemistry Prize awardees in 2013 "for the development of multiscale models for complex chemical systems."(Figure I.13).[83]

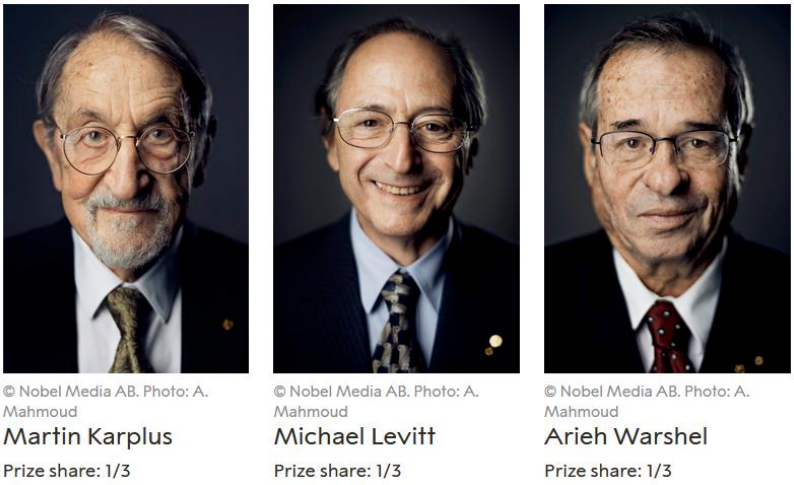

Figure I.13. The Nobel Prize in Chemistry 2013 was awarded jointly to Martin Karplus, Michael Levitt and Arieh Warshel "for the development of multiscale models for complex chemical systems".[83]

Being able to narrow the candidates down to the most promising lead for clinical testing is still an intricate challenge in drug discovery. Conventionally, the search for potential lead structures is done mainly by high-throughput screening of an existing internal library. Since the late 1990s, virtual screening methods have made possible the screening of compounds that do not physically exist in the investigators library but that can be readily obtained through purchase or synthesis. What's more, computational prediction of binding affinity obtained from molecular docking models assists in prioritizing the synthesis and test of newly designed analogues. Molecular docking protocols are used to mimic the binding of a ligand into the binding pocket of the protein of interest. During docking, an extensive set of the ligand conformations (or ligand poses) is sampled within the binding cavity of the target protein (Figure I.14). The binding affinity of the ligand is estimated rapidly for all sampled conformations with a scoring function. In principle, docking will predict the most favorable conformations of ligands and reveal key groups or atoms for binding. Once a protein target and suitable compound libraries are selected, molecular docking-based

virtual high-throughput screening is used to identify the compounds with higher affinities to the active site of the protein.[84]

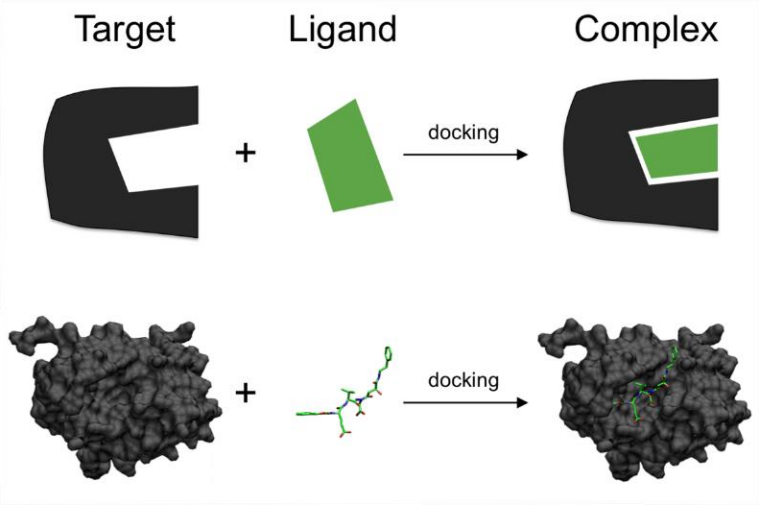

Figure I.14. Schematic illustration of docking a small molecule ligand (green) to a protein target (black) producing a stable complex.[85] Reprinted from REF 81 under the terms and conditions of the Creative Commons Attribution (CC BY) license.

For the last 30 years, computer-aided drug design (CADD) has been typically used during early drug discovery for hit identification, hit-to-lead development, and lead optimization of other pharmaceutical properties (Figure I.15).[86,87]

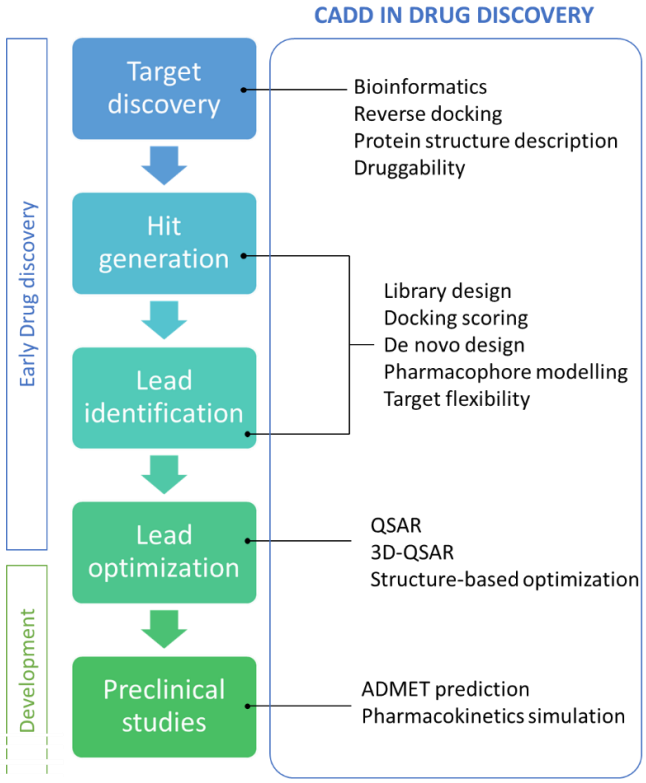

Figure I.15. Applications of CADD to the various stages of drug development.

CADD encompasses a set of techniques aimed at identifying novel potential leads that will interact with the target proteins. Among various existing computational approaches, the most notable are:

- Structure-based drug design (SBDD), that relies on the knowledge of the three-dimensional structure of the biomolecular target.
- Ligand Based drug design (LBDD), that relies on the knowledge of active and inactive ligands.
- Quantitative Structure Activity Relationship (QSAR), that relates molecular descriptors to biological activity.

In principle, the aim of such computational methods is to predict the affinity of a compound for a protein target to guide and prioritize the synthesis of compounds for *in vitro* testing. Thus, saving time and cost in early drug discovery.[88] As such, these tools have proved to accelerate drug discovery by reducing the number of iterations required and have often provided novel structures.

In the past two decades, tremendous gain in computational capabilities have enabled us to expedite early drug discovery via *in silico* approaches. The main advances have been in the field of artificial intelligence and in the combination of empirical CADD methods with biophysics, notably molecular mechanics and molecular dynamics.[89]

I.2.2.1 Artificial intelligence and deep learning

It is estimated that the chemical space comprises more than 10^{60} molecules, which potentially contains structurally diverse hits for the development of drug molecules. In addition, as of February 2021, the RCSB Protein Data Bank[90] (rcsb.org) contained more than 170.000 protein structures obtained from X-ray, NMR and electron microscopy techniques. Among them, 29% correspond to human proteins and therefore constitute a wide range of potential targets for human diseases: Notwithstanding, computational models of unknown proteins can also be constructed by homology modelling, threading and de novo design.[91] Moreover, the general increase in data digitalization in the pharmaceutical sector inextricably comes with the challenge of processing and analyzing all that knowledge to solve complex clinical problems.[92]

Artificial intelligence (AI) can be applied at different stages of drug discovery (Figure I.16).[96] AI methods are used to handle increasingly large volumes of data with enhanced automation.[92] AI technologies involve advanced tools and networks that are designed to mimic human intelligence. Even though, it is clear that AI will not replace humans (yet), they utilize systems and softwares that can interpret data and learn from input data to make independent decisions for accomplishing specific objectives.[93,94] According to the McKinsey Global Institute, advances in AI-guided automation are likely to change the work culture of society.[95] These still growing developments have opened a new landscape for CADD, entering the realm of Big Data.

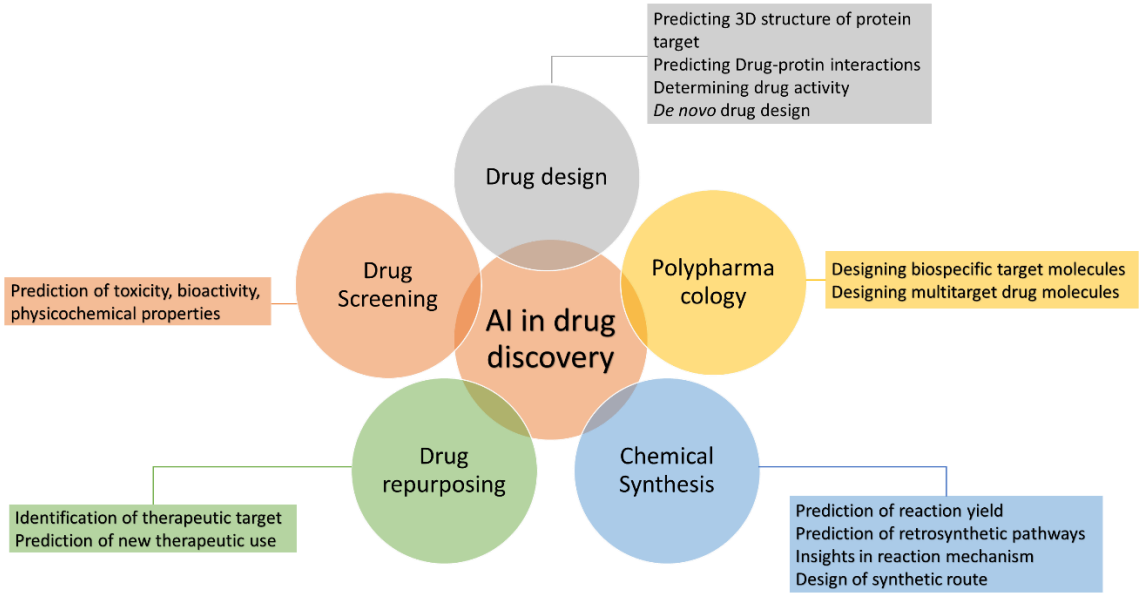

Figure I.16. Role of AI in drug discovery.[96] AI can be used effectively in different parts of drug discovery, including drug design, chemical synthesis, drug screening, polypharmacology and drug repurposing.

Quantitative structure-activity relationship (QSAR) methods started out as linear relationship methods. QSAR was first introduced as Free Wilson method and Hansch analysis[97] in the end of the 1980s. More recently, these methods have evolved into machine learning (ML) methods, a sub-field of artificial intelligence.

In the last two decades, ML methods have achieved great successes in the field of chemoinformatics, to design and discover new drugs. Recently, scientists are looking to extend the capabilities of machine learning to the prediction of additional properties such as toxicity, microsomal permeability, ADME, etc. A subfield of the ML is deep learning (DL) which has gained popularity with the development of advanced neural network

architectures for the pharmaceutical research. DL engages artificial neural networks (ANNs), an algorithm that achieves problem-solving by mimicking brain function. In other words, in the same way that our brain will apply information obtained from past experiences to solve new problems, a neural network will construct a system of „neurons“ that can reach decisions, classifications and predictions based on previous data.[98]

An important innovation is the combination of ML methods and big data analysis to predict more extensive biological features. A recent example is the development of a deep learning approach for the prediction of human hERG[v] (human ether-a-go-go-related gene) blockers.[99] Another important application of this technology is in the field of polypharmacology.[100] Indeed, virtual screening and high throughput screening (HTS) techniques may be efficient to assess the biological effect of a molecule toward the target; yet, they do not account for potential „off-target“effects. With over 20 000 proteins in the human body and every one of them having the potential to interact with an exogenous small molecule, a given drug may have hundreds of off-targets interactions, a phenomenon called polypharmacology (Figure I.17).[100] Polypharmacology causes toxicity and other adverse effects, most of which are only discovered much further down the development pipeline, after the drug has already been heavily invested in. Such adverse effects can be discovered in animal studies, clinical trials, or worst of all, when the drug is already on the market and widely used.

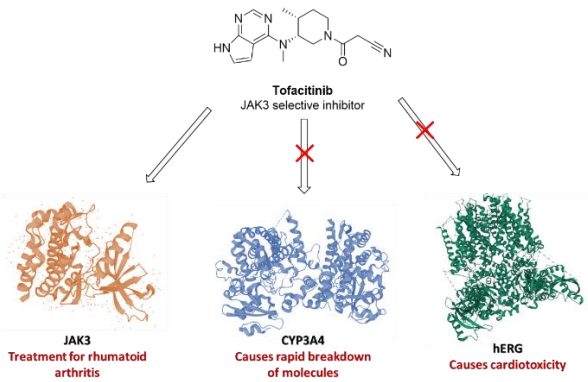

Figure I.17. Schematic depiction of polypharmacology.[101] The same molecule may interact with a number of off-target, leading to various adverse effects. hERG pdb code: 5VA1; CYP3A4 pdb code : 6UNE; JAK3 pdb code : 6DUD.

[v] hERG (the human *Ether-à-go-go*-Related Gene) is a gene that codes for a protein known as $K_v11.1$, the alpha subunit of a potassium ion channel. This ion channel (sometimes simply denoted as 'hERG') is best known for its contribution to the electrical activity of the heart. Interaction of drug with hERG can result in decreased channel function and drug-induced (acquired) long QT syndrome, which can subsequently lead to sudden death.

Over the past few years, numerous start-ups and companies have applied ML to large databases of known experimental drug binding data with the aim of assessing the viability of a drug along the drug development pipeline.

Ligand Express™ by Cyclica takes on the challenge of proteome-wide screening, an approach that proposes the screening of hundreds of thousands of proteins for the binding of an individual molecule. The algorithm expands the prediction potential of protein-drug interactions by 10- to 100-fold over conventional machine learning approaches, offering a panoramic view of a small molecule and enabling a comprehensive understanding of a drug's effect, both on- and off-target across the entire structurally characterized proteome.[101]

Similarly, LigandScout proposes a "parallel screening" workflow supplied with a high quality collection of 3D pharmacophores.[102] The pharmacophore database covers approximately 300 clinically relevant pharmacological targets originating from major therapeutical classes, such as anti-infective, cardiovascular, endocrine, gastrointestinal, immunologic, metabolic, neurologic, oncolytic, renal-urologic, and respiratory agents[101]. According to the targets encoded by these models, a pharmacological profile for the compound will emerge.

I.2.2.2 Combination with biophysics methods

Biophysical methods are defined as a set of techniques to study the structure, properties, dynamics or function of biomolecules at an atomic or molecular level.[103] They encompass a range of techniques including microscopy, spectroscopy, electrophysiology, single-molecule methods and molecular modelling. As above-mentioned, experimental techniques to „solve"[vi] novel protein structures or macromolecules (NMR, X-ray crystallography, etc.) are on the rise and steadily outperforming each other in precision and accuracy. However, these tools only assert a static vision of the macromolecular complex: Because proteins are dynamic and can undergo various conformational changes, protein conformation is one of the biggest approximations in ligand design. Thus, protein flexibility crucially affects the range of possible target conformational states for ligand binding. Experimentally, fluorescence and NMR methods enable to partially depict the protein dynamics on

[vi] "solving" a structure refers to the processes of determining tridimensional coordinates of the atoms of the protein of interest, generally in physiological solution

timescale of femtosecond to microseconds.[104–111] A promising alternative or complement to studies of protein dynamics is to simulate these movements *in silico* via molecular modelling and molecular dynamics.

In general, the purpose of a computer simulation is to gain insight into the behavior of an actual physical system or process. To achieve that specific objective, a model system is developed that represents or emulates the given physical system. A suitable algorithm subsequently generates a time series or an ensemble of states (observations) for the model system. Molecular dynamics allows, starting from classical mechanics, to simulate the trajectory of atoms in a molecular system in solution, in crystalline phase or in gaseous phase, in order to provide information on the evolution of the system over time.

Molecular dynamics (MD) simulations can provide important information on the dynamic character of the target regarding drug design and have become increasingly useful in modern drug discovery. Recently, MD simulation techniques have been successfully applied to uncover the conformational dynamics of challenging drug target. For example, KRAS is a driver oncogene that is observed particularly in pancreatic, colorectal and lung cancers.[112] Recently, the study of the interaction between its oncogenic mutant KRAS(G12) and its covalent inhibitor AMG 512 by classical all-atom MD simulations shed light on the biology of KRAS and the binding mode of AMG512.[113] Comprehensive reviews explain how the new understanding of KRAS dynamics helped deciphering the mutant functionality and pointed out vulnerabilities of these oncoproteins at the atomic level.[114]
Another example, intrinsically disordered proteins (IDPs), which are a type of proteins that do not possess a stable tertiary structural arrangement and thus only exist as conformation ensembles. In this case, MD represents a major asset to gain insights into inhibitor binding sites, internal protein dynamics or more elaborate mechanistic views. A number of proteins of interest have already been investigated with such methods with very promising results e.g. c-Myc[115] (oncoprotein, anti-canter target), tau protein[116] (microtubule-associated protein, neurodegenerative diseases, Alzheimer's), stahmin[117] (microtubule-regulating protein, anticancer target and more.[118–123]

MD can offer significant insights into ligand-receptor interactions and in the best cases can even predict the behavior of the system in defined conditions (temperature and pressure). As such it is can be used to complement molecular docking with accurate estimation of

protein-ligand binding energy[124–126] and can also predict binding kinetics.[127–129] Moreover, MD has also found relevance in combination with molecular docking techniques for virtual screening.[130] Indeed, molecular docking is probably the most widespread method to simulate protein ligand binding.[131] However, the inability to handle protein's flexibility is considered as the main drawback of docking methods. Despite the rise of seemingly „dynamic" strategies such as soft docking,[132] rotamer libraries,[133] and local optimization of side chains,[134] this downside remains substantial. A practical approach to simulate the receptor's plasticity during docking is by the parallel screening multiple receptor conformations.[130] In this case, MD is a convenient way to generate receptor conformations.[135] The „relaxed complex scheme" described by the McCammon's group is an emblematic example of this strategy for drug design (Figure I.18).[136]

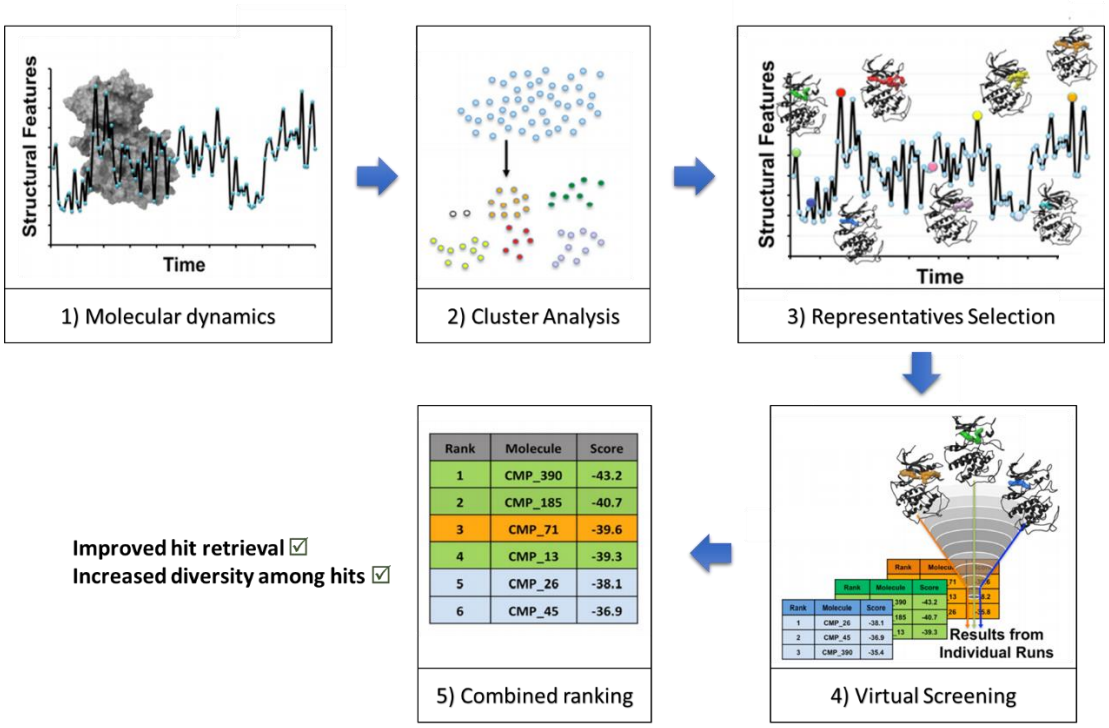

Figure I.18. Fundamental steps in a virtual screening workflow combining docking and MD simulations.[130] **(1)** An MD trajectory is used to explore the receptor conformational space. **(2)** From the trajectory, several snapshots are extracted, and redundancy is eliminated by means of cluster analysis. **(3)** From each cluster, a representative structure (e.g., medoid) is selected. **(4)** Virtual ligand screening is independently carried out at each representative conformation. **(5)** Activity predictions returned by independent runs are combined together in a global ranking. Adapted from REF 122 authorized under ACS AuthorChoice agreement.

Later on, Shoichet's group was able to demonstrate that some conformers perform better than others in retrieving active ligands.[137] Various reports published over the years strongly demonstrate that limited conformational ensemble can improve both the final enrichment

and the chemical diversity of the hits.[138–143] A comparative study published by Nichols and co-workers emphasized that MD-generated receptor variants can match and outperform crystal structures in virtual screening experiments.[144] Finally, in a review reported by Mc Cammon and Ivetac, the authors report the successful identification of several modulators of relevant pharmaceutical targets owing to the relaxed complex method.[145]

I.3 HEPATITIS B VIRUS

I.3.1 BACKGROUND

I.3.1.1 A global health threat

Hepatitis B is a viral infection of the liver and the world's most common serious liver infection.[146] A HBV infection either develops into acute or chronic hepatitis. Acute hepatitis B will cause an acute inflammation of the liver and hepatocellular necrosis. Chronic hepatitis B (CHB) is a persistent HBV infection which complications lead to cirrhosis and hepatocellular carcinoma, the most common form of liver cancer. According to the Global Hepatitis Report released by the World Health Organization (WHO) in 2017,[147] an estimated 257 million people worldwide were living with hepatitis B virus (HBV) infection in 2015. During the same year, the United Nations included combating viral hepatitis in the Sustainable Development Goals.[148] The aim is a reduction in hepatitis related mortality of 65% and a 90% reduction in new infections by 2030. In 2016, World Health Assembly passed the Global Health Sector Strategy on Viral Hepatitis which aims to eliminate HBV and HCV by 2030.[149]

With the COVID-19 pandemic still raging worldwide, HBV programs render difficult to maintain, in particular in low- and middle- income countries.[150] Recent alarming reports state that the current disruption to HBV initiative threatens the 2030 elimination goals as it will increase the global burden of chronic infection in the long term and provide a source of onward transmission to future generations.[151]

I.3.1.2 A bit of history

In around 400 *b.c*, cases of jaundice had been documented by Hippocrates who named it „epidemic jaundice".[152] It is only within the last half century that HBV started to be

described from a molecular perspective. The „Australia antigen" (HbsAg) is the surface antigen of the hepatitis B virus. HbsAg was discovered by Blum and co-workers in 1965. Four years later, Dane's group were able to describe the morphology of HBV viral particles thanks to electron microscopy (EM).[153] During the next decade, work performed by Summers and collaborators lead to the identification of the viral genome[154] and followed by its complete sequencing by Galbert *et al.* in 1979.[155] These efforts combined have fostered the development of an effective vaccine in 1981, perceived as the first „anticancer" vaccine considering the evolution of HBV infection into fibrosis, cirrhosis and hepatocellular carcinoma.[156]

I.3.1.3 Epidemiology

The chronic form of the disease is diagnosed when HbsAg persists in the host after 6 months. According to WHO, more than 400 million people are chronic carriers of HBV. Among them, 78% live in Asia, 16% in Africa, 3% in South America and the 3% remaining are dispersed in Europe, North America and Oceania. It is estimated that 650.000 people die each year due to a complication of the virus infection.[157]

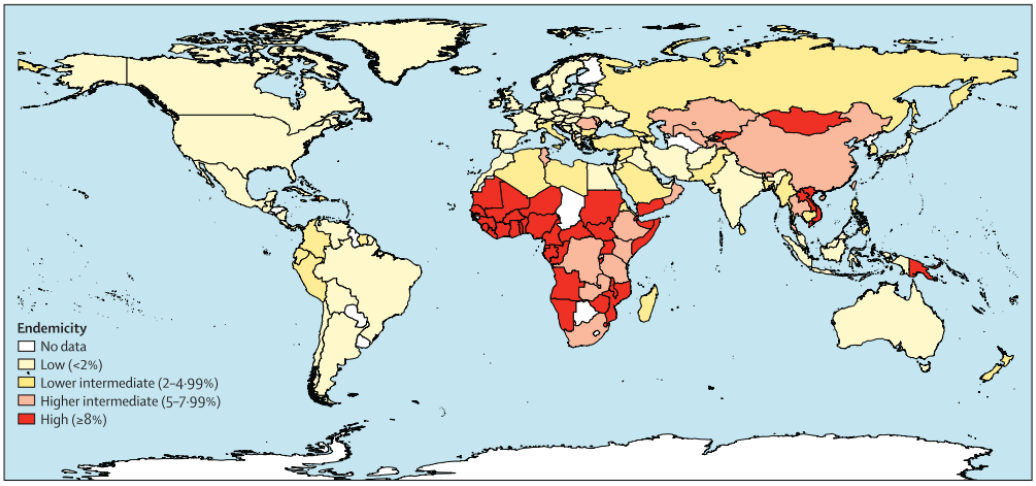

Figure I.19. HbsAg endemicity (1957-2013).[159] Reprinted from The Lancet, Vol. 386, A. Schweitzer *et al.*, Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013, Pages 1546-1555, Copyright 2015, with permission from Elsevier.

Global distribution of HBsAg carriers in adults is highlighted in 3 regions (Figure I.19):

- Low endemic area (< 2% HBsAg): Wes Europe, Australia, North America and some countries of Latin America;

- Medium endemic area (2 to 7% HBsAg): Eastern Europe, Middle East, Mediterranean countries, South-East Asia, Russia and some countries in South America;
- High endemic area (8 to 20% HBsAg): Sub-saharan Africa, China, some East European, South American and North American countries.[158]

I.3.1.4 Transmission

HBV has a very high transmission potential: though enveloped it is very resistant and can survive more than 7 days without a host.[160] It is considered 100 times more contagious than HIV. The main transmission modes are through infected blood, sexual (horizontal transmission) or perinatal (vertical transmission or mother-to-child transmission). Transmission via infected blood typically happens after contact with needles or infected medical equipment. Sexual transmission may occur during unprotected sexual intercourse, subjects with multiple partners (hetero- or homosexuals) being the main risk group. Infection in adulthood leads to chronic hepatitis in less than 5% of cases. Perinatal transmission from mother to infant is the major route of HBV transmission in many parts of the world. The risk of developing chronic infection is 90% following perinatal infection (up to 6 months of age).[161] Horizontal transmission (from person to person) are generally mediated by body fluids generally saliva when there is a repeated exposure.[161] In low endemic areas, infection mainly occurs in adulthood. The infection is asymptomatic in 80% of the cases. For the remaining 20% of the cases, acute hepatitis can arise after an incubation period from one to six months. In more than 90% of the cases patients will naturally recover from HBV infection. In highly endemic areas, HBV infection ends up being a chronic infection in 80% of the cases.[161]

I.3.2 STRATEGIES FOR A CURE

I.3.2.1 HBV replication cycle

The human virus HBV is the prototype of a virus family named Hepadnaviridae (for “hepatotropic DNA virus”). This family typically contains small spherical virus essentially hepatotropes, enveloped, with high host specificity.[162] With the *Spumaviridate* and *Caulimoviridae* families, they are the only DNA viruses that replicates their genome via a reverse transcription step from the viral RNA. HBV is a small, enveloped DNA virus that

contains a 3.2-kb, partially double-stranded, relaxed circular (rc) DNA genome and replicates via an RNA intermediate. It is organized into four frame-shifted overlapping open-reading frames encoding the core protein (HBcAg, nucleocapsid); polymerase (P); the precore protein, which is processed to secreted hepatitis B e antigen (HBeAg); hepatitis B surface antigens (HBsAg, consisting of the large, medium, and small envelope proteins); and the X protein (HBx) (Figure I.20).[163-164]

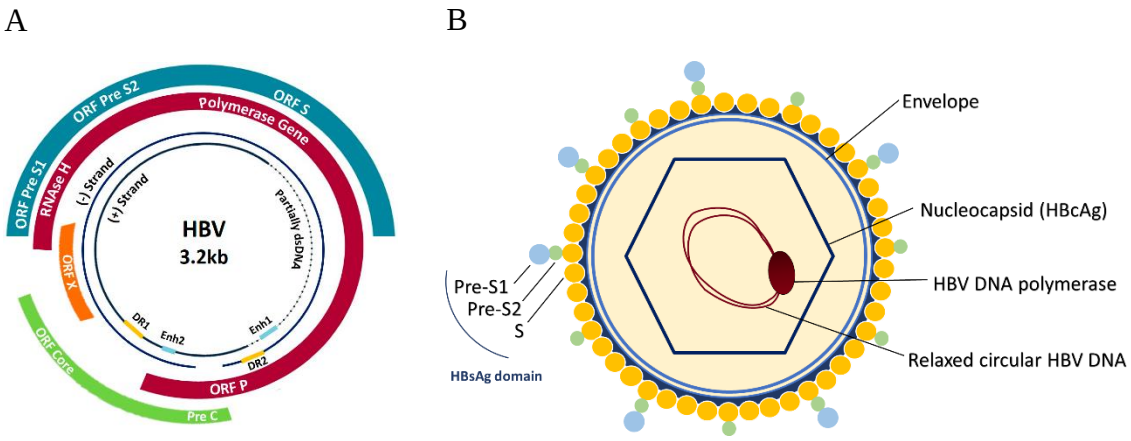

Figure I.20. (A) Schematic representation of the HBV genome.[164] The gene S is a long open reading frame (ORF) that encodes for HBsAg. The gene is divided in sections pre-S1, pre-S2, and S. The core gene consists of the pre-core and core regions, which encode for the HBV e antigen (HBeAg) and core protein, respectively. The polymerase (P) gene overlaps the entire S gene and encodes the viral DNA polymerase. Hepatitis B x antigen (HBxAg) is the smallest gene and is associated with the activation of transcription. Reprinted from REF 154 under the terms and conditions of the Creative Commons Attribution (CC BY) license. **(B) Schematic representation of hepatitis B virus (HBV).**[165] The structure of the virion is composed of a rcDNA, enclosed by a nucleocapsid, comprised of HBcAg and surrounded by a lipid envelope containing large (L)-HBsAg, middle (M)-HBsAg and small (S)-HBsAg. The virus also expresses two non-particulate proteins X protein and HBeAg.

Hepatocytes are the main target of viral replication. While the replication cycle of HBV has been well characterized over the years, the biogenesis, homoeostasis, and turnover of the cccDNA reservoir remains understudied.[166]

The replication cycle is initiated by the specific binding of the preS1 domain of the large envelope protein to the sodium-taurocholate cotransporting polypeptide (NTCP) receptor on the hepatocyte plasma membrane.[167] The viral and cellular membranes fuse to deliver the viral capsid to the cytoplasm. The viral capsid is addressed to the cell nucleus where the DNA/polymerase complex is released. Then, the nucleocapsid disassembles to release its content of partially rcDNA. The conversion of the rcDNA into covalently closed circular DNA (cccDNA) is mediated by a host DNA repair machinery.[168] cccDNA acts as a transcriptional template and DNA reservoir that will persists in the hepatocyte.[5] It is found packaged into chromatin-like mini chromosomes sitting by histones.[169,170] Transcription of

mRNAs from cccDNA is operated by RNA polymerase II, monitored by virus-encoded promoter and enhancer elements.[171] The pregenomic RNA (pgRNA), precore mRNA, preS1 and pre S2/S mRNA and HBx mRNA are translated into the main viral proteins thanks to the host cell translation machinery. Encapsidation of the pgRNA is done by the viral polymerase (P). Finally, mature nucleocapsids are either packaged with envelope proteins and exported as infectious virions or recycled back to the nucleus for cccDNA replenishment (Figure I.21).[172]

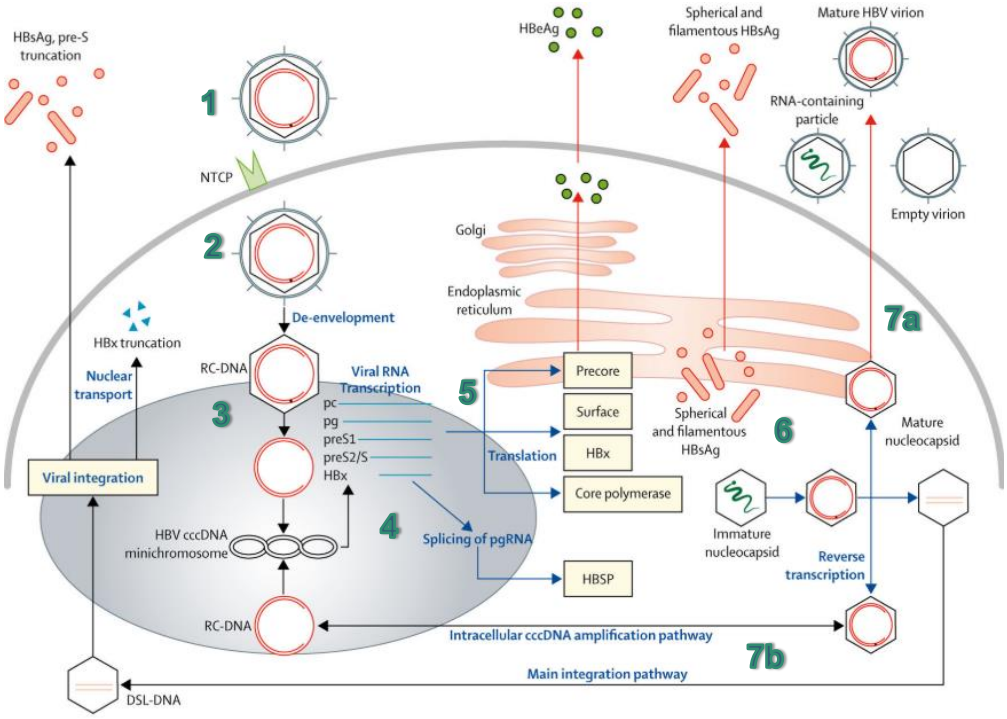

Figure I.21. The hepatitis B virus replication cycle.[172] **(1)** Viral entry via binding to NTCP receptor **(2)** The nucleocapsid is transported to the nucleus **(3)** The nucleocapsid disassembles to release rcDNA **(4)** The host's machinery converts the incoming DNA to cccDNA **(5)** The five major mRNAs are translated into the seven viral proteins **(6)** pgRNA is encapsidated by RNA polymerase **(7a)** Mature nucleocapsid are packaged with envelope proteins and exported or **(7b)** recycled to the nucleus. Reprinted from The Lancet, Vol. 4, P. Revill *et al.*, A global scientific strategy to cure hepatitis B, Pages 545-558, Copyright 2009, with permission from Elsevier.

I.3.2.2 Current treatments

Antiviral therapies currently available to treat chronic hepatitis B virus are based on interferon-alpha (IFNα) and its pegylated form (Peg-IFNα) and nucleos(t)ide analogs (NUCs). IFNα is an innate immunity cytokine which, in the case of HBV operates via a complex pathway that: activates natural killer (NK) cells; inhibits the viral genome transcription; destabilized viral nucleocapsids and degrades cccDNA.[173–175] Overall, the

antiviral effect of interferons remains modest in CHB patients for reasons that are mainly unknown.[176] On another hand, NUCs will mimic natural nucleoside and interrupt the transcription of HBV proteins. FDA-approved NUCs include Lamivudine (LMV), Adefovir Dipivocil (ADV), Tenofovir disoproxil fumarate (TDF) and the most potent to date: Entecavir (ETV).

Inhibition of the viral polymerase activity by NUCs results in the decreased production of virions and reduced recycling of viral nucleocapsids to the nucleus of the infected cells. However, NUCs do not inhibit the *de novo* formation of cccDNA in newly infected cells, meaning that despite therapy, the persistent residual viremia can always infect new hepatocytes and re-establish a viral cccDNA reservoir.[168] Additionally, cccDNA pool is maintained via the recycling of viral nucleocapsids to the nucleus of infected cells.[168] To date, none of the available treatment is able to fully eliminate HBV from hepatocytes, which means lifelong therapies are often required.[177]

I.3.2.2 Potential targets

As of now, the persistence of cccDNA is a key obstacle for a cure of chronic hepatitis B. To tackle this hurdle, a number of strategies are being explored by targeting different steps of the viral replication cycle (Figure I.22).[172]

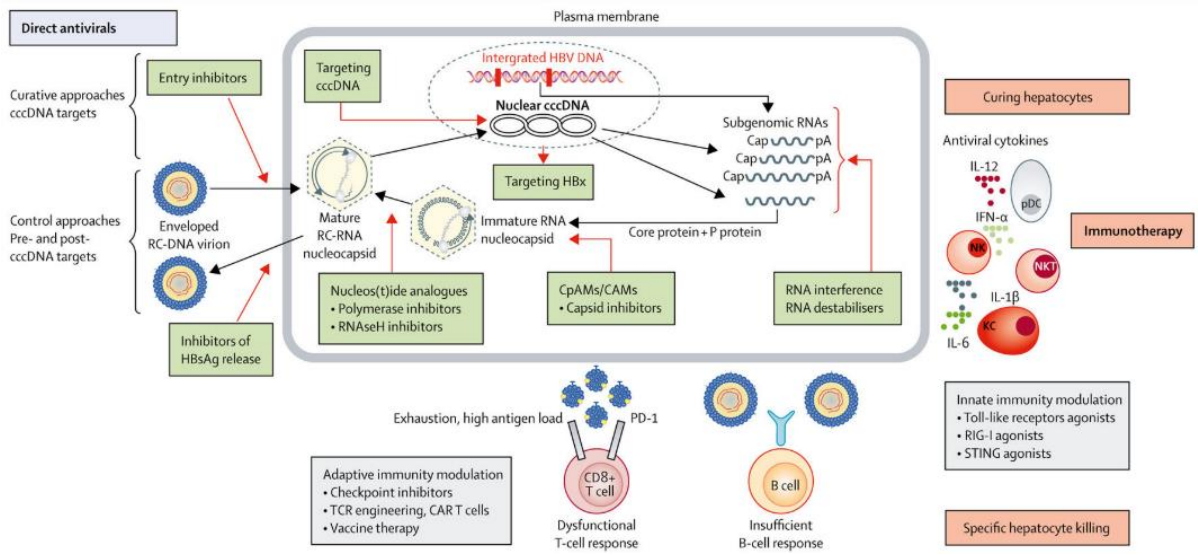

Figure I.22. Current and future virological and immunological targets for the treatment and cure of chronic hepatitis B.[172] All steps of the HBV replication cycle (viral entry, cccDNA formation, chromatinization and transcription, viral mRNAs, envelope protein secretion, core proteins and the capsid, Pol enzymatic activities). Reprinted from The Lancet, Vol. 4, P. Revill *et al.*, A global scientific strategy to cure hepatitis B, Pages 545-558, Copyright 2009, with permission from Elsevier.

HBV cure strategies can be classified in four functional categories depending on their mode of action. A first approach is to completely inhibit HBV replication to prevent *de novo* infection and to block the recycling of capsids in the nucleus and avoid replenishment of the cccDNA pool. Entry inhibitors, target the HBV entry receptor NTCP to stop new HBV infections and viral spreading to healthy hepatocytes. The most advanced entry inhibitor to date is Myrcludex B which is giving very promising results in combination with tenofovir.[178–181]

In parallel, capsid assembly modulators (CAMs) have been identified, showing the potential to efficiently eliminate HBV DNA from infected liver cells.[182] Two main classes of CAMs currently in development are heteroaryldihydropyrimidines[183] (HAP) and sulfamoyl benzamides[184] (SBA). Notably, the former lead to the formation of aberrant capsids and the latter to normally shaped but empty capsids.[185] In addition to capsid assembly modulators and entry inhibitors, new Pol and RNAse H inhibitors, NAPs and si/shRNA-based approaches have the potential to achieve a complete inhibition of HBV replication.[186]

A second approach is to restore the innate and adaptive immunity of the host with the aim of stimulating antiviral immuno-mediated pathways without triggering anti-HBV flare.[186] This can be achieved by inhibition of HBV gene expression at the post-transcriptional level and by relieving HBsAg-mediated immunosuppression. Recent clinical studies using RNA interference (RNAi) are showing liver-specific knockdown replication and protein expression.[187–190] Nucleic acid polymers (NAPs) such as REP9-AC inhibits both HBV entry and HBsAg release from infected hepatocytes.[191–193] Checkpoint molecule inhibitors have exhibited a potent immunostimulatory effect by rescuing virus specific cytotoxic T cells.[194] Specifically, PD-1 inhibitors nivolumab and pembrolizumab are monoclonal antibodies currently in phase 1 studies for CHB patients.[195–197] Bioengineered HBV-specific T cells is another promising strategy to build up immunity in CHB patients which is currently supported by preclinical data and an initial patient case report. [198–200] Finally, therapeutic vaccines[201,202] and agonists of toll like receptors (TLRs) are also being actively pursued.[203–207]

A third approach is to selectively sensitize HBV infected hepatocytes to immune elimination. To this end, SMAC mimetic antagonizes cellular inhibitors of apoptosis proteins (cIAPs) to promote hepatitis B virus clearance.[208] Recently, SMAC mimetic birinapant has shown effective control of HBV replication in preclinical models.[208–210]

Finally, the fourth approach is to directly target cccDNA by preventing its formation, destroying existing cccDNA, or silencing cccDNA transcription. The main strategy used in this case if by using direct-acting antivirals (DAAs),[211,212] host-targeting agents (HTAs),[202,213,214] and immune-modulatory agents.

In conclusion, the mechanism behind HBV persistence is complex and therefore challenging to address. Several strategies are being applied currently underway to reach a relevant therapy for CHB patients as soon as possible. With the combined efforts of academic and industry-based research, coupled with a robust drug development pipeline, the promise of a viable cure arises. Together with mass HBV vaccination and improved access to existing DAAs in highly endemic areas, a real hope for the total elimination of HBV can exist.

I.4 MAIN OBJECTIVES

The present thesis work was conceived as part of the VIRO-FLOW project. VIRO-FLOW is an innovative training network (ITN) aiming at the fast and efficient identification of new curative agents for the Hepatitis B virus (HBV). In particular, novel class of capsid assembly modulators (CAMs) would be synthesized, integrating the advantages of continuous flow chemistry with *in vitro* microfluidic bioassay technologies (Figure I.23).

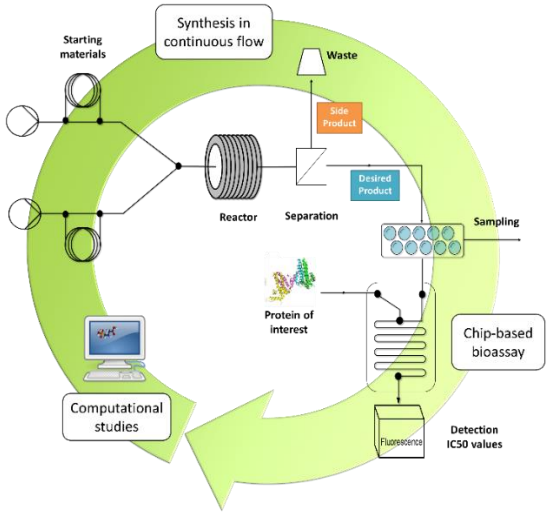

Figure I.23. Integrated system for the generation of SAR data envisioned by the VIROFLOW project. The synthesis of new compounds in continuous flow is driven by computational studies. The desired molecules are analysed and purified online before being supplied to the chip-based bioassay in flow. The IC_{50} values can be used to rationalize structure-activity relationship and to inform the design of new more active compounds.

As part of the VIRO-FLOW project, the overall goal of the present thesis is the development of new synthetic and computational methodologies that will effectively hasten the discovery of novel HBV inhibitors. As such, this work entails:

- the development of efficient synthetic methodologies in flow to support the generation of a focused library of compounds.
- the development of computational tools for the rapid identification of novel potentially active CAMs.

Following these guidelines, the goals of each chapter are:

- Chapter II. The development of three different continuous flow processes that will facilitate the synthesis of relevant building blocks for a novel CAM chemotype.
- Chapter III. The study of a focused library of HBV CAMs: understanding of structure-activity relationship and evaluation of their mode of action *in vitro*.
- Chapter IV. The elaboration of a workflow *in silico* to drive hit-to-lead and lead optimization synthetic plans of a CAMs series.
- Chapter V. The investigation of a synthetic process in continuous flow that leads to chemically relevant building blocks supported by mechanistic elucidation.
- Chapter VI. The investigation of CAMs mode of action *in silico* via molecular dynamics to propose new potential capsid assembly modulators with the predicted phenotype.

I.5 REFERENCES

1. Farlex. Segen's Medical Dictionary.
2. FW, D. The True History of the Discovery of Penicillin, with Refutation of the Misinformation in the Literature. *Br J Biomed Sci.* **1999**, *56* (2), 83–93.
3. Moffat, J. G.; Vincent, F.; Lee, J. A.; Eder, J.; Prunotto, M. Opportunities and Challenges in Phenotypic Drug Discovery: An Industry Perspective. *Nat. Rev. Drug Discov.* **2017**, *16* (8), 531–543.
4. G., B. Mother Nature — an Inexhaustible Source of Drugs and Lead Molecules. *Nat. Prod. Chem.* **2009**, *Biochemist* (Narosa Publishing House), 1–20.
5. Brahmachari, G. Natural Products in Drug Discovery: Impacts and Opportunities – an Assessment. *Bioact. Nat. Prod. Oppor. Challenges Med. Chem.* **2011**, 1–199.
6. Newman DJ, Cragg GM, S. K. Natural Products as Sources of New Drugs over the Period 1981–2002. *J Nat Prod* **2003**, *66*, 1022–1037.
7. Salazar, D. E.; Gormley, G. *Modern Drug Discovery and Development*; Elsevier Inc., 2017; Vol. 26.
8. AG, B. Aspirin flask.
9. Rolinson, G. . Forty Years of SS-Lactam Research. *J. Antimicrob. Chemother.* **1998**, No. 2641, 589–603.
10. Salazar, D. E.; Gormley, G. *Modern Drug Discovery and Development*; Elsevier Inc., 2017; Vol. 26.
11. Duelen, R.; Corvelyn, M.; Tortorella, I.; Leonardi, L.; Chai, Y. C.; Sampaolesi, M. Medicinal Biotechnology for Disease Modeling, Clinical Therapy, and Drug Discovery and Development. In *Introduction to Biotech Entrepreneurship: From Idea to Business*; 2019.
12. Hughes, J. P.; Rees, S. S.; Kalindjian, S. B.; Philpott, K. L. Principles of Early Drug Discovery. *Br. J. Pharmacol.* **2011**, *162* (6), 1239–1249.
13. Mahlich, J.; Bartol, A.; Dheban, S. Can Adaptive Clinical Trials Help to Solve the Productivity Crisis of the Pharmaceutical Industry ? - A Scenario Analysis. **2021**, 1–10.
14. Scannell, J. W.; Blanckley, A.; Boldon, H.; Warrington, B. Diagnosing the Decline in Pharmaceutical R&D Efficiency. *Nat. Rev. Drug Discov.* **2012**, *11* (3), 191–200.
15. Wouters, O. J.; McKee, M.; Luyten, J. Estimated Research and Development

Investment Needed to Bring a New Medicine to Market, 2009-2018. *JAMA - J. Am. Med. Assoc.* **2020**, *323* (9), 844–853.

16. Paul, S. M.; Mytelka, D. S.; Dunwiddie, C. T.; Persinger, C. C.; Munos, B. H.; Lindborg, S. R.; Schacht, A. L. How to Improve RD Productivity: The Pharmaceutical Industry's Grand Challenge. *Nat. Rev. Drug Discov.* **2010**, *9* (3), 203–214.
17. Gioiello, A.; Piccinno, A.; Lozza, A. M.; Cerra, B. The Medicinal Chemistry in the Era of Machines and Automation: Recent Advances in Continuous Flow Technology. *J. Med. Chem.* **2020**, *63* (13), 6624–6647.
18. Blanco, M.-J.; Gardinier, K. M. New Chemical Modalities and Strategic Thinking in Early Drug Discovery. *ACS Med. Chem. Lett.* **2020**, No. November, acsmedchemlett.9b00582.
19. Mennen, S. M.; Alhambra, C.; Allen, C. L.; Barberis, M.; Berritt, S.; Brandt, T. A.; Campbell, A. D.; Cherney, A. H.; Christensen, M.; Damon, D. B.; Diego, J. E. De; Janey, J.; Leitch, D. C.; Garc, S.; Garc, P.; Li, L.; Liu, F.; Lobben, P. C.; Macmillan, D. W. C.; Magano, J.; Mcintur, E.; Monfette, S.; Post, R. J.; Schultz, D.; Sitter, B. J.; Stevens, J. M.; Strambeanu, I. I.; Twilton, J.; Wang, K.; Zajac, M. A. The Evolution of High-Throughput Experimentation in Pharmaceutical Development and Perspectives on the Future ́ Castan ̃ o N. **2019**.
20. Schmink, J. R. Scientist-Led High-Throughput Experimentation (HTE) and Its Utility in Academia and Industry Scientist-Led High-Throughput Experimentation (HTE) and Its Utility in Academia and Industry Cross-Linked Enzyme Aggregates (CLEAs) in Organic Synthesis. **2013**, No. January.
21. Santanilla, A. B.; Regalado, E. L.; Pereira, T.; Shevlin, M.; Bateman, K.; Campeau, L. C.; Schneeweis, J.; Berritt, S.; Shi, Z. C.; Nantermet, P.; Liu, Y.; Helmy, R.; Welch, C. J.; Vachal, P.; Davies, I. W.; Cernak, T.; Dreher, S. D. Nanomole-Scale High-Throughput Chemistry for the Synthesis of Complex Molecules. *Science (80-.).* **2015**.
22. Inhibitors, A.; Cernak, T.; Gesmundo, N. J.; Dykstra, K.; Yu, Y.; Wu, Z.; Shi, Z.; Vachal, P.; Sperbeck, D.; He, S.; Murphy, B. A.; Sonatore, L.; Williams, S.; Madeira, M.; Verras, A.; Reiter, M.; Lee, C. H.; Cu, J.; Sherer, E. C.; Goble, S.; Perrotto, N.; Pinto, S.; Shen, D.; Nargund, R.; Balkovec, J.; Devita, R. J.; Dreher, S. D. Microscale High-Throughput Experimentation as an Enabling Technology in Drug Discovery: Application in the Discovery of (Piperidinyl)Pyridinyl - 1 H -

Benzimidazole Diacylglycerol Acyltransferase 1 Inhibitors. **2016**.

23. Godfrey, A. G.; Masquelin, T.; Hemmerle, H. A Remote-Controlled Adaptive Medchem Lab: An Innovative Approach to Enable Drug Discovery in the 21st Century. *Drug Discovery Today*. 2013.
24. Reizman, B. J.; Wang, Y. M.; Buchwald, S. L.; Jensen, K. F. Suzuki-Miyaura Cross-Coupling Optimization Enabled by Automated Feedback. *React. Chem. Eng.* **2016**.
25. Wang, Y.; Shaabani, S.; Ahmadianmoghaddam, M.; Gao, L.; Xu, R.; Kurpiewska, K.; Kalinowska-Tluscik, J.; Olechno, J.; Ellson, R.; Kossenjans, M.; Helan, V.; Groves, M.; Dömling, A. Acoustic Droplet Ejection Enabled Automated Reaction Scouting. *ACS Cent. Sci.* **2019**, *5* (3), 451–457.
26. Ellson, R. Picoliter: Enabling Precise Transfer of Nanoliter and Picoliter Volumes. *Drug Discov. Today* **2002**.
27. Baumann, M.; Moody, T. S.; Smyth, M.; Wharry, S. A Perspective on Continuous Flow Chemistry in the Pharmaceutical Industry. *Org. Process Res. Dev.* **2020**.
28. Gutmann, B.; Cantillo, D.; Kappe, C. O. Continuous-Flow Technology—A Tool for the Safe Manufacturing of Active Pharmaceutical Ingredients. *Angew. Chemie Int. Ed.* **2015**, *54* (23), 6688–6728.
29. Porta, R.; Benaglia, M.; Puglisi, A. Flow Chemistry: Recent Developments in the Synthesis of Pharmaceutical Products. *Org. Process Res. Dev.* **2016**, *20* (1), 2–25.
30. Fülöp, Z.; Szemesi, P.; Bana, P.; Éles, J.; Greiner, I. Evolution of Flow-Oriented Design Strategies in the Continuous Preparation of Pharmaceuticals. *React. Chem. Eng.* **2020**.
31. Plutschack, M. B.; Pieber, B.; Gilmore, K.; Seeberger, P. H. The Hitchhiker's Guide to Flow Chemistry. *Chem. Rev.* **2017**, *117* (18), 11796–11893.
32. Mason, B. P.; Price, K. E.; Steinbacher, J. L.; Bogdan, A. R.; McQuade, T. D. Greener Approaches to Organic Synthesis Using Microreactor Technology. *Chem. Rev.* **2007**, *107* (6), 2300–2318.
33. McQuade, D. T.; Seeberger, P. H. Applying Flow Chemistry: Methods, Materials, and Multistep Synthesis. *J. Org. Chem.* **2013**, *78* (13), 6384–6389.
34. Cole, K. P.; Groh, J. M. C.; Johnson, M. D.; Burcham, C. L.; Campbell, B. M.; Diseroad, W. D.; Heller, M. R.; Howell, J. R.; Kallman, N. J.; Koenig, T. M.; May, S. A.; Miller, R. D.; Mitchell, D.; Myers, D. P.; Myers, S. S.; Phillips, J. L.; Polster, C. S.; White, T. D.; Cashman, J.; Hurley, D.; Moylan, R.; Sheehan, P.; Spencer, R. D.; Desmond, K.; Desmond, P.; Gowran, O. Kilogram-Scale Prexasertib

Monolactate Monohydrate Synthesis under Continuous-Flow CGMP Conditions. *Science (80-.).* **2017**, *356* (6343), 1144–1151.

35. Bogdan, A. R.; Dombrowski, A. W. Emerging Trends in Flow Chemistry and Applications to the Pharmaceutical Industry. *J. Med. Chem.* **2019**, No. Table 1.
36. Gomollón-bel, F. Ten Chemical Innovations Hed That Will Change Our World. *Chem. Int.* **2019**, No. June, 12–17.
37. Stanton, D. Janssen and Rugers Expand R&D as Continuous Manufacturing Picks up Steam. *https://www.outsourcing-pharma.com/Article/2015/05/20/J-J-and-Rutgers-expand-R-D-as-continuous-manufacturing-picks-up-steam*. 2015, p Accessed January 2021.
38. Palmer, E. GSK Opens $95M Continuous Production Operation in Singapore. *https://www.fiercepharma.com/manufacturing/gsk-opens-130m-continuous-production-facilities-singapore*. 2019, p Accesed January 2021.
39. Dan Stanton. Lilly Takes to Continuous Manufacturing with $40m Irish Investment. *https://www.outsourcing-pharma.com/Article/2016/04/06/Lilly-takes-to-continuous-manufacturing-with-40m-Irish-investment*. 2016, p Accessed January 2021.
40. Editors, P. T. Vertex Receives FDA Approval for Continuously Manufactured Drug Product. *https://www.pharmtech.com/view/vertex-receives-fda-approval-continuously-manufactured-drug-product*. 2018, p Accessed January 2021.
41. Brennan, Z. FDA Calls on Manufacturers to Begin Switch from Batch to Continuous Production. *https://www.outsourcing-pharma.com/Article/2015/05/01/FDA-calls-on-manufacturers-to-begin-switch-from-batch-to-continuous-production*. 2015, p Accessed January 2021.
42. Hughes, D. L. Applications of Flow Chemistry in the Pharmaceutical Industry—Highlights of the Recent Patent Literature. *Org. Process Res. Dev.* **2020**, *24* (10), 1850–1860.
43. Baumann, M.; Moody, T. S.; Smyth, M.; Wharry, S. A Perspective on Continuous Flow Chemistry in the Pharmaceutical Industry. *Org. Process Res. Dev.* **2020**.
44. López E, Linares ML, A. J. Flow Chemistry as a Tool to Access Novel Chemical Space for Drug Discovery. *Futur. Med Chem.* **2020**, *12* (17), 1547–1563.
45. Bogdan, A. R.; Dombrowski, A. W. Emerging Trends in Flow Chemistry and Applications to the Pharmaceutical Industry. *J. Med. Chem.* **2019**.
46. Lévesque, F.; Rogus, N. J.; Spencer, G.; Grigorov, P.; McMullen, J. P.;

Thaisrivongs, D. A.; Davies, I. W.; Naber, J. R. Advancing Flow Chemistry Portability: A Simplified Approach to Scaling Up Flow Chemistry. *Org. Process Res. Dev.* **2018**, *22* (8), 1015–1021.

47. de Souza, J. M.; Galaverna, R.; de Souza, A. A. N.; Brocksom, T. J.; Pastre, J. C.; de Souza, R. O. M. A.; de Oliveira, K. T. Impact of Continuous Flow Chemistry in the Synthesis of Natural Products and Active Pharmaceutical Ingredients. *An. Acad. Bras. Cienc.* **2018**, *90* (1), 1131–1174.
48. Vrijdag, J. L.; Delgado, F.; Alonso, N.; De Borggraeve, W. M.; Pérez-Macias, N.; Alcázar, J. Practical Preparation of Challenging Amides from Non-Nucleophilic Amines and Esters under Flow Conditions. *Chem. Commun.* **2014**, *50* (95), 15094–15097.
49. Gustafsson, T.; Pontén, F.; Seeberger, P. H. Trimethylaluminium Mediated Amide Bond Formation in a Continuous Flow Microreactor as Key to the Synthesis of Rimonabant and Efaproxiral. *Chem. Commun.* **2008**, No. 9, 1100–1102.
50. Len, C.; Bruniaux, S.; Delbecq, F.; Parmar, V. S. Palladium-Catalyzed Suzuki–Miyaura Cross-Coupling in Continuous Flow. *Catalysts*. 2017.
51. Shu, W.; Pellegatti, L.; Oberli, M. A.; Buchwald, S. L. Continuous-Flow Synthesis of Biaryls Enabled by Multistep Solid-Handling in a Lithiation/Borylation/Suzuki-Miyaura Cross-Coupling Sequence. *Angew. Chemie - Int. Ed.* **2011**.
52. Noal, T.; Kuhn, S.; Musacchio, A. J.; Jensen, K. F.; Buchwald, S. L. Suzuki-Miyaura Cross-Coupling Reactions in Flow: Multistep Synthesis Enabled by a Microfluidic Extraction. *Angew. Chemie - Int. Ed.* **2011**.
53. Hamper, B. C.; Tesfu, E. Direct Uncatalyzed Amination of 2-Chloropyridine Using a Flow Reactor. *Synlett* **2007**.
54. Charaschanya, M.; Bogdan, A. R.; Wang, Y.; Djuric, S. W. Nucleophilic Aromatic Substitution of Heterocycles Using a High-Temperature and High-Pressure Flow Reactor. *Tetrahedron Lett.* **2016**.
55. Jaman, Z.; Logsdon, D. L.; Szilágyi, B.; Sobreira, T. J. P.; Aremu, D.; Avramova, L.; Cooks, R. G.; Thompson, D. H. High-Throughput Experimentation and Continuous Flow Evaluation of Nucleophilic Aromatic Substitution Reactions. *ACS Comb. Sci.* **2020**.
56. Falus, P.; Boros, Z.; Hornyánszky, G.; Nagy, J.; Darvas, F.; Ürge, L.; Poppe, L. Reductive Amination of Ketones: Novel One-Step Transfer Hydrogenations in Batch and Continuous-Flow Mode. *Tetrahedron Lett.* **2011**.

57. Suveges, N. S.; de Souza, R. O. M. A.; Gutmann, B.; Kappe, C. O. Synthesis of Mepivacaine and Its Analogues by a Continuous-Flow Tandem Hydrogenation/Reductive Amination Strategy. *European J. Org. Chem.* **2017**.
58. Artiukha, E. A.; Nuzhdin, A. L.; Bukhtiyarova, G. A.; Zaytsev, S. Y.; Plyusnin, P. E.; Shubin, Y. V.; Bukhtiyarov, V. I. One-Pot Reductive Amination of Aldehydes with Nitroarenes over an Au/Al2O3 Catalyst in a Continuous Flow Reactor. *Catal. Sci. Technol.* **2015**.
59. Liu, J.; Fitzgerald, A. E.; Mani, N. S. Reductive Amination by Continuous-Flow Hydrogenation: Direct and Scalable Synthesis of a Benzylpiperazine. *Synth.* **2012**.
60. Bogdan, A. R.; Charaschanya, M.; Dombrowski, A. W.; Wang, Y.; Djuric, S. W. High-Temperature Boc Deprotection in Flow and Its Application in Multistep Reaction Sequences. *Org. Lett.* **2016**, *18* (8), 1732–1735.
61. Li, B.; Li, R.; Dorff, P.; McWilliams, J. C.; Guinn, R. M.; Guinness, S. M.; Han, L.; Wang, K.; Yu, S. Deprotection of N-Boc Groups under Continuous-Flow High-Temperature Conditions. *J. Org. Chem.* **2019**, *84* (8), 4846–4855.
62. Greb, A.; Poh, J. S.; Greed, S.; Battilocchio, C.; Pasau, P.; Blakemore, D. C.; Ley, S. V. A Versatile Route to Unstable Diazo Compounds via Oxadiazolines and Their Use in Aryl–Alkyl Cross-Coupling Reactions. *Angew. Chemie - Int. Ed.* **2017**.
63. Murray, P. R. D.; Browne, D. L.; Pastre, J. C.; Butters, C.; Guthrie, D.; Ley, S. V. Continuous Flow-Processing of Organometallic Reagents Using an Advanced Peristaltic Pumping System and the Telescoped Flow Synthesis of (E/Z)-Tamoxifen. *Organic Process Research and Development*. 2013.
64. Zhang, J.; Gong, C.; Zeng, X.; Xie, J. Continuous Flow Chemistry: New Strategies for Preparative Inorganic Chemistry. *Coordination Chemistry Reviews*. 2016.
65. Degennaro, L.; Carlucci, C.; De Angelis, S.; Luisi, R. Flow Technology for Organometallic-Mediated Synthesis. *Journal of Flow Chemistry*. 2016.
66. Cambie, D.; Bottecchia, C.; Straathof, N. J. W.; Hessel, V.; Noe, T. Applications of Continuous-Flow Photochemistry in Organic Synthesis , Material Science , and Water Treatment. **2016**.
67. Knowles, J. P.; Elliott, L. D.; Booker-Milburn, K. I. Flow Photochemistry: Old Light through New Windows. *Beilstein Journal of Organic Chemistry*. 2012.
68. Gilmore, K.; Seeberger, P. H. Continuous Flow Photochemistry. *Chem. Rec.* **2014**.
69. Noël, T. A Personal Perspective on the Future of Flow Photochemistry. *J. Flow Chem.* **2017**.

70. Sambiagio, C.; Noël, T. Flow Photochemistry: Shine Some Light on Those Tubes! *Trends in Chemistry*. 2020.
71. Abdiaj, I.; Bottecchia, C.; Alcazar, J.; Noël, T. Visible-Light-Induced Trifluoromethylation of Highly Functionalized Arenes and Heteroarenes in Continuous Flow. *Synth.* **2017**.
72. Straathof, N. J. W.; Gemoets, H. P. L.; Wang, X.; Schouten, J. C.; Hessel, V.; Noël, T. Rapid Trifluoromethylation and Perfluoroalkylation of Five-Membered Heterocycles by Photoredox Catalysis in Continuous Flow. *ChemSusChem* **2014**.
73. Jensen, K. F.; Becker, R.; Delville, M. M. E.; Fekete, M.; Fülöp, F.; Glasnov, T.; Hamlin, T. A.; Harmel, R. K.; Kappe, C. O.; Koch, K.; Leadbeater, N. E.; Löwe, H.; Macchi, A.; Mándity, I. M.; Nieuwland, P.; Ötvös, S. B.; Plouffe, P.; Roberge, D.; Rutjes, F.; Yoshida, J. 2. Fundamentals of Flow Chemistry. In *Fundamentals*; 2014.
74. Jensen, K. F.; Becker, R.; Delville, M. M. E.; Fekete, M.; Fülöp, F.; Glasnov, T.; Hamlin, T. A.; Harmel, R. K.; Kappe, C. O.; Koch, K.; Leadbeater, N. E.; Löwe, H.; Macchi, A.; Mándity, I. M.; Nieuwland, P.; Ötvös, S. B.; Plouffe, P.; Roberge, D.; Rutjes, F.; Yoshida, J. 3. Principles of Controlling Reactions in Flow Chemistry. In *Fundamentals*; 2014.
75. Salehi Marzijarani, N.; Snead, D. R.; McMullen, J. P.; Lévesque, F.; Weisel, M.; Varsolona, R. J.; Lam, Y. H.; Liu, Z.; Naber, J. R. One-Step Synthesis of 2-Fluoroadenine Using Hydrogen Fluoride Pyridine in a Continuous Flow Operation. *Org. Process Res. Dev.* **2019**.
76. Stueckler, C.; Hermsen, P.; Ritzen, B.; Vasiloiu, M.; Poechlauer, P.; Steinhofer, S.; Pelz, A.; Zinganell, C.; Felfer, U.; Boyer, S.; Goldbach, M.; De Vries, A.; Pabst, T.; Winkler, G.; Lavopa, V.; Hecker, S.; Schuster, C. Development of a Continuous Flow Process for a Matteson Reaction: From Lab Scale to Full-Scale Production of a Pharmaceutical Intermediate. *Org. Process Res. Dev.* **2019**.
77. Köbrich, G.; Akhtar, A.; Ansari, F.; Breckoff, W. E.; Büttner, H.; Drischel, W.; Fischer, R. H.; Flory, K.; Fröhlich, H.; Goyert, W.; Heinemann, H.; Hornke, I.; Merkle, H. R.; Trapp, H.; Zündorf, W. Chemistry of Stable A-Halogenoorganolithium Compounds and the Mechanism of Carbenoid Reactions. *Angew. Chemie Int. Ed. English* **1967**.
78. Hafner, A.; Mancino, V.; Meisenbach, M.; Schenkel, B.; Sedelmeier, J. Dichloromethyllithium: Synthesis and Application in Continuous Flow Mode. *Org. Lett.* **2017**.

79. Schneider, G. Automating Drug Discovery. *Nat. Rev. Drug Discov.* **2018**, *17* (2), 97–113.
80. Baranczak, A.; Tu, N. P.; Marjanovic, J.; Searle, P. A.; Vasudevan, A.; Djuric, S. W. Integrated Platform for Expedited Synthesis-Purification-Testing of Small Molecule Libraries. *ACS Med. Chem. Lett.* **2017**, *8* (4), 461–465.
81. Parry, D. M. Closing the Loop: Developing an Integrated Design, Make, and Test Platform for Discovery. *ACS Med. Chem. Lett.* **2019**, *10* (6), 848–856.
82. Desai, B.; Dixon, K.; Farrant, E.; Feng, Q.; Gibson, K. R.; Van Hoorn, W. P.; Mills, J.; Morgan, T.; Parry, D. M.; Ramjee, M. K.; Selway, C. N.; Tarver, G. J.; Whitlock, G.; Wright, A. G. Rapid Discovery of a Novel Series of Abl Kinase Inhibitors by Application of an Integrated Microfluidic Synthesis and Screening Platform. *J. Med. Chem.* **2013**.
83. 2021, N. M. A. The Nobel Prize in Chemistry 2013. *Accessed Feb 2021* **2021**, No. https://www.nobelprize.org/prizes/chemistry/2013/summary.
84. E., G. Building a Virtual Ligand Screening Pipeline Using Free Software: A Survey. *Br. Bioinform.* **2016**, *17* (2), 352–366.
85. Scigenis. https://en.wikipedia.org/wiki/Docking_(molecular).
86. Hammer, B. Recurrent Networks for Structured Data - A Unifying Approach and Its Properties. *Cogn. Syst. Res.* **2002**.
87. Kore, P. P.; Mutha, M. M.; Antre, R. V.; Oswal, R. J.; Kshirsagar, S. S. Computer-Aided Drug Design: An Innovative Tool for Modeling. *Open J. Med. Chem.* **2012**.
88. Leelananda, S. P.; Lindert, S. Computational Methods in Drug Discovery. *Beilstein J. Org. Chem.* **2016**, *12*, 2694–2718.
89. Liu, X.; Shi, D.; Zhou, S.; Liu, H.; Liu, H.; Yao, X. Molecular Dynamics Simulations and Novel Drug Discovery. *Expert Opin. Drug Discov.* **2018**, *13* (1), 23–37.
90. H.M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T.N. Bhat, H. Weissig, I.N. Shindyalov, P. E. B. The Protein Data Bank. *Nucleic Acids Res.* **2000**, *28*, 235–242.
91. Dorn M, E Silva MB, Buriol LS, L. L. Three-Dimensional Protein Structure Prediction: Methods and Computational Strategies. *Comp. Biol. Chem.* **2014**, *52* (B), 251–276.
92. Hamet, P.; Tremblay, J. Artificial Intelligence in Medicine. *Metabolism.* **2017**.
93. Yang, Y.; Siau, K. A Qualitative Research on Marketing and Sales in the Artificial Intelligence Age. *Midwest Assoc. Inf. Syst. Conf. Inf. Syst. Conf.* **2018**.
94. Wirtz, B. W.; Weyerer, J. C.; Geyer, C. Artificial Intelligence and the Public

Sector—Applications and Challenges. *Int. J. Public Adm.* **2019**.

95. Smith, R. G.; Farquhar, A. Road Ahead for Knowledge Management an AI Perspective. *AI Mag.* **2000**.
96. Paul, D.; Sanap, G.; Shenoy, S.; Kalyane, D.; Kalia, K.; Tekade, R. K. Artificial Intelligence in Drug Discovery and Development. *Drug Discov. Today* **2021**, *26* (1), 80–93.
97. Kubinyi, H. Free Wilson Analysis. Theory, Applications and Its Relationship to Hansch Analysis. *Quant. Struct.-Act. Relat.* **1988**, *7*, 121–133.
98. Mandlik, V.; Bejugam, P. R.; Singh, S. Application of Artificial Neural Networks in Modern Drug Discovery. In *Artificial Neural Network for Drug Design, Delivery and Disposition*; 2016.
99. Cai, C.; Guo, P.; Zhou, Y.; Zhou, J.; Wang, Q.; Zhang, F.; Fang, J.; Cheng, F. Deep Learning-Based Prediction of Drug-Induced Cardiotoxicity. *J. Chem. Inf. Model.* **2019**.
100. Martinez-Mayorga, K.; Madariaga-Mazon, A.; Medina-Franco, J. L.; Maggiora, G. The Impact of Chemoinformatics on Drug Discovery in the Pharmaceutical Industry. *Expert Opin. Drug Discov.* **2020**, *15* (3), 293–306.
101. Inte:ligand. pharmDB.
102. Steindl, T. M.; Schuster, D.; Laggner, C.; Langer, T. Parallel Screening: A Novel Concept in Pharmacophore Modeling and Virtual Screening. *J. Chem. Inf. Model.* **2006**.
103. Definition of Biophysical Methods. *Nat. Portf.* No. https://www.nature.com/subjects/biophysical-methods.
104. Ishima, R.; Torchia, D. A. Protein Dynamics from NMR. *Nature Structural Biology*. 2000.
105. Kay, L. E. NMR Studies of Protein Structure and Dynamics. In *Journal of Magnetic Resonance*; 2005.
106. Kleckner, I. R.; Foster, M. P. An Introduction to NMR-Based Approaches for Measuring Protein Dynamics. *Biochimica et Biophysica Acta - Proteins and Proteomics*. 2011.
107. Zuiderweg, E. R. P. Mapping Protein-Protein Interactions in Solution by NMR Spectroscopy. *Biochemistry* **2002**.
108. Mittermaier, A.; Kay, L. E. New Tools Provide New Insights in NMR Studies of Protein Dynamics. *Science*. 2006.

109. Lippincott-Schwartz, J.; Snapp, E.; Kemvorthy, A. Studying Protein Dynamics in Living Cells. *Nature Reviews Molecular Cell Biology*. 2001.
110. Ebbinghaus, S.; Dhar, A.; McDonald, J. D.; Gruebele, M. Protein Folding Stability and Dynamics Imaged in a Living Cell. *Nat. Methods* **2010**.
111. Schröder, G. F.; Alexiev, U.; Grubmüller, H. Simulation of Fluorescence Anisotropy Experiments: Probing Protein Dynamics. *Biophys. J.* **2005**.
112. Mo, S. P.; Coulson, J. M.; Prior, I. A. RAS Variant Signalling. *Biochemical Society Transactions*. 2018.
113. Pantsar, T. KRAS(G12C)–AMG 510 Interaction Dynamics Revealed by All-Atom Molecular Dynamics Simulations. *Sci. Rep.* **2020**.
114. Pantsar, T. The Current Understanding of KRAS Protein Structure and Dynamics. *Computational and Structural Biotechnology Journal*. 2020.
115. Jin, F.; Yu, C.; Lai, L.; Liu, Z. Ligand Clouds around Protein Clouds: A Scenario of Ligand Binding with Intrinsically Disordered Proteins. *PLoS Comput. Biol.* **2013**.
116. Fichou, Y.; Heyden, M.; Zaccai, G.; Weik, M.; Tobias, D. J. Molecular Dynamics Simulations of a Powder Model of the Intrinsically Disordered Protein Tau. *J. Phys. Chem. B* **2015**.
117. Missimer, J. H.; Steinmetz, M. O.; Van Gunsteren, W. F.; Dolenc, J. Influence of 63Ser Phosphorylation and Dephosphorylation on the Structure of the Stathmin Helical Nucleation Sequence: A Molecular Dynamics Study. *Biochemistry* **2012**.
118. Cino, E. A.; Wong-ekkabut, J.; Karttunen, M.; Choy, W. Y. Microsecond Molecular Dynamics Simulations of Intrinsically Disordered Proteins Involved in the Oxidative Stress Response. *PLoS One* **2011**.
119. Chong, S. H.; Chatterjee, P.; Ham, S. Computer Simulations of Intrinsically Disordered Proteins. *Annual Review of Physical Chemistry*. 2017.
120. Lindorff-Larsen, K.; Trbovic, N.; Maragakis, P.; Piana, S.; Shaw, D. E. Structure and Dynamics of an Unfolded Protein Examined by Molecular Dynamics Simulation. *J. Am. Chem. Soc.* **2012**.
121. Apicella, A.; Marascio, M.; Colangelo, V.; Soncini, M.; Gautieri, A.; Plummer, C. J. G. Molecular Dynamics Simulations of the Intrinsically Disordered Protein Amelogenin. *J. Biomol. Struct. Dyn.* **2017**.
122. Henriques, J.; Cragnell, C.; Skepö, M. Molecular Dynamics Simulations of Intrinsically Disordered Proteins: Force Field Evaluation and Comparison with Experiment. *J. Chem. Theory Comput.* **2015**.

123. Qiao, Q.; Bowman, G. R.; Huang, X. Dynamics of an Intrinsically Disordered Protein Reveal Metastable Conformations That Potentially Seed Aggregation. *J. Am. Chem. Soc.* **2013**.
124. Karplus, M.; Petsko, G. A. Molecular Dynamics Simulations in Biology. *Nature*. 1990.
125. Durrant, J. D.; McCammon, J. A. Molecular Dynamics Simulations and Drug Discovery. *BMC Biology*. 2011.
126. Zhao, H.; Caflisch, A. Molecular Dynamics in Drug Design. *European Journal of Medicinal Chemistry*. 2015.
127. Upadhyay, S. K. *Chemical Kinetics and Reaction Dynamics*; 2006.
128. Votapka, L. W.; Amaro, R. E. Multiscale Estimation of Binding Kinetics Using Brownian Dynamics, Molecular Dynamics and Milestoning. *PLoS Comput. Biol.* **2015**.
129. Buch, I.; Giorgino, T.; De Fabritiis, G. Complete Reconstruction of an Enzyme-Inhibitor Binding Process by Molecular Dynamics Simulations. *Proc. Natl. Acad. Sci. U. S. A.* **2011**.
130. De Vivo, M.; Masetti, M.; Bottegoni, G.; Cavalli, A. Role of Molecular Dynamics and Related Methods in Drug Discovery. *Journal of Medicinal Chemistry*. 2016.
131. Matter, H.; Sotriffer, C. Applications and Success Stories in Virtual Screening; 2011.
132. Jiang, F.; Kim, S. H. "Soft Docking": Matching of Molecular Surface Cubes. *J. Mol. Biol.* **1991**.
133. Leach, A. R. Ligand Docking to Proteins with Discrete Side-Chain Flexibility. *J. Mol. Biol.* **1994**.
134. Davis, I. W.; Baker, D. RosettaLigand Docking with Full Ligand and Receptor Flexibility. *J. Mol. Biol.* **2009**.
135. Morra, G.; Genoni, A.; Neves, M.; Merz Jr., K.; Colombo, G. Molecular Recognition and Drug-Lead Identification: What Can Molecular Simulations Tell Us? *Curr. Med. Chem.* **2009**.
136. Lin, J. H.; Perryman, A. L.; Schames, J. R.; McCammon, J. A. Computational Drug Design Accommodating Receptor Flexibility: The Relaxed Complex Scheme. *J. Am. Chem. Soc.* **2002**.
137. McGovern, S. L.; Shoichet, B. K. Information Decay in Molecular Docking Screens against Holo, Apo, and Modeled Conformations of Enzymes. *J. Med. Chem.* **2003**.
138. Amaro, R. E.; Baudry, J.; Chodera, J.; Demir, Ö.; McCammon, J. A.; Miao, Y.;

Smith, J. C. Ensemble Docking in Drug Discovery. *Biophysical Journal*. 2018.

139. Zacharias, M. Accounting for Conformational Changes during Protein-Protein Docking. *Current Opinion in Structural Biology*. 2010.
140. Huang, S. Y.; Zou, X. Ensemble Docking of Multiple Protein Structures: Considering Protein Structural Variations in Molecular Docking. *Proteins Struct. Funct. Genet.* **2007**.
141. Hospital, A.; Goñi, J. R.; Orozco, M.; Gelpí, J. L. Molecular Dynamics Simulations: Advances and Applications. *Advances and Applications in Bioinformatics and Chemistry*. 2015.
142. Campbell, A. J.; Lamb, M. L.; Joseph-McCarthy, D. Ensemble-Based Docking Using Biased Molecular Dynamics. *J. Chem. Inf. Model.* **2014**.
143. Osguthorpe, D. J.; Sherman, W.; Hagler, A. T. Exploring Protein Flexibility: Incorporating Structural Ensembles from Crystal Structures and Simulation into Virtual Screening Protocols. *J. Phys. Chem. B* **2012**.
144. Nichols, S. E.; Baron, R.; McCammon, J. A. On the Use of Molecular Dynamics Receptor Conformations for Virtual Screening. *Methods Mol. Biol.* **2012**.
145. Ivetac, A.; Andrew McCammon, J. Molecular Recognition in the Case of Flexible Targets. *Curr. Pharm. Des.* **2011**.
146. WHO. Hepatitis B facts sheet http://www.who.int/news-room/fact-sheets/detail/hepatitis-b.
147. WHO. *Global Hepatitis Report, 2017*; 2017.
148. UN. Transforming our world: the 2030 Agenda for Sustainable Development https://sustainabledevelopment.un.org/post2015/transformingourworld (accessed Dec 5, 2015).
149. WHO. Global Health Sector Strategies for viral hepatitis 2016–21. http://www.who.int/hepatitis/strategy2016-2021/ghss-hep/en (accessed Jul 1, 2016).
150. Lemoine, M.; Kim, J. U.; Ndow, G.; Bah, S.; Forrest, K.; Rwegasha, J.; Bouyou, M.; Napon, D.; Somda, S.; Sawadogo, A.; Sombie, R.; Shimakawa, Y. Effect of the COVID-19 Pandemic on Viral Hepatitis Services in Sub-Saharan Africa. *Lancet Gastroenterol. Hepatol.* **2020**, *5* (11), 966–967.
151. Pley, C. M.; McNaughton, A. L.; Matthews, P. C.; Lourenço, J. The Global Impact of the COVID-19 Pandemic on the Prevention, Diagnosis and Treatment of Hepatitis B Virus (HBV) Infection. *BMJ Glob. Heal.* **2021**, *6* (1), e004275.
152. Cooke, G. S.; Andrieux-Meyer, I.; Applegate, T. L.; Atun, R.; Burry, J. R.;

Cheinquer, H.; Dusheiko, G.; Feld, J. J.; Gore, C.; Griswold, M. G.; Hamid, S.; Hellard, M. E.; Hou, J.; Howell, J.; Jia, J.; Kravchenko, N.; Lazarus, J. V; Lemoine, M.; Lesi, O. A.; Maistat, L.; McMahon, B. J.; Razavi, H.; Roberts, T.; Simmons, B.; Sonderup, M. W.; Spearman, C. W.; Taylor, B. E.; Thomas, D. L.; Waked, I.; Ward, J. W.; Wiktor, S. Z.; Abdo, A.; Aggarwal, R.; Aghemo, A.; Al-Judaibi, B.; Al Mahtab, M.; Altaf, A.; Ameen, Z.; Asselah, T.; Baatarkkhuu, O.; Barber, E.; Barnes, E.; Boulet, P.; Burrows, L.; Butsashvili, M.; Chan, E.; Chow, C.; Cowie, B.; Cunningham, C.; de Araujo, A.; Diap, G.; Dore, G.; Doyle, J.; Elsayed, M.; Fajardo, E.; Gane, E.; Getahun, A.; Goldberg, D.; Got, T.; Hickman, M.; Hill, A.; Hutchinson, S.; Jones, C.; Kamili, S.; Khan, A.; Lee, A.; Lee, T. Y.; Malani, J.; Morris, T. M.; Nayagam, S.; Njouom, R.; Ocama, P.; Pedrana, A.; Peeling, R.; Reddy, A.; Sacks, J.; Sarin, S.; Shimakawa, Y.; Silva, M.; Skala, P.; Taylor-Robinson, S.; Thompson, A.; Thursz, M.; Tonganibeia, A.; Wallace, J.; Ward, J.; Wolff, F.; Vickerman, P.; Yau, J. Accelerating the Elimination of Viral Hepatitis: A Lancet Gastroenterology & Hepatology Commission. *Lancet Gastroenterol. Hepatol.* **2019**, *4* (2), 135–184.

153. Dane, D. S.; Cameron, C. H.; Briggs, M. Virus-like Particles in Serum of Patients with Australia-Antigen-Antigen-Associated Hepatitis. *Lancet* **1970**.
154. Summers, J.; O'Connell, A.; Millman, I. Genome of Hepatitis B Virus: Restriction Enzyme Cleavage and Structure of DNA Extracted from Dane Particles. *Proc. Natl. Acad. Sci. U. S. A.* **1975**.
155. Seeger, C.; Mason, W. S. Molecular Biology of Hepatitis B Virus Infection. *Virology*. 2015.
156. Ott, M. J.; Aruda, M. Hepatitis B Vaccine. *J. Pediatr. Heal. Care* **1999**.
157. Summers, J.; Mason, W. S. Replication of the Genome of a Hepatitis B-like Virus by Reverse Transcription of an RNA Intermediate. *Cell* **1982**, *29* (2), 403–415.
158. Razavi-Shearer, D.; Gamkrelidze, I.; Nguyen, M. H.; Chen, D.-S.; Van Damme, P.; Abbas, Z.; Abdulla, M.; Abou Rached, A.; Adda, D.; Aho, I.; Akarca, U.; Hasan, F.; Al Lawati, F.; Al Naamani, K.; Al-Ashgar, H. I.; Alavian, S. M.; Alawadhi, S.; Albillos, A.; Al-Busafi, S. A.; Aleman, S.; Alfaleh, F. Z.; Aljumah, A. A.; Anand, A. C.; Anh, N. T.; Arends, J. E.; Arkkila, P.; Athanasakis, K.; Bane, A.; Ben-Ari, Z.; Berg, T.; Bizri, A. R.; Blach, S.; Brandão Mello, C. E.; Brandon, S. M.; Bright, B.; Bruggmann, P.; Brunetto, M.; Buti, M.; Chan, H. L. Y.; Chaudhry, A.; Chien, R.-N.; Choi, M. S.; Christensen, P. B.; Chuang, W.-L.; Chulanov, V.; Clausen, M. R.; Colombo, M.; Cornberg, M.; Cowie, B.; Craxi, A.; Croes, E. A.; Cuellar, D. A.;

Cunningham, C.; Desalegn, H.; Drazilova, S.; Duberg, A.-S.; Egeonu, S. S.; El-Sayed, M. H.; Estes, C.; Falconer, K.; Ferraz, M. L. G.; Ferreira, P. R.; Flisiak, R.; Frankova, S.; Gaeta, G. B.; García-Samaniego, J.; Genov, J.; Gerstoft, J.; Goldis, A.; Gountas, I.; Gray, R.; Guimarães Pessôa, M.; Hajarizadeh, B.; Hatzakis, A.; Hézode, C.; Himatt, S. M.; Hoepelman, A.; Hrstic, I.; Hui, Y.-T. T.; Husa, P.; Jahis, R.; Janjua, N. Z.; Jarčuška, P.; Jaroszewicz, J.; Kaymakoglu, S.; Kershenobich, D.; Kondili, L. A.; Konysbekova, A.; Krajden, M.; Kristian, P.; Laleman, W.; Lao, W. C.; Layden, J.; Lazarus, J. V; Lee, M.-H.; Liakina, V.; Lim, Y.-S. S.; Loo, C. K.; Lukšić, B.; Malekzadeh, R.; Malu, A. O.; Mamatkulov, A.; Manns, M.; Marinho, R. T.; Maticic, M.; Mauss, S.; Memon, M. S.; Mendes Correa, M. C.; Mendez-Sanchez, N.; Merat, S.; Metwally, A. M.; Mohamed, R.; Mokhbat, J. E.; Moreno, C.; Mossong, J.; Mourad, F. H.; Müllhaupt, B.; Murphy, K.; Musabaev, E.; Nawaz, A.; Nde, H. M.; Negro, F.; Nersesov, A.; Nguyen, V. T. T.; Njouom, R.; Ntagirabiri, R.; Nurmatov, Z.; Obekpa, S.; Ocama, P.; Oguche, S.; Omede, O.; Omuemu, C.; Opare-Sem, O.; Opio, C. K.; Örmeci, N.; Papatheodoridis, G.; Pasini, K.; Pimenov, N.; Poustchi, H.; Quang, T. D.; Qureshi, H.; Ramji, A.; Razavi-Shearer, K.; Redae, B.; Reesink, H. W.; Rios, C. Y.; Rjaskova, G.; Robbins, S.; Roberts, L. R.; Roberts, S. K.; Ryder, S. D.; Safadi, R.; Sagalova, O.; Salupere, R.; Sanai, F. M.; Sanchez-Avila, J. F.; Saraswat, V.; Sarrazin, C.; Schmelzer, J. D.; Schréter, I.; Scott, J.; Seguin-Devaux, C.; Shah, S. R.; Sharara, A. I.; Sharma, M.; Shiha, G. E.; Shin, T.; Sievert, W.; Sperl, J.; Stärkel, P.; Stedman, C.; Sypsa, V.; Tacke, F.; Tan, S. S.; Tanaka, J.; Tomasiewicz, K.; Urbanek, P.; van der Meer, A. J.; Van Vlierberghe, H.; Vella, S.; Vince, A.; Waheed, Y.; Waked, I.; Walsh, N.; Weis, N.; Wong, V. W.; Woodring, J.; Yaghi, C.; Yang, H.-I.; Yang, C.-L.; Yesmembetov, K.; Yosry, A.; Yuen, M.-F.; Yusuf, M. A. M.; Zeuzem, S.; Razavi, H. Global Prevalence, Treatment, and Prevention of Hepatitis B Virus Infection in 2016: A Modelling Study. *Lancet Gastroenterol. Hepatol.* **2018**, *3* (6), 383–403.

159. Schweitzer, A.; Horn, J.; Mikolajczyk, R. T.; Krause, G.; Ott, J. J. Estimations of Worldwide Prevalence of Chronic Hepatitis B Virus Infection: A Systematic Review of Data Published between 1965 and 2013. *Lancet* **2015**, *386* (10003), 1546–1555.

160. Spradling, P. R.; Hu, D. J.; McMahon, B. J. Epidemiology and Prevention. In *Viral Hepatitis: Fourth Edition*; 2013.

161. Beasley, R. P.; Lee, G. C. Y.; Roan, C. H.; Hwang, L. Y.; Lan, C. C.; Huang, F. Y.; Chen, C. L. Prevention of Perinatally Transmitted Hepatitis B Virus Infections with

Hepatitis B Immune Globulin and Hepatitis B Vaccine. *Obstet. Gynecol. Surv.* **1984**, *39* (6), 367–369.

162. Seeger C, Zoulim F, M. W. Hepadnaviruses. *Fields Virol. 6th ed.* **2013**, 2185–2221.
163. Messageot, F.; Salhi, S.; Eon, P.; Rossignol, J. M. Proteolytic Processing of the Hepatitis B Virus e Antigen Precursor: Cleavage at Two Furin Consensus Sequences. *J. Biol. Chem.* **2003**.
164. Al-Sadeq, D. W.; Taleb, S. A.; Zaied, R. E.; Fahad, S. M.; Smatti, M. K.; Rizeq, B. R.; Al Thani, A. A.; Yassine, H. M.; Nasrallah, G. K. Hepatitis B Virus Molecular Epidemiology, Host-Virus Interaction, Coinfection, and Laboratory Diagnosis in the MENA Region: An Update. *Pathogens*. 2019.
165. Stevan A. Gonzalez, M.D., M. S. Hepatitis B Virus. *Microbes*.
166. Steven, A. C.; Conway, J. F.; Cheng, N.; Watts, N. R.; Belnap, D. M.; Harris, A.; Stahl, S. J.; Wingfield, P. T. Structure, Assembly, and Antigenicity of Hepatitis B Virus Capsid Proteins. *Advances in Virus Research*. 2005.
167. Yan, H.; Zhong, G.; Xu, G.; He, W.; Jing, Z.; Gao, Z.; Huang, Y.; Qi, Y.; Peng, B.; Wang, H.; Fu, L.; Song, M.; Chen, P.; Gao, W.; Ren, B.; Sun, Y.; Cai, T.; Feng, X.; Sui, J.; Li, W. Sodium Taurocholate Cotransporting Polypeptide Is a Functional Receptor for Human Hepatitis B and D Virus. *Elife* **2012**, *2012* (1), 1–28.
168. Nassal, M. HBV CccDNA: Viral Persistence Reservoir and Key Obstacle for a Cure of Chronic Hepatitis B. *Gut* **2015**, *64* (12), 1972–1984.
169. Newbold, J. E.; Xin, H.; Tencza, M.; Sherman, G.; Dean, J.; Bowden, S.; Locarnini, S. The Covalently Closed Duplex Form of the Hepadnavirus Genome Exists in Situ as a Heterogeneous Population of Viral Minichromosomes. *J. Virol.* **1995**.
170. Bock, C. T.; Schwinn, S.; Locarnini, S.; Fyfe, J.; Manns, M. P.; Trautwein, C.; Zentgraf, H. Structural Organization of the Hepatitis B Virus Minichromosome. *J. Mol. Biol.* **2001**.
171. Donello, J. E.; Beeche, A. A.; Smith, G. J.; Lucero, G. R.; Hope, T. J. The Hepatitis B Virus Posttranscriptional Regulatory Element Is Composed of Two Subelements. *J. Virol.* **1996**.
172. Revill, P. A.; Chisari, F. V.; Block, J. M.; Dandri, M.; Gehring, A. J.; Guo, H.; Hu, J.; Kramvis, A.; Lampertico, P.; Janssen, H. L. A.; Levrero, M.; Li, W.; Liang, T. J.; Lim, S. G.; Lu, F.; Penicaud, M. C.; Tavis, J. E.; Thimme, R.; Arbuthnot, P.; Boonstra, A.; Chang, K. M.; Chen, P. J.; Glebe, D.; Guidotti, L. G.; Fellay, J.; Ferrari, C.; Jansen, L.; Lau, D. T. Y.; Lok, A. S.; Maini, M. K.; Mason, W.;

Matthews, G.; Paraskevis, D.; Petersen, J.; Rehermann, B.; Shin, E. C.; Thompson, A.; van Bömmel, F.; Wang, F. S.; Watashi, K.; Yang, H. C.; Yuan, Z.; Yuen, M. F.; Block, T.; Miller, V.; Protzer, U.; Bréchot, C.; Locarnini, S.; Peters, M. G.; Schinazi, R. F.; Zoulim, F. A Global Scientific Strategy to Cure Hepatitis B. *Lancet Gastroenterol. Hepatol.* **2019**, *4* (7), 545–558.

173. Lucifora, J.; Xia, Y.; Reisinger, F.; Zhang, K.; Stadler, D.; Cheng, X.; Sprinzl, M. F.; Koppensteiner, H.; Makowska, Z.; Volz, T.; Remouchamps, C.; Chou, W. M.; Thasler, W. E.; Hušer, N.; Durantel, D.; Liang, T. J.; Muñk, C.; Heim, M. H.; Browning, J. L.; Dejardin, E.; Dandri, M.; Schindler, M.; Heikenwalder, M.; Protzer, U. Specific and Nonhepatotoxic Degradation of Nuclear Hepatitis B Virus CccDNA. *Science (80-.).* **2014**.
174. Thimme, R.; Dandri, M. Dissecting the Divergent Effects of Interferon-Alpha on Immune Cells: Time to Rethink Combination Therapy in Chronic Hepatitis B? *Journal of Hepatology*. 2013.
175. Micco, L.; Peppa, D.; Loggi, E.; Schurich, A.; Jefferson, L.; Cursaro, C.; Panno, A. M.; Bernardi, M.; Brander, C.; Bihl, F.; Andreone, P.; Maini, M. K. Differential Boosting of Innate and Adaptive Antiviral Responses during Pegylated-Interferon-Alpha Therapy of Chronic Hepatitis B. *J. Hepatol.* **2013**.
176. Zoulim, F.; Lebossé, F.; Levrero, M. Current Treatments for Chronic Hepatitis B Virus Infections. *Current Opinion in Virology*. 2016.
177. Zeisel, M. B.; Lucifora, J.; Mason, W. S.; Sureau, C.; Beck, J.; Levrero, M.; Kann, M.; Knolle, P. A.; Benkirane, M.; Durantel, D.; Michel, M. L.; Autran, B.; Cosset, F. L.; Strick-Marchand, H.; Trépo, C.; Kao, J. H.; Carrat, F.; Lacombe, K.; Schinazi, R. F.; Barré-Sinoussi, F.; Delfraissy, J. F.; Zoulim, F. Towards an HBV Cure: State-of-the-Art and Unresolved Questions-Report of the ANRS Workshop on HBV Cure. *Gut* **2015**.
178. Urban, S.; Bartenschlager, R.; Kubitz, R.; Zoulim, F. Strategies to Inhibit Entry of HBV and HDV into Hepatocytes. *Gastroenterology*. 2014.
179. Bogomolov, P.; Alexandrov, A.; Voronkova, N.; Macievich, M.; Kokina, K.; Petrachenkova, M.; Lehr, T.; Lempp, F. A.; Wedemeyer, H.; Haag, M.; Schwab, M.; Haefeli, W. E.; Blank, A.; Urban, S. Treatment of Chronic Hepatitis D with the Entry Inhibitor Myrcludex B: First Results of a Phase Ib/IIa Study. *J. Hepatol.* **2016**.
180. Blank, A.; Markert, C.; Hohmann, N.; Carls, A.; Mikus, G.; Lehr, T.; Alexandrov, A.; Haag, M.; Schwab, M.; Urban, S.; Haefeli, W. E. First-in-Human Application of

the Novel Hepatitis B and Hepatitis D Virus Entry Inhibitor Myrcludex B. *J. Hepatol.* **2016**.

181. Donkers, J. M.; Appelman, M. D.; van de Graaf, S. F. J. Mechanistic Insights into the Inhibition of NTCP by Myrcludex B. *JHEP Reports* **2019**.
182. Nijampatnam, B.; Liotta, D. C. Recent Advances in the Development of HBV Capsid Assembly Modulators. *Curr. Opin. Chem. Biol.* **2019**, *50*, 73–79.
183. Qiu, Z.; Lin, X.; Zhang, W.; Zhou, M.; Guo, L.; Kocer, B.; Wu, G.; Zhang, Z.; Liu, H.; Shi, H.; Kou, B.; Hu, T.; Hu, Y.; Huang, M.; Yan, S. F.; Xu, Z.; Zhou, Z.; Qin, N.; Wang, Y. F.; Ren, S.; Qiu, H.; Zhang, Y.; Zhang, Y.; Wu, X.; Sun, K.; Zhong, S.; Xie, J.; Ottaviani, G.; Zhou, Y.; Zhu, L.; Tian, X.; Shi, L.; Shen, F.; Mao, Y.; Zhou, X.; Gao, L.; Young, J. A. T.; Wu, J. Z.; Yang, G.; Mayweg, A. V.; Shen, H. C.; Tang, G.; Zhu, W. Discovery and Pre-Clinical Characterization of Third-Generation 4-H Heteroaryldihydropyrimidine (HAP) Analogues as Hepatitis B Virus (HBV) Capsid Inhibitors. *J. Med. Chem.* **2017**, *60* (8), 3352–3371.
184. Campagna, M. R.; Liu, F.; Mao, R.; Mills, C.; Cai, D.; Guo, F.; Zhao, X.; Ye, H.; Cuconati, A.; Guo, H.; Chang, J.; Xu, X.; Block, T. M.; Guo, J.-T. Sulfamoylbenzamide Derivatives Inhibit the Assembly of Hepatitis B Virus Nucleocapsids. *J. Virol.* **2013**, *87* (12), 6931–6942.
185. Cole, A. G. Modulators of HBV Capsid Assembly as an Approach to Treating Hepatitis B Virus Infection. *Curr. Opin. Pharmacol.* **2016**, *30*, 131–137.
186. Levrero, M.; Testoni, B.; Zoulim, F. HBV Cure: Why, How, When? *Curr. Opin. Virol.* **2016**, *18*, 135–143.
187. McCaffrey, A. P.; Nakai, H.; Pandey, K.; Huang, Z.; Salazar, F. H.; Xu, H.; Wieland, S. F.; Marion, P. L.; Kay, M. A. Inhibition of Hepatitis B Virus in Mice by RNA Interference. *Nat. Biotechnol.* **2003**.
188. Chen, Y.; Cheng, G.; Mahato, R. I. RNAi for Treating Hepatitis B Viral Infection. *Pharmaceutical Research*. 2008.
189. Wooddell, C. I.; Rozema, D. B.; Hossbach, M.; John, M.; Hamilton, H. L.; Chu, Q.; Hegge, J. O.; Klein, J. J.; Wakefield, D. H.; Oropeza, C. E.; Deckert, J.; Roehl, I.; Jahn-Hofmann, K.; Hadwiger, P.; Vornlocher, H. P.; McLachlan, A.; Lewis, D. L. Hepatocyte-Targeted RNAi Therapeutics for the Treatment of Chronic Hepatitis B Virus Infection. In *Molecular Therapy*; 2013.
190. Wooddell, C. I.; Yuen, M. F.; Chan, H. L. Y.; Gish, R. G.; Locarnini, S. A.; Chavez, D.; Ferrari, C.; Given, B. D.; Hamilton, J.; Kanner, S. B.; Lai, C. L.; Lau, J. Y. N.;

Schluep, T.; Xu, Z.; Lanford, R. E.; Lewis, D. L. Rnai-Based Treatment of Chronically Infected Patients and Chimpanzees Reveals That Integrated Hepatitis b Virus DNA Is a Source of Hbsag. *Sci. Transl. Med.* **2017**.

191. M.A., M.; M., B.; A., V. REP 9AC': A Second Generation HBsAg Release Inhibitor with Improved Tolerability. *Hepatology* **2012**.
192. M.A., M.; M., B.; A., V. REP 9AC: A Potent HBsAg Release Inhibitor That Can Rapidly Restore Immunocompetence in Patients with Chronic Hepatitis B. *Hepatology* **2010**.
193. M., A.-M.; M., B.; A., V. REP 9AC: A Potent HBsAg Release Inhibitor That Rapidly Elicits Sustained Virologic Responses in Patients with Chronic Hepatitis B. *Hepatol. Int.* **2011**.
194. Guzik, K.; Zak, K. M.; Grudnik, P.; Magiera, K.; Musielak, B.; Törner, R.; Skalniak, L.; Dömling, A.; Dubin, G.; Holak, T. A. Small-Molecule Inhibitors of the Programmed Cell Death-1/Programmed Death-Ligand 1 (PD-1/PD-L1) Interaction via Transiently Induced Protein States and Dimerization of PD-L1. *J. Med. Chem.* **2017**.
195. Gane, E.; Verdon, D. J.; Brooks, A. E.; Gaggar, A.; Nguyen, A. H.; Subramanian, G. M.; Schwabe, C.; Dunbar, P. R. Anti-PD-1 Blockade with Nivolumab with and without Therapeutic Vaccination for Virally Suppressed Chronic Hepatitis B: A Pilot Study. *J. Hepatol.* **2019**.
196. Kothapalli, A.; Khattak, M. A. Safety and Efficacy of Anti-PD-1 Therapy for Metastatic Melanoma and Non-Small-Cell Lung Cancer in Patients with Viral Hepatitis: A Case Series. *Melanoma Res.* **2018**.
197. Pu, D.; Yin, L.; Zhou, Y.; Li, W.; Huang, L.; Cai, L.; Zhou, Q. Safety and Efficacy of Immune Checkpoint Inhibitors in Patients with HBV/HCV Infection and Advanced-Stage Cancer: A Systematic Review. *Medicine (Baltimore).* **2020**.
198. Krebs, K.; Böttinger, N.; Huang, L. R.; Chmielewski, M.; Arzberger, S.; Gasteiger, G.; Jäger, C.; Schmitt, E.; Bohne, F.; Aichler, M.; Uckert, W.; Abken, H.; Heikenwalder, M.; Knolle, P.; Protzer, U. T Cells Expressing a Chimeric Antigen Receptor That Binds Hepatitis B Virus Envelope Proteins Control Virus Replication in Mice. *Gastroenterology* **2013**.
199. Kruse, R. L.; Shum, T.; Tashiro, H.; Barzi, M.; Yi, Z.; Whitten-Bauer, C.; Legras, X.; Bissig-Choisat, B.; Garaigorta, U.; Gottschalk, S.; Bissig, K. D. HBsAg-Redirected T Cells Exhibit Antiviral Activity in HBV-Infected Human Liver

Chimeric Mice. *Cytotherapy* **2018**.

200. Boni, C.; Barili, V.; Acerbi, G.; Rossi, M.; Vecchi, A.; Laccabue, D.; Penna, A.; Missale, G.; Ferrari, C.; Fisicaro, P. HBV Immune-Therapy: From Molecular Mechanisms to Clinical Applications. *International Journal of Molecular Sciences*. 2019.
201. Wang, W.; Zhou, X.; Bian, Y.; Wang, S.; Chai, Q.; Guo, Z.; Wang, Z.; Zhu, P.; Peng, H.; Yan, X.; Li, W.; Fu, Y. X.; Zhu, M. Dual-Targeting Nanoparticle Vaccine Elicits a Therapeutic Antibody Response against Chronic Hepatitis B. *Nat. Nanotechnol.* **2020**.
202. Lin, C. L.; Yang, H. C.; Kao, J. H. Hepatitis B Virus: New Therapeutic Perspectives. *Liver International*. 2016.
203. Jones, M.; Cunningham, M. E.; Wing, P.; DeSilva, S.; Challa, R.; Sheri, A.; Padmanabhan, S.; Iyer, R. P.; Korba, B. E.; Afdhal, N.; Foster, G. R. SB 9200, a Novel Agonist of Innate Immunity, Shows Potent Antiviral Activity against Resistant HCV Variants. *J. Med. Virol.* **2017**.
204. Korolowicz, K. E.; Iyer, R. P.; Czerwinski, S.; Suresh, M.; Yang, J.; Padmanabhan, S.; Sheri, A.; Pandey, R. K.; Skell, J.; Marquis, J. K.; Kallakury, B. V.; Tucker, R. D.; Menne, S. Antiviral Efficacy and Host Innate Immunity Associated with SB 9200 Treatment in the Woodchuck Model of Chronic Hepatitis B. *PLoS One* **2016**.
205. Sato, S.; Li, K.; Kameyama, T.; Hayashi, T.; Ishida, Y.; Murakami, S.; Watanabe, T.; Iijima, S.; Sakurai, Y.; Watashi, K.; Tsutsumi, S.; Sato, Y.; Akita, H.; Wakita, T.; Rice, C. M.; Harashima, H.; Kohara, M.; Tanaka, Y.; Takaoka, A. The RNA Sensor RIG-I Dually Functions as an Innate Sensor and Direct Antiviral Factor for Hepatitis B Virus. *Immunity* **2015**.
206. Lanford, R. E.; Guerra, B.; Chavez, D.; Giavedoni, L.; Hodara, V. L.; Brasky, K. M.; Fosdick, A.; Frey, C. R.; Zheng, J.; Wolfgang, G.; Halcomb, R. L.; Tumas, D. B. GS-9620, an Oral Agonist of Toll-like Receptor-7, Induces Prolonged Suppression of Hepatitis B Virus in Chronically Infected Chimpanzees. *Gastroenterology* **2013**.
207. Boni, C.; Vecchi, A.; Rossi, M.; Laccabue, D.; Giuberti, T.; Alfieri, A.; Lampertico, P.; Grossi, G.; Facchetti, F.; Brunetto, M. R.; Coco, B.; Cavallone, D.; Mangia, A.; Santoro, R.; Piazzolla, V.; Lau, A.; Gaggar, A.; Subramanian, G. M.; Ferrari, C. TLR7 Agonist Increases Responses of Hepatitis B Virus–Specific T Cells and Natural Killer Cells in Patients With Chronic Hepatitis B Treated With

Nucleos(T)Ide Analogues. *Gastroenterology* **2018**.

208. Ebert, G.; Allison, C.; Preston, S.; Cooney, J.; Toe, J. G.; Stutz, M. D.; Ojaimi, S.; Baschuk, N.; Nachbur, U.; Torresi, J.; Silke, J.; Begley, C. G.; Pellegrini, M. Eliminating Hepatitis B by Antagonizing Cellular Inhibitors of Apoptosis. *Proc. Natl. Acad. Sci. U. S. A.* **2015**.
209. Pan, W.; Luo, Q.; Yan, X.; Yuan, L.; Yi, H.; Zhang, L.; Li, B.; Zhang, Y.; Sun, J.; Qiu, M. Z.; Yang, D. J. A Novel SMAC Mimetic APG-1387 Exhibits Dual Antitumor Effect on HBV-Positive Hepatocellular Carcinoma with High Expression of CIAP2 by Inducing Apoptosis and Enhancing Innate Anti-Tumor Immunity. *Biochem. Pharmacol.* **2018**.
210. Ebert, G.; Allison, C.; Preston, S.; Cooney, J.; Silke, J.; Pellegrini, M. A Potent Clinical Stage Smac Mimetic Antagonizes Cellular Inhibitors of Apoptosis and Promotes Hepatitis B Virus Clearance in Vivo. *J. Hepatol.* **2017**.
211. Lanini, S.; Ustianowski, A.; Pisapia, R.; Zumla, A.; Ippolito, G. Viral Hepatitis: Etiology, Epidemiology, Transmission, Diagnostics, Treatment, and Prevention. *Infectious Disease Clinics of North America*. 2019.
212. Martinez, M. G.; Villeret, F.; Testoni, B.; Zoulim, F. Can We Cure Hepatitis B Virus with Novel Direct-Acting Antivirals? *Liver International*. 2020.
213. Baumert, T. F.; Verrier, E. R.; Nassal, M.; Chung, R. T.; Zeisel, M. B. Host-Targeting Agents for Treatment of Hepatitis B Virus Infection. *Current Opinion in Virology*. 2015.
214. Ji, X.; Li, Z. Medicinal Chemistry Strategies toward Host Targeting Antiviral Agents. *Medicinal Research Reviews*. 2020.
215. Pouillard, J. Une Découverte Oubliée : La Thèse de Médecine Du Docteur Ernest Duchesne. *Hist. Sci. Med.* **2002**, *XXXVI* (1), 13.

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VII.1 GENERAL CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

Overall, this PhD thesis encompasses methodologies and tools -whether experimental or computational - aiming at supporting the medicinal chemist in finding new drugs efficiently, in particular against hepatitis B virus.

In Chapter II, three flow processes were developed that were effectively used to accelerate the generation of relevant building blocks for a focused library of HBV inhibitors. Some of the most used reactions in drug discovery were successfully adapted in continuous flow. The batch and flow conditions are respectively depicted in Table VII.1, Table VII.2, Table VII.3 and Table VII.4, highlighting how flow processing:

- Allowed selectivity issues to be alleviated compared to batch.
- Yielded higher compared to batch.
- Reduced reaction time compared to batch.
- Simplified the downstream processing.
- Granted a high reaction diversity.

Table VII.1.

Table VII.2.

Table VII.3.

Table VII.4.

In this specific project, the next step was necessarily to combine such process into an automatized platform to accomodate several transformations in a fully continuous manner (Scheme VII.1). A prototype for such system was imagined to be focused on diversification of the indole and the terminal amine moiety. The complete system must be monitored by a computer interface and coding will also intervene in such development. It is a large-scale endeavour that encompasses several disciplines *e.g.* continuous flow, robotics, automatization, online analysis, etc.

Scheme VII.1. Concept of automated platform for the fast production of oxalamides analogues.

Yet, those processes already have undeniable advantages as single operations. Practically, even when setting up the system can take several hours, the gain in time remained significant. With an average productivity of 7 g per day using a lab scale system, all these processes can be easily reused in a fully reproducible way at later stages of drug development to synthesize gram to kilogram scale of one or several lead to supply preclinical or clinical studies. This application confirms flow chemistry a versatile tool that supports synthesis at several steps of the drug development process.

In Chapter III, batch and flow processes were combined judiciously combined to foster chemical diversity and efficiently lead to a library of oxalyl-amide scaffolds. A total of five synthetic routes were optimized and adapted to tolerate a variety of substitutions in a smart and convenient manner. Analysis of structure activity-relationship supported the rationalization of activity trends. The potency of the initial hit **1** (EC_{50} = 2.0 µM) was improved by 40-fold thanks to an acute understanding of SAR, leading to **18j** with an EC_{50} of 0.046 µM (Scheme VII.2). Finally, confirmation of the mode of action *in vitro* of few analogues series was informed, placing the series in the class of SBA-type CAMs (CAM class I) with their associated properties *in vitro*.

Scheme VII.2. New hit **18j** identified from starting from hit **1**. SAR mapping.

From there, next step has been to take advantage of this newly acquired knowledge to feed the development of an *in silico* approach that would allow to further optimize this compound series. This work was conducted in the following chapter.

Chapter IV reports the validation of docking methods and the development of new in silico tools to drive lead optimization synthetic plans. The crystal structure obtained for one potent oxalamides analogues (Figure VII.1) continued the rationalization of the observed SAR described in Chapter III. The crystallized model could also be used as a starting point for docking studies and structure-based pharmacophore modelling.

Figure VII.1. Binding pocket at the interface of two HBV Cp149 protein chain (chain E in yellow and chain B in green). The hydrogen bonds are shown as orange dashed lines.

A pharmacophore-based workflow was developed and validated to use as a yes/no assessment tool for new virtually designed oxalamides compounds. The study focused on two points of diversity. A virtual library of 1037 molecules were virtually synthesized according to existing synthetic pathways and tested against the validated pharmacophore model. The development of these tools supported the progress of the hit-to-lead efforts and rewarded very promising results (Figure VII.2). Notably, compound I**V4a** was found to be 4-fold more potent than the initial lead (**18j**) (Figure VII.2).

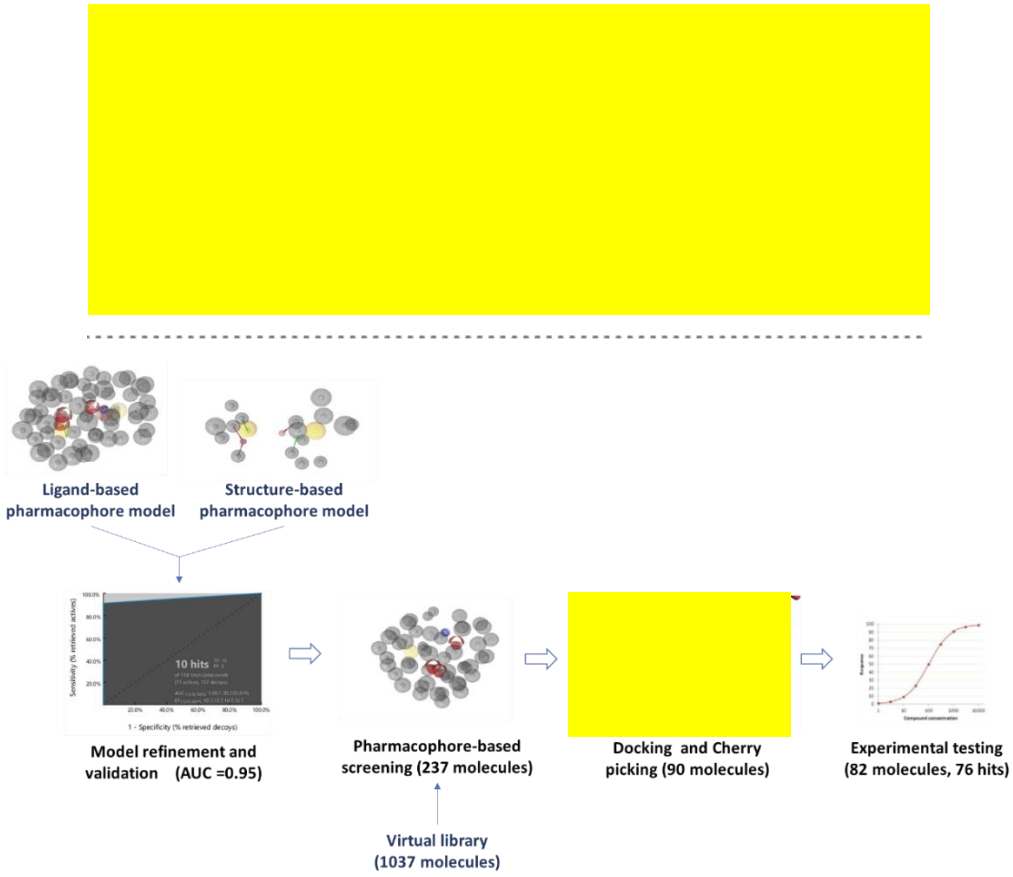


Figure VII.2. Overall results from HVLS workflow.

While a number of molecules are still pending evaluation in cellular assay, the best hits were taken to the first stage of pre-clinical studies: solubility, hERG toxicity and microsomal stability are assessed. From this point onward, QSAR modelling could be used to predict various ADMET properties and inform further lead optimization.

In total, above 150 new compounds from this chemotype were synthesized with competing bioactivity. While competing series (Figure VII.3, GLPs) also display excellent bioactivity values, they may differ in preclinical stages. Initial results in microsomal stability assay and solubility assay for our compound series were already very promising.

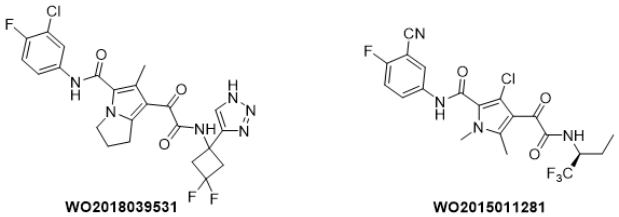
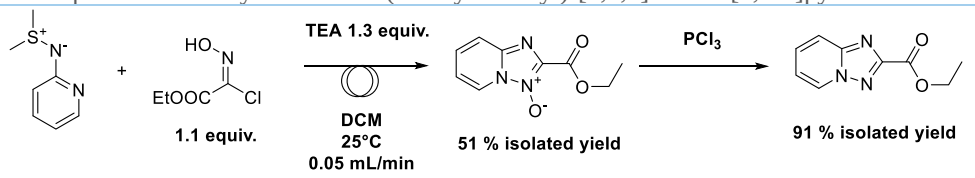


Figure VII.3. Glyoxamides (GLPs) from competitors.

In Chapter V, a continuous flow process was applied for the validation of a novel synthetic methodology of a hardly accessible heterobicycle in batch (Table VII.5). Investigations in both batch and flow also lead to the elucidation of a complex reactional mechanism via DFT calculations.

Table VII.5. Optimization of synthesis of 2-(ethoxycarbonyl)-[1,2,4]triazolo[1,5-*a*]pyridine 3-oxide **.**



	Batch	Flow
Reaction time	4 h	Residence time = 5-20 min
Temperature	-20°C	25°C
Yield	25-31%	48-53%

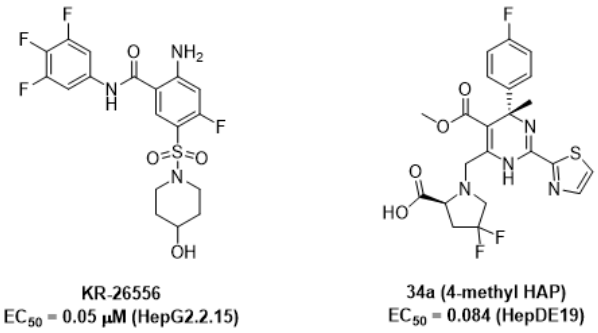
The reaction rate was mildly increased in continuous flow (ca. 25-31% in batch *vs* 48-53% in flow) and reaction time (Table VII.5). The use of continuous flow proved very valuable in handling unstable intermediates such as nitrile oxide. Once set up, the flow system provided an uncomparable ease of handling as well as highly reproducible conditions. More than fifty reactions in total were conducted in flow during this study, with a rate of up to 12 “reaction-sampling-analysis” cycle per day. In this regard, the integration of an online process analytical tool (PAT) could have been even beneficial to reproducibility. The results obtained from the computational investigation revealed the occurrence of kinetically highly competitive intermediates that explained the selectivity issues that were faced and hardly overcame. To go further, a thorough kinetic study would allow to quantitatively assess the reaction rate constant and reaction orders relative to the desired product and the side products formation. In this area, flow chemistry can again reveal to be extremely useful with numerous reports praising the use of continuous-flow reactors for the rapid evolution and validation of kinetic motifs.

Obtaining these additional reaction parameters can set the basis of an accurate computational modelization of the reaction system with opportunities of putative optimization to guide additional experimentation. An exciting opportunity would be to tackle this challenge from a pure process chemistry point of view and elaborate a custom

design reactor that could give the desired product in sufficient amount. Transcending the limits of competitive kinetics thanks to reactor design has been done successfully before thanks to the collaborative efforts of chemists, process chemists, flow modelling experts and 3D printing capacity.

Finally, the structure-based approach developed in chapter VI focuses on the identification of new CAMs chemotypes belonging specifically to class II, *i.e* new molecules possessing a HAP-type MOA.

The choice to use two structurally dissimilar representative molecules that can trigger a class II MOA offers to cover a larger conformational space and promises to increase the diversity of novel chemotypes that could be retrieved. Ideally, to obtain new potentially active compound that could combine the most relevant pharmacophore features of both reference molecules (Scheme VII.5).



Scheme VII.5. Reference molecules used for the development of the MD-driven study. Both compounds belonged to class II CAMs.

A molecular dynamics approach was particularly interesting to use in that case for two main reasons:

1) it is known that crystallized protein-ligand complex (Cp149_Y132A crystallization system) represents only a subset of WT dimer formation;

2) independent report highlights that the dynamics of Cp early assembly intermediates (dimers, tetramers or hexamers) are a key factor in the subsequent capsid assembly process.

Fifty nanoseconds molecular dynamics simulation were conducted on ligand-bound Cp tetramers providing multiple snapshots of the protein-ligand state at different point in time, thus rendering the dynamic of the Cp tetramer upon binding of either **HAP-34a** or **KR-26556**.

The MD snapshots were clustered thanks to CHA filtering tools to identify representative pharmacophore models *i.e* pharmacophore models with common binding interactions and protein-ligand conformations.

A set of MD pharmacophores from **HAP-34a** and **KR-26556** were selected to run a 65 million molecules virtual screening that retrieved a total of 115 molecules. The docking study that followed helped assessing the quality of the hits and the plausibility of interacting with the binding pocket. A final evaluation of druglikeness by calculating relevant physico-chemical properties helped finalize a selection of 30 molecules (Figure VII.4).

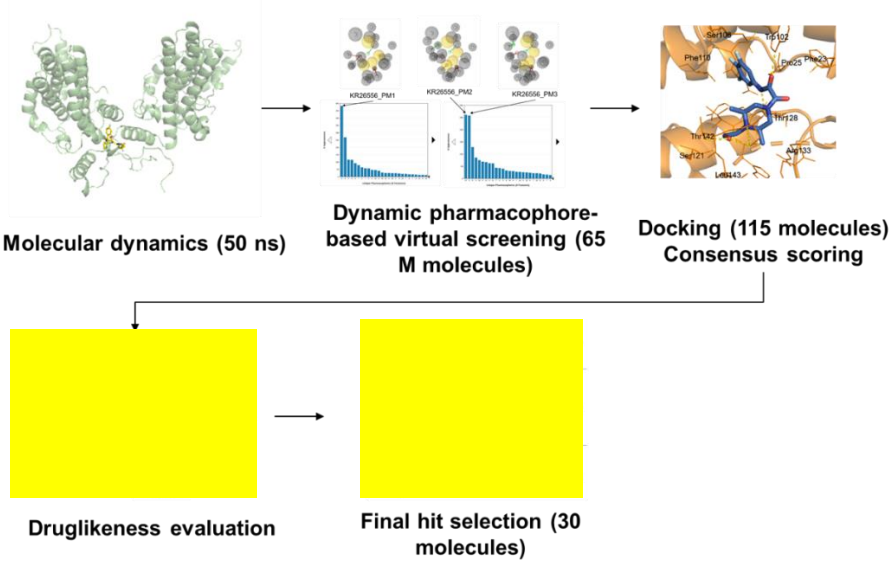


Figure VII.4. Structure-based workflow for discovering potent inhibitors of HBV Cp.

Unequivocally, the limitation of this work is the lack of experimental validation. Yet, the final hit list potentially contains active compounds with a likelihood to belong to class II CAMs. The selected molecules were also assessed by their druglikeness or leadlikeness making them appropriate for further optimization. As such, it can be used for researchers willing to develop novel HBV CAMs.

This thesis work raises an argument in favor of the use of flow chemistry in drug discovery:

- In medicinal chemistry programs as an enabling synthetic methodology for fast generation of relevant building blocks in a focused library.
- In fundamental research as an investigative tool for understudied chemical reactions.

- In methodology development for the synthesis of species difficult to access.

In addition, this thesis work aims to demonstrate the potential of pharmacophore modelling in *in silico* predictions:

- As a pre-docking filter to mitigate virtual libraries of new potentially active compounds in lead optimization.
- As an analysis tool to characterize protein-ligand dynamics.
- As a precise screening procedure to identify new potential molecules of interest driven by molecular dynamics.

VII.2 DRAWING A BIGGER PICTURE

Intensifying drug discovery is a continuous challenge that will require the cooperation of numerous institutions, academic, industrial and governmental. Moreover, scientists must be willing to shift their perspective and be open to novel technologies and to give on their old ways. In other words, it also requires a consensual commitment of individual scientists to stay in touch with increasing challenges. Especially, in the era of the 21st century that has seen the emergence of numerous hurdles on societal and environmental level.
Growing awareness of the imminence of an ecological threat is also a part if these challenges and is essential to stimulate the shift toward a more circular economy. Flow chemistry is just one of a wide variety of novel technology that contributes to a collective effort towards sustainability. Computational modelling rationalises drug discovery and prioritizes the synthetic efforts, thus limiting the waste of resources.

As such, the VIRO-FLOW project follows in line by promoting the use of innovative technologies and tools – whether experimental or computational – to bring solution to the R&D productivity gap. The present PhD dissertation that resulted from this programme intends to be an evidence-based testimonial of the advantages of continuous flow for lab bench medicinal chemists, especially as it goes further into demonstrating real-life applications of drug development in the case of HBV.

In parallel, this PhD thesis is also the result of a program that required to cross the frontiers of scientific disciplines, countries, type of institutions. Indeed, European Industrial Doctorates as part of Marie Skłodowska-Curie Innovative Training Networks effectively

blends international collaboration, sustainability, and new technologies in an applied environment. Thus, this framework encompasses principles that are at the core of the quest towards a sustainable drug development model. This type of initiative must keep being supported and encouraged.

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