Fragment-based Discovery Aiming at a Novel Modulation of Malate Dehydrogenase and Beyond

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Chapter 1

General Introduction and Scope of the Thesis

A perspective review, carried out in 2002, estimated that the percentage of the human genome associated to the "druggable" proteins is approximately between 10% and 14% corresponding roughly to 500 – 1500 members distributed in six major classes of proteins [1] (**Figure 1**).

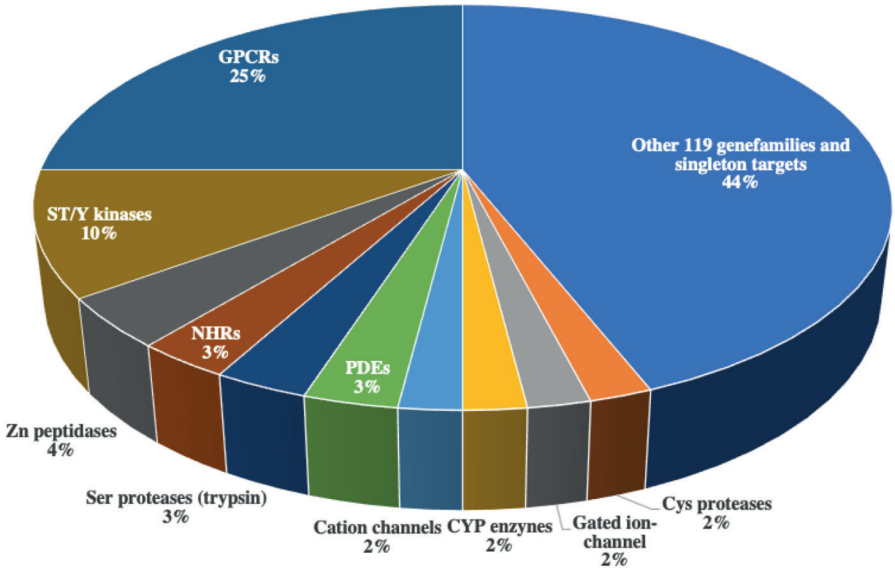

Figure 1 Pie chart of the most represented protein families within the druggable targets. The picture is adapted from Hopkins AL & Groom CR, The druggable genome. *Nat. Rev. Drug. Disc.* **1**, 727-30 (2002).

Later on, a subsequent update covering the two-year period 2010 – 2012 identified 364 successful targets, 286 clinical trial targets and 1331 research targets [2]. Five years later, the number of proteins that bind small molecules was 550 – precisely, 670 including biologicals [3]. This growing trend follows a joint interest of both academies and pharmaceutical industries to explore targets typically considered not druggable, such as protein-protein interactions (PPI). In physiological conditions, PPIs are involved in various intra- and inter-cellular networks related to maintaining cell homeostasis and signal transduction [4]. The importance of PPIS is exemplified by the fact that the majority of viral and bacterial diseases require contacts between different protein surfaces in order to initiate the infection. This aspect is also particularly relevant in many promising targets for anticancer therapy, like the mixed lineage leukemia 1 and its activator WD Repeat Domain 5, bromodomain-containing

protein 4 /Histone H4, , BCL2 Associated Transcription Factor 1/ L3MBTL Histone Methyl-Lysine Binding Protein 3, β-Catenin/ T-cell factor, and 14-3-3 interactions [5].

However, targeting PPIs is challenging, because they consist of flat molecular surfaces with few cavities where molecules can bind. Moreover, the binding epitopes are distant from each other and they are distributed along the interface. A tight network of weak interactions is concentrated on these areas and it contributes to the total binding affinity. On the other hand, druggable pockets are shallow, deep and mostly hydrophobic, implying that a classic small-molecule approach cannot be applied for PPIs [6]. Current strategies to target PPIs include macrocycles from chemical synthesis or natural sources [7], covalent inhibitors and stapled peptides [8].

Within this context the reader is firstly introduced to oligomeric surfaces as a specific PPI class. Next, the focus moves to a benchmark of a novel algorithm for conformational analysis of macrocycles. The job has been carried out with Moloc, one of the first open access molecular modeling packages. Finally, the thesis ends by reporting an interesting case of inhibition of Protein Tyrosine Phosphatase 1B (PTP1B) followed by a computational docking of gliptins in the catalytic pockets of the SARS-Cov-2 main and papain-like proteases.

OLIGOMERIC INTERFACES

Shape complementarity, electrostatics, flexibility, hydration and allostery represents the most conventional approaches to modulate on-target selectivity [9]. However, the discovery of allosteric pockets remains challenging due to their cryptic nature and to the co-existence of fluctuant protein conformations in equilibrium, implying thus a tailored strategy to disclose them [10].

Chapter 2 presents the crystal structure of 4-(3,4-difluorophenyl) thiazol-2-amine bound to malate dehydrogenase of *Plasmodium Falciparum* (PfMDH). This class of oxidoreductases is highly represented in human cancer and infectious diseases with a strongly conserved binding site for dicarboxylic acids, like malate and oxaloacetate. So far, the strategy of modulation of this class of enzyme is still based on dual target inhibitors or structural analogues of the substrate/cofactor. However, the high potency of these compounds is followed by a lack of selectivity among homologues. Within this context we have chosen

*Pf*MDH as a well-characterized model protein whose catalytic activity can be modulated at the oligomeric interface. Moreover, the enzyme is involved in the aspartate metabolism of *Plasmodium Falciparum*, an essential metabolite for the proliferation of the parasite. This biochemical pathway consists of two steps of a transamination into glutamate and oxaloacetate and next oxidation into malate and it is headed by aspartate aminotransferase (PfAspAt) and malate dehydrogenase (*Pf*MDH) [14]. The crystal structures of *Pf*AspAt [15] and *Pf*MDH [16] disclose a homo-dimeric and homo-tetrameric assembly in their quaternary structures with a low degree of aminoacidic conservation at the oligomeric interface, making them a potential candidate for drug target validation. While the main focus remains the antimalaria drug discovery, a significant part is dedicated to the discovery and validation of 4-(3,4-difluorophenyl) thiazol-2-amine through a fragment-based high throughput screening. Despite a weak K_D (equilibrium dissociation constant), the kinetics experiments of its scaffold derivative, namely 4-phenylthiazol-2-amine, show that oxidation of malate can be allosterically modulated. Inhibition of its activity is followed by a thermal destabilization of the tetrameric assembly in presence of the compound. Such alternative modality of inhibition might be eventually extended to MDHs of other organisms involved in relevant diseases. Actually, the primary advantage of targeting oligomeric surface relies on the fact that 60% of the proteins deposited in the Protein Data Bank (PDB, [17]) are oligomers with dimers being the majority [18], meaning that the structural data are already available. Oligomerization leads to the formation of a bio-functional complex regulating the gene transcription [19], ion influx/efflux across the membrane, cell-to-cell adhesion [20], transduction events in cellular regulation [21] as well as in the enzyme activity [22]. Finally, the evidence presented in this chapter suggests that future chemical optimization of 4-(3,4-difluorophenyl) thiazol-2-amine should aim to expand towards from the meta position of the benzene ring since it's the moiety of the molecule with higher scores in the context of the cooperativity binding network of molecular interaction. Based on this result, new derivatives should contain functional groups creating new interface, hence interfering with the correct formation of the tetramer.

MACROCYCLES

Drug discovery is a risky, long-term project. It has been estimated that bringing a new chemical entity to the market ranges between several billion to tens of billions of dollars with a time frame of 3 – 20 years and an average cost of $2.7 billion, according to a research

survey among 106 new drugs developed by ten pharmaceutical companies [23]. Additionally, the risk of rejection increases during post-marketing surveillance as the data collected come from a wider community of consumers.

Computer-Aided Drug Design (CADD) technologies serve the scientific community with the aim of predicting *in silico* some of the most relevant causes of drugs failure. Among these, the most relevant organ-related toxicities there is the cardiotoxicity caused by the binding to hERG potassium channel, leading to arrhythmia and, finally, to death [24]. Several methods allow the prediction of hERG toxicity. For instance, a well-accepted pharmacophore model consists in a basic nitrogen center flanked by aromatic or hydrophobic groups [25]. Other methodologies are 3D and 2D Quantitative Structure Activity Relationships (QSAR) [26-28] and Machine Learning methods (ML) [29,30]. Nevertheless, all these computational approaches require a dataset of conformations with structural diversity. In that respect, many algorithms have been developed along the years based on different principles, for instance distance geometry [31] with experimental torsional-angle constrains [32], stochastic sampling [33,34], inverse kinematics [35] or molecular dynamics in combination with low- or normal modes approaches [36].

The benchmark of Moloc is presented in **Chapter 3**. Moloc is one of the first molecular modeling package which has been developed since 1986 and it still counties to be, in close collaboration with drug designers and crystallographers from the Roche Biostructural community. The accuracy, the diversity, the speed and the exhaustiveness of 207 macrocycles, an emerging class of molecules representing a mature area of medicinal chemistry, are systematically assessed. Moloc was identified as having the highest sampling efficiency and exhaustiveness without producing thousands of conformations, random ring splitting into two half-loops and possibility to interactively produce globular or flat conformations with diversity similar to Prime, MacroModel and Molecular Dynamics.

COVALENT INHIBITORS

Binding to the pharmacological target represents one of the several main features required for a molecule in order to be useful as a drug. Other appropriate properties are necessary, though. Multiparameter optimization aims to get a reasonable balance between the pharmacokinetics (absorption, distribution, metabolism and excretion) and the

pharmacodynamics (potency, efficacy and on-target selectivity). Finding a compromise among all of these parameters is often conflicting and it represents a bottleneck during an early-stage drug discovery. Among the pharmacodynamic properties, the binding affinity represents a generally well accepted parameter for ranking the hits during the initial high throughput screening and it is quantified by the K_D. In this phase, thousands of molecules are tested in order to converge on only a small fraction of molecules only to be subsequentially optimized. To date, there are several biophysical techniques for measuring K_D, for instance isothermal titration calorimetry, microscale thermophoresis, titration with nuclear magnetic resonance, surface plasmon resonance and fluorescence polarization with related advantages and disadvantages according to which aspect of the protein/ligand interaction one wants to assess [37,38]. Considering the implication of kinetics in the ligand mode of action, this parameter can also provide also further insight into the association and dissociation rates.

With the growing interest of fragment-based drug design, the attention of the scientific community is focused on the detection limit of weak binders by modern methodologies [39]. In contrast, the covalent binders fix this problem in a glance by conferring unlimited or very high affinity, even for a small compound, while leaving a main issue for selectivity/off-target labeling [40]. So far, the FDA have approved 40 drugs containing a covalent warhead [41]. Most important, some of them have become blockbusters in the pharmaceutical market, for example the beta lactams antibiotics, aspirin and omeprazole (**Figure 2**). Within this context an efficient synthetic methodology for access to acrylamides using MCR will be described. Finally docking studies of covalent drugs is presented.

Chapter 4 will focus primarily on a rapid access to complex acrylamides across several scales of production. These molecules are important scaffolds in pharmaceutical chemistry and many drugs for clinical use carry them in order to form a covalent bond with the side chains of aminoacids like cysteine, serine, threonine and histidine. In the second part of the chapter, the biological activity of a small library of about 90 derivatives is presented and the covalent inhibition of human protein tyrosine phosphatase 1B (PTP1B) will be explained.

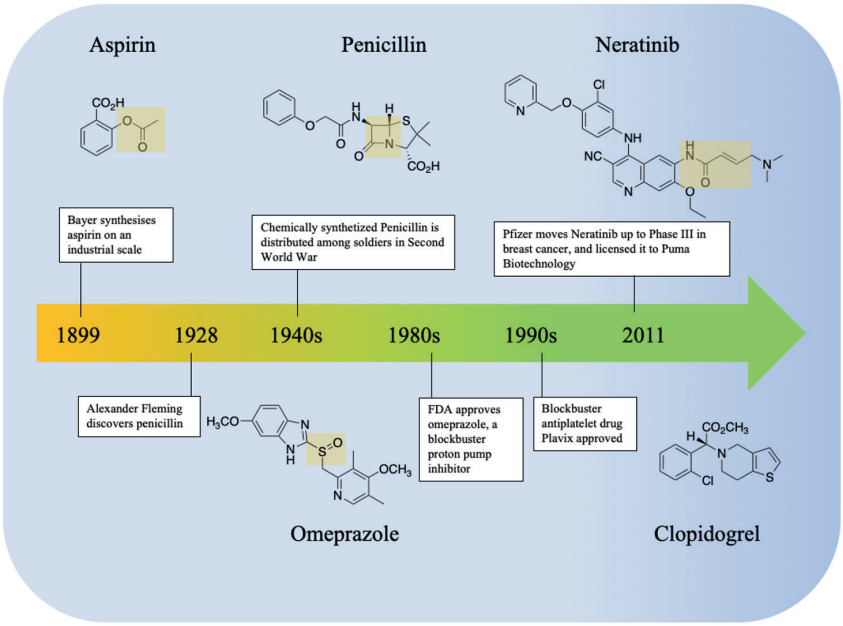

Figure 2 Many medications that have become blockbuster in the last century act as covalent inhibitors. The picture is readapted from "The Resurgence of Covalent Drugs" Singh, Juswinder, Russell C. Petter, Thomas A. Baillie, e Adrian Whitty. *Nature Reviews Drug* Discovery 10, n. 4 (April 2011): 307–17.

The medical significance of this protein is related to its overexpression and uncontrolled high activity in adipose tissues and skeletal muscles [42] which leads to obesity and type 2 diabetes mellitus. So far, PTP1B covalent inhibitors are phosphate mimetic carrying sulfate, nitrate, arsenate or selelinate which cannot penetrate the lipidic cellular membrane due to the negative charge of the inhibitors [43]. Trypsin digestion followed by mass spectrometry indicates that the acrylamide compounds do bind covalently to the solvent exposed cysteines with no specificity among and time-dependent inhibition in the colorimetric assay.

Secondly, **Chapter 5** presents a study case of nitrile containing gliptins as a potential anti-COVID19 drug for the inhibition of coronavirus main protease (3CLpro) and papain-like protease (PLpro). Based on covalent computational docking, a strong relationship between the coronavirus 3C-like proteases inhibition and nitrile-based broad-spectrum was studied in 2011 with four co-crystal structures peptidomimetic structures bound to 3CLpro and its effectiveness against 3CL-pro from six different strains of coronavirus was proved [44]. The drug repurposing consists in the use of already approved drugs for original medical

investigations. This strategy is undoubtedly favored by the low risk failure due to the fairly complete understanding of the pharmacokinetic and pharmacodynamic properties in preclinical and clinical models [45]. The orally bioavailable antidiabetic drug class of gliptins is safe and it has been clinically available (and used by millions of patients) since 2006 [46].

Finally, **Chapter 6** is a summary of the thesis. A short overview of possible improvements of the algorithm for macrocycle conformational sampling will be discussed. In the end, future perspectives to improve the 4-phenylthiazol-2-amine scaffold will follow.

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