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Structure-based drug discovery aiming at human-diseases related protein targets

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**Structure-Based Drug Discovery
Aiming at Human-Diseases Related
Protein Targets**

Kai Gao

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Structure-Based Drug Discovery Aiming at Human-Diseases Related Protein Targets

PhD thesis

to obtain the degree of PhD at the
University of Groningen
on the authority of the
Rector Magnificus Prof. C. Wijmenga
and in accordance with
the decision by the College of Deans.

This thesis will be defended in public on

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General Introduction and Scope of this Thesis

Introduction

Human history is also an evolutionary history full of stories about fighting against emergent diseases. Our ancestors utilized botanical herbs, mineral elements, animal products, or even devotional religious and spiritual beliefs¹ to arm the body and combat both environment and diseases. There are numerous medicinal recipe books left behind recording the success cases of dealing with various diseases, including the experiences written in alchemy, which was later developed into modern chemistry.^{2,3} The impact of medicines was quite obvious, for example a minor wound may kill a man through bacterial infection hundreds of years ago. However, after Penicillin was discovered in 1928, just a few years later this antibiotic saved thousands of soldiers from severe wound infection during World War II.^{4,5} Like antibiotics, many other important discoveries in medicine showed their profound capabilities in changing the situation when facing fatal diseases over our long history, the discovery of vaccines dramatically defeated smallpox,⁶ rabies,⁷ measles⁸ and so on, artemisinin, a chemical molecule from herbs has been used against malaria for half a century and is still of benefit to the world.^{9,10}

Good hygienic environments and medicine breakthroughs contribute impressively to increase human life expectancy,¹¹ now over 78 years old on average globally and will break 90 by 2030.¹² This longer lifespan also comes with related challenges, as normal ageing processes and/or age-related diseases remain the most prevalent cause of mortality worldwide.¹³ Thousands of newly named diseases emerged in recent decades, especially related to genetic defects, or poor nutrition (such as excess fat or sugar), and the accumulation of irreparable cellular damage in senescent cells plays an increasing role in modern diseases.^{14,15} So people have searched and discovered numerous drugs to cure or treat these diseases. However, in this long marathon, the human being is always one step behind the illness. Moreover, in the

traditional field where humans have been previously victorious, these wins are now revealed to be temporary victories. Bacteria have gained resistance as antibiotics were overused in medicine, and after less than one century, the superbugs return and threaten the public with the possibility of no efficient antibiotics available.¹⁶ Mosquitos also keep spreading malaria with strong resistance to artemisinin and causing 400 thousand deaths in 2018 alone,¹⁷ a cocktail strategy combined with multiple anti-malaria drugs somehow can slow down the speed of mutations, but cannot eradicate malaria completely. In addition, the outbreak of recent coronavirus (COVID-19)^{18,19} is now sweeping across China and the world, causing more than 2000 deaths in one month, and an effective drug is needed more than ever. All these contribute to the need to find new drugs urgently, either to catch up with the diseases that have suddenly appeared, or maintain efficiently treatment of “traditional” sicknesses.

In terms of drug discovery, nowadays two main pathways are widely used in both industry and academia: phenotypic screening, which historically was the basis for new drug discovery; and target-based screening, which mainly appeared after modern molecular biology was introduced in the mid-1980s.²⁰

Phenotypic screening tracks physiological responses in cell, organism or animal models.²¹ These were systematically screened with small compounds, peptides or RNAi using a target-agnostic assay. Only after the substances have been evaluated as potential therapeutic candidates, were methods to identify the targets or the mechanism behind used.²² It represents a historically developed screening method, and the basis for drug discovery before modern molecular biology was invented.²³ While we were confident that genomic techniques will lead us a better way in healing diseases, subsequently, the trend in screening methods has turned to target-based screening, which mainly relied on the theory that genes or gene products have a key role in disease pathogenesis.²⁴ Phenotypic screening now seems to be an outdated technique in the face of target-based screening. In 2011, a review analyzed new drugs approved by the US Food and Drug Administration (FDA) between 1999 and 2008,²⁵ from the 50 first-in-class drugs with new molecular mechanism of action (MMOA), 28 (56%) small molecules were discovered by phenotypic screening, more than the 17 (34%) drugs that were selected based on target-based screening, the unexpected comparison result highlighted the interest of phenotypic screening in an era where the major focus has relied on target screening since the 1990s. As indicated, a specific molecular hypothesis driven by a target-based approach may not show

molecular function in disease pathogenesis, or provided enough therapeutic index, because the molecular interaction (between drug and the target) generally cannot guarantee the success of desired pharmacological response.²⁵ Both the pharmaceutical industry and academic scientists then quickly started to move back towards phenotypic screening, and began to evaluate what cells or which phenotypes were worth testing.²⁶ Even though later analysis claimed that most first-in-class drugs were gained from target-based approach - only 7% were theoretically found by phenotypic screening,²⁰ the limitations and high attrition rate in clinical trials of target-based screening already shook pharmaceutical companies' belief in target-based screening. Despite the concerns above, phenotypic screening undoubtedly plays an important role in modern pharmaceutical research, and it is complementary to target-based-screening.

Target-based screening is heavily dependent on the prior understanding of the target – typically a protein target –for which treatments are sought that may inhibit or reverse disease progression.^{27,28} The emergence of molecular biology and the human genome project have been attributed to the foundation of target-based screening. Large numbers of compounds or fragments were subjected to screening assays, where the targets are hypothesized as the key factor in certain diseases. Dependent on the compound size, and the structural information of target of interest, there gradually formed two main streams in target-based screening. One mainly focuses on small fragments selection in systematically probing the active sites of protein targets, which later developed into the fragment based drug discovery (FBDD) approach.^{29,30} Another prefers relatively larger scaffold compounds and filters potent chemicals in a rapid manner, which then formed the basis of high throughput screening (HTS).^{31,32} There have been enormous numbers of articles comparing these two approaches, listing the advantages and disadvantages of both methods. Concepts, such as rule of 3 (RO3),^{33,34} rule of 5 (RO5),^{35,36} lead-like,^{37,38} druglike³⁹ and reduced complexity⁴⁰ have been developed to classify the properties of compounds under screening. Fragments screening assembles the information from fragment hits against known structure targets, followed by subsequent linking, merging or fragment growth strategy to create the lead compounds.

High throughput screening (HTS), especially for a novel target, can rapidly identify a chemical for target protein modulation, or cell validation. The chemical diversity of hits, either scaffolds or function groups, facilitate the likelihood to get potential hits in a fast way while the structure of targets may yet be unclear. Recently to combat coronavirus, both academia and industry

joined the Xchem screening project at UK's Diamond Light Source to chase anti-COVID drugs. Fragment screening based on the novel *SARS-CoV-2* main protease (Mpro) crystal structure have yielded over 60 hits. Now scientists with expertise from all over the world work together and keep uploading modified compounds oriented from the hits, or newly designed compounds to the Cloud platform. Massive numbers of chemicals then will be processed to virtual or experimental screening to accelerate the speed of reliable drug identification. This new kind of HTS combined efforts from all the pharmacy fields provides a good example when we are facing a public emergency, and also it may lead to a new trend for later HTS modes.

Overall, there is not a distinct barrier between the two methods FBDD and HTS, and screening also can be applied in both ways - the key factor to success of both is deeply reliant on the diversity and scale of library used. In this thesis we provide a good example by applying both fragment-based and HTS-based screening on the same leukemia-associated protein menin. The resulted chemical hits from two methods provide a close-up view of differences in ligand scaffold, function group property, affinity and library scale. Despite the screening types, we obtain good hits with co-crystals in both approaches and this shows that there is no clear distinction in screening approaches if the library is designed appropriately.

Aim and Scope of this thesis

This thesis mainly focuses on the discovery of small molecule ligands and the subsequent investigation of the interactions with their protein targets by protein X-ray crystallography. Fragment based drug discovery (FBDD) and high throughput screening (HTS) were used to screen compound libraries and identify initial hit compounds. Multiple biophysical approaches like differential scanning fluorimetry (DSF), microscale thermophoresis (MST), surface plasmon resonance (SPR) were used to identify the hit compounds during screening. The confirmed hit-compounds from screening then support fragment growth index for later lead-like compounds optimization. Finally, the detailed atomic structure gained by protein X-ray crystallography was used to investigate interactions of the peptides, small fragments or larger compounds that were found in the screening.

In **chapter 1**, I present a review that comprehensively illustrates the theory of differential scanning fluorimetry (DSF). With intrinsic or extrinsic fluorescence dyes used currently, DSF is now widely spread and used in either protein buffer optimization for storage and

crystallization, or screening ligand libraries for hits. The low sample consumption and fast screening rate suits both FBDD and HTS. Meanwhile, the cellular thermal shift assay (CETSA) which targets cells instead of protein, makes it possible to validate ligand:target interaction downstream directly on the cellular level.

In **chapter 2**, malaria remains a big threat to human health so that uncovering the mechanisms of its metabolism would be beneficial to antimalarial drugs design. Here I report the protein Pyridoxine/pyridoxal kinase (PdxK) crystal structure together with ligands ANP (Phosphoaminophosphonic acid-adenylate ester), PL (pyridoxal) and Mg^{2+} by X-ray diffraction. The complex illustrated the binding mode of ANP and PL as substances participating in the vitamin B6 metabolism pathway in PdxK from *P. falciparum*, and it may also provide the basis for further development of a novel pro-drug class.

In **chapter 3**, for the high throughput screening, we constructed a library based on the Groebke-Blackburn-Bienaymè reaction (GBB), a nanoliter scale dispensing system using acoustic energy made it possible to create a massive library with chemical diversity in a short time. Reaction mixtures without further purification were selected for DSF screening against the acute-leukemia related cancer cofactor menin, and three compounds out of 1536 reaction mixtures were found to have μM binding affinity to menin validated by MST, one has been validated by X-ray diffraction.

In **chapter 4**, with the same protein menin, we utilized another target-based fragment screening (FBDD) strategy to screen a library with 700 small fragments. DSF together with MST identified 30 hits, finally X-ray diffraction confirmed two fragments bound in the active site pocket. Here we also showed the experience that dramatically increase the menin crystal quality, from a low resolution of 3.1 Å to a stable to higher resolution that reached 1.98 Å, this might help for other protein targets for which quality of crystals is critical to ligand screening.

In **chapter 5**, we describe a peptide designed to interfere the interaction between human-Arginases and the substrate L-arginine, in order to regulate the arginine metabolism. The peptide was part of C terminal of human-Arginase I, and its existence is critical to the structural conformation, as well as the enzymatic activity. Our peptide showed a similar inhibition of both

human Arginase I and II and we suggest that peptide deriving from protein itself can also be a good strategy to discover novel peptide-like analogues in drug discovery.

In summary, this thesis aims to encompass my efforts to apply both fragment-based and HTS-based screening for identifying new hit compounds in the early stages drug discovery. The peptide and chemical compounds selected from these two types of screening showed good consistency with theory of target-based screening. As a practical and comprehensive method, target-based screening will bring us more advances in the battle against diseases in the future.

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