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Proteins in harmony: Tuning selectivity in early drug discovery

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Chapter one

General Introduction

About this thesis

This thesis describes the importance of being able to control the selectivity of potential drug candidates. It explains how computational models are employed to predict and rationalize compound-protein binding and therewith, selectivity of compounds. Moreover, it shows that selectivity can purposely be tuned to target either a single protein or an entire panel of proteins. The challenges of selectivity modeling are addressed based on case studies in the sodium-dependent glucose co-transporters, G protein-coupled receptors, and kinases.

Background

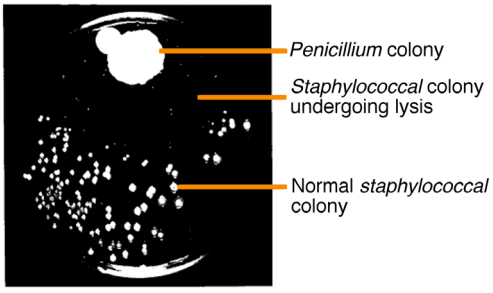
Medicine discovery

The development of new medicines is essential as not every disease can be treated yet and successful treatments that do exist may become ineffective over time due to resistance.[1,2] Moreover, some medications trigger harmful side effects and thus require optimization or development of an alternative therapy.[3] Where in former times medicines were occasionally developed by serendipity,[4] the current era requires a more structured approach to medicine discovery. However, on average it takes about 13 years to develop a new medicine and a total of ~1.8 billion dollar.[5] A typical drug discovery pipeline involves an early drug discovery phase, a preclinical phase, and three subsequent clinical phases. Most of the development costs are made during the (pre-) clinical phases (~1.1 billion dollar) because of failure of drug-candidates due to e.g. unforeseen toxicity or lack of efficacy.[5] By assessing efficacy and toxicity (i.e. side effects) in the early drug discovery phase, issues during the clinical phases may be avoided.

Medicines are chemicals

One can refer to a medicine as many things: a drug, a ligand, a compound, a chemical, and a molecule. Although all of these are correct, the meaning of the words slightly varies and can therefore not always be used interchangeably. To illustrate: all medicines are molecules, but not all molecules are medicines. Although by basis molecules and chemicals do carry the same meaning, they are not always perceived as being similar because of the misconception that naturally-occurring molecules are not chemicals. Furthermore, the terms medicines and drugs

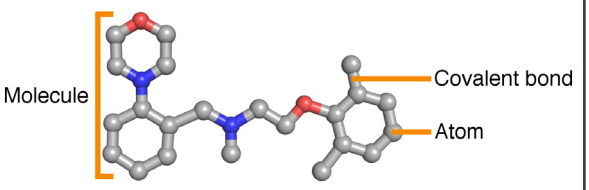
Box 1.1. In 1928, Alexander Fleming discovered the well-known antibiotic penicillin thanks to serendipity. He coincidentally noticed that an unintended mold (penicillium notatum) that was growing on his bacteria cell-culture prevented the bacteria from growing in the space next to the fungus. When he analyzed the fungus more closely he discovered that a substance, which he named penicillin, exhibited anti-bacterial effects that killed the bacteria.[24] *The photograph from A. Fleming (adapted) displays a culture plate showing the dissolution of staphylococcal colonies in the neighbourhood of a penicillium colony.*



can be exchanged naturally as well - although one should be aware that a drug might suggest a stimulant rather than a pharmaceutical. The words ligand and compound are more nuanced and cannot directly be converted into each other. The difference between the two is essential in drug discovery: a compound refers to a chemical that may or may not display any activity, whereas a ligand does exhibit activity. When a ligand has proven its effect *in silico*, *in vitro* or *in vivo*, it may also be referred to as hit compound or lead compound depending on the stage of the process. A ligand that exerts a therapeutic effect and has a good pharmacological profile may eventually become a drug.

The characteristics of compounds are described as physicochemical properties. Such properties include the size, the weight, solubility, and the electrostatic charge of compounds. Furthermore, it describes the number of structural features such as the number of atoms, bonds, rings, and functional groups. The functional groups are important features of compounds as these are able to establish interactions, which include aromatic interactions, interactions through hydrogen bond formation, ionic interactions, or even alteration of covalent bonds.

Box 1.2. A compound is a molecule that is built up by atoms that are connected through covalent bonds. How the atoms are connected through these bonds matters, as different connections result in different molecules. A molecule can contain structural elements such as ring systems: a chain of atoms connected to each other, which results in a ring-shaped construction upon ring closure when the last atom is connected to the first atom of the chain.



Proteins as drug targets

Medicines are molecules. These molecules interact with other, usually bigger, molecules in our bodies, which we call proteins. The human body encompasses over 20,000 different protein-encoding genes, resulting in many proteins that all play a unique role in human physiology.[6] Distortion of a single protein species may already result in pathophysiology, or disease. Early drug discovery focuses on the identification of these pathophysiological proteins and enables the development of compounds that are promising drug candidates. The main protein classes that are targeted by drugs today comprise receptors, enzymes, and transporter proteins. Receptors are responsible for transmission of signals, and encompass the main drug target family: G protein-coupled receptors (GPCRs).[7] It is estimated that about 36% of the current drugs on the market target these receptors, showing their potential in drug discovery.[8] Furthermore, enzymes make up for the second largest drug target class, encompassing 29% of the total amount of drug targets.[7] Some enzymes are responsible for metabolism of compounds, which can be of both natural (endogenous ligands) and synthetic nature (medicines). In contrast to receptors and enzymes, transporters are less explored. Transporters are considered to be more complex, as binding pockets are not always clearly defined. Furthermore, since transporters carry their substrates from inside the cell to the extracellular space or vice versa, they are located within the cell membrane, making it more challenging to elucidate their structure using crystallographic techniques. Consequently, this results in a lack of structural protein information. Although GPCRs are located within the cell membrane as well, many of these protein structures have

been elucidated using a ligand to stabilize the complex. Furthermore, GPCRs have been crystalized by inducing stabilizing mutations and by linking them to easily crystallizable proteins such as T4 lysozyme and apocytochrome b_{562}RIL.[9,10] However, despite of this success for membrane receptors, transporters still remain challenging to crystallize due to instability issues.[11] Nevertheless, upcoming techniques such as cryo-electron microscopy (cryo-EM) may shed light on the structure of transporter proteins as this technique is able to capture proteins whilst avoiding the crystallization issues that occur with commonly used X-ray crystallography.[12]

The three addressed protein classes can be subdivided into protein families. These families are grouped based on protein similarity and function. In general proteins that resemble each other in sequence (**Box 1.3**) and topology (structurally similar), function in a similar matter and are subsequently categorized as family members. Although different proteins may exert a similar function, this does not imply that they are located in the same area. Proteins can be expressed at different locations in the human body, regardless of the location of their family members. Moreover, the same protein may also be expressed in multiple locations. Nevertheless, the location of a protein is usually related to its function: e.g., digestive enzymes are generally present in high amounts in the liver and may occur in lower amounts in the lungs.

Box 1.3. Proteins are constructed from a fixed set of building blocks, which are called amino acids. The human body comprises 20 standard amino acids and one additional non-standard residue named selenocysteine. The residues are linked to each other, creating a chain that facilitates the primary structure of the protein, which is called the protein sequence. The residue type and order of the residues in the sequence define the protein that is formed. When a protein is mutated (e.g., in the case of cancer) one or more residues have been replaced by a different residue type.

Drug-protein interactions

A medicine that acts on a protein makes physical contact with that specific protein: this is called "drug binding". Drugs bind to so-called "binding pockets" on proteins, of which the orthosteric binding pocket is the location where also the natural, or endogenous, ligand or substrate can bind. Any other sites on the protein that allow for ligand binding are called allosteric pockets. When a drug binds to a protein binding pocket, interactions are constituted between the drug and the protein (**Figure 1.1**). These interactions can be of electrostatic, aromatic, or hydrophobic nature, or enable hydrogen bond formation. Moreover, water molecules can additionally form hydrogen bonds with the ligand and the protein. The interactions that are formed between the ligand and the protein are based on the physicochemical properties of both the ligand and the amino acids in the binding pocket. For example, a positive charge on a ligand that is in close proximity to a negatively charged amino acid will result in an electrostatic interaction. In general, an increase in the number of drug-protein interactions improves drug binding. The measure of how well a drug binds to a protein is described as affinity, where a high affinity means good binding and low affinity means poor binding. When a compound is unable to bind to a protein, it has no affinity for that particular protein and is thus not considered a ligand for the respective target.

Binding of a ligand to a protein may result in different effects. Stimulation of proteins can be measured as activation, and blocking as inhibition. In the case of GPCRs, activators are also called agonists and inhibitors antagonists. Additionally, an inverse agonist can deactivate a GPCR and a partial agonist is able to partially activate the receptor instead of triggering full activation as (full) agonists do. The resulting effect depends on the properties of the particular ligand and the protein it is binding to. It is thus possible that a ligand may activate protein A, but inhibits protein

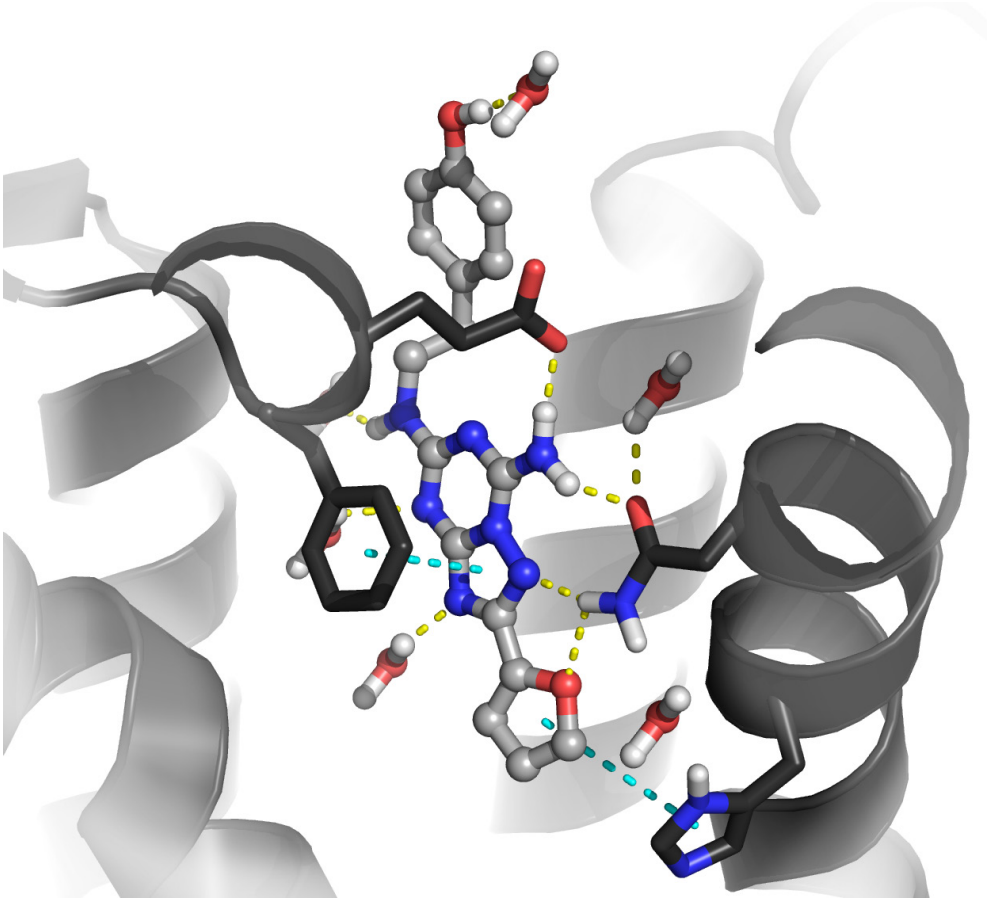


Figure 1.1. A compound (ZM241385) in the binding pocket of the A_{2A} adenosine receptor.[13] Hydrogen bond interactions between the compound (grey), water molecules (red/white), and the protein (black) are displayed in yellow. The aromatic interactions are shown in cyan.

B. Although it might seem contradictory in the case of inhibition, both effects are considered as a result of a compound being bioactive, as in both cases binding of the compound results in a biological effect. The higher the bioactivity of a compound, the stronger is its stimulating or inhibiting effect.

Drugs may compete with natural entities

The properties of a ligand and its target protein are not the only factor influencing drug binding, and therefore its efficacy. When a drug is administered it enters an environment containing many natural ligands and substrates. Some of these natural ligands and substrates may bind to the intended drug target. This may subsequently lead to competition between the drug and the endogenous entity when they both try to bind to the same binding pocket. As a consequence, higher concentrations of the drug, and thus a higher dose, are required to reach the same effect compared to when it would not have to compete. A resolution could be to target the allosteric binding site instead of the orthosteric binding pocket. However, this may face other challenges,

such as tweaking the ability to cross the cell membrane (in case of transmembrane proteins), which may not be feasible for every drug. Moreover, in general orthosteric binding pockets are better explored than allosteric pockets, providing us with less information about allosteric pockets. This lack of data can obstruct the development of certain ligands, as more knowledge may be required to design highly effective drugs.

Selectivity, promiscuity, and polypharmacology

A drug should target the protein that is involved in disease, as targeting of any other protein may result in adverse effects. The more specificly a protein is targeted, the higher the selectivity of a drug. When a drug is not very selective and therefore binds to miscellaneous proteins, it is called promiscuous. Selectivity of a compound can be tuned by changing its molecular structure and properties. For instance, a molecule can be grown into a certain subpocket by adding extra atoms to the molecule. When this subpocket is not present in the anti-target (the protein that should not be bound) the compound does not fit anymore, resulting in achieved selectivity for the on-target over the anti-target. Selectivity of compounds is not limited to specific targeting of only a single protein. In more complex diseases such as cancer, it can be advantageous to target multiple proteins with a single drug to limit the onset of resistance. Multi-target drug design can also be coined as polypharmacology. Although polypharmacological drugs tend to be more promiscuous, selectivity over multiple off-targets can still be of interest. In this case, multiple on-targets can be collectively targeted, while drug binding to off-targets is prevented.

Computers in drug discovery

Computational methods are an important aspect in early drug discovery, as they allow for quick and cost-efficient validation of millions of compounds. Moreover, they can provide insight into ligand-protein binding and guide medicinal chemists in drug design and optimization. Considering that the amount of possible molecules is endless, it would be very inefficient to physically create every molecule and test them all for bioactivity. Even if we would limit the number of molecules to only the medicine-like molecular space by filtering on Lipinski's rule of five[14], still an astonishing 10^{33} compounds would remain.[15] Using computational methods we can create molecules digitally, instead of physically, this saves us a lot of time and resources. Moreover, the compounds can also be screened for bioactivity using the computer in a process called virtual screening. By using computational models, the time to test a potential medicine can be decreased significantly.[16,17] With automated biological assays (high-throughput screening) 10,000 compounds per day can be measured,[18] whereas a relatively slow three-dimensional computational model already is capable of testing ~170,000 compounds per day (24 cores)[i]. For comparison, a traditional (dual-core processor) laptop contains two cores. Faster systems (1500 cores) have reported a virtual screening speed of 115 million compounds per day.[19] A core can be assigned to execute a single task. However, with hyper-threading a core can be split virtually to assign multiple tasks to a single core.

Box 1.4. The Lipinski's rule of five is used to evaluate drug-likeness of compounds. Counter-intuitively the rule includes four guidelines that are based on the physicochemical properties of medicines. Based on these guidelines a compound is considered as a potential drug when: 1) the compound has no more than 5 hydrogen bond donors, 2) the compound has a maximum of 10 hydrogen bond acceptors, 3) the molecular weight of the compound is less than 500 daltons, and 4) the log octanol-water partition coefficient is less than 5.[14]

i Chapter 5 from this thesis

Virtual screening requires models that represent ligand-protein binding. The most comprehensible models contain a three-dimensional representation of the protein (**Figure 1.2**). The affinity of a compound can subsequently be predicted by fitting the compound into the binding pocket of the protein, where a better fit indicates a higher affinity. The three-dimensional protein structures can be derived using crystallography or cryo-EM. With crystallography the protein can be visualized by measuring electron densities of the crystalized protein. The success of the procedure is heavily dependent on the stability of the protein, which can be influenced by the acidity of the solution (pH) that the protein is in, and by co-crystalized factors such as stabilizers and ligands. The stability issue has as a consequence that not all proteins can easily be crystalized. Nevertheless, crystalization of proteins may not be necessary as protein structures may also be derived with cryo-EM, which uses frozen proteins instead. Although this is a viable solution to derive protein structures, resolutions are generally better with crystallography. Proteins for which no protein structure is available (yet) may be structurally modeled if the structure of a close homolog is available. However, if this is not the case, other modeling techniques should be used that do not require information on the protein structure.

Methods based on machine learning or artificial intelligence can predict ligand-protein binding without direct knowledge about the protein structure. In the context of drug discovery, these models are called quantitative structure-activity relationship (QSAR) models and proteochemometric (PCM) models. QSARs are constructed based on compound information only and are commonly used for single targets, whereas PCMs include additional protein sequence data and can encompass multiple proteins in one model. The models rely on statistics and trends in data and are therefore also known as statistical models. Statistical models require a training phase in which they extract trends from data. These trends can subsequently be applied in the test and screening phases to label new compounds. In bioactivity modeling of compounds, models are trained on experimentally measured bioactivities. Therefore, this requires prior knowledge

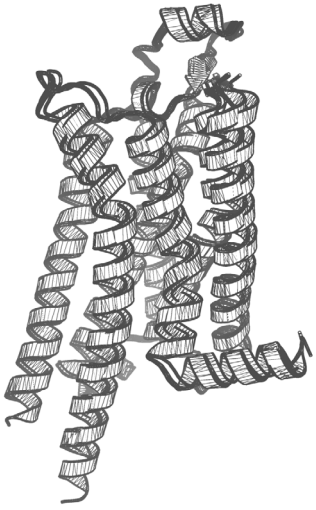


Figure 1.2. Overlay of 3D representations of the A_{2A} adenosine receptor. Protein crystal structures of the exact same protein may vary, as proteins are dynamic and can be influenced by the crystallization procedure. Protein structure PDB codes[20] (pH 5.0-5.5): 4EIY[13], 5IU4[21], 5IU7[21], 5K2C[21], 5NM2[23], 5NM4[23], 5OLG[24], and 5OLZ[24].

on ideally many examples of ligand-protein binding. It is therefore no surprise that lack of data can be one of the major bottlenecks in statistical modeling.

Bioactivity predictions made by computational models are rarely 100% correct. Therefore, it is important to perform experimental validation of the obtained results. This experimental validation commonly includes *in vitro* assessment of the predicted bioactivities of compounds. The hit rate indicates the number of true actives, and thus the rate of good predictions, that results from the *in vitro* biological assay. The expected hit rate can be estimated prior to virtual screening by validation of the computational models with known ligands. These ligands are not used in model training and are therefore considered as new compounds. The better the model is able to retrieve the bioactive compounds, the better the estimated hit rate will be.

Objectives of this thesis

Selectivity of drugs is essential as side effects can be prevented. Therefore, this thesis encompasses multiple approaches to model selectivity in early drug discovery. Additionally, the challenges that are faced in selectivity modeling are discussed. The compound-protein modeling techniques that can be applied in selectivity modeling are thoroughly described in **Chapter 2**. Moreover, this chapter also describes how potential anti-targets can be identified, which allows us to anticipate side effects and enables the design of more selective compounds. **Chapter 3** presents a case study on sodium-dependent glucose co-transporters, where selectivity can be achieved by changing the area of treatment from the kidneys to the small intestine. Additionally, it is shown that novel ligands for these transporters can be found using a machine learning approach in virtual screening. Furthermore, **Chapter 4** describes that machine learning can be applied to model selectivity in the adenosine receptors; a subfamily of the GPCRs. Using this approach, a quantitative measure of selectivity between two proteins can be provided. **Chapter 5** illustrates the concept of polypharmacology in kinases. It presents a workflow in which fifteen kinases are carefully curated and validated, followed by a virtual screen for novel inhibitors using both machine learning and structure-based methods. **Chapter 6** addresses the lack of binding type data (orthosteric or allosteric binder), which influences the performance of computational models that predict compound bioactivities and consequently selectivity. In this chapter text mining is employed to label orthosteric and allosteric compounds. These binding types are subsequently used to improve bioactivity predictions and thus illustrate the necessity of filling data gaps in computational chemistry.

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