Allosteric modulation and ligand binding kinetics at the Kv11.1 channel

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Chapter 1

General introduction

Ion channels are transmembrane proteins that create a gated and water-filled pore to allow the flow of ions down their electrochemical gradient between the intracellular and extracellular environment.[1, 2] They are selective for a wide range of cations and anions, such as Na^+, K^+, Ca^{2+}, and Cl^- ions. The distribution of ion channels has been found in cell membranes as well as the membranes of intracellular mitochondria, endoplasmic reticulum, and nucleus, which suggests their crucial physiological functions in a plethora of excitable and non-excitable tissues. Accordingly, ion channels are important targets for many drugs in current use. Indeed, application of ion channel modulators as medications was operative long before their existence had been demonstrated.[3] For instance, in the 19th century cocaine extraction from coca leaves gave rise to a pharmaceutical class of local anesthetics, which are selective sodium channel blockers.[4] In addition to the widespread therapeutic properties, ion channels are also crucially related to human toxicology due to their involvement in human physiology. K_v11.1 (hERG) channel induced cardiotoxicity has become a thorny issue for the pharmaceutical industry in the past years. Blockade of the K_v11.1 channel leads to the prolongation of cardiac action potential duration (APD) and long QT syndrome, which in some individuals can degenerate into ventricular arrhythmias and sudden cardiac death.[5] It has been pointed out that 50% of drugs recalled by the FDA from 1995 to 2000 were also K_v11.1 blockers.[6] In this thesis, I, therefore, focus on parameters that might determine the K_v11.1 toxicity of compounds at the molecular level, as well as possible strategies for relieving the K_v11.1-related cardiac side effects of drugs. In order to provide some background for the research and concepts that are discussed in this thesis, a brief overview of ion channels has been outlined. Subsequently, the target receptor, the K_v11.1 channel, is introduced, followed by a summary of objectives and contents of this thesis.

Ion channels

Several hundred genes in the human genome encode the pore-forming ion channels across plasma membranes.[1] According to the precise control of channel gating, ion channels are classified as ligand- and voltage-gated channels, of which the opening and closure are mediated by a specific ligand and membrane voltage, respectively.[7]

Ligand-gated ion channels

Ligand-gated ion channels (LGICs) are oligomeric protein assemblies that span the cell membrane and form both the binding site for an endogenous ligand and an ion-conducting pore, which converts a chemical signal into an ion flux through

the membrane.[8, 9] Based on homology in their amino acid sequence and topology of their component subunits, LGICs are divided into three superfamilies, including P2X, ionotropic glutamate, and Cys-loop receptors.[10] They play an important role in fast synaptic transmission and in the modulation of cellular activity, in particular for basic brain functions like learning, memory and attention.

P2X receptors

P2X receptors are non-selective cationic channels gated by extracellular ATP, which are permeable to Na^+, K^+, Ca^{2+}, and in some cases also to Cl^- ions.[11] A P2X channel contains three subunits, and each subunit is composed of two transmembrane domains connected by a large, glycosylated extracellular loop, and intracellular C- and N-terminus (**Figure 1A**).[9] At present, there are seven P2X receptor subtypes found in mammals, which are termed $P2X_1$ to $P2X_7$.[12] These channels play paramount roles in various physiological processes such as nerve transmission, pain sensation, the response to inflammation, multiple facets of diabetes, and tumor cell growth, making them attractive drug targets in recent years.[12-14]

Ionotropic glutamate receptors

Ionotropic glutamate receptors are cation-selective ion channels that open their ion-conducting pores in response to the binding of glutamate.[15] This channel is made up of four homologous subunits with each subunit containing three transmembrane segments, an intracellular C-terminus and extracellular N-terminus (**Figure 1B**).[9] The ionotropic glutamate receptors are grouped into three subtypes according to their distinct responses to certain small molecule agonists: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic-acid (AMPA) and kainate (KA) receptors.[10, 15, 16] These distinct ion channels are implicated in almost all aspects of nervous system development and function, and their dysfunctions can be linked to epilepsy or chronic neurodegenerative conditions like schizophrenia, Alzheimer's and Parkinson's diseases, as well as stroke.[15, 16]

Cys-loop receptors

The Cys-loop receptors, named from the conserved cysteine loop in their extracellular domain, constitute the largest subfamily amongst all LGICs.[9, 17] The best characterized members of the Cys-loop receptor superfamily are nicotinic acetylcholine receptors (nAChRs), serotonin type 3 receptors ($5\text{-}HT_3Rs$), GABA receptors type A ($GABA_ARs$), glycine receptors (GlyRs) and zinc activated chan-

nels (ZACs).[18] Although these ion channels exhibit diverse kinetic and pharmacological profiles, they share a common structure of five identical subunits that are pseudo-symmetrically arranged to form a rosette with a central ion-conducting pore for either anion ($GABA_A$Rs and GlyRs) or cation (nAChRs, 5-HT_3Rs, and ZACs) flux.[17, 19] As shown in **Figure 1C**, each subunit of the channel is made up of an extensive N-terminus and a short C-terminus on the extracellular side, and four transmembrane segments.[8] All these receptors are very important targets for a wide variety of drugs, and their dysfunctions are associated with different kinds of diseases. For example, nAChRs have been demonstrated to play many critical roles in brain and body functions, indicating their therapeutic modulation of Alzheimer's disease, epileptic disorders and chronic pain.[20-22] Likewise, $GABA_A$Rs have been unraveled to regulate the controlling neuronal activity in distinct brain regions, and dysfunction of this ion channel involves a range of central nervous system diseases such as anxiety, insomnia, muscle spasms, Alzheimer's disease and schizophrenia.[23, 24]

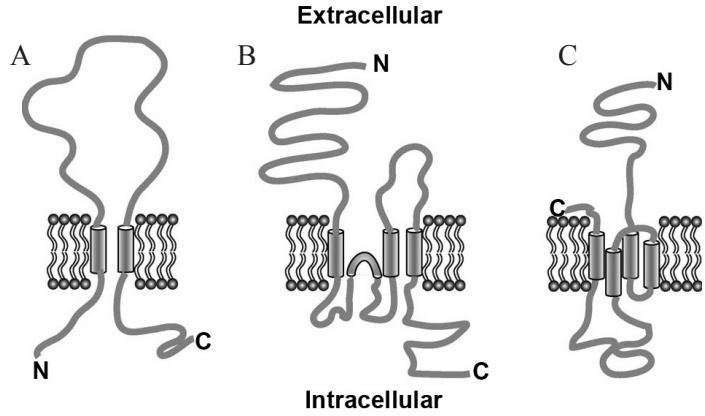

Figure 1. Schematic representation of a single subunit from the three superfamilies of ligand-gated ion channels across the membrane: **(A)** P2X receptors, **(B)** Ionotropic glutamate receptors, and **(C)** Cys-loop receptors.[9]

Voltage-gated ion channels

Voltage-gated ion channels (VGICs) are integral transmembrane proteins that conduct selected inorganic ions at high rates across the membrane in response to changes in transmembrane voltage.[25] The VGIC superfamily, with 143 members in the human genome, forms the third largest family of signal-transduction proteins, following the G protein-coupled receptors and protein kinases.[26, 27] As shown in **Figure 2**, ten families are included in this VGIC superfamily: voltage-gated sodium/calcium/potassium channels (Na_v/Ca_v/K_v), calcium-activated

potassium channels (K_{Ca}), cyclic nucleotide-modulated (CNG) ion channels, hyperpolarization-activated cyclic nucleotide-modulated (HCN) channels, transient receptor potential (TRP) channels, two-pore channels (TPCs), inwardly rectifying potassium channels (K_{ir}) and two-pore potassium channels (K_{2P}).[10, 28] All these channels are composed of a common cation-selective pore-forming module containing two transmembrane segments and an intervening pore loop.[10] Except for K_{ir} and K_{2P} channels, all other ion channels consist of four subunits symmetrically arranged around the central ion-conducting pore, and each subunit encompasses six transmembrane segments (S1-S6).[10, 26, 27] Amongst these segments, S1-S4 segments form the voltage-sensor domain with four to eight positively charged residues in the S4 segment conferring voltage dependence, whereas S5 and S6 make up the pore domain including the ionic pore with the selectivity filter.[1] Movement of the voltage sensors and pore domains constitutes three main conformational states of the VGICs: closed, open and inactivated states. On the other hand, K_{ir} and K_{2P}, which are composed of four and three subunits respectively, are structurally the simplest VGICs with only pore-forming segments for each subunit. These two potassium channels are regulated by membrane lipids and intracellular 'ligands', as diverse as G proteins, Mg^{2+} ions, polyamines and ATP.[10] Apart from the main subunits, VGICs also possess some smaller, auxiliary subunits to modify their functions, increasing the diversity and complexity of this superfamily. VGICs play a fundamental role in most aspects of cell physiology and underlie complex integrative processes like learning and memory in the brain and coordinated movements of the muscles, which makes them the molecular target for a number of therapeutic agents and also toxins.[27]

Potassium-selective channels with 78 members are the most diverse and widespread group of ion channels, whereas K_v channels, in humans encoded by 40 genes and divided into 12 subfamilies (K_v1-12), form the largest family among these potassium channels.[29, 30] All K_v channels have a high level of structural similarity, and play a pivotal role in a variety of cellular processes, such as functioning of excitable cells, cell apoptosis, growth and differentiation, the release of neurotransmitters and hormones, and maintenance of cardiac activity.[31] Therefore, K_v channels constitute potential drug targets and provide tremendous opportunities for treating cancer, autoimmune diseases, metabolic, neurological and cardiovascular disorders. However, K_v channels also present a challenge to the development of K_v-targeted medications, of which the K_v11.1 channel is an explicit example with its promiscuity to drug binding, resulting in drug-induced ventricular arrhythmias.[2]

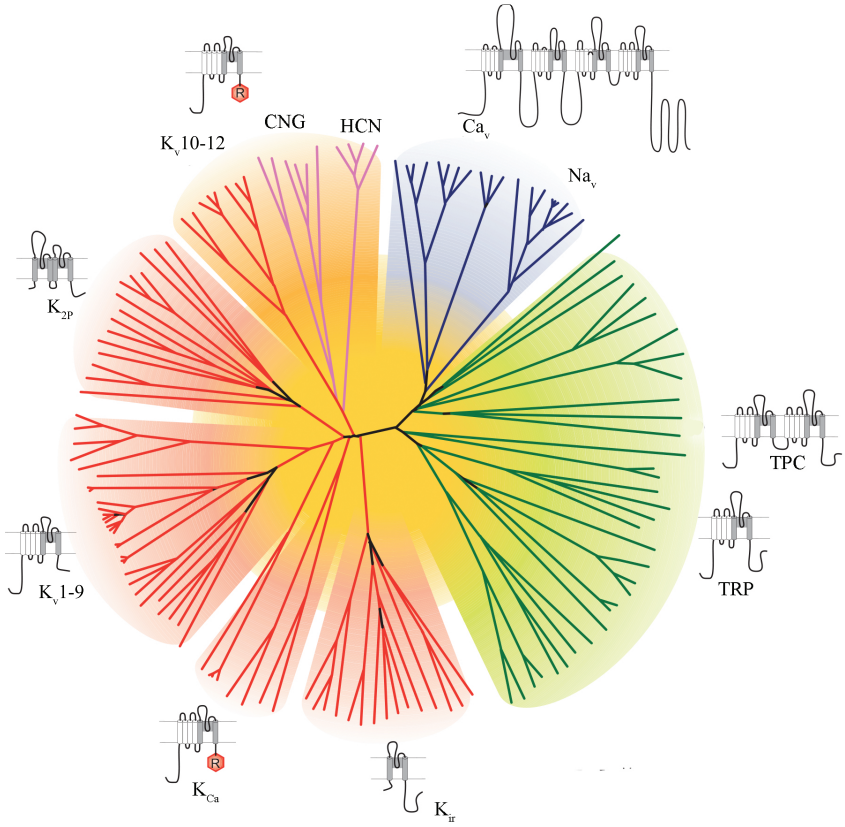

Figure 2. A phylogenetic tree of voltage-gated ion channels, and a schematic representation of the structure of each voltage-gated ion channel within this superfamily.[28]

The K_v11.1 channel

Overview of the K_v11.1 channel

The K_v11.1 channel, of which the pore-forming α-subunits are encoded by the human ether-à-go-go-related gene (hERG), carries a rapidly activating delayed rectifier potassium current (I_{Kr}) underlying its essential role in cardiac action potential repolarization.[32] In addition, the K_v11.1 channel has been found to be expressed in various brain regions, smooth muscle cells, endocrine cells, and a wide range of tumor cell lines, which implicates the channel's therapeutic opportunities beyond the cardiac system.[32, 33] However, due to its involvement in life-threatening cardiac arrhythmias, the terminology "hERG" or "K_v11.1" has become notorious in the drug discovery community. Hence, much attention has been paid to K_v11.1-induced cardiotoxicity over the past decades, which makes

its role in the heart best characterized.

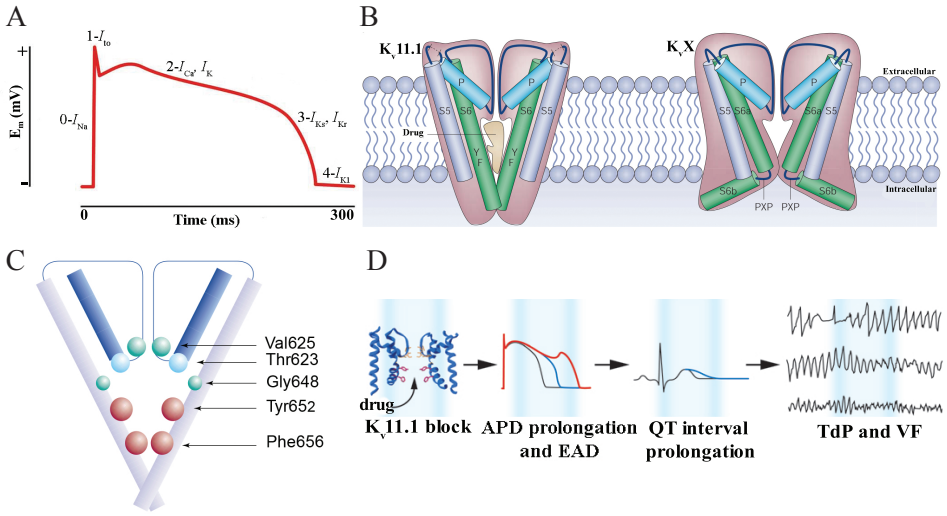

Figure 3. (A) The ventricular action potential waveform indicating the major ionic currents that contribute to its morphology and duration (I_{Na}: inward sodium current, I_{to}: transient outward potassium current; I_{Ca}: L-type inward calcium current; I_K: outward repolarizing potassium current; I_{Ks}: slowly activating delayed rectifier potassium current; I_{Kr}: rapidly activating delayed rectifier potassium current; I_{K1}: inward rectifier potassium current).[34, 35] **(B)** Structure of the pores of the K$_v$11.1 channel and other K$_v$ channels: the presence of a Pro-X-Pro (PXP) motif in the S6 helix of other K$_v$ channels causes a kink and thereby reduces the size of the channel pore cavity.[36] **(C)** Amino acids that are crucial for high-affinity binding of prototypical K$_v$11.1 blockers.[37] **(D)** Mechanism of cardiac arrhythmias induced by K$_v$11.1 blockade.[38]

The K$_v$11.1 channel displays unusual gating properties that impact cardiac electrical function. Upon membrane depolarization the K$_v$11.1 channel opens slowly but inactivates rapidly, while it recovers rapidly from inactivation but deactivates slowly during repolarization.[5, 34] Therefore, the K$_v$11.1 channel conducts robust outward currents (I_{Kr}) relatively late in the ventricular action potential, accelerating the phase 3 repolarization without significantly disturbing the preceding plateau phase 2 (**Figure 3A**). Nevertheless, the K$_v$11.1 channel lacks a Pro-X-Pro (PXP) motif existing in other K$_v$ channels (**Figure 3B**), resulting in a larger cavity that can accommodate a wide range of drugs.[36] Furthermore, the K$_v$11.1 channel has multiple aromatic rings lining the pore (**Figure 3C**), which facilitates the binding of drugs containing aromatic rings at the channel.[37] Collectively, a number of drugs including cardiac and non-cardiac medications can bind to the K$_v$11.1 channel. As depicted in **Figure 3D**, drug block of the K$_v$11.1 channel at the molecular level induces APD prolongation, early afterdepolariza-

tions (EADs) and heterogeneity of repolarization.[38] This generates a lengthening of the QT interval in the electrocardiogram at the tissue level, which can further lead to life-threatening arrhythmias like Torsade de Pointes (TdP) and ventricular fibrillation (VF). Therefore, it has become obligatory routine practice for all pharmaceutical and biotechnology companies to screen for K_v11.1 liability of drug candidates in the early stage of drug development. More details on the structural features and physiological functions of K_v11.1 channel will be stated in the chapter 2.

Ligands for the K_v11.1 channel

Blockers and modulatory activators

A large number of structurally diverse compounds have been shown to bind to the K_v11.1 channel. In most cases these drugs reduce the I_{Kr} by directly blocking the conduction pore or less frequently by interfering with channel trafficking to the surface membrane, and these drugs are referred to as K_v11.1 blockers.[32, 39, 40] In addition, some blockers like APETx1 exert their inhibitory effects on the K_v11.1 current by modulating the gating properties of the channel.[41, 42] On the other hand, a small amount of compounds have also been characterized as K_v11.1 activators, which have the opposite activity of blockers and thus enhance the I_{Kr} current.[39, 43]

Most K_v11.1 blockers share a common binding region within the aqueous inner cavity at the pore of the channel, where residues Tyr652 and Phe656 are found to be crucial for drug binding via hydrophobic and electrostatic interplays, including π–π and π–cation interactions.[37, 39] Apart from the intracellular binding pocket, various scorpion, spider and sea anemone toxins inhibit the K_v11.1 channel from the extracellular side or by binding to the voltage-sensor domain.[42] With regard to the K_v11.1 activators, different binding locations at the channel have been identified, depending on the mechanisms by which they potentiate the I_{Kr}. According to the primary action model, these K_v11.1 activators are separated into types 1 and 2 classes.[32, 39] Type 1 K_v11.1 channel activators attenuate the inactivation and deactivation rates of the channel, whereas type 2 activators primarily impair the channel's inactivation rate. Distinct from each other as well as blockers, type 1 and type 2 K_v11.1 activators bind to a site between the pore and voltage sensor and a region that is closer to the selectivity filter, respectively. Additionally, several type 2 K_v11.1 activators like NS1643 and PD307243 have been proposed to interact with the channel at the extracellular face.[43, 44] Taken together, multiple binding sites exist at the K_v11.1 channel, which illustrates the potential for different ligands to display either competitive or allosteric interactions depending on their binding sites relative to one another (**Figure 4A**).[45]

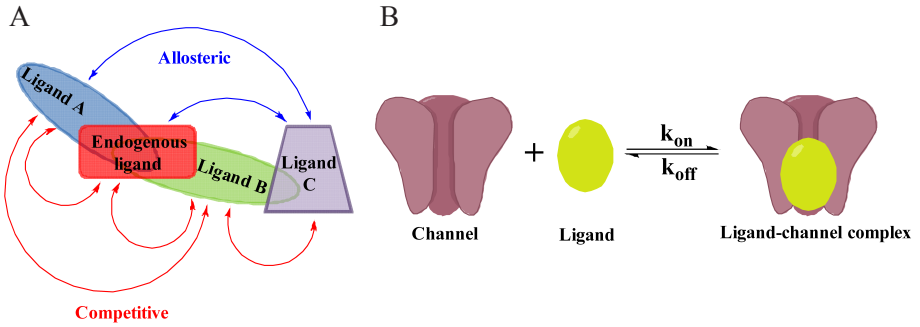

Figure 4. Simple schematic representations of the ligand-channel interaction: **(A)** Arrows demonstrate a competitive (endogenous ligand with ligands A and B, ligand B with ligands A and C) or allosteric interaction (ligand C with ligand A and endogenous ligand) of different ligands based on their binding sites relative to one another,[45] and **(B)** Single-step binding of a ligand to the K_v11.1 channel .

Binding kinetics of ligands

The specific ligand-receptor interaction is the chemical basis of virtually all biological activities of compounds.[46] As shown in **Figure 4B**, binding of a K_v11.1 ligand to the channel can be considered as a single-step reaction in the simplest scenario. At first, the ligand binds to the K_v11.1 channel with a second-order association rate (k_{on}), and then detaches from the channel with a first-order dissociation rate (k_{off}). In a closed system, the extent of ligand-channel binding is commonly quantified in terms of the equilibrium dissociation constant (K_D) defined as the value of k_{off}/k_{on} or its (often inhibitory) affinity (IC_{50} or K_i). To date, more and more evidence from a wide range of receptors has addressed the concept that *in vivo* efficacy of a ligand is not only described by the *in vitro* measured equilibrium dissociation constant, but also depends on its association and, more critically, dissociation rates.[47-49] A ligand's residence time (RT), i.e. the reciprocal of its dissociation rate, may be at the basis of the ligand's duration of action and target selectivity.[48] As for the K_v11.1 channel, a 30-fold safety margin, quantified as the ratio of IC_{50} and maximum plasma concentration (C_{max}), has been proposed to define the cardiac safety of K_v11.1 blockers.[50] However, the binding of a K_v11.1 ligand to the channel remains a dynamic process in which binding (association) and unbinding (dissociation) events develop over time.[51] Moreover, the approach in which only the IC_{50} value is considered, is rather arbitrary and may not satisfactorily account for the degree of ion channel inhibition reached in various conditions, leading to unsatisfactory quantitative predictions.[51, 52] Importantly, K_v11.1 blockers with similar IC_{50} values but different binding kinetics at the channel

have been reported to pose distinct proarrhythmic risks.[52, 53] Therefore, binding kinetics of ligand-K_v11.1 interactions constitute a critical component in understanding the mechanism of action, and thus should be incorporated within the present screening strategy to improve the risk profiles of drug candidates.[51, 52, 54]

Objectives and outline of this thesis

Drug-induced arrhythmia due to blockade of the K_v11.1 channel has emerged as an unanticipated side effect of many pharmacological agents and is a major obstacle for drug development. Although much attention has been paid to screening K_v11.1 liability of drug candidates over the past decades, the current testing paradigm has important limitations and may have led to withdrawal of blockbusters on the present market or stopping the development of potentially valuable therapeutics. Therefore, investigating the mechanism of action of ligands at the K_v11.1 channel, where in particular the ligand binding kinetics may yield comprehensive understanding of K_v11.1-related cardiotoxicity, may further provide new strategies for circumventing this side effect in drug development. In addition, compounds that can relieve the unintended K_v11.1 blockade of drugs, e.g., allosteric modulators, are of considerable interest because of their potential to prevent drug-induced arrhythmia via combination therapy.

In **Chapter 2**, the gating kinetics and ligand binding characteristics at the K_v11.1 channel are extensively discussed, following the basic introduction of the channel biosynthesis, structure and function, and pharmacological target and anti-target applications in drug development.

At the start of this project, high-throughput assays had not been reported to characterize allosteric modulators of the K_v11.1 channel, nor had ligand binding kinetics been determined at the channel. Thus, [^{3}H]astemizole and [^{3}H]dofetilide binding assays were developed and utilized to identify positive and negative allosteric modulators of the K_v11.1 channel in **Chapter 3**.

Based on the work in chapter 3, a novel negative allosteric modulator (LUF7244) was identified in several different [^{3}H]dofetilide binding assays in **Chapter 4**. LUF7244 was found to reduce the K_v11.1 affinity of dofetilide, astemizole, sertindole and cisapride in a [^{3}H]dofetilide competitive displacement assay, and more importantly, to prevent the proarrhythmic effects induced by astemizole, sertindole and cisapride in a newly validated neonatal rat ventricular myocyte model. This research raises the possibility to resume the clinical use of unintended K_v11.1 blockers via pharmacological combination therapy. Following this, in **Chapter 5** the synthesis of a series of possible allosteric modulators and the evaluation of their allosteric effects on [^{3}H]dofetilide binding at the chan-

nel are reported. Compared to LUF7244, several more potent negative allosteric modulators were obtained via small chemical modifications.

In **Chapter 6**, a novel [^{3}H]dofetilide competition association assay was developed and validated to evaluate the kinetic parameters of fifteen prototypical K_v11.1 blockers at the channel. Next to that, an immobilized artificial membrane column was applied to measure the membrane affinity of these blockers to elucidate the effect of membrane affinity of ligands on their affinity and kinetic parameters. In addition to varied affinity values and association rates of K_v11.1 ligands at the channel**, Chapter 7** focuses on the discovery of blockers with diversified dissociation rates or RTs. Compounds with very short (< 1 min) and much longer RTs (> 100 min) were disclosed in this chapter, which enabled the construction of a "k_{on}-k_{off}-K_D" kinetic map for all these compounds. Additionally, results from patch clamp studies implicated the importance of RTs in regulating the functional IC_{50} values of K_v11.1 blockers.

Overall, allosteric modulation and binding kinetics of ligands at the K_v11.1 channel have been thoroughly studied in this thesis. The general conclusions and future perspectives for this field of research have been summarized in **Chapter 8**. Hopefully, the findings in this thesis will add to the current understanding in ligand-K_v11.1 interactions, and also provide effective opportunities for abrogating K_v11.1-induced cardiotoxicity.

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