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

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Chapter 8

Conclusions and perspectives

About this chapter

In this thesis I have explored the allosteric modulation and the kinetics of ligands at the $K_v11.1$ channel. The research in this thesis may provide valuable information and potential approaches for relieving the $K_v11.1$-induced cardiotoxicity of drug candidates in the future. This chapter will summarize the major findings from these studies, and propose the future directions of this research field.

Conclusions of this thesis

Allosteric modulations of the K_v11.1 channel

Nowadays, K_v11.1 blockade by potential drug candidates has received much attention since it potentially causes proarrhythmic side effects. However, at the start of this research allosteric modulation of the K_v11.1 channel as an alternative way of drug interaction had not been studied in any significant detail. The unique structural features of the K_v11.1 channel, which were thoroughly reviewed in **Chapters 1** and **2**, imply the presence of multiple ligand binding sites at the channel with possible allosteric interactions between them. In **Chapter 3**, the allosteric effects of a plethora of compounds as well as potassium ions on the K_v11.1 channel were evaluated in both [^{3}H]astemizole and [^{3}H]dofetilide binding assays. LUF6200 was identified as a negative allosteric modulator, whereas potassium ions behaved as positive allosteric modulators. Moreover, LUF6200 and potassium ions acted on distinct allosteric sites at the K_v11.1 channel, and these binding sites showed positive cooperativity. This investigation is the first to provide direct evidence for allosteric modulation of the K_v11.1 channel using radioligand binding assays.

Subsequently, a novel negative allosteric modulator LUF7244 was characterized with several different [^{3}H]dofetilide binding assays (**Chapter 4**). LUF7244 was found to decrease the affinity of the prototypical K_v11.1 blockers dofetilide, astemizole, sertindole and cisapride. Moreover, the antiarrhythmic propensity of this compound was revealed in a newly validated neonatal rat ventricular myocyte (NRVM) model. Pretreatment of the NRVM monolayers with LUF7244 relieved the arrhythmic events induced by astemizole, sertindole and cisapride. Importantly, LUF7244 *per se* did not significantly affect the action potential durations of NRVMs. Concomittantly, LUF7244 displayed no obvious cytotoxicity upon a long exposure to NRVMs, and exerted negligible effects on astemizole at its intentional target (the human histamine H_1 receptor). Taken together, this study has introduced allosteric modulation of the K_v11.1 channel as a new and effective strategy to reduce the unintended binding of drugs to the channel's central cavity and to further prevent the proarrhythmic changes associated with this binding. This opens a promising avenue for abolishment of K_v11.1-induced cardiotoxicity of drug candidates via pharmacological combination therapy. Following this chapter, a series of compounds with the same scaffold as LUF7244 were synthesized and further assessed for their allosteric effects on [^{3}H]dofetilide binding to the K_v11.1 channel in **Chapter 5**. Structure-activity relationships were derived for these compounds, which offers important information for future op-

timization of allosteric modulators of the K_v11.1 channel. Specifically, compared to LUF7244 several more potent negative allosteric modulators like **7f** and **7p** were obtained, implicating improved potency in subsequent *ex vivo* and *in vivo* antiarrhythmic studies.

Kinetics studies of ligands at the K_v11.1 channel

Previous studies on K_v11.1 blockers have primarily focused on their equilibrium parameters such as affinity or potency at the channel. The kinetics of the interaction between a drug and the K_v11.1 channel has rarely been investigated though, albeit that its importance has been reported with respect to other targets, like enzymes and G protein-coupled receptors. In **Chapter 6**, a novel [^{3}H]dofetilide competition association assay was successfully developed and validated to characterize the kinetic binding parameters of unlabeled compounds at the K_v11.1 channel. The association rates of fifteen prototypical, structurally diverse K_v11.1 blockers obtained in this assay were extremely divergent, with e.g., a 50,000-fold difference between moxifloxacin and clofilium, and correlated excellently to their affinity values at the channel. Meanwhile, membrane affinity determined in an immobilized artificial membrane column and other calculated physiochemical properties (molecular weight, pK_a, and logD) did not correlate with the binding affinity and kinetics of these blockers at the channel. Collectively, these results lead to the conclusion that a compound's affinity at the K_v11.1 channel is mainly regulated by its association rate instead of its dissociation rate. This finding is against the general dogma that affinity values of ligands for their targets are mainly dominated by their dissociation rates with diffusion limited association rates. In this case, association rates can therefore be used to evaluate the K_v11.1 liability of drug candidates, apart from the affinity. Since the dissociation rates of the selected K_v11.1 blockers in **Chapter 6** only exhibited a 10-fold difference between the fastest and slowest dissociating compounds, we therefore aimed to search for and design compounds with a wide range of RTs, i.e. reciprocal of dissociation rates at the channel, in **Chapter 7** in order to explore the role of the dissociation process in K_v11.1 blockade.

In **Chapter 7**, the association rates and RTs as well as affinity values of four compound series (dofetilide, E-4031, clofilium and pyridinium derivatives) at the K_v11.1 channel were determined and evaluated. Based on the results, the structure-kinetics relationships of these K_v11.1 blockers were explored next to their structure-affinity relationships. Interestingly, the RTs of these compounds, in particular the newly synthesized series of pyridinium derivatives, were up to 300-fold different, in addition to dramatic differences in affinity and association

rates. For instance, compound **38** had a RT of 105 ± 4 min at the K_v11.1 channel, while **37** displayed a short RT that was only 0.34 ± 0.04 min. Subsequently, a "k_{on}-k_{off}-K_D" kinetic map was constructed to putatively categorize all compounds based on their potential K_v11.1 liability, together with three reference drugs (positive control astemizole, negative control ranolazine, and dofetilide). Lastly, two representative compounds were tested in patch clamp assays, and their patch clamp IC_{50} values and current inhibition rates were in agreement with the RTs and association rates derived from radioligand binding experiments, respectively. Altogether, the importance of incorporating kinetic binding parameters (association and dissociation rates or RTs) into the current affinity-dominated paradigm is strongly suggested by our studies in order to more comprehensively and accurately evaluate the K_v11.1 liability of drug candidates. Overall, this work offers a new avenue for drug researchers to efficiently design ligands with less K_v11.1-induced cardiac side effects by considering ligand binding kinetics in the drug development trajectory.

Future perspectives

Allosteric modulator as a therapeutic agent

Although the antiarrhythmic property of a negative allosteric modulator (LUF7244) was evidenced in a NRVM model (**Chapter 4**), the capability of this modulator to counteract the proarrhythmic effects of unintentional K_v11.1 blockers should be further investigated in human subjects due to a species difference in K_v11.1 characteristics between humans and rats.[1-3] Recent access to human-induced pluripotent stem cell (hiPSC) derived cardiomyocytes (hiPSC-CMs) has offered a window of opportunity to overcome this bottleneck induced by species-to-species differences. hiPSC-CMs have been demonstrated to exhibit the expected genomic, biochemical, mechanical and electrophysiological behavior of human cardiomyocytes as evidenced by RT-PCR, patch clamp, and/or immunofluorescence techniques.[2, 4-6] Most importantly, the I_{Kr} current mediated by the K_v11.1 channel has been observed in these hiPSC-CMs.[2, 5, 6] To date, different techniques have been explored to assess the drug-induced electrophysiological alterations of hiPSC-CMs, including whole-cell patch clamp, impedance-based cellular and multi-electrode array (MEA) assays.[7, 8] For example, the cardiotoxic effects of selective K_v11.1 blockers E-4031 and dofetilide were successfully detected with the label-free xCELLigence RTCA Cardio system,[9] and the MEA assays have been widely utilized to screen the K_v11.1 liability of compounds as well.[8, 10] In this context, the impedance-based xCELLigence system or MEA

technique can be introduced to further investigate the antiarrhythmic effects of LUF7244 in a hiPSC-MC model. In addition, a simple and low-cost integrated platform has been recently reported to mimic the functional properties of native heart by creating large areas of aligned hiPSC-CMs.[11] Thus, the latter platform may produce more clinically and physiologically relevant data for drug screening, which can be applied to the follow-up studies on allosteric modulation of the K_v11.1 channel. Interestingly, K_v7.1 and K_v11.1 activators have shown compensatory effects on long QT syndromes (LQTSs) caused by dysfunctions of either channel type.[13-15] Recently, Zhang *et al.* established a patient- and disease-specific model with hiPSC-CMs from family members affected by the K_v7.1-mediated LQTS type 1, and found that modulation of the K_v11.1 channel by ML-T531 restored the action potential prolongation caused by this inherited dysfunction of the K_v7.1 channel.[16] As ML-T531 was identified as a negative allosteric modulator in **Chapter 4** of this thesis, K_v11.1 modulators are supposed to possess antiarrhythmic roles in congenital cardiac diseases.[16] Therefore, hiPSC-CM models with different patient-specific LQTSs can be used to evaluate the proarrhythmic propensities of LUF7244 and other more potent modulators like **7f** and **7p**. Taken together, negative allosteric modulators of the K_v11.1 channel can enable the rescue of inherited LQTSs alone as well as prevent drug-induced arrhythmias via combination therapy, making them an attractive and promising therapy in the future.

The hiPSC-CM differentiation system mentioned above has brought unique *in vitro* cellular- or tissue-based assays for the future development of K_v11.1 modulators as a novel alternative to current antiarrhythmic treatments. However, a myriad of *ex vivo* and *in vivo* preclinical studies like Langendorff and animal experiments as well as clinical evaluations are still required to eventually translate our negative allosteric modulators into a new class of therapeutic agents for both acquired and inherited arrhythmias. Currently, a zebrafish model of K_v11.1-mediated LQTS type 2 has been used as a high-throughput screening approach for compounds that are able to reverse the lengthened myocardial repolarization.[12] This high-throughput *in vivo* functional whole-organism model provides an efficient opportunity for the future preclinical investigations of K_v11.1 modulators.

Of note, the current generation of negative allosteric modulators of the K_v11.1 channel are not very potent (active in the micromolar range), which may raise concerns about their specificity and potential for cardiac and non-cardiac side effects. Thus, the characterization of the binding site of these modulators at the channel will be of particular interest in follow-up studies to facilitate the development of more potent and specific modulators. Here, computational methods, mutational studies and crystallization of the K_v11.1 channel will bring useful in-

formation for such research in the future.[17, 18] Furthermore, more potent allosteric modulators can be synthesized according to the structure-activity relationships concluded in **Chapter 5**.

Binding kinetics of K_v11.1 blockers

Ligand binding kinetics in patch clamp assays

Radioligand binding assays are high-throughput and useful in the early assessment of ligand binding to the K_v11.1 channel, yet they do not provide a functional endpoint.[19, 20] Conversely, voltage patch clamp assays have capabilities to produce the highest quality and most physiologically relevant data of precise and detailed activity of the channel function among all available methods, albeit with relatively low throughput.[19, 21] Historically, potency of K_v11.1 blockers has been measured using patch clamp techniques due to the mandatory requirements of the ICH S7B guideline, while the binding kinetics of drugs at the channel were not.[19, 22-24] Thus, it appears attractive and meaningful to establish patch clamp assays that can be used to directly determine the binding and unbinding kinetics of ligands at the K_v11.1 channel. In **Chapter 7**, two compounds with distinguished kinetic parameters, in particular RTs, were selected to evaluate their binding kinetics in the whole-cell patch clamp assay. Although the K_v11.1 current inhibition rates of tested compounds were in the same rank order as their association rates derived from the radioligand binding assay, the I_{Kr} currents displayed insignificant recovery from the inhibition by both ligands during the washout experiments (data not shown). Subsequently, whole-cell radioligand binding assays, which are different from membrane radioligand binding assays used in the whole thesis, were performed in cells with or without expression of the K_v11.1 channel. In this case, [^{3}H]dofetilide exhibited comparable binding at empty HEK293 cells to that at HEK293 cells expressing the K_v11.1 channel (data not shown). Collectively, we speculated that these K_v11.1 blockers with an intracellular binding site at the channel could be trapped within the cell after diffusing through the cell membrane, which made the direct measurement of ligand's unbinding kinetics impossible. Therefore, a so-called inside-out patch clamp recording without drug trapping phenomena will be particularly useful to verify our speculation, as this technique will allow for accurately determining the "true" binding kinetics of ligands with an intracellular binding pocket at the K_v11.1 channel.[25]

Ex vivo and *in vivo* investigations

The categorization of all K_v11.1 blockers in **Chapter 7** is relatively arbitrary due

to the lack of arrhythmogenic data for the screened compounds. Monophasic action potentials of isolated Langendorff-perfused heart preparations can be recorded to assess *ex vivo* proarrhythmic liability of K_v11.1 blockers,[26-28] and various animal models such as AV dogs and rabbits are widely applied to evaluate the *in vivo* arrhythmic propensities of drug candidates.[29, 30] Accordingly, the K_v11.1 blockers from **Chapter 7** should be further assessed in these different *ex vivo* and *in vivo* experiments in order to define a more educated and reliable "k_{on}-k_{off}-K_D" map that allows for a better indication of torsadogenic risks of compounds. Notably, *ex vivo* and *in vivo* preclinical studies are commonly carried out on animal hearts or whole animals that are unlikely to mimic diseased human subjects and therefore might not be predictive of the clinical situation.[3, 27, 31, 32] Hence, this kinetic map could be even further improved by incorporating affinity and kinetic parameters of more reference drugs like astemizole, ranolazine and dofetilide, which are selective K_v11.1 blockers and have already been assessed for their proarrhythmic profiles in clinical trials. Ultimately, a more complete translational model based on all *in vitro*, *ex vivo* and *in vivo* data can hopefully be elucidated to improve the predictive index of K_v11.1 liability of drug candidates in the future.

Rechanneling the K_v11.1 liability paradigm

The current assessment of proarrhythmic risks of K_v11.1 blockers is predominantly dependent on their IC_{50} values.[3, 33] However, this approach does not take into account drug binding kinetics, which can have a major impact on cardiac arrhythmias of drug candidates.[24, 34, 35] In 2013 a Comprehensive *in vitro* Proarrhythmia Assay (CiPA) was proposed as a new paradigm to replace the current regulatory strategy by the Cardiac Safety Research Consortium, and the CiPA was expected to be operational worldwide by July 2015.[34, 36, 37] In this new CiPA paradigm *in silico* modeling is one of the most important components. In this regard, the best simulation model is suggested to be selected before the release of the CiPA assay, and more sophisticated models that include both simple channel blockade affinity and additional drug binding kinetics are inferred to significantly improve the proarrhythmic assessment for drug candidates.[36] Thus, incorporating association and dissociation rates of e.g., the compounds from **Chapter 6** and **7** with their binding affinity may lead to a more efficient, readily applicable computational model for the forthcoming CiPA implementation. As a branch of this extended research, a large library of K_v11.1 blockers with varied affinity and kinetic parameters should also be synthesized and evaluated to provide enough data for the representative *in silico* model in the near future. Hopefully, the ultimate, optimized CiPA paradigm will demonstrate much better predictive value for proarrhythmic risks of pharmaceutical products, compared to the traditional

strategy recommended by ICH S7A and S7B guidelines.[38, 39]

Final note

K_v11.1-induced cardiotoxicity has emerged as an unanticipated adverse effect of many pharmacological agents and has become a major obstacle in drug development over the past decades. In this thesis, allosteric modulation of the K_v11.1 channel has been extensively explored, and negative allosteric modulators were shown to relieve the proarrhythmic effects of structurally and therapeutically diverse K_v11.1 blockers. The most potent modulators can be further assessed in *in vitro* assays based on hiPSC-CM models, *ex vivo* and *in vivo* studies, and may be developed as a new class of antiarrhythmic medications in the future. On the other hand, kinetic binding parameters of a wide range of K_v11.1 blockers at the channel have been thoroughly investigated in this thesis. Association and dissociation rates or residence times are strongly suggested to be integrated with equilibrium affinity values in the future strategies for a better and more comprehensive evaluation of K_v11.1 liability of drug candidates. The "k_{on}-k_{off}-K_D" kinetic map provides a first and promising classification of K_v11.1 blockers, which could be beneficial and indicative for drug researchers to design compounds with less K_v11.1-mediated cardiac side effects in the early stage of drug development. Functionally determining the kinetic parameters of K_v11.1 blockers at the channel, e.g., by using inside-out patch clamp assays, will facilitate the incorporation of ligand-channel binding kinetics into the novel CiPA paradigm. Hopefully, all findings in this thesis have brought new insights into K_v11.1-induced cardiac arrhythmias, and will offer opportunities for restoring or preventing this kind of arrhythmias in the near future.

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