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„Examination of polyunsaturated fatty acids modulating the hERG and hEAG Potassium Channel“

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Declaration in lieu of oath

I hereby declare in lieu of an oath that this Diploma thesis has been written only by the undersigned and without any assistance from third parties. I confirm that thoughts taken directly or indirectly from external source are indicated with references in the text.

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Vienna on 18.10.2021

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1. TABLE OF CONTENT

2. ABSTRACT (ENGLISH)

The hERG channel (referred to as human ether-a-go-go related gene), is a voltage dependent, inward rectifiying potassium channel in cardiomyocytes and neurons of the nucleus subthalamicus, which has an important role in cardiac action potential repolarization. Because of the high cardiotoxic potential of hERG blockers, it is fundamental to understand the molecular mechanisms involved in the blocking of hERG channels. Drugs that interact and block the hERG channel are leading to a longer repolarization of the action potential and to long-QT-syndrome, which may result in torsade the pointes arrythmia. Many drugs have been withdrawn from the market, nowadays all drugs need to be tested during the registration process.

The current working hypothesis for inhibiting the hERG channel function is based on results obtained from molecular dynamic simulations based on the cryo-EM structure of hERG from Wang and McKinnon published in 2017. In 2020 the first cyro-EM structure of the hERG in a complex with the blocker astemizole was proposed by Asai et al.

Overall, however putative binding sites as well as structure-activity relationships of hERG-drug-interactions often remain incompletely characterized and contradictory in some cases.

An interesting study by Guizy et al in 2005 showed that arachidonic acid inhibits the hERG channel but activates the hEAG channel, which is also a voltage gated potassium channel with similarity to the hERG channel.

In this diploma thesis computational methods like docking have been used to investigate the possible binding site and interactions of arachidonic acid on the hERG channel.

As the putative binding site of arachidonic acid in Kv1.5, another voltage gated potassium channel has already been reported, it has been investigated in this study using multiple sequence alignment, if arachidonic acid could bind to the same site in the hERG (Kv11.1) and hEAG (Kv10.1), but the conservation of this binding site was very poor, therefore the next step was to simulate the docking of arachidonic acid on Kv10.1 and Kv11.1.

The results showed no correlation of the amino acids in the binding site in Kv1.5 and Kv10.1 or Kv11.1. An alignment of Kv11.1 and Kv10.1 showed that the conservation of the binding site of arachidonic acid is well. The putative binding site of arachidonic acid in Kv11.1 seems to be a bit more conserved than the putative binding site in the Kv10.1.

Within the frame of this diploma thesis the possible binding site and interactions of acid arachidonic acid on the hERG and hEAG channels using docking have been predicted. Lack of conservation of 4 amino acids in the predicted binding site, provide a possible explanation for the different effects of arachidonic acid on the two channels.

Further research is needed to validate the modelling predictions of this diploma thesis.

3. KURZFASSUNG (DEUTSCH)

Der hERG-Kanal (human ether-a-go-go related gene) ist ein spannungsabhängiger, nach innen gleichrichtender Kaliumkanal in Kardiomyozyten und Neuronen des Nucleus subthalamicus, er spielt eine wichtige Rolle bei der Repolarisation des Herzaktionspotentials. Wirkstoffe, die den hERG-Kanal blockieren, führen zu einer verlängerten Repolarisation des Aktionspotentials und dadurch zum Long-QT-Syndrom, welches zur schwerwiegenden Torsade der Pointes-Arrythmie führen kann. Aufgrund des hohen kardiotoxischen Potenzials von hERG-Blockern ist es von grundlegender Bedeutung, die molekularen Mechanismen zu verstehen, die an der Blockierung von hERG-Kanälen beteiligt sind. Da viele Medikamente aufgrund der Blockierung des hERG-Kanals nachträglich vom Markt genommen werden mussten ist heutzutage eine Testung aller Wirkstoffkandidaten vor der Marktzulassung vorgeschrieben.

Die aktuellen Forschungshypothesen zur Hemmung der hERG-Kanalfunktion basieren auf Ergebnissen molekulardynamischer Simulationen, basierend auf der 2017 veröffentlichten Kryo-EM-Struktur vom hERG von Wang und McKinnon. 2020 wurde die erste Cyro-EM-Struktur des hERG-Kanals in einem Komplex mit dem Blocker Astemizol von Asai et al veröffentlicht.

Insgesamt sind mutmaßliche Bindungsstellen sowie Struktur-Aktivitäts-Beziehungen von hERG-Arzneistoff-Interaktionen in einigen Fällen noch unvollständig charakterisiert und widersprüchlich. Eine interessante Studie von Guizy et al. aus dem Jahr 2005 zeigte, dass Arachidonsäure den hERG-Kanal hemmt, aber den hEAG-Kanal aktiviert, welcher ebenfalls ein spannungsgesteuerter Kaliumkanal ist und dem hERG-Kanal ähnelt.

In dieser Diplomarbeit wurden Berechnungsmethoden wie das Docking verwendet, um die mögliche Bindungsstelle und Wechselwirkungen von Arachidonsäure auf dem hERG-Kanal zu untersuchen. Da über die mutmaßliche Bindungsstelle von Arachidonsäure im Kv1.5, einem anderen spannungsgesteuerten Kaliumkanal bereits berichtet wurde, wurde in dieser Studie durch Multisequence-Alignment untersucht, ob Arachidonsäure an derselben Stelle im hERG (Kv11.1) als auch hEAG (Kv10.1) binden könnte, dies wurde durch die schlechte Konservierung der Bindungsstelle jedoch ausgeschlossen. Daher war der nächste Schritt, das Andocken von Arachidonsäure an Kv10.1 und Kv11.1 durch Docking zu simulieren. Die Ergebnisse des Dockings zeigten keine Korrelation der Aminosäuren an der Bindungsstelle in Kv1.5 und Kv10.1 oder Kv11.1. Ein Alignment von Kv11.1 und Kv10.1 zeigte jedoch, dass die Bindungsstelle von Arachidonsäure gut konserviert ist. Die mutmaßliche Bindungsstelle von Arachidonsäure in Kv11.1 scheint etwas konservierter zu sein als die mutmaßliche Bindungsstelle in Kv10.1. Im Rahmen dieser Diplomarbeit wurden mögliche Bindungsstellen und Wechselwirkungen von Arachidonsäure mit dem hERG-Kanal mittels Docking vorhergesagt. Die fehlende Konservierung von 4 Aminosäuren innerhalb der mutmaßlichen Bindungsstelle könnte eine mögliche Erklärung für die unterschiedlichen Effekte von Arachidonsäure auf diese zwei Kanäle bieten. Um die Ergebnisse der Molekül-Simulationen in dieser Diplomarbeit zu bestätigen, sind weitere Untersuchungen notwendig.

4. ABBREVIATIONS

Cryo-EM	cryogenic electron microscopy
hERG	human ether a go go related gene
Kv10.1	hEAG channel
Kv11.1	hERG channel
Ca^{2+}	calcium ions
LQTS	long QT syndrome
ECG	electrocardiogram
AA	arachidonic acid
TdP	torsade de pointes
SIDS	sudden infant death syndrome
K^{+}	potassium
I_{Kr}	delayed rectifier potassium channel
ERG	EAG-related gene
ELK	EAG-like potassium
PAS	per-Arnt-Sim
CaM	calmodulin
EMBL-EBI	the European Bioinformatics Institute
bEAG	Bovine ether-a-go-go

4.1. AMINO ACID ABBREVIATIONS

A	Ala	Alanine
R	Arg	Arginine
N	Asn	Asparagine
D	Asp	aspartic acid
C	Cys	Cysteine
Q	Gln	Glutamine
E	Glu	glutamic acid
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
L	Leu	Leucine
K	Lys	Lysine
M	Met	Methionine
F	Phe	Phenylalanine
P	Pro	Proline
S	Ser	Serine
T	Thr	Threonine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
V	Val	Valine

5. INTRODUCTION

hERG potassium channels play a critical role in the normal electrical activity of the heart and have been an interesting anti-target in order to develop safer drugs without the propensity to cause cardiac arrhytmias. Pharmaceutical companies have to discard a large number of potentially useful compunds during the late development stages and the screening-based approach is very expensive and consumes a lot of chemistry resources. In this diploma thesis the theory that lipid-based drugs could compensate the blocking effect of hERG blocking drugs is closer investigated. The aim of this diploma thesis is to discover the putative binding site of arachidonic acid in the hERG channel and to find out the important role of unsaturated fatty acids which modulate the hERG channel.

5.1. Kv channels

Potassium selective channels have a large molecular channel diversity which is mirrored by many different physiological functions such as modulation of the resting potential and action potential duration of neurons, myocytes and endocrine cells. Kv channels stabilize the membrane potential of excitable and nonexcitable cells. Differental cellular expression of K^+ channels determine the specific electrical responses to stimuli in a particular cell or tissue. Mutations in genes encoding K^+ channels cause congenital disorders such as long QT syndrome, Bartters syndrome and episodic ataxia with myokymia.

5.1.1. The hEAG channel/Kv10.1

The hEAG (human ether-à-go-go gene) channel is a voltage-dependent K^+ channel, also referred to as human Kv10.1 and belongs to the ether à go-go potassium channel family besides the EAG-related gene (ERG), and EAG-like potassium (ELK) channels. EAG channels play a crucial role in cardiac repolarization, but relatively less is known about their physiological role. The Kv10.1 channel is mainly expressed in the mammalian brain, particularly the hippocampus, cerebellum, and brain stem and its ectopic expression occurs in 70% of human cancers.

Like other voltage-gated K^+ channels, the central pore of hEAG channels is formed by the tetrameric assembly of S5–S6 helices and is surrounded by voltage sensor domains formed by S1–S4. The N terminus of hEAG1 contains an eag domain, which is unique to the EAG channel family and contains a Per-Arnt-Sim (PAS) homology domain, which plays a key role in mediating protein-protein interactions [1].

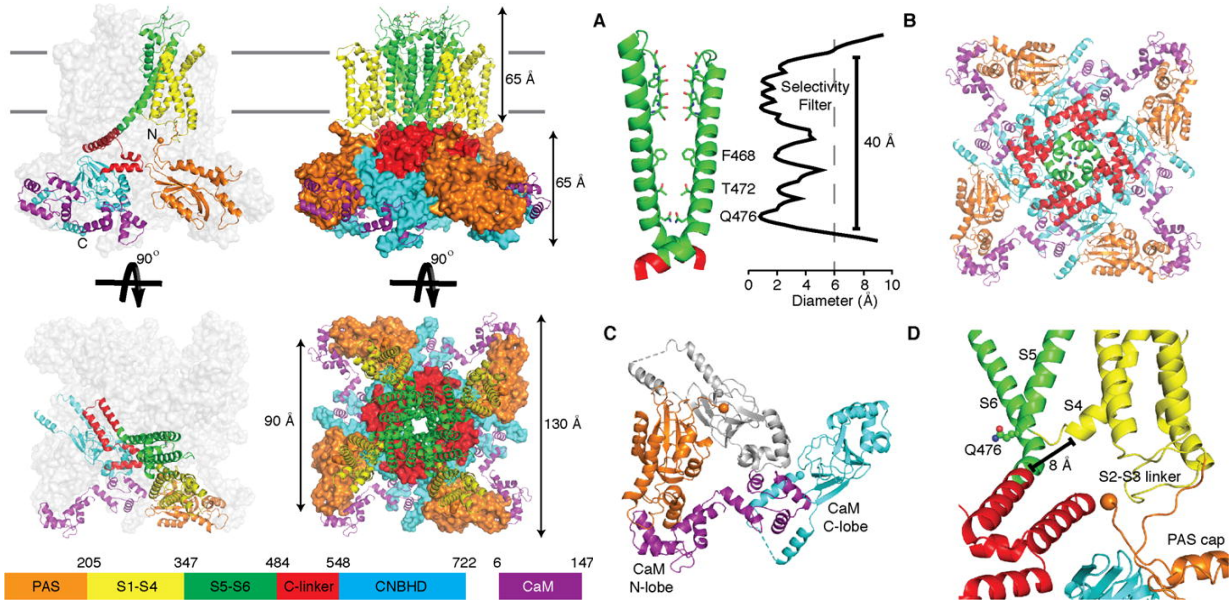

Figure 1: Model of hEAG1 bound to CaM [2] https://science.sciencemag.org/content/353/6300/664 with permission from the American Association for the Advancement of Science

In the search for novel anticancer drugs, a new group of Kv10.1 inhibitors have been reported, the purpurealidin analogs, which are bromotyrosine alkaloids produced by the Verongida sponges. In a study published in 2017 from Moreels et al, 5 potent analogs were closer investigated to the mechanism of action and their effects on cancerous and non-cancerous cell lines. Compound 5, a 3-chloro-4-methoxyphenyl derivative, the most potent Kv10.1 modulator in this study showed to be cytotoxic and induced apoptosis in all evaluated cell lines. The inhibitory effect of compound 5 is concentration- and voltage-dependent and indicates that compound 5 induces an apparent open-state inactivation upon prolonged depolarizations and also affects the gating of the channel at hyperpolarized potentials. The binding site has been suggested in the S1-S4 voltage sensor module, near the binding site of mibefradil, which is also a gating modifier of Kv10.1 and in addition a calcium channel antagonist. Similar effects like the shift of the activation curve to the left, a decreased rate limiting step of Kv10.1 activation and facilitation of activation, were observed between Compound 5 and mibefradil, but the hypothesis that indicates that the binding site of compound 5 on Kv10.1 overlaps with the binding site of mibefradil still needs to be confirmed by site-directed mutagenesis studies [3].

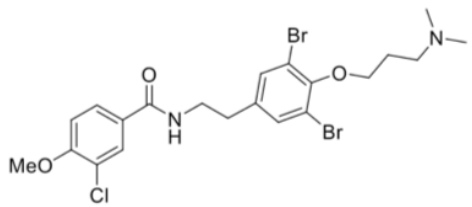

Figure 2: Compound 5 (3-Chloro-N-[3,5-dibromo-4-[3-(dimethylamino)propoxy]phenethyl]- 4-methoxybenzamide), a concentration- and voltage dependent inhibitor of Kv10.1 and possible lead compound for anticancer drugs. [3] https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0188811 with the permission from Moreels et al. (2017)

An interesting study by Garg et al in 2013 discovered that the compound ICA-105574 (3-Nitro-N-(-4-phenoxyphenyl)-benzamide or ICA) which causes a shortening in the duration of cardiac action potentials, has opposite effects on the inactivation of the hEAG and hERG channel. When ICA binds to the hERG channel, its inactivation is attenuated by a shifting of its voltage dependence to more positive potentials while if ICA binds to the hEAG its inactivation is enhanced without an alteration in its voltage dependence. The inhibitory effect of ICA on hEAG is more potent than the activation effect of ICA on hERG channels. The ICA binding sites of both channels were investigated by molecular modeling and site directed mutagenesis. The putative binding site of ICA have been suggested in a hydrophobic pocket in the S5/pore helix /S6 of one subunit and S6 segment neighboring subunit. Experiments with hERG/bEAG chimeras showed that if the pore region (S45 linker-S6) was contributed by hERG, ICA was an agonist, while if the same pore region was contributed by bEAG, ICA was an antagonist. This indicates that the pore domain (S5-S6) region determines the different response to ICA. Because all amino acids except of three amino acids (M431, M458, L463) in the binding site of ICA are conserved in the hEAG channel, it is very likely that ICA binds to the same binding site while causing different effects. But the binding pockets for ICA have a different shape due to differences in the sequence of hEAG and hERG, for example ICA protrudes deeply into the binding pocket in the hERG, while in the hEAG Tyr464 forms a barrier at the S6-S6 interface, not allowing a deeper binding mode.
Mutations of the 3 key residues M431F, M458L, L463M were introduced into the hEAG to render the shape of the binding site to be more hERG-like, however, the functional response of ICA didn't change. All findings of the Garg et al study came to the conclusion that, not differences in the binding determine whether ICA is a channel antagonist (hEAG) or agonist (hERG), but intrinsic differences in the mechanism of slow (hEAG) versus fast (hERG) inactivation gating [4].

5.1.2. The hERG channel/Kv11.1

The Kv11.1 or hERG (referred to as human ether-a-go-go related gene), is a voltage dependent, inward rectifiying potassium channel in cardiomyocytes and neurons of the nucleus subthalamicus. It has an important role in cardiac action potential repolarization, because it forms the major portion of the rapid delayed rectifier current (I_{Kr}), which is critical in correctly timing the repolarization of cardiac action potentials, if the repolarization is changed by mutations or pharmacological inhibitions of the hERG, it can cause long- or short-QT-syndrome, which leads to torsade the pointes, a lethal cardiac arrythmia [5]. The molecular structure of hERG has a central pore domain surrounded by four voltage-sensor domains, each subunit of this tetramer has 6 transmembrane helices, the S5-S6 are the pore forming regions and the S1-S4 helix bundles constitute the peripheral voltage-sensor domains, which sense the changes in the transmembrane potential and trigger the conformational change responsible for opening and

closing the pore. There are 4 to 7 positively charged amino acids in the S4, that sense the electric field and move in response to it. Under depolarizing conditions, S4 transfers upwards, resulting in the channel's activation and open conformation of the channel. When the membrane is hyperpolarized, the electric field causes S4 to move outwards, leading to the channel's deactivation and pore closure.

A cryo-electron microscopy structure of the open gated conformation by Wang et al [5] may explain the unusual sensitivity to many drugs. There are four elongated, relatively hydrophobic pockets that extend from the central cavity, and are not present in other K+ channels, and do only exist in hERG because the S6 inner helix is displaced to create a separation between the pore helix and S6 helix. Particular amino acids which apparently form the high-affinity drug binding site in hERG, according to mutagenesis studies, are located on the surface of these four deep hydrophobic pockets in the central cavity. The hydrophobic pockets are roughly cylindrical in shape with a diameter of about 8 Å and a depth about 11 Å. The substituted aromatic rings of astemizole and dofetilide, which are hERG blocking drugs, fit snugly into the pockets. Although astemizole and dofetilide are more than 20 Å in the longest dimension and around 3~5 Å in the narrowest dimension, it occurs, these drugs occupy the center of the cavity and insert a functional group into one of the hydrophic pockets [5].

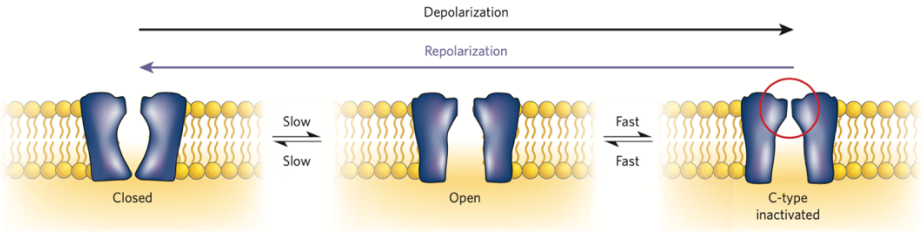

Figure 3: Conformation of a single hERG channel is voltage dependent (Sanguinetti M.C., 2006) [6] https://www.researchgate.net/publication/236016633_HERG_potassium_channels_and_cardiac_arrhythmia with the permission from Springer Nature

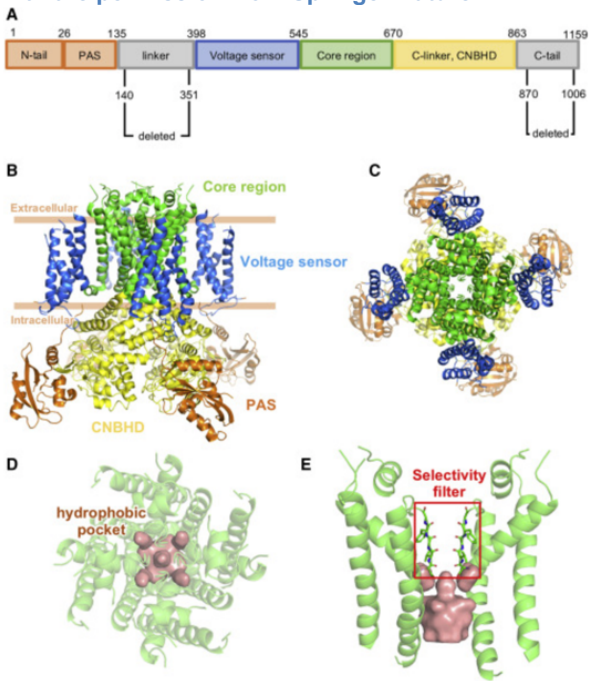

Figure 4: Construct and overall architecture of hERGT solved in previous studies (Asai et al., 2021) [7] https://pubmed.ncbi.nlm.nih.gov/33450182/ with the permission from Elsevier Ltd. (2020)

5.2. Long QT syndrome (LQTS)

The long QT syndrome is a life-threatening disease and predisposes to fatal cardiac arrythmias. The basic defect is a delay in ventricular repolarization that leads to a prolongation of the QT interval on electrocardiogram (ECG). Patients can have intermittent ventricular arrhytmias, in particular torsade de pointes (TdP) and ventricular fibrillation resulting in syncope or sudden death, but many individuals remain completely asymptomatic. Torsade de pointes is a polymorphic ventricular tachycardia associated with LQTS, it is characterized by a twisting of the QRS axis around the isoelectric line of the body surface ECG.

LQTS can be triggered, among other things, by pharmacological side effects on cardiac ion channels, especially the hERG channel is often affected. The congenital LQTS is an autosomal dominant inherited disorder and one of the best studied cardiac dysfunctions. The congenital LQTS is caused by mutations in cardiac voltage gated sodium or potassium channels. These mutations can develop spontaneously, but in the most cases the mutation is being inherited. The hERG was mapped to human chromosome 7 which is linked to LQTS-2. Missense mutations in the hERG such as A561V, G628S and N470D have a dominant negative effect on I_{Kr} channels, N470D is also associated with LQTS-2. Seven forms of congenital LQTS (LQTS-1 to LQTS-7) have been discovered yet [Finlayson K. 2004]. The risk for drug induced LQTS is higher for patients with lower pulse rates, females, patients with hypokalemia, hypertrophy of the heart, hypertension, heart failure and higher drug levels due to pharmacogenetic causes. LQTS is a relatively common cause of often unexplained cases of sudden cardiac death and sudden infant death syndrome (SIDS). [6]

5.3. Free fatty acids

Free fatty acids play a major role as constituents in the lipid bilayer and the cellular adaption to the environment, but they also have, depending to their structure, the ability to modulate protein functions. The activity of many membrane proteins is determined by the lipid composition of the membrane and the physicochemical properties of free fatty acids.

Karnovsky used fluorescence measurements to find out that free fatty acids can induce membrane alterations, which can lead to conformational changes in membrane embedded proteins. Free fatty acids with a kink in the molecule as the cis-unsaturated fatty acids disorder the membranes interior, whereas saturated and trans-unsaturated fatty acids with a linear structure did not alter the bilayer core [8].

The high increase of free fatty acids under some pathological conditions, particularly in the brain, is assumed to be a protective mechanism, which is potentially operative on ion channels. [9] In an event of ischemia, hypoxia and kainite-induced epilepsy the intracellular pH is decreased and intracellular free fatty acids and Ca^{2+} concentrations are increased, which lead to the activation of phospholipases. A Study of Lauritzen et al in animal models has proved that

linolenic acid has neuroprotective and antiepileptical properties by increasing both, the intracellular pH and the free fatty acid concentration and cause a hyperpolarization and reduction of the Ca^{2+} influx, therefore it averts excitatory glutamatergic transmission and neuronal death. [10]
The effect of free fatty acids on ion channels correlates with the chemical structure. Examples of ion channels that are affected by unsaturated fatty acids, or both, saturated and unsaturated fatty acids or of which are only affected by cis-isomers or both, cis- and trans-isomers can be found in references Meyes et al [11], Ordway et al [12] and Sumida et al [13]. Although there are many studies concerning the relationship between free fatty acids and ion channels, a common mechanism of action has not been found, because in some cases the free fatty acids stimulate the functional properties of the protein and in other cases it inhibits the function, it is also not always relevant to a specific structure of free fatty acids. The modulation of ion channel proteins can be either affected directly, if the free fatty acid interacts with the ion channel protein, or indirectly, if the free fatty acid activates an intracellular cascade, after it is transformed into a biologically active metabolite. [12]

5.3.1. Arachidonic acid

Arachidonic acid (Figure 5) is an essential polyunsaturated omega-6 fatty acid which is found in animal and human fat as well as in the liver, brain, and glandular organs, and is a constituent of animal phosphatides. The all cis-5,8,11,14-eicosatetraenoic acid is formed by the synthesis from dietary linoleic acid and is a precursor in the biosynthesis of prostaglandins, thromboxanes, and leukotrienes.
As an integral constituent of biological cell membranes arachidonic acid conferes fluidity, flexibility and selective permeability to cell membranes of all mammalian cells. The highest proportion of arachidonic acid in phospholipids is up to 25% in platelets, mononuclear cells, neutrophils, liver, brain and muscle. Arachidonic acid has a proper function in the brain and in muscles as it has a protective potential against infections such as Schistosoma mansoni and S. haematobium infection and also against tumor initiation, development, and metastasis.
The concentration of free arachidonic acid in cells is at a very low level, which limits their availability to oxidation due to reacylation/diacylation cycles in cell membranes.
Oxidated arachidonic acid metabolites namely eicosanoids contribute to inflammation and lead to the generation of mediators such as prostaglandins PGE_2, PGI_2, lipoxin A_4 and leukotriene B_4 responsible for resolving inflammation and wound healing.
Endocannabinoids, arachidonic acid derivatives, play an important role for brain reward signaling, motivational processes, emotion, stress responses, pain, and energy balance.
Free arachidonic acid modulates the function of many voltage-gated ion channels including Na^+, K^+, Ca^{2+} and Cl^- ion channels and several receptors and enzymes via activation as well as

inhibition. As an example, arachidonic acid is an activator of the hEAG potassium channel, of the NADPH oxidase and membrane-associated, magnesium-dependent, neutral sphingomyelinases (nSMase), but it is an inhibitor of the hERG potassium channel. Arachidonic acid was also discovered to greatly enhance the functional activity of ligand-gated ion channels like the c-amino butyric acid receptor (GABA-R) located on the neuronal membrane, via modulating the GABA-R interaction characteristics with its ligands. Another study discovered that free arachidonic acid can inhibit the muscle and neuronal nicotinic acetylcholine receptor (nAChR) by displacing lipids in the plasma membrane and direct acting as an antagonist at the PUFA-protein interface [14].

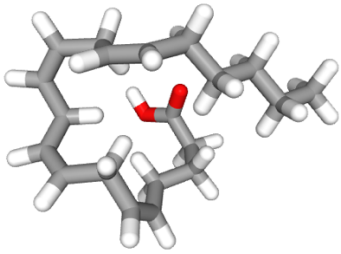

Figure 5: 3D structure image of arachidonic acid (PubChem Identifier CID: 444899) https://pubchem.ncbi.nlm.nih.gov/compound/Arachidonic-acid#section=3D-Conformer&fullscreen=true with permission from PubChem

5.4. the hERG interacts with unsaturated fatty acids

An early suggestion that PUFAs may bind directly to voltage-gated ion channels came from experiments on NaV channels in which the PUFA eicosapentaenoic acid inhibited the binding of a radio-labeled toxin to cardiac NaV channels [15]. Further evidence that PUFAs have direct ion channel effects is provided by the demonstration that single point mutations in various voltage-gated ion channels also affects the ability of PUFAs to modulate those channels [16]. Furthermore, five different PUFA-binding sites in voltage-gated ion channels have been suggested, which can exist in a single ion channel and the overall effect is determined by the relative contribution of the five sites. [Figure 6] The first PUFA site is in the intracellular cavity, where the binding of PUFA reduces the current, sometimes as a time-dependent block, inducing an apparent inactivation. The second PUFA site is at the extracellular entrance to the pore, it also leads to a block of the channel. The third PUFA site is the intracellular gate, the binding to this site can bend the gate open and increase the current. The fourth PUFA site is the interface between the extracellular leaflet of the lipid bilayer and the voltage-sensor domain, binding to this site leads to an opening of the channel via an electrostatic attraction between the negatively charged PUFA and the positively charged voltage sensor. The fifth PUFA site, which is the interface between the extracellular leaflet of the lipid bilayer and the pore domain can regulate slow inactivation by acting on distance. [17]

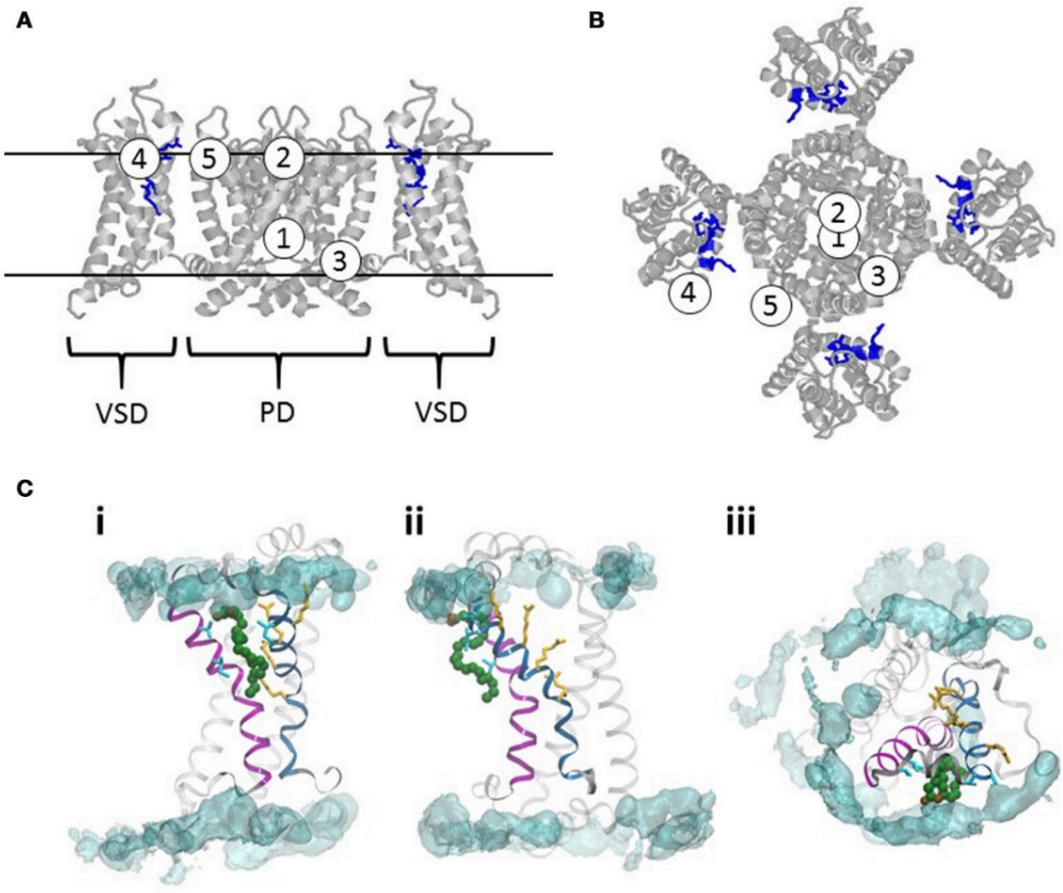

Figure 6: Sites of actions of PUFAs on voltage-gated ion channels
(A): A homology model of the Shaker Kv channel based on the structure of the KV2.1/1.2 chimera (Long et al., 2007; Henrion et al., 2012). The Figures 1–5 denote five proposed sites of actions of PUFA. (B): Top view of the channel in (A). (C) Interaction site for a DHA molecule with the VSD of the Shaker Kv channel. The helix in magenta is S3 and the helix in blue is S4. The four yellow amino acid residues are the four gating charges [R362 (in the top), R365, R368, and R371]. The four residues in cyan (two in S3, residues 325 and 329; two in S4, 359 and 360) are the residues identified to be close to the PUFA binding site (Börjesson and Elinder, 2011). A typical binding pose for a DHA molecule in green is from Yazdi et al. (2016). [17]
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5292575/ with permission from Elinder and Liin (2017)

In a study from Guizy et al [18] Arachidonic acid and docosahexaenoic acid induced a use-dependent inhibition of hERG channels stably expressed in Chinese hamster ovary cells by using the whole cell patch-clamp technique. The observed blocking effect of hERG was depending on time, voltage and channel conformational state. At 10 μM, arachidonic acid and docosahexaenoic acid blocked hERG channels, at the end of 5-s pulses to -10 mV, to a similar extent. The arachidonic acids nonmetabolizable analog 5,6,11,14-eicosatetrayenoic acid inhibited hERG current similar to the inhibition produced by arachidonic acid at the same concentration. Moreover, the kinetics of block were also similar, therefore there must be a direct mechanism and not due to arachidonic acid metabolites. The general properties of fatty acids which are often described to induce the same effects as the described PUFAs are at least two double bonds in the acyl tail with cis-geometry and a negative charge of the carboxyl group. Saturated fatty acids and monounsaturated fatty acids or unsaturated fatty acids with trans-geometry double bonds were ineffective. Docosahexaenoic acid and arachidonic acid accelerated the deactivation kinetics and slowed the inactivation kinetics at potentials positive to +40 mV. The block induced by docosahexaenoic acid was higher when measured after applying

a pulse to -120 mV. As a result of docosahexaenoic acid induced a higher block when measured at the maximum peak current than at the end of 5-s depolarizing pulses, an open channel block is suggested. Another piece of evidence of an open channel interaction between both PUFAs and hERG is a negative shift of the activation curve, but due to changes in the channel gating parameters, it is advocated, that these free fatty acids also interact in the closed state. Both polyunsaturated fatty acids produced a positive shift in the inactivation curve, which could be explained either by stabilization of the open state of hERG channels or destabilizing the inactivation process. Generally, all these results suggest that both arachidonic acid and docosahexaenoic acid preferentially bind to the open state of hERG channels, and that DHA exhibits a higher affinity for this state of the channel. The inhibition of hERG could be the explanation of the antiarrythmic effects of arachidonic acid and docosahexanoic acid [18]. Contrary to the blocking effect of arachidonic acid on hERG, it has been reported that arachidonic acid activated human ether à go-go (hEAG) potassium channels expressed in CHO cells and 5,8,11,14-eicosatetraynoic acid (ETYA) also potentiated hEAG currents. The reason why free fatty acids inactivate hERG channels but activate hEAG channels must be in the different structure of the pore mouth. The activating effect of arachidonic acid on EAG channels has been associated with an increased tumor proliferative rate as hEAG channels are expressed in various tumoral tissues. [19]

6. AIM OF THE STUDY

The aim of the diploma thesis was to predict the putative binding site and possible interactions of arachidonic acid on the Kv11.1 using docking and to investigate why Kv10.1 and Kv11.1 are regulated differently by arachidonic acid, despite sharing high sequence and structural similarity.

7. METHODS

7.1. Docking

Docking is a method which predicts the preferred binding modes of a ligand with a protein of known three-dimensional structures. Docking programs use scoring functions and search algorithms to calculate and rank the preferred poses by the highest binding affinity, therefore docking can be used to find the best binding pose with one ligand and a protein, but also to find the best ligand, if more ligands are being docked.

7.2. Description of used programs

7.2.1. Clustal Omega

Clustal Omega is a multiple sequence alignment program that uses seeded guide trees and HMM profile-profile techniques to generate alignments between three or more sequences. It was used to align the putative binding site from AA in Kv1.5, Kv10.1 and Kv11.1.

7.2.2. GOLD

GOLD stands for “Genetic Optimization for Ligand Docking” and is a genetic algorithm for docking flexible ligands into protein binding sites. GOLD supports a lot of methods for modelling protein flexibility, like ensemble docking can be used to reflect different receptor binding site conformations and it provides receptor flexibility through side-chain flexibility. It is also used for virtual screening. The visualization of the results are being displayed with Hermes, a visualization program, which provides the graphical user interface for GOLD. All docking tasks in this diploma thesis were carried out by using GOLD.

7.2.3. PyMOL

PyMOL is an Open-Source molecular visualization system for 3D structures of biomolecules. I used it to display the 3D structures of the hERG and hEAG channel, while altering the unmodified PDB-files 5VA1 (Kv11.1) and 5K7L (Kv10.1) to prepare the structures for the docking with GOLD by removing unnecessary atoms or making a whole tetrameric structure out of 1 subunit.

7.2.4. VMD

VMD stands for “Visual Molecular Dynamics” and is a visual molecular visualizations program for displaying, modeling and analyzing large biomolecular structures and systems, such as proteins, nucleic acids, lipid bilayer assemblies, etc. It has a useful user interface and provides a wide range of options for highlighting, coloring and rendering a molecule: simple points and lines, CPK spheres and cylinders, licorice bonds, backbone tubes and ribbons, cartoon drawings, and many others. VMD can also be used to animate and analyze the trajectory of a molecular dynamics (MD) simulation by acting as a graphical front end for an external MD program.
VMD was used in this thesis for visualizing the docking results and to get the figures of arachidonic acid binding to the hERG and hEAG channel.

8. RESULTS

Because arachidonic acid also inhibits Kv1.5 channels and putative binding sites for AA on the human cardiac Kv1.5 channel have already been reported in experimental studies in 2015 [20], the first step to identify the binding site in hERG was to compare these amino acids and see their conservation in Kv10.1 and Kv11.1. The involved amino acids in the pore domain mediating the blocking action of AA on Kv1.5 channels are H463, T480, R487, I502, I508, V512 and V516. To compare a possible conservation of binding residues between Kv1.5, Kv10.1 and Kv11.1, a multiple sequence alignment has been performed with the protein sequences of the channels from UniProt and the multiple sequence alignment program Clustal Omega from the EMBL-EBI. Because the data was saved from UniProtKB (The European Bioinformatics Institute protein knowledgebase), where the entry names of the channels constists of the gene name and the organism, the alignment also shows the gene numbers and not the protein numbers.

Interestingly, the multiple sequence alignment showed that the binding site of arachidonic acid in Kv1.5 is not conserved, as seen in Figure 7, below. Remarkably, not a single amino acid, reported to be important for AA, is identical between Kv1.5 and the erg-like channels. However, the sequence conservation between the latter two is much higher, with only few non-conserved residues.

```
sp|P22460|KCNA5_HUMAN      LGLLIFFLFIGV----ILFSSAVYFAEA----------------------DNQGT----- 462
sp|O95259|KCNH1_HUMAN      YGAAVLVLLVCVFGLAAHWMACIWYSIGDYEIFDEDTKTIRNNSWLYQLAMDIGTPYQFN 433
sp|Q12809|KCNH2_HUMAN      YGAAVLFLLMCTFALIAHWLACIWYAIGNMEQPHMD----SRIGWLHNLGDQIGKPYNSS 600
                            *  ::.*:: .      : :.:::: .                      : *.

sp|P22460|KCNA5_HUMAN      ----------HFSSIPDAFWWAVVTMTTVGYGDMRPITVGGKIVGSLCAIAGVLTIAL 510
sp|O95259|KCNH1_HUMAN      GSGSGKWEGGPSKNSVYISSLYFTMTSLTSVGFGNIAPSTDIEKIFAVAIMMIGSLLYAT 493
sp|Q12809|KCNH2_HUMAN      GL------GGPSIKDKYVTALYFTFSSLTSVGFGNVSPNTNSEKIFSICVMLIGSLMYAS 654
                                        .    :::::. ::*:**:*:: * *   **..    : * *  *

sp|P22460|KCNA5_HUMAN      PVPVIVSNFNYFYHRETD-HEEPAVLKEEQGTQSQGPGLDRG----VQRKVSGSRGSFCK 565
sp|O95259|KCNH1_HUMAN      IFGNVTTIFQQMYANTNRYHEMLNSVRDFLKLYQVPKGLSERVMDYIVSTWSMSRGI--- 550
sp|Q12809|KCNH2_HUMAN      IFGNVSAIIQRLYSGTARYHTQMLRVREFIRFHQIPNPLRQRLEEYFQHAWSYTNGI--- 711
                            .  : : :: :*      *     :::     .    * .     .    * :.*
```

Figure 7: The alignment of the binding site in the Kv1.5 (KCNA5) with the Kv10.1 (KCNH1) and Kv11.1 (KCNH2)

Kv1.5	H463	T480	R487	I502	I508	V512	V516
Kv10.1	K446	S463	A470	M485	Y491	F495	T499
Kv11.1	I607	S624	S631	L646	Y652	F656	S660

Table 1: amino acids in the binding site of the Kv1.5 compared to the aligned amino acids in the Kv10.1 and Kv11.1

8.1. Docking simulation of arachidonic acid on Kv10.1 and Kv11.1

Nevertheless, we hypothesized that the central cavity could be the binding site for AA in Kv10.1 and Kv11.1. Thus, in a second step, the program GOLD was used for assessing the putative binding of arachidonic acid to the central cavity of Kv10.1 and Kv11.1 using docking. At first the cryo-EM structure of Kv11.1 (5va1) and Kv10.1 (5k7l) from the PDB were modified with the Text

Editor and in the Terminal to fully assemble the channels (4-fold symmetry). The ligand arachidonic acid was downloaded from the DrugBank (DB04557) as mol2-file. As binding site, the cavity was choosen, since this has been shown to be the binding site fo arachidonic acid in the related Kv1.5 channels. In the settings 30 runs have been set for both channels, the best PLP fitness in hERG was 70.47 and 59.23 in Kv10.1.

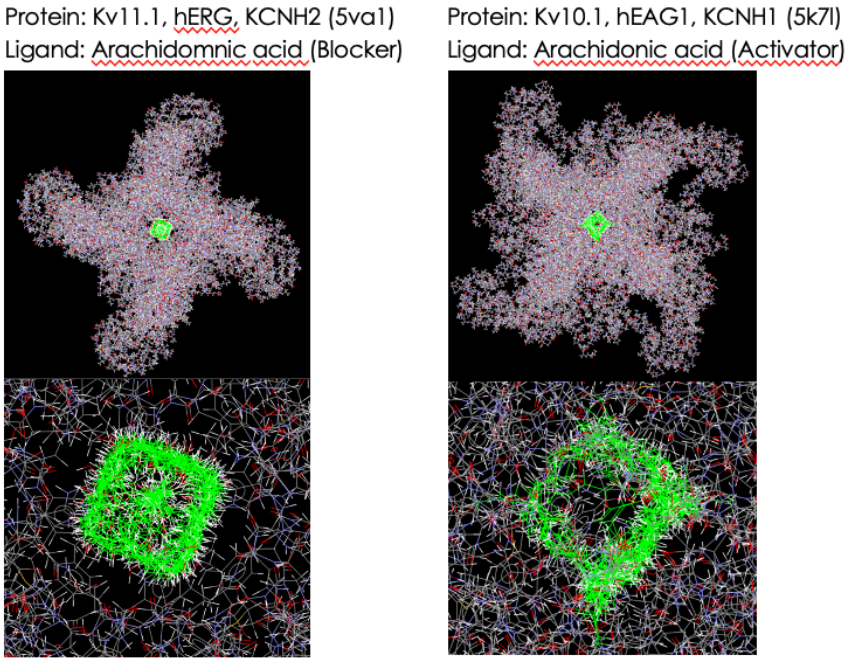

Figure 8: Docking simulation in GOLD of arachidonic acid in the hERG (left) and in the hEAG (right) channel

channel	Docking scores (highest pose)
Kv11.1	71.17
Kv10.1	59.23

Table 2: Docking scores of the highest poses from the docking simulation with GOLD in the Kv11.1 and Kv10.1

Overall, the drug fits well into the cavity of both cryo-EM structures, with very high docking scores of 71.17 in Kv11.1 and 59.23 in Kv10.1 respectively. Figure 7 shows all 30 poses in light green.

The docking showed other amino acids in the predicted binding site of arachidonic acid in the hERG and hEAG channel than in the Kv1.5, as shown in table 3 below. Residues predicted to form hydrogen bonds are shown in bold.

Kv10.1:	THR473, **GLN476**, GLN477, MET478, TYR479, A480, ASN481, **ASN483**, ARG484, HIS486, GLU487
Kv11.1:	**THR623**, **SER624**, SER649, TYR652, ALA653, PHE656, GLY657, SER660

Table 3: amino acids in the binding site of the hEAG (10.1) and the hERG (Kv11.1) channel

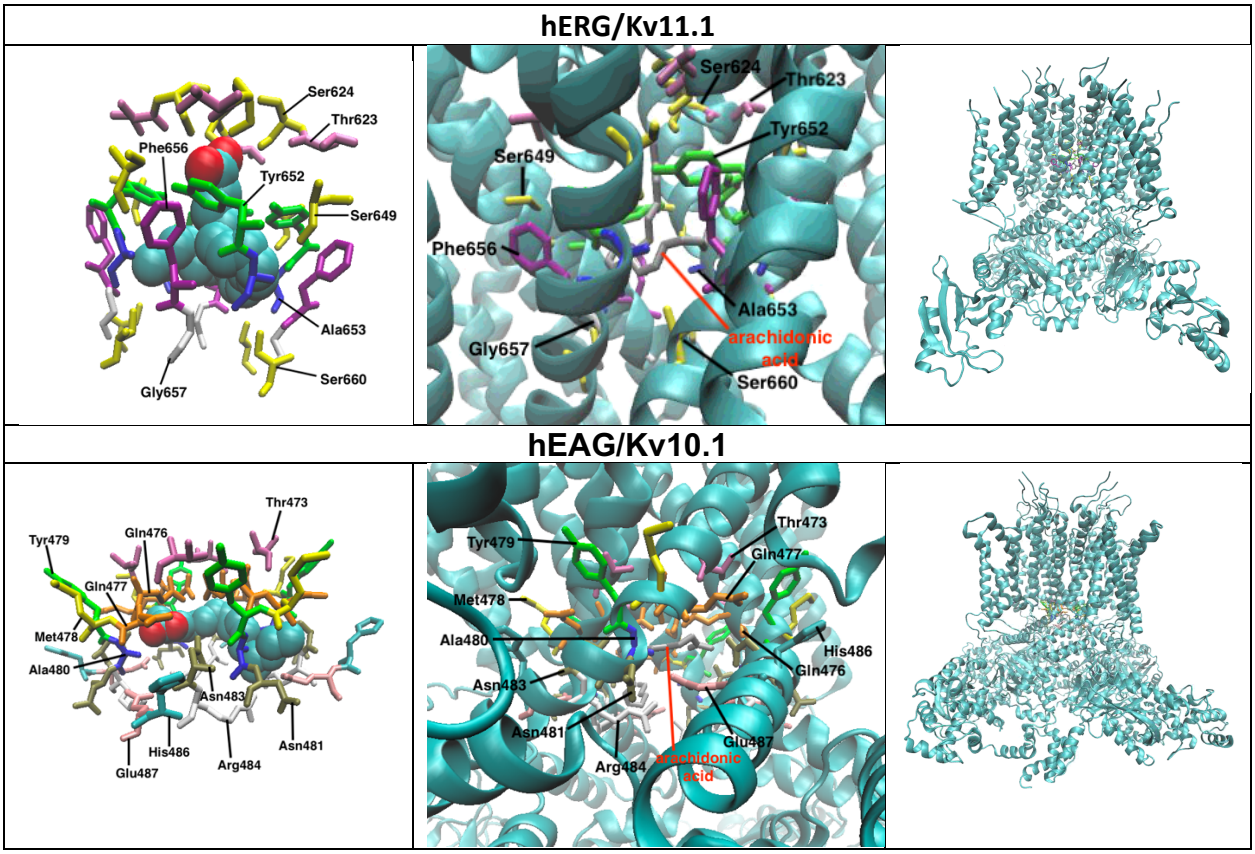

Figure 9: Visualisation with VMD of the Docking of arachidonic acid in the hERG and hEAG channel

To compare the conservation of the binding sites an alignment of the Kv11.1 and Kv10.1 with all amino acids in the binding site within 5Å was accomplished.

```
sp|Q12809|KCNH2_HUMAN      CIWYAIGNMEQPHMD----SRIGWLHNLGDQIGKPYNSSGL------GGPSIKDKYVTAL 615
sp|O95259|KCNH1_HUMAN      CIWYSIGDYEIFDEDTKTIRNNSWLYQLAMDIGTPYQFNGSGSGKWEGGPSKNSVYISSL 454
                           ****:**: *  . *     . .**::*. :**.**: .*       **** :. *:::*

sp|Q12809|KCNH2_HUMAN      YFTFSSLTSVGFGNVSPNTNSEKIFSICVMLIGSLMYASIFGNVSAIIQRLYSGTARYHT 675
sp|O95259|KCNH1_HUMAN      YFTMTSLTSVGFGNIAPSTDIEKIFAVAIMMIGSLLYATIFGNVTTIFQQMYANTNRYHE 514
                           ***::**********::*.*: ****::.:*:****:**:*****::*:*::*:.* ***
```

Figure 10: The conservation in Kv11.1 and Kv10.1 of both binding sites

In the binding site of the Kv11.1 all amino acids within 5A from the docking, except Ser660, are conserved: Thr623, Ser624, Ser649, Tyr652, Ala653, Phe656 and Gly657. The conserved amino acids in the Kv10.1 binding site are: Thr473, Gln476, Gln477, Met478, Tyr479, Arg484, His486 and Glu487, not conserved are Ala480, Asn48 and Asn483.

Because the used cryo-EM structure 5K7L of the Kv10.1 is from both organisms Rattus norvegicus and Homo sapiens, some of the amino acids (Gln476-E476, Gln477–Lys477, Met478-Ile478, Tyr479-Phe479, Arg484-Met484, His486-Ile486, Glu487-Gly487, Asn481-Val481 and Asn483-Ille483) differ from the human sequence in the alignment.

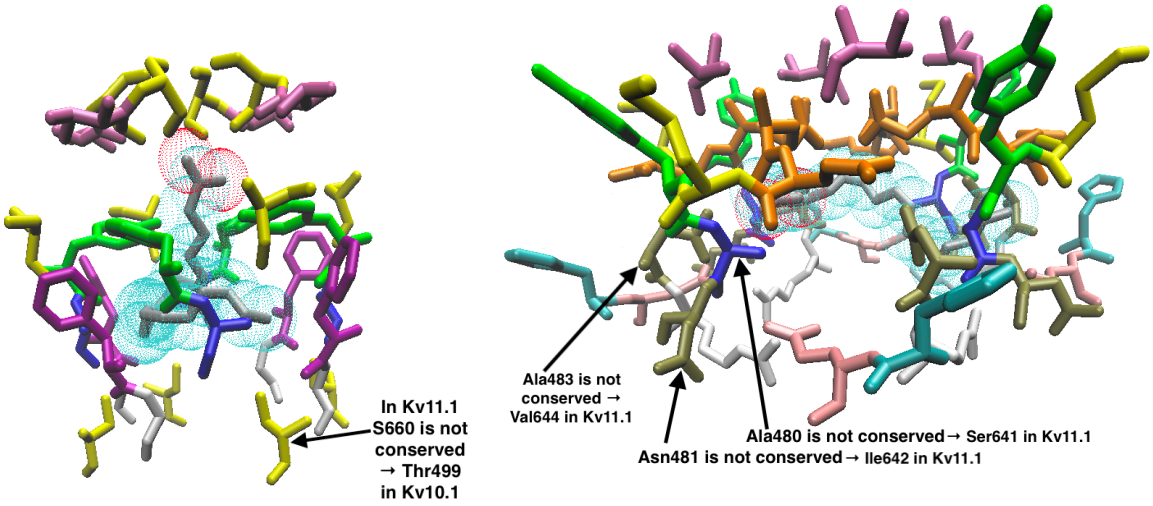

Figure 11: binding sites in Kv11.1 and Kv10.1 with the non-conserved amino acids

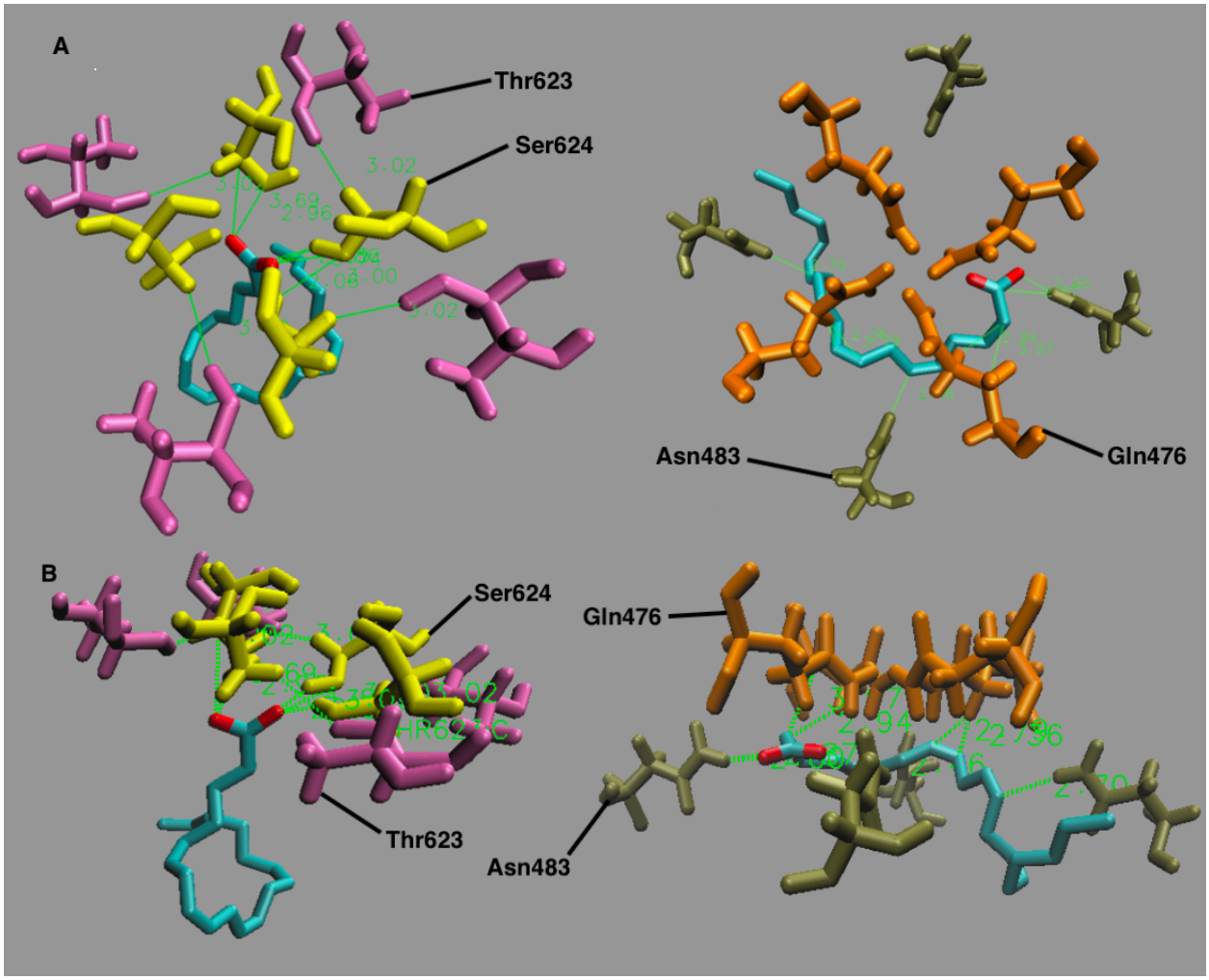

Figure 12: hydrogen-bonds between arachidonic acid and hERG (left) and between arachidonic acid and hEAG (right) A: overview B: side view

Interestingly, 4 amino acids, predicted to be in close contact with the lipid, are non-conserved between the two channels. Thr499 is replaced with the smaller Ser660 in Kv11.1, while Ala483 is replaced with the slightly bulkier Val644 in Kv11.1 and the polar side-chain of Asn481 (Kv10.1) predicted to form a hydrogen bond with AA is replaced by the hydrophobic Ile642 in Kv11.1, as shown in Figure 11.

This might explain the different functional effects on the channel. This of course requires further studies, such as mutagenesis, which could confirm the modelling predictions.

9. DISCUSSION

In the present diploma thesis binding of arachidonic acid to Kv10.1 and Kv11.1 channels was compared, using molecular modeling. A particular interesting question that was addressed in the present work concerns the different behavior of the lipid in the closely related K+ channels.

Since the binding site of arachidonic acid in neither Kv10.1 nor Kv11.1 channels is currently known, the first aim was to identify the putative binding site in this channels. Given the high structural conservation of Kv channels, the Kv1.5 channels served as starting point for these investigations in this diploma thesis. This channel has been shown to be inhibited by AA, via blockade of the central cavity [20].

Surprisingly, a sequence alignment performed in this work revealed that none of the binding residues are conserved in Kv10.1 or Kv11.1. (Fig. 7, Tab.1)

Thus, a docking study was performed to investigate, if the lipid could fit into the central cavity of the two channels. Indeed docking strongly supports binding of arachidonic acid to both channels with high affinity. Thus, the binding site seems to be in an identical region in all three Kv channels. Sequence conservation between the predicted Kv10.1 and Kv11.1 binding sites is reasonably high, with 4 important amino acids being non-conserved. The experimentally reported difference in arachidonic acid behavior seems to be caused by these amino acid changes in the binding site. This is in line with previous studies, reporting opposite effects of small molecule compounds interacting with Kv10.1 and 11.1. For example, Garg et al [4] reported that the compound ICA-105574 (3-Nitro-N-(-4-phenoxyphenyl)-benzamide or ICA) has opposite effects on the inactivation of the hEAG and hERG channel. When ICA binds to the hERG channel, its inactivation is attenuated by a shifting of its voltage dependence to more positive potentials while if ICA binds to the hEAG its inactivation is enhanced without an alteration in its voltage dependence. The inhibitory effect of ICA on hEAG is more potent than the activation effect of ICA on hERG channels. The reported ICA binding site seems to be in identical regions in both channels. The difference in behavior seems to be caused by changes of 3 amino acids in the binding site.

A similar effect is suggested in this diploma thesis for arachidonic acid, based on the modelling performed. Of course, further investigations, including mutagenesis studies and MD simulations should be performed to get a better understanding of how arachidonic acid blocks the hERG and hEAG channels.

10. LIST OF FIGURES

11. REFERENCES

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