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

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Chapter 6

K_v11.1 (hERG)-induced cardiotoxicity: a molecular insight from a binding kinetics study of prototypic K_v11.1 inhibitors

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Abstract

Drug-induced arrhythmia due to blockade of the $K_v11.1$ channel (also known as the hERG K^+ channel) is a frequent side effect and arouses major safety concerns during the drug development process. Previous studies have primarily focused on equilibrium parameters, i.e. affinity or potency, of drug candidates at the channel. The aim of this study was to determine the kinetics of the interaction with the channel for a number of known $K_v11.1$ blockers and to explore a possible correlation with the affinity or physicochemical properties of these compounds. In this context, the affinity and kinetic parameters of fifteen prototypic $K_v11.1$ inhibitors were evaluated in a number of [3H]dofetilide binding assays. The lipophilicity ($\log K_{w-C8}$) and membrane partitioning ($\log K_{w-IAM}$) of these compounds were determined by means of HPLC analysis. Accordingly, a novel [3H]dofetilide competition association assay was set up and validated, which allowed us to determine the binding kinetics of the $K_v11.1$ blockers used in this study. Interestingly, the compounds' affinity (K_i values) were correlated to their association rates rather than dissociation rates. Overall lipophilicity or membrane partitioning of the compounds were not correlated to their affinity and rate constants for the channel. In conclusion, a compound's affinity for the $K_v11.1$ channel is determined by the rate of association with the channel, while overall lipophilicity and membrane affinity are not. In more general terms, our findings provide novel insights into the mechanism of action for a compound's activity at the $K_v11.1$ channel. This may yield further understanding in how $K_v11.1$ -induced cardiotoxicity is governed and how it can be circumvented in the future.

Introduction

The $K_v11.1$ channel, a voltage-gated potassium channel previously known as hERG (human ether-à-go-go related gene), encodes the pore-forming subunit of the rapid component of the delayed rectifier K^+ channel, I_{Kr} , which contributes to phase 3 repolarization in cardiac action potentials¹⁻⁴. Genetic defects or drug blockade of the $K_v11.1$ channel normally cause delayed repolarization of cardiac action potentials and Torsades de Pointes (TdP)⁵. In some cases, TdP can degenerate into ventricular fibrillation and lead to sudden death^{6,7}. Currently, a number of drugs have been withdrawn from the market due to their blockade of the $K_v11.1$ channel, including astemizole and cisapride⁸. In addition, either ‘black box’ warnings or significant restrictions have been rendered for several other drugs, such as droperidol and ibutilide⁸. Withdrawing or restricting drugs due to their $K_v11.1$ liability in less than 1% of the patient population causes unpredictable and huge costs to industry, and brings troubles for patients as well owing that some effective drugs would be beneficial for the vast majority of patients^{8,9}.

In the past decades, a series of techniques including whole cell patch-clamp technologies, radioligand binding assays and ion flux assays have been developed and used to screen for $K_v11.1$ liability of compounds^{5,10}. From these, equilibrium radioligand displacement assays have been suggested as a low-cost and high throughput technology alternative to other available methods and are widely employed to obtain the affinity of compounds at the $K_v11.1$ channel¹¹⁻¹³. These studies provide equilibrium parameters, such as IC_{50} or K_i values. For instance, a 30-fold margin between $K_v11.1$ IC_{50} and maximum free plasma concentration (C_{max}) has been recommended to assess drug safety with respect to arrhythmogenesis¹⁴. However, the kinetics of the interaction between a drug and the $K_v11.1$ channel may be equally, or even more, important. Knowledge of association and dissociation rate constants has already led to better insights in the action of drugs at other targets, such as enzymes and G protein-coupled receptors^{15,16}.

In the present study, we hypothesized that a slow association rate (k_{on}) to and/or a fast dissociation rate constant (k_{off}) from the channel may be beneficial to reduce or avoid $K_v11.1$ -related cardiovascular side effects. Hence, a detailed understanding of a ligand’s binding kinetics at the channel may provide clues to enable the optimization of its kinetic profiles, and potentially ‘rescue’ it from removal of the drug discovery pipeline. In this context, we describe the development and validation of a [3H]dofetilide competition association assay in HEK293 $K_v11.1$ cell membranes to determine the kinetic binding parameters of 15 unlabelled reference compounds from different “Redfern” categories¹⁴ (**Table 1**). We systematically investigated both equilibrium affinity and kinetic binding

data of those compounds, and compared these parameters with the compounds' physicochemical properties. Since the interaction of drugs with membrane phospholipids is not only driven by lipophilicity, but also electrostatic interactions with both acidic and basic moieties on phosphate head groups^{17, 18}, a regular column and an immobilized artificial membrane (IAM) column, were used to derive two hydrophobic parameters for these compounds, i.e. $\log K_{W-C8}$ and $\log K_{W-IAM}$, respectively. Finally, both hydrophobic parameters were compared with the compounds' affinity and kinetic data. Taken together, the present study provides a novel approach to study the kinetics of the interaction between a drug and the $K_v11.1$ channel, which may help to more precisely assess $K_v11.1$ liability of drug candidates in the future.

Methods

Materials

Astemizole, sertindole, terfenadine, moxifloxacin, amiodarone, chlorpromazine, ibutilide, clofilium, pimozone, cisapride, ranolazine, sotalol, thioridazine and all the solutes for HPLC determinations were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands). Dofetilide and E-4031 were synthesized in our own laboratory^{19, 20}. Tritium-labeled dofetilide (specific activity 65~87 Ci·mmol⁻¹) was purchased from Perkin Elmer (Groningen, The Netherlands). Bovine serum albumin (BSA, fraction V) was purchased from Sigma (St. Louis, MO, USA). G418 was obtained from Stratagene (Cedar Creek, USA). The chemicals for HPLC were of HPLC grade; all the other chemicals were of analytical grade and achieved from standard commercial sources. HEK293 cells stably expressing the $K_v11.1$ channel (HEK293 $K_v11.1$) were kindly provided by Dr Eckhard Ficker (University of Cleveland, USA). The molecular target nomenclature ($K_v11.1$) conforms to 'The Concise Guide to PHARMACOLOGY 2013/14: Ion Channels'²¹.

Cell culture and membrane preparation

HEK293 $K_v11.1$ cells were cultured and membranes were prepared and stored as described previously²².

Table 1. Chemical structures and information on the use of 15 $K_v11.1$ blockers examined in this study.

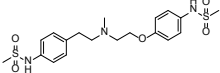
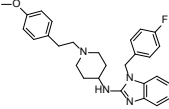
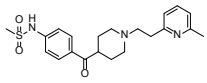
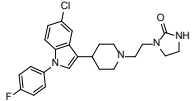
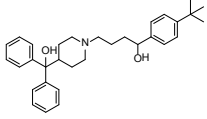
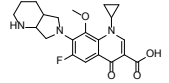
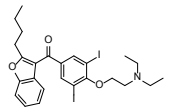
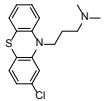
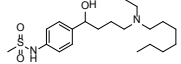
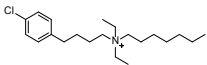
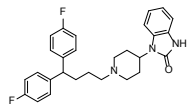
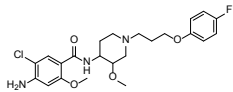
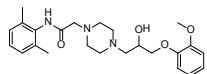
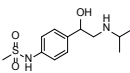
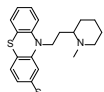
Name	Chemical structure	Therapeutic class	TdP ^a	Redfern category ^b	Status in the US ^a
Dofetilide		antiarrhythmic	++	1	restricted
Astemizole		antihistamine	++	2	withdrawn
E-4031		antiarrhythmic	++	1*	not approved
Sertindole		antipsychotic	+	2	not approved
Terfenadine		antihistamine	++	2	withdrawn
Moxifloxacin		antibacterial	++	3*	restricted
Amiodarone		antiarrhythmic	++	1	restricted
Chlorpromazine		antipsychotic	++	3*	restricted
Ibutilide		antiarrhythmic	++	1	restricted
Clofilium		antiarrhythmic	++	1*	not approved
Pimozide		antipsychotic	++	3	restricted

Table 1 continued

Name	Chemical structure	Therapeutic class	TdP ^a	Redfern category ^b	Status in the US ^a
Cisapride		gastroprokinetic	++	2	withdrawn
Ranolazine		antianginal	+	5*	restricted
Sotalol		antiarrhythmic	++	1	restricted
Thioridazine		antipsychotic	++	3	restricted

^a+ drugs with possible TdP risk, and ++ drugs with known TdP risk. Information on these compounds was retrieved from CredibleMeds[®] available at: <http://crediblemeds.org/> (accessed 10 April 2014).

^bRedfern categories for most compounds were derived from the literature (Redfern *et al.*, 2003); they are 1: Class Ia and III antiarrhythmics; 2: Withdrawn from market due to TdP; 3: Measurable incidence / numerous reports of TdP in humans; 4: Isolated reports of TdP in humans; 5: No reports of TdP in humans. * Redfern categories for these compounds were deduced according to the definition for different categories.

Radioligand saturation assay

Membrane aliquots containing 20 µg of protein were incubated in a total volume of 100 µL of incubation buffer (10 mM HEPES, 130 mM NaCl, 60 mM KCl, 0.8 mM MgCl₂, 1 mM EGTA, 10 mM Glucose, 0.1% BSA, pH 7.4) at 25 °C for 120 min to ensure that the equilibrium was reached at all concentrations of radioligand. Total binding was determined at a range of concentrations (0.2 ~ 22 nM) of [³H]dofetilide, whereas nonspecific binding was determined at three different concentrations of radioligand in the presence of 10 µM astemizole and analyzed by linear regression. Incubations were terminated by dilution with ice-cold wash buffer (25 mM Tris-HCl, 130 mM NaCl, 60 mM KCl, 0.8 mM MgCl₂, 0.05 mM CaCl₂, 0.05% BSA, pH 7.4). Separation of bound from free radioligand was performed by rapid filtration through Whatman GF/B filters using a Brandel harvester. Filters were subsequently washed 6 times with 2 mL ice-cold wash buffer. Filter-bound radioactivity was determined by scintillation spectrometry using a liquid Scintillation Analyzer (Tri-Carb 2900TR) after addition of 3.5 mL

of Packard Emulsifier Safe and 2 h extraction.

Radioligand association and dissociation assay

Kinetic association experiments were performed by incubating membrane aliquots containing 20 μg of protein in a total volume of 100 μL incubation buffer at 25 °C for 120 min with 16 different concentrations (0.7–16 nM) of [^3H]dofetilide. The amount of radioligand bound to the receptor was measured at various time intervals during the incubation. Incubations were terminated, and samples were obtained and analyzed as described in “*radioligand saturation assay*”. Further traditional association and dissociation assays were performed as described previously²².

Radioligand displacement assay

The [^3H]dofetilide binding assay for the $\text{K}_{\text{v}}11.1$ channel was performed as described previously²². In short, membrane aliquots containing 20 μg protein were incubated in a total volume of 100 μL incubation buffer at 25 °C for 60 min. Radioligand displacement experiments were conducted using 11 concentrations of the competing ligand in the presence of 5 nM [^3H]dofetilide. At this concentration, total radioligand binding did not exceed 10% of the radioligand added to prevent ligand depletion. Nonspecific binding was determined in the presence of 10 μM astemizole and represented approximately 15% of the total binding. [^3H]Dofetilide did not bind to membranes prepared from empty HEK293 cells lacking the $\text{K}_{\text{v}}11.1$ channel (data not shown). Total binding was determined in the presence of incubation buffer and was set at 100% in all experiments, whereas nonspecific binding was set at 0%. Incubations were terminated by dilution with ice-cold wash buffer. Separation of bound from free radioligand was performed by rapid filtration through a 96-well GF/B filter plate using a Perkin Elmer Filtermate-harvester (Perkin Elmer, Groningen, The Netherlands). Filters were subsequently washed 12 times with ice-cold wash buffer. The filter-bound radioactivity was determined by scintillation spectrometry using the P-E 1450 Microbeta Wallac Trilux scintillation counter (Perkin Elmer) after addition of 25 μL Microscint and 2 h extraction.

Radioligand competition association assay

The binding kinetics of unlabelled reference compounds were determined at 25 °C using the competition association assay according to a previously published method²³. In a standard assay, three different concentrations (0.3, 1- and 3-fold

of their K_i values) of unlabelled dofetilide, astemizole and E-4031 were tested. We assessed the binding kinetics of all other unlabelled reference compounds in a simplified one-concentration competition association assay based on Guo *et al.*²⁴. The experiments were initiated by incubating membrane aliquots containing 20 μg of protein in a total volume of 100 μL of incubation buffer in the absence (control) or presence of a certain concentration of unlabelled ligands at 25 °C for 120 min with 5 nM [^3H]dofetilide. The amounts of radioligand bound to the receptor were measured at various time intervals during the incubation. Incubations were terminated, and samples were obtained and analyzed as described in “*radioligand displacement assay*”.

Determination of $\log K_{\text{W-C8}}$ and $\log K_{\text{W-IAM}}$ parameters by HPLC

$\log K_{\text{W-C8}}$ values were measured on a Supelcosil LC-ABZ, 5 cm $\times$ 4.6 mm, 5 μm column according to a methodology described previously^{25, 26}. In short, retention times of the compounds were determined at three different methanol percentages. These retention times were converted to k values by using the formula $k = (t_{\text{R}} - t_0)/t_{\text{R}}$ in which t_{R} is the retention time and t_0 the retention time of a “nondelayed” compound (pure methanol). The calculated $\log k$ values were plotted against the methanol concentrations and extrapolated to a 0% methanol situation yielding the $\log K_{\text{W-C8}}$ values for 15 reference compounds (intercept of y axis).

An isocratic method was applied to measure the $\log K_{\text{W-IAM}}$ values of all tested compounds on a 10 cm $\times$ 4.6 mm, 10 μm Regis IAM PC DD2 column²⁷. Retention times of the compounds were determined at three different concentrations of acetonitrile. The k_{IAM} values were calculated by the equation $k_{\text{IAM}} = (t_{\text{R}} - t_0)/t_{\text{R}}$ in which t_{R} represents retention times of tested compounds, whereas t_0 is determined by injecting a sodium nitrate solution in the HPLC system. The $\log k_{\text{IAM}}$ values for a compound were plotted against the applied acetonitrile concentrations. The intercept with the Y-axis of the straight line through these data points yielded the extrapolated $\log K_{\text{W-IAM}}$ values for the 15 reference compounds.

Data analysis

All data of radioligand binding assays were analyzed using the non-linear regression curve fitting program Prism v. 5.1 (GraphPad, San Diego, CA, USA). K_{D} and B_{max} values of [^3H]dofetilide at HEK293K_v11.1 membranes were obtained by computational analysis of saturation curves. Apparent inhibitory binding constants (K_i values) were derived from the IC_{50} values according to the Cheng and Prusoff equation $K_i = \text{IC}_{50}/(1 + [\text{L}^*]/K_{\text{D}})$, where $[\text{L}^*]$ was the concentration of radioligand and K_{D} was its dissociation constant from the saturation assay²⁸. In

the kinetic association experiments, the on- and off-rates were derived from the linear regression analysis using the equation $k_{\text{obs}} = k_{\text{on}}[L^*] + k_{\text{off}}$, where the k_{obs} value was obtained by computer analysis of the exponential association of [^3H] dofetilide bound to the receptor with $[L^*]$ being the concentration of radioligand. The association and dissociation rates were used to calculate the kinetic K_D value using the following equation $K_D = k_{\text{off}}/k_{\text{on}}$. The association and dissociation rates for unlabelled compounds were calculated by fitting the data into the competition association model using “kinetics of competitive binding”²³:

$$K_A = k_1[L] + k_2$$

$$K_B = k_3[I] + k_4$$

$$S = \sqrt{(K_A - K_B)^2 + 4k_1k_3LI10^{-18}}$$

$$K_F = 0.5 (K_A + K_B + S)$$

$$K_S = 0.5 (K_A + K_B - S)$$

$$Q = \frac{B_{\text{max}}k_1L10^{-9}}{K_F - K_S}$$

$$Y = Q \left(\frac{k_4(K_F - K_S)}{K_F K_S} + \frac{k_4 - K_F}{K_F} e^{(-K_F X)} - \frac{k_4 - K_S}{K_S} e^{(-K_S X)} \right)$$

Where X is the time (min), Y the specific binding of [^3H]dofetilide, k_1 and k_2 are the k_{on} ($\text{M}^{-1} \cdot \text{min}^{-1}$) and k_{off} (min^{-1}) of [^3H]dofetilide obtained from the traditional association and dissociation assay, L the concentration of [^3H]dofetilide (nM), B_{max} the maximum specific binding (dpm) and I the concentration of the unlabelled compound (nM). Fixing these parameters allowed the following parameters to be calculated: k_3 , which is the k_{on} value ($\text{M}^{-1} \cdot \text{min}^{-1}$) of the unlabelled compound and k_4 , which is the k_{off} value (min^{-1}) of the unlabelled compound. $\text{Log}K_{\text{W-C8}}$ and $\text{log}K_{\text{W-IAM}}$ values were derived from linear regression analysis as mentioned above. The molecular weight (M.W.), logP and $\text{p}K_a$ values were calculated using a structure-based calculation plug-in provided by ChemAxon (Budapest, Hungary). All values obtained from radioligand binding assays in this study are means of at least three independent experiments performed in duplicate. Statistical analysis was performed with a two-tailed unpaired Student's t-test.

Results

[^3H]Dofetilide saturation assay

The binding of [^3H]dofetilide to HEK293K $_{\text{V}}$ 11.1 cell membranes was saturable and best described by a one-site binding model. A representative saturation curve and the averaged data of three independent experiments performed in duplicate are shown in **Figure 1** and **Table 2**, respectively. The K_{D} and B_{max} values obtained from this assay were 2.4 ± 0.1 nM and 1.6 ± 0.1 pmol $\cdot$ mg $^{-1}$ protein, respectively. The K_{D} value for [^3H]dofetilide from this assay was used to calculate K_{i} rather than IC_{50} values from the displacement assay for 15 reference compounds.

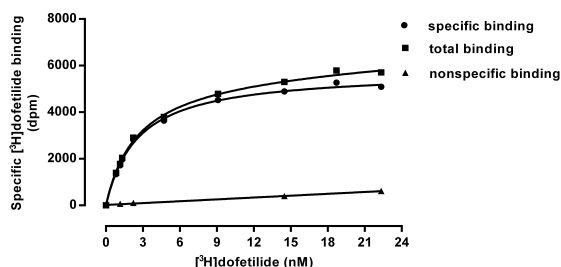


Figure 1. Saturation of [^3H]dofetilide binding to HEK293K $_{\text{V}}$ 11.1 membranes. Total binding was determined at increasing concentrations of [^3H]dofetilide. Nonspecific binding was determined at three concentrations of [^3H]dofetilide, and nonspecific binding at other concentrations of radioligand was extrapolated by linear regression. Specific binding was calculated as the difference between the total and nonspecific binding. The K_{D} value was 2.4 ± 0.1 nM, and the B_{max} value was 1.6 ± 0.1 pmol $\cdot$ mg $^{-1}$ protein. Experiments were performed at 25 °C using 20 μg of HEK293K $_{\text{V}}$ 11.1 membrane protein.

[^3H]Dofetilide association and dissociation assay

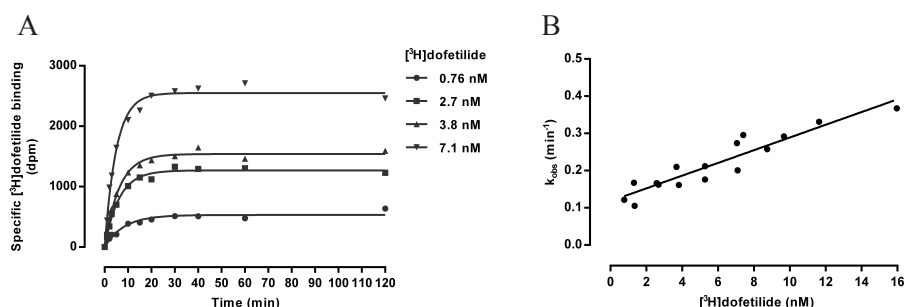


Figure 2: Characterization of the association and dissociation rates of [^3H]dofetilide to HEK293K $_{\text{V}}$ 11.1 membranes in the kinetic association assay. **(A)** Representative association curves of [^3H]dofetilide at four different concentrations; **(B)** A plot of k_{obs} values versus the concentration of [^3H]dofetilide. Experiments were performed at 25 °C using 20 μg of HEK293K $_{\text{V}}$ 11.1 membrane protein.

Initial experiments were performed to fully characterize the association and dissociation rates of [^3H]dofetilide to and from HEK293K $_{\text{V}}$ 11.1 membranes, respec-

tively. As the association rate of a ligand is dependent upon the concentration used, kinetic association experiments with a range of [^3H]dofetilide concentrations were conducted. In **Figure 2A**, curves are shown for four of such concentrations (0.76, 2.7, 3.8 and 7.1 nM). A plot of the k_{obs} values against more, including higher, concentrations of [^3H]dofetilide (**Figure 2B**) was consistent with a linear correlation ($r^2 = 0.86$, $P < 0.0001$), indicating that the binding of [^3H]dofetilide to the $K_v11.1$ channel followed the law of mass action for a simple bimolecular interaction and that the equation $k_{\text{on}} = (k_{\text{obs}} - k_{\text{off}})/[L^*]$ was applicable in this study. The k_{on} and k_{off} values obtained from this plot were $0.017 \text{ nM}^{-1} \cdot \text{min}^{-1}$ and 0.12 min^{-1} , respectively (**Table 2**). When k_{off} was divided by k_{on} , a kinetically derived K_D value of 7.1 nM was obtained. These values were in agreement with values for apparent on- and off-rates and kinetic K_D of [^3H]dofetilide assessed at one (5 nM) concentration ($k_{\text{on}} = 0.032 \pm 0.003 \text{ nM}^{-1} \cdot \text{min}^{-1}$, $k_{\text{off}} = 0.20 \pm 0.03 \text{ min}^{-1}$ and $K_D = 6.4 \pm 1.3 \text{ nM}$) derived from the traditional association and dissociation assays published previously²².

Table 2. Binding parameters of dofetilide from different equilibrium and kinetic binding assays.

Binding parameters	Saturation assay	Kinetic association assay ^a	Traditional association and dissociation assay ^b	Displacement assay	Competition association assay
$k_{\text{on}} (\text{nM}^{-1} \cdot \text{min}^{-1})$	-	0.017	0.032 ± 0.003	-	0.048 ± 0.011
$k_{\text{off}} (\text{min}^{-1})$	-	0.12	0.20 ± 0.03	-	0.13 ± 0.02
$K_D (\text{nM})$	2.4 ± 0.1	7.1 ^c	6.4 ± 1.3^c	-	2.7 ± 0.3^c
$K_i (\text{nM})$	-	-	-	5.4 ± 0.8	-

Values are means ($\pm$ S.E.M.) of three independent assays performed in duplicate.

^aData were derived from linear regression of one independent association assay of [^3H]dofetilide at different concentrations.

^bData from our previous study²².

^cKinetic $K_D = k_{\text{off}}/k_{\text{on}}$.

[^3H]Dofetilide displacement assay

Competition binding assays were performed to generate K_i values for 15 reference compounds. Compounds were selected based on structural diversity and having a wide range of $K_v11.1$ binding affinity, and included both anti-arrhythmic drugs and drugs for other therapeutic areas (e.g., astemizole and terfenadine for the treatment of allergic conditions). All compounds produced a concentration-dependent inhibition of specific [^3H]dofetilide binding, and their displace-

ment curves were best described by a one-site competition model (**Figure 3**). All K_i values are listed in **Table 3**. Among the 15 compounds, clofilium had the highest affinity to the $K_v11.1$ channel, displacing [3 H]dofetilide with a K_i value of 0.55 ± 0.09 nM, whereas moxifloxacin exhibited the lowest affinity of 252 ± 121 μ M. Ranolazine and sotalol showed similar and relatively weak inhibition of the channel with K_i values of 21 ± 6 and 25 ± 1 μ M, respectively. Additionally, amiodarone, thioridazine and chlorpromazine displayed modest $K_v11.1$ blockade with K_i values from 0.3 to 3 μ M. All other compounds demonstrated relatively high affinity to the $K_v11.1$ channel, between 2.5 ± 0.2 nM (astemizole) and 63 ± 4 nM (terfenadine).

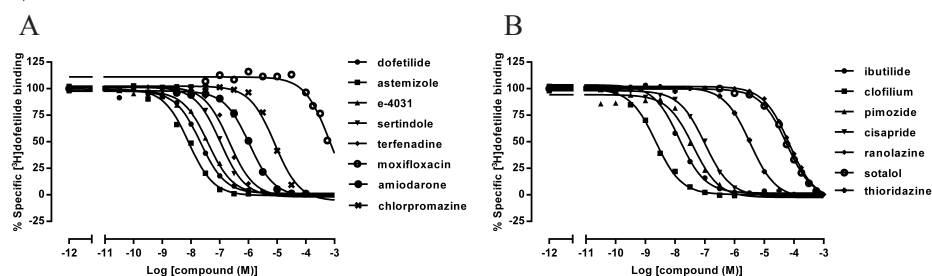


Figure 3. Displacement curves of [3 H]dofetilide from HEK293 $K_v11.1$ membranes by different known $K_v11.1$ channel blockers. **(A)** Dofetilide, astemizole, E-4031, sertindole, terfenadine, moxifloxacin, amiodarone and chlorpromazine; **(B)** Ibutilide, clofilium, pimozone, cisapride, ranolazine, sotalol and thioridazine. Experiments were performed at 25 °C using 20 μ g of HEK293 $K_v11.1$ membrane protein.

[3 H]Dofetilide competition association assay

With the k_{on} (k_1) and k_{off} (k_2) values of [3 H]dofetilide obtained from the traditional association and dissociation assays, it was possible to determine the k_{on} (k_3) and k_{off} (k_4) values of unlabelled compounds by performing so-called competition association experiments. Firstly, we validated the competition association assay at the $K_v11.1$ channel using three concentrations of ‘cold’ dofetilide equivalent to 0.3-, 1- and 3-fold of its K_i value. A representative experiment is shown in **Figure 4**. The k_{on} (k_3) and k_{off} (k_4) values for dofetilide determined in this assay were 0.048 ± 0.011 nM $^{-1}$ ·min $^{-1}$ and 0.13 ± 0.02 min $^{-1}$, respectively, and in good agreement with the kinetic parameters determined in the traditional association and dissociation assays, as shown in **Table 2**. Furthermore, the kinetic K_D value (2.7 ± 0.3 nM) derived from this assay was similar to the K_D value (2.4 ± 0.1 nM) obtained from the [3 H]dofetilide saturation assay. In addition, these K_D values were in the same range as dofetilide’s affinity constant that stemmed from the displacement assay ($K_i = 5.4 \pm 0.8$ nM) and the kinetically derived K_D values from kinetic association assay and traditional kinetic experiments (7.1 nM or 6.4

± 1.3 nM, respectively). Overall, the results presented here demonstrated that the competition association assay could be applied to determine the association and dissociation rates of other unlabeled ligands at the $K_v11.1$ channel. It is noteworthy that a good experimental window was achieved using a concentration of dofetilide at 1-fold its K_i value in the competition association assay, as displayed in **Figure 4**. Reassuringly, when astemizole and E-4031 were tested in this standard three-concentration assay, similar findings were observed as well (data not shown). Thus, the other unlabelled ligands were only tested at 1-fold their K_i values rather than three different concentrations in the further competition association experiments in order to improve the throughput of this method.

Table 3. The affinity constants and kinetic parameters of 15 compounds at the $K_v11.1$ channel obtained from the [3 H]dofetilide displacement and competition association assay.

Compound	K_i (nM) ^a	k_{on} (nM ⁻¹ ·min ⁻¹) ^b	k_{off} (min ⁻¹) ^b	K_D (nM) ^c
Dofetilide	5.4 ± 0.8	0.048 ± 0.011	0.13 ± 0.02	2.7 ± 0.3
Astemizole	2.5 ± 0.2	0.17 ± 0.03	0.083 ± 0.003	0.53 ± 0.07
E-4031	13 ± 0.7	0.026 ± 0.003	0.27 ± 0.02	10 ± 1
Sertindole	34 ± 5	0.048 ± 0.007	0.86 ± 0.17	18 ± 1
Terfenadine	63 ± 4	0.0071 ± 0.0025	0.25 ± 0.03	39 ± 7
Moxifloxacin	252347 ± 120995	$(4.7 \pm 1.0) \times 10^{-6}$	0.28 ± 0.06	64531 ± 10276
Amiodarone	308 ± 33	$(6.0 \pm 0.7) \times 10^{-4}$	0.23 ± 0.02	387 ± 37
Chlorpromazine	2518 ± 301	$(1.3 \pm 0.2) \times 10^{-4}$	0.36 ± 0.05	2714 ± 154
Ibutilide	5.1 ± 0.4	0.046 ± 0.006	0.20 ± 0.02	4.6 ± 1.1
Clofilium	0.55 ± 0.09	0.23 ± 0.07	0.10 ± 0.01	0.54 ± 0.17
Pimozide	28 ± 7	0.071 ± 0.020	0.22 ± 0.07	3.2 ± 0.4
Cisapride	54 ± 10	0.031 ± 0.009	0.59 ± 0.14	20 ± 2
Ranolazine	21379 ± 5776	$(1.5 \pm 0.4) \times 10^{-5}$	0.23 ± 0.04	16672 ± 1820
Sotalol	24663 ± 1379	$(1.7 \pm 0.3) \times 10^{-5}$	0.32 ± 0.03	19740 ± 1591
Thioridazine	1065 ± 41	$(2.3 \pm 0.4) \times 10^{-4}$	0.24 ± 0.05	1050 ± 48

Values are means ($\pm$ S.E.M.) of three independent assays performed in duplicate.

^a K_i values were derived from the [3 H]dofetilide displacement assay.

^b k_{on} (k_3) and k_{off} (k_4) values of unlabelled compounds were determined in the [3 H]dofetilide competition association assay.

^cKinetic $K_D = k_{off}/k_{on}$.

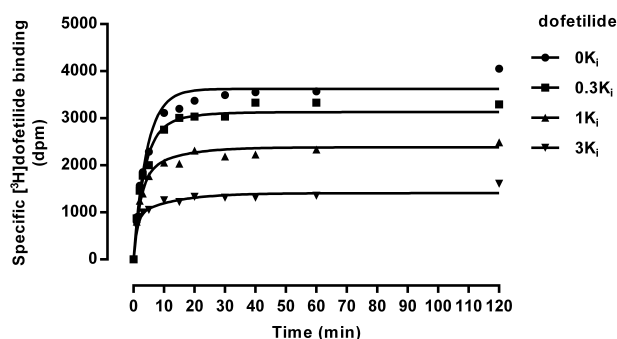


Figure 4. Competition association assay of [^3H]dofetilide in the absence (control) or presence of 0.3-, 1- and 3-fold of unlabeled dofetilide's K_i value. Experiments were performed at 25 °C using 20 μg of HEK293K $_{v11.1}$ membrane protein.

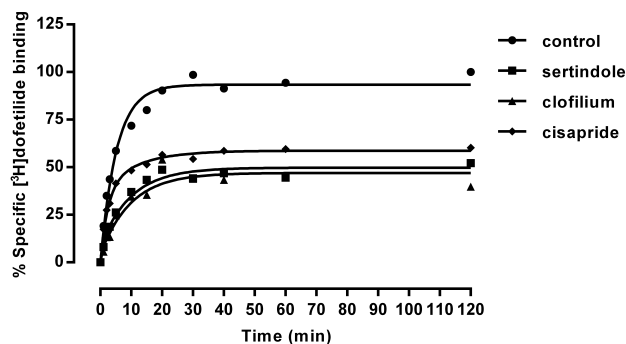


Figure 5. Representative competition association curves of [^3H]dofetilide in the absence (control) or presence of unlabelled sertindole, clofilium and cisapride at a concentration of 1-fold their K_i values. Experiments were performed at 25 °C using 20 μg of HEK-293K $_{v11.1}$ membrane protein.

Subsequently, kinetic parameters of 12 other known K $_{v11.1}$ blockers were evaluated and representative normalized curves for several compounds are depicted in **Figure 5**. The on- and off-rates of all compounds determined by these experiments are shown in **Table 3**. The association rates for all the compounds were quite distinct with k_{on} values ranging from $(4.7 \pm 1.0) \times 10^{-6} \text{ nM}^{-1} \cdot \text{min}^{-1}$ (moxifloxacin) to $0.23 \pm 0.07 \text{ nM}^{-1} \cdot \text{min}^{-1}$ (clofilium), i.e. an almost 50,000-fold difference between the fastest and slowest associating compounds. On the other hand, the dissociation rates of these 15 compounds were more similar, with the highest value of $0.86 \pm 0.17 \text{ min}^{-1}$ for sertindole and lowest k_{off} of $0.083 \pm 0.003 \text{ min}^{-1}$ for astemizole, i.e. only a 10-fold difference. Considering the kinetically-derived K_D values shown in **Table 3**, clofilium was the most potent inhibitor to

the $K_v11.1$ channel with a kinetic K_D value of 0.54 ± 0.17 nM, while moxifloxacin had the lowest affinity ($K_D = 65 \pm 10$ μ M) to the channel. These results were in the same rank order as the K_i values derived from the equilibrium displacement assay.

Correlations of equilibrium K_i with kinetic K_D , k_{on} , and k_{off} values

A plot of the logarithms of kinetic K_D values (i.e. k_{off}/k_{on}) derived from the [3 H] dofetilide competition association assays and the logarithms of equilibrium K_i values obtained from the displacement experiments was made, and a significant correlation (**Figure 6A**) was observed. This showed an excellent consistence of the results from two different methods and indicated a high reliability of the [3 H] dofetilide competition association assay. More interestingly, a significant inverse relationship was also found between pk_{on} and pK_i values for unlabelled compounds in **Figure 6B**. On the contrary, there was no significant linear relationship between pk_{off} and pK_i values ($r^2 = 0.15$, $P = 0.15$, data not shown). Together, this suggested that the [3 H]dofetilide competition association assay was successfully validated for assessing the kinetics of other unlabelled competitive compounds, and that the affinity of these compounds at the $K_v11.1$ channel was mainly controlled by their on-rates rather than off-rates.

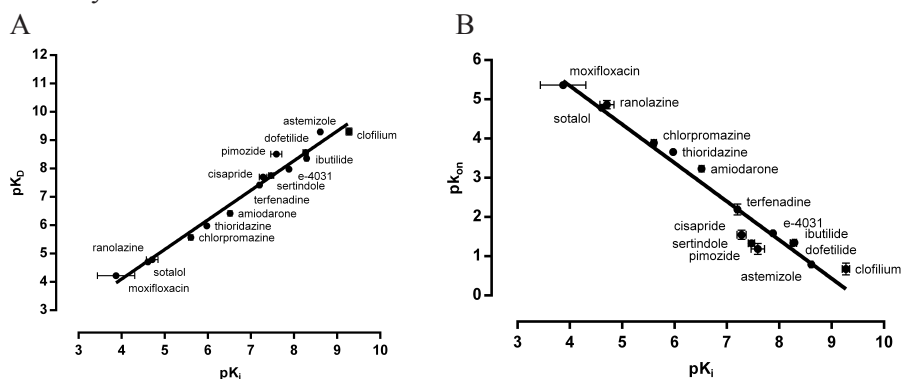


Figure 6. Correlations between the affinity constant (K_i) and **(A)** the kinetically derived equilibrium dissociation constants, K_D ($r^2 = 0.98$, $P < 0.0001$) and **(B)** the association rates, k_{on} for unlabelled ligands at the $K_v11.1$ channel ($r^2 = 0.95$, $P < 0.0001$). K_i , K_D and k_{on} values are listed in **Table 3**.

Lipophilicity ($\log K_{W-C8}$) and membrane partition coefficient ($\log K_{W-IAM}$) of $K_v11.1$ blockers

The isocratic $\log K_{W-C8}$ values ('lipophilicity') were evaluated at pH 7.4 and are detailed in **Table 4**. The lipophilicity of 15 reference compounds covered a wide numerical range, varying from 0.56 (sotalol) to 5.52 (amiodarone). We also calculated $\log P$ values as a measure for lipophilicity, and plotted these against the $\log K_{W-C8}$ data (**Table 4**). A significant correlation was found between them ($r^2 = 0.81$, $P < 0.0001$), which implied that for this series of compounds calculated $\log P$ values can be used interchangeably with the experimentally determined values.

Table 4. The lipophilicity, membrane partition coefficients and other physicochemical properties of 15 K_v11.1 blockers.

Compound	$\log K_{W-C8}$ ^a	$\log K_{W-IAM}$ ^b	M.W. ^c	$\log P$ ^c	pK_a ^c
Dofetilide	0.84	2.08	441.57	0.59	8.99
Astemizole	3.52	3.40	458.57	5.39	8.75
E-4031	1.29	1.98	401.52	1.73	8.01
Sertindole	3.97	3.38	440.94	3.77	8.59
Terfenadine	4.05	4.01	471.67	6.48	9.02
Moxifloxacin	1.12	1.57	401.43	1.97	9.42
Amiodarone	5.52	3.30	645.31	7.64	8.47
Chlorpromazine	3.39	3.36	318.86	4.54	9.20
Ibutilide	0.90	2.53	384.58	3.25	10.40
Clofilium	2.00	-0.35	338.98	2.91	n.a. ^d
Pimozide	4.69	3.80	461.55	5.83	8.38
Cisapride	3.12	2.66	465.95	2.49	8.24
Ranolazine	2.17	2.39	427.54	2.83	7.17
Sotalol	0.56	0.66	272.36	0.05	9.43
Thioridazine	3.53	3.80	370.58	5.47	8.93

^a $\log K_{W-C8}$ values were derived from HPLC experiments on a Supelcosil LC-ABZ, 5 cm × 4.6 mm, 5 μ m column.

^b $\log K_{W-IAM}$ values were derived from HPLC experiments on a 10 cm x 4.6 mm, 10 μ m Regis IAM PC DD2 column.

^cValues were derived from the structure-based calculation plug-in by ChemAxon.

^dn.a. not applicable; this compound is permanently charged.

To mimic the interactions of our ligands with membrane phospholipids, an IAM HPLC column that is a reflection of the lipid environment of a fluid cell membrane on a solid matrix was used to determine membrane partition coefficients ($\log K_{W-IAM}$) for all reference compounds. Their $\log K_{W-IAM}$ values were measured at pH 7.4 and are summarized in **Table 4**. Terfenadine had the highest $\log K_{W-IAM}$ value of 4.01, indicating that this compound possessed the highest affinity for membrane phospholipids. On the contrary, the $\log K_{W-IAM}$ for clobilium was only -0.35, most likely due to its quaternary ammonium moiety, which demonstrated that this compound hardly interacted with phospholipid membranes.

Subsequently, the possible correlation between $\log K_{W-C8}$ and $\log K_{W-IAM}$ was studied, and the result is shown in **Figure 7**. A significant linear relationship ($r^2 = 0.52$, $P < 0.0024$) was observed for these two parameters even when including the outlier clobilium. Obviously, a similar significant correlation was also found between calculated $\log P$ and $\log K_{W-IAM}$ values ($r^2 = 0.52$, $P = 0.0022$, data not shown). Next, the relationship between affinity constants or kinetic rate constants of the 15 $K_v 11.1$ inhibitors and their membrane interactions were investigated, as shown in **Figure 8A – 8C**. Apparently, no relationships were found for any of them ($P > 0.05$), demonstrating that membrane interactions did not affect affinity and binding kinetics of these ligands at the $K_v 11.1$ channel. Similarly, there were no correlations between $\log K_{W-C8}$ and pK_i , pK_{on} or pK_{off} values (data not shown).

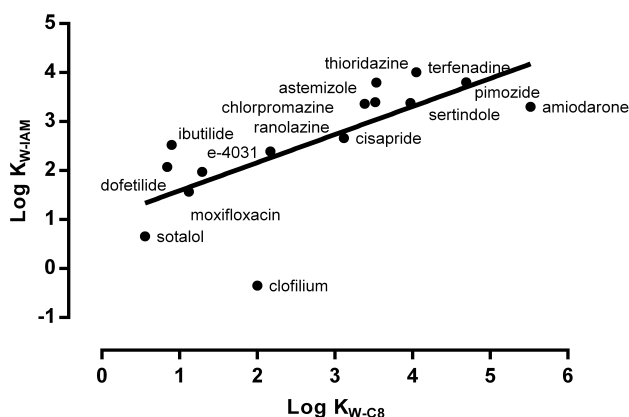


Figure 7. Correlation between $\log K_{W-C8}$ and $\log K_{W-IAM}$ values at pH 7.4 for 15 $K_v 11.1$ inhibitors ($r^2 = 0.52$, $P < 0.0024$). $\log K_{W-C8}$ and $\log K_{W-IAM}$ values are listed in **Table 4**.

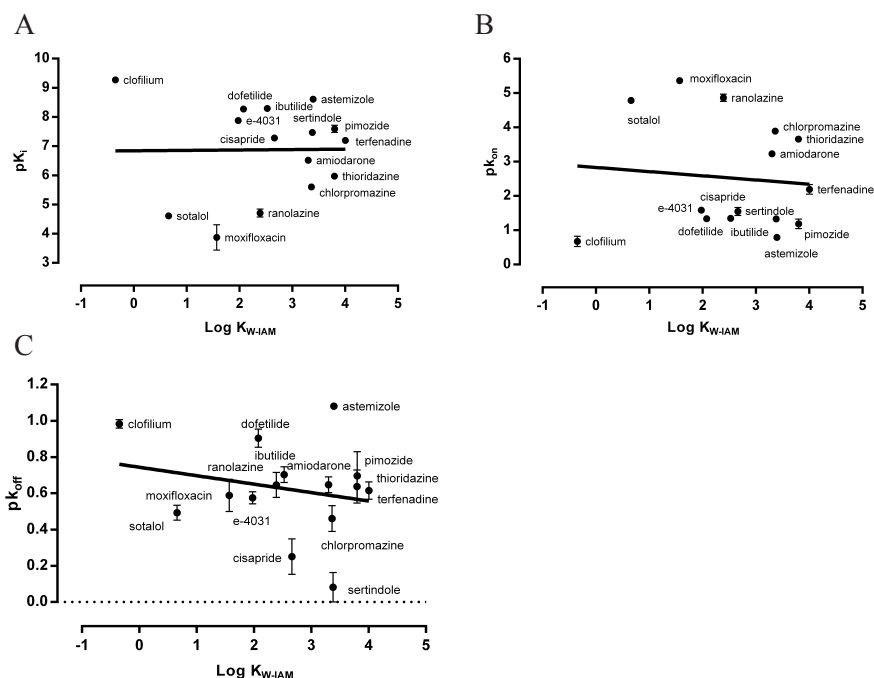


Figure 8. Correlation of $\log K_{w-IAM}$ values and binding parameters of 15 $K_v11.1$ inhibitors. No significant relationship between $\log K_{w-IAM}$ and values of (A) pK_i ($r^2 = 0.00011$, $P = 0.97$), (B) pK_{on} ($r^2 = 0.0087$, $P = 0.74$) or (C) pK_{off} was observed ($r^2 = 0.051$, $P = 0.42$). All data used in these plots are listed in **Table 3** and **Table 4**.

Role of other calculated physicochemical properties in ligand-receptor binding kinetics

Lastly, two other physicochemical properties of the unlabelled compounds, molecular weight (M.W.) and acid/base constant (pK_a) (**Table 4**), were compared with their on-/off- rates and dissociation constants. As depicted in **Figure 9**, no significant correlations were observed between the logarithms of on-rates of the compounds in this study and their molecular properties. Moreover, there were no obvious relationships between the logarithms of off-rates or equilibrium K_i values and these physicochemical properties either ($P > 0.05$, data not shown). These results implied that the binding kinetics of compounds at the $K_v11.1$ channel was not governed by their overall, macroscopic, physicochemical properties.

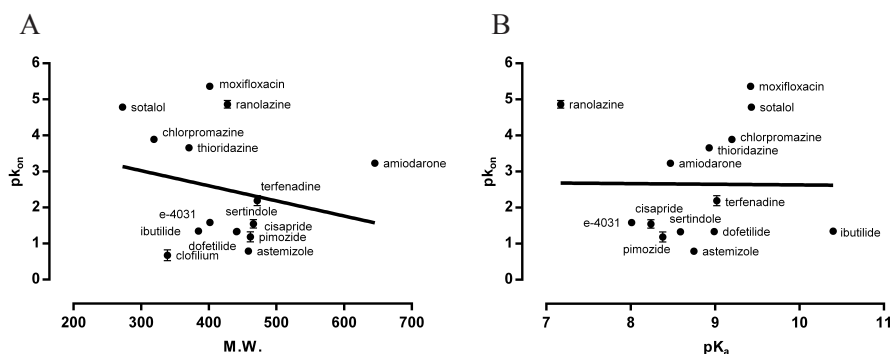


Figure 9. Lack of correlation between the association rates (pK_{on} values) of selected compounds and their physicochemical properties. **(A)** No significant correlation was observed with M.W. ($r^2 = 0.049$, $P = 0.43$); **(B)** No significant correlation was observed with pK_a ($r^2 = 0.000076$, $P = 0.98$). M.W. and pK_a values are shown in **Table 4**.

Discussion and conclusions

Since the introduction of the competition association assay²³, more and more researchers have utilized this method to study the binding kinetics of unlabelled ligands at their targets, such as muscarinic receptors and the adenosine A_{2A} receptor^{24, 29, 30}. In addition, a few examples in literature have been reported to apply this technique for the determination of ligand binding kinetics at ligand-gated ion channels³¹. To the best of our knowledge, the current study is the first to assess binding kinetics of ligands interacting with voltage-gated ion channels, in particular the $K_v11.1$ channel.

It might be argued that our study has some limitations. The stably-transfected HEK293 $K_v11.1$ cells lack the (co-)expression of two ancillary β -subunits, minK and MiRP1³. Discrepancies could also exist between membrane binding assays and whole cell experiments, which are more relevant to the channel's natural orientation. For instance, the kinetics of the drug- $K_v11.1$ interaction have been reported to be use- and frequency-dependent in whole cell patch clamp assays due to special gating kinetics of the channel^{32, 33}. In our membrane binding assays, we speculate that the channels maintain the same configurations, and thus the kinetic parameters in this investigation should resemble the actual binding process between $K_v11.1$ blockers and the channel. To perform straightforward and accurate kinetic determinations, experiments were carried out at 25 °C with membrane preparations of stably-transfected HEK293 $K_v11.1$ cells. We reason that all parameters obtained at 25 °C in this investigation lead to a similar compound rank order as those at physiological temperature. In fact, this has been shown for

another target based on van't Hoff and Eyring equations³⁴.

The linear plot of k_{obs} values obtained at increasing concentrations of [³H] dofetilide (**Figure 2B**) strongly supported a pseudo-first-order kinetic behavior of its binding to the $K_v11.1$ channel, which provided the theoretical foundation for all kinetic analyses in our present study. Other mechanisms of action such as induced-fit or conformational selection would have resulted in deviations from linearity; in such cases, the Motulsky and Mahan model and equations would not have been applicable^{35, 36}. All 15 reference compounds had a pseudo-Hill coefficient close to unity in the [³H]dofetilide displacement assay (data not shown), which indicated a competitive mode of inhibition with regard to the radioligand. This is another indication of the simple bimolecular interaction model and corroborated a pseudo-first-order kinetic behavior of the binding to the $K_v11.1$ channel. This finding was in accordance with previous studies describing the presence of a single affinity state for $K_v11.1$ inhibitors at the channel^{37, 38}. Subsequently, a newly developed [³H]dofetilide competition association assay was applied to study the binding kinetics of 15 unlabelled reference compounds with diverse chemical structures at the $K_v11.1$ channel (**Table 1**). The k_{on} , k_{off} and K_D values of dofetilide from this assay were comparable to the values derived from saturation and kinetic association experiments and traditional association and dissociation assays²², indicative of the accuracy and reliability of this new assay. Moreover, the excellent linear correlation between these kinetically derived K_D values of 15 reference compounds and their K_i values from the equilibrium displacement assay further supported the latter (**Figure 6A**). Taken together, this led us to conclude that a novel [³H]dofetilide competition association assay was successfully developed and validated.

Correlations between the affinity data (K_i) and the kinetic parameters (on- and off-rates) were also investigated in the present study. Surprisingly, there was a significant inverse correlation between pK_i and pk_{on} values for reference compounds (**Figure 6B**), whereas no relationship was found between pK_i and pk_{off} values (data not shown). Although this correlation between affinity and association rates has been shown to be the case for β_2 -adrenoceptor agonists³⁹ and OX_2 -receptor antagonists⁴⁰, this phenomenon is supposed to be unusual and counter-intuitive. Dogma has it that the collision theory sets the maximum value for k_{on} as the diffusion limit for a ligand and a target, which is about $10^8 \sim 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ ⁴¹. In this view, there can only be small variations in the on-rate constants and thus the equilibrium affinity changes are mainly dictated by the off-rates of ligands^{15, 41, 42}. In contrast to these classical assumptions, association rates of our selected structurally diverse compounds varied around 50,000-fold, whereas their dissociation rates differed only 10-fold. This clearly indicated that apart from

off-rates, on-rates also play a pivotal role in regulating affinity of ligands to their targeted receptors, at least for the $K_v11.1$ channel.

Recently, it has been reported that drug-receptor association rates and corresponding affinity were enhanced due to concentrating effects and lateral diffusion of membrane associated β_2 -adrenoceptor ligands and k_{on} values were positively correlated to their lipophilicity and membrane interactions^{18, 43}. Furthermore, Sykes *et al.*¹⁸ observed that dissociation rates were much more correlated to the corrected 'true' affinity, which considers drug concentration gradients in the local environment due to membrane affinity, than the apparent affinity. Additionally, it was recommended that any prediction of pharmacodynamic properties should take membrane interactions as well as lipophilicity into account¹⁷. Hence, the potential influence of membrane affinity on ligand binding to the $K_v11.1$ channel was investigated by the determination of both $\log K_{w-C8}$ and $\log K_{w-IAM}$ values. However, when pK_i and pK_{on} were compared to $\log K_{w-C8}$ and $\log K_{w-IAM}$, no significant relationships were observed in our case (**Figure 8**). This indicated that membrane interactions of our ligands did not influence their association rates and binding affinity to the $K_v11.1$ channel, and that the apparent affinity of these reference compounds is their 'true' affinity. Interestingly, when $\log K_{w-C8}$ and $\log K_{w-IAM}$ were compared to each other, clofilium was observed to deviate from the significant linear correlation (**Figure 7**). Herein, we hypothesized that the lipophilic alkyl chains of clofilium compensated for its hydrophilic quaternary ammonium group and thus dominated its lipophilicity in the octanol-water system, while the positive charge at the nitrogen played a more critical role in the IAM system and weakened its membrane interactions. With regard to dissociation rates, no correlations were found to either $\log K_{w-C8}$ or $\log K_{w-IAM}$ values as well (data not shown). This was in accordance with previous findings^{18, 44} and demonstrated that the dissociation rate of a drug was not affected by its local concentrations and thus independent of membrane affinity. Of note, typical $K_v11.1$ inhibitors are known to bind to the inner cavity in the intracellular part of the channel³. However, all experiments in the present study were performed with HEK293 $K_v11.1$ membranes instead of intact cells and were thus independent of transmembrane transport of ligands. From **Figure 6B**, it follows that a significant relationship between affinity and association rates for 15 $K_v11.1$ inhibitors was found, which showed that ligand- $K_v11.1$ rather than ligand-membrane association controlled their affinity. Apparently, an aqueous entry pathway predominates the binding of $K_v11.1$ blockers to the channel, and an additional compartment induced by the lipid membranes does not play a significant role.

We questioned whether it would be possible to correlate other general physicochemical properties of these molecules to their biological profile (**Figure 9**).

This was not the case, since typical features such as molecular weight and the basicity of the $K_v11.1$ blockers were not correlated to any of the equilibrium or kinetic binding parameters tested. However, it has been stated previously that physicochemical properties of drugs are relevant for their kinetic parameters^{41, 45}. For instance, Miller *et al.*⁴⁶ reported that molecular weight was one of the most important factors to affect the dissociation kinetics of ligands from their biological targets including enzymes (kinases) and G protein-coupled receptors. From the present study, it follows that the effects of general molecular properties on the binding kinetics of a ligand to $K_v11.1$ channel are negligible compared to other targets.

Previous studies have also suggested that k_{off} values, or drug-target residence times (RTs, the reciprocal of k_{off}), play an important role in the duration of pharmacological effects^{15, 24, 42}. In other words, an increased drug–target RT could elicit an improved or prolonged drug effect⁴⁷. Nevertheless, when adverse effects result directly from drug occupancy at the pharmacological target, such as chlorpromazine binding to D_2 receptor or clopidogrel to $P2Y_{12}$ receptor, a long RT would result in so-called ‘on-target’ drug toxicity, and thus a short RT is favored⁴⁸. In our hands, however, the dissociation rates and hence residence times of selected 15 $K_v11.1$ inhibitors were very similar (**Table 3**). On the contrary, association rates of our reference compounds varied widely, and are therefore potentially more important to predict the safety of $K_v11.1$ inhibitors.

Previously, Redfern *et al.*¹⁴ have assigned drugs into five categories of torsadogenic propensity and proposed a “30-fold safety margin” between C_{max} and $K_v11.1$ IC_{50} values as a marker to predict TdP. However, it is known that TdP are not only induced by inhibition of the $K_v11.1$ channel but also regulated by other potassium channels, sodium and calcium channels^{14, 49}. Therefore, a Comprehensive *in vitro* Proarrhythmia Assay (CiPA) has recently been introduced to assess drug-induced proarrhythmic risk more efficiently and accurately^{49, 50}. The kinetics of drug block and unblock were suggested to be incorporated together with drug potency at ion channels in arrhythmia evaluation during the interpretation of this paradigm⁴⁹. Although the present study focuses on the $K_v11.1$ channel only, it provides a new medium throughput method to determine the association and dissociation rates of $K_v11.1$ blockers, which can be used subsequently for other ion channels as well, if a radioligand is available. Interestingly, Veroli *et al.*⁵¹ derived from mathematical models that fast binding $K_v11.1$ blockers in the untrapped configuration and trapped blockers induced greater action potential and QT prolongation. This strengthens the application of our method in the future, and suggests that using slow association and/or fast dissociation characteristics as a “novel marker” might be beneficial for reducing $K_v11.1$ cardiotoxicity of drug

candidates.

In conclusion, a novel [^3H]dofetilide competition association assay has been successfully developed and validated to characterize the kinetic binding parameters of unlabelled compounds at the $\text{K}_v11.1$ channel. Importantly, association rates of $\text{K}_v11.1$ blockers were divergent, i.e. not diffusion limited, and excellently correlated to their affinity values. In addition, membrane interactions and other molecular properties do not influence the affinity and kinetic binding parameters of ligands at the $\text{K}_v11.1$ channel. Altogether, this is quite unlike the mechanisms of interaction proposed for other drug target classes, such as kinases and G protein-coupled receptors. Hence, we postulate that association rates can be used to assess a compound's $\text{K}_v11.1$ liability, apart from its affinity. However, further studies involving compounds with a wide range of k_{off} values are required to assess the effect of residence time on drug-induced $\text{K}_v11.1$ cardiotoxicity. Overall, we believe that this research provides novel insights into the kinetic study of ion channels, which can hopefully help to avoid $\text{K}_v11.1$ -induced cardiotoxicity of drug candidates in the future.

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