Use- and state-dependent $Na_V1.5$ blockers on QPatch X and *in vivo* and *in vitro* assays.

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Introduction

The cardiac voltage dependent sodium channel ($Na_V1.5$) is responsible for the upstroke and directed propagation of action potentials in the heart, and is therefore a central ion channel in safety assessment and drug discovery. It is often important to determine the mode of action of a drug candidate, and this requires high-quality recordings and careful experiment planning. Such experiments usually do not lend themselves well to testing of large drug libraries. We show that the QPatch X in multi-hole mode can be successfully used in such a screening scenario. Furthermore, since $Na_V1.5$ inhibition *per se* is not necessarily arrhythmogenic or cardiotoxic as is hERG inhibition, integrated *in vitro* and *in vivo* studies are necessary to assess a potential risk of cardiotoxicity including proarrhythmia. QPatch data combines relevantly with the data from Langendorff-perfused rabbit hearts and the ECG assay and effectively discriminate the cardiovascular profiles of compounds. For drug screening, we established a protocol for QPatch X that both tests the decay of the sodium current (30 Hz pulsetrain), and the recovery of the current from this pulse-train induced decay. The easy assay set-up in the QPatch Assay Software allows the combination of these elements into a single protocol, thereby shortening experimental time and costs.

Materials & Methods

QPatch experiments
Cells: CHO $Na_V1.5$ QCells, grown according to Sophion SOP. The cells were supplied by B'SYS (Switzerland). **Ringer's solutions**: Extracellular (in mM): 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 4 KCl, 145 NaCl, 10 glucose, 10 TEA-Cl. pH 7.4, 310 mOsm. Intracellular (in mM): 135 CsF, 1 EGTA (solubilized in CsOH, to a total Cs concentration of 140 mM), 10 HEPES, 10 NaCl. pH 7.3. Adjusted to approx. 310 mOsm with sucrose. **Compounds**: Lidocaine, Flecainide (Sigma – dissolved in ethanol to a stock solution x1000 of the highest final concentration. Quinidine (Sigma) – dissolved directly in extracellular Ringer's solution. TTX (Alomone labs, Israel), dissolved in H_2O to a stock solution x1000 of the highest final concentration. **Experimental setup**: All compounds were tested in six concentrations with increasing concentration of compound applied to the same measurement site. Experiments were done on QPatch X in multi-hole mode. QPatch operation in single-hole mode is a classic patch clamp experiment where one cell is in whole-cell configuration, whereas multi-hole mode comprises 10 cells in whole-cell configuration. The multi-hole mode therefore measures the summed current of ten cells. The voltage protocol (Figure 1) consisted of a 30 Hz pulsetrain (50 depolarizations to -20 mV) followed by a interpulse for recovery at V_{hold} and a final depolarization at -20 mV. V_{hold} was either -80 mV or -115 mV. The interpulse duration for recovery lasted 200 ms, increasing by 60% per sweep to max. 3355 ms (7 sweeps) in the screening experiments. Another, similar, voltage protocol with finer increments in time (25%, starting at 50 ms), thus giving more datapoints in this segment, was used to determine time constants of recovery after the pulsetrain more precisely ("extended voltage protocol").

Figure 1.

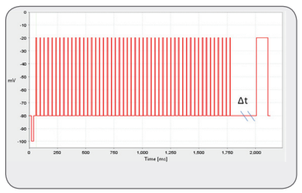

Figure 1. Voltage protocol for QPatch screening experiments. Here V_{hold} of -80 mV is shown.

Data analysis: All data analysis for QPatch experiments was done using QPatch Assay Software 3.4 in combination with Origin 7.5. Time constants of recovery were calculated with a monoexponential fit. Data is represented as mean ± standard error.

Langendorff heart model
Animals: New Zealand White female rabbits (Oriental Yeast) at 8-13 weeks. Commercial diet (RC 4, Oriental Yeast) and chlorinated tap water was given ad libitum.

Compounds: Lidocaine, quinidine and flecainide (Sigma). **Experimental procedure:** The rabbit was initially injected with heparin (500 U/kg i.v. from the marginal ear vein) and anesthetized with sodium thiopental (30 mg/kg iv from the marginal ear vein). After exsanguination by carotid artery dissection, the heart was excised via an anterolateral thoracotomy and immediately immersed in ice-cold modified Krebs-Henseleit solution containing (in mM)118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 11.1 glucose. The aorta was quickly cannulated to the Langendorff apparatus (Physio-Tech), and the heart was retrogradely perfused using a peristaltic pump (WPI) with warm (37 °C) Krebs-Henseleit solution equilibrated with a mixture of 95% O_2 and 5% CO_2 at a constant flow that was adjusted between 20-30 mL/min thus maintaining an initial perfusion pressure of about 70 mmHg. The electrocardiogram (ECG) (one electrode (-) was placed on the carotid artery and the second (+) on the cardiac apex with a spring against the epicardium) and monophasic action potential (MAP: a pressure-contact MAP (monophasic action potential) electrode placed on the left ventricular epicardial surface) were continuously recorded. After the stabilization period, the test compound was applied in a cumulative manner of increasing concentrations with 20-min intervals. Measurement parameters: Heart rate (HR: beats/min), PR (msec), QRS (msec), QT (msec), QT_cB (msec/√sec), MAP_{90} (msec), and MAP_{90} corrected ($MAP_{90}c$: msec/√sec). **Data analysis:** Data is represented as mean.

Anesthetized rabbit model
Animals: New Zealand White female rabbits (Oriental Yeast) at 10-12 weeks were used. Commercial diet (RC 4, Oriental Yeast) and chlorinated tap water was given ad libitum. **Compounds:** Lidocaine, quinidine and flecainide (Sigma). **Experimental procedure:** The rabbits were initially anesthetized with intra-muscular sodium ketamine hydrochloride (ketamine: 35 mg/0.7 mL/kg) + xylazine hydrochloride (xylazine: 5 mg/0.25 mL/kg), and then followed by ketamine (10 mg/mL) + xylazine (1 mg/mL) at a volume of 5 mL/hr continuous i.v. infusion from the marginal ear vein. A tracheotomy was performed, and the animal was artificially ventilated with room air (35 strokes/min, about 5~10 mL/kg) using a ventilator (SN-480-5 or -6, Shinano-Seisakusho). Cannulae were implanted in the femoral artery and femoral vein for recording of arterial blood pressure (BP) and infusion of the test compounds, respectively. The electrocardiogram (ECG, Lead II) was monitored via subcutaneous needle electrodes. After baseline recordings were obtained over a period of 30 minutes, the test compound was intravenously administered at a speed of 12 mL/kg/hr for 60 minutes. Following the start of the i.v. infusion the BP and electrocardiogram (ECG) were continuously monitored on a polygraph (RMP-6004M, NIHON KOHDEN), and concurrently the BP and ECG output from the polygraph was transmitted to an ECG PROCESSOR (Softron) during the test period. The appearance of arrhythmia, with a special reference to lethal arrhythmia including the ventricular tachycardia (VT), polymorphic ventricular tachycardia (Torsades de pointes;TdP) and ventricular fibrillation (VF), were monitored for 60 minutes after the start of test compound infusion. **Data analysis:** Data is represented as mean.

Conclusion

The results obtained convincingly show that: 1) Flecainide is a use-dependent blocker of open-channels, as demonstrated by a slow decay of I_{Na} (30 Hz pulsetrain), and a delayed recovery from the use-dependent block in the QPatch experiments. Flecainide produces ECG changes (marked PR and QRS prolongation, and slight QTc prolongation), and lethal arrhythmia (VT ~Vf) in both Langendorff-hearts and anesthetized rabbits at lower doses. 2) Lidocaine is a state-dependent blocker of inactivated channels, in that lidocaine produces a fast decay of I_{Na} (30 Hz pulsetrain) and a fast recovery. Lidocaine produces slight ECG changes (PR and QRS prolongation), but no arrhythmia in Langendorff-hearts or anesthetized rabbits. 3) Quinidine demonstrates a slow decay of I_{Na} (30 Hz pulsetrain), and delayed recovery from use-dependent block. Quinidine shows moderate PR and QRS prolongation, and severe morphological changes in ECGs. The present results suggest that: 1) The QPatch X can offer a time- and cost-effective unique screening scenario to select safe compounds with $Na_V1.5$ blocking activity and no proarrhythmic activity, and 2) the pro-arrhythmic activity of the $Na_V1.5$ blockers flecainide and quinidine might be attributable to their marked delay of recovery from use-dependent block.

QPatch experiments

Figure 2.

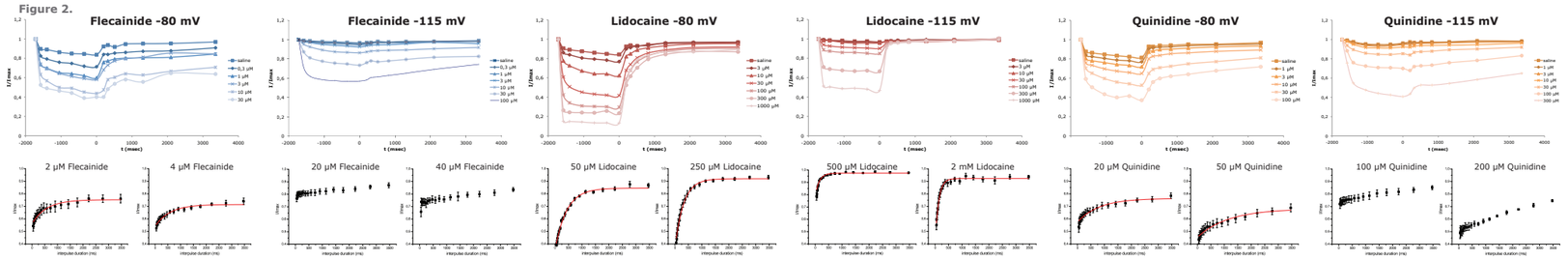

Table 1	V_{hold} -115 mV		V_{hold} -80 mV	
IC_{50} (µM)	Resting block (1st peak)	Use-dependent block (50th peak)	Resting block (1st peak)	Use-dependent block (50th peak)
Flecainide	40.3 ± 10.4	22.6 ± 6.6	4.1 ± 0.5	2.2 ± 0.3
Lidocaine	1915.3 ± 434.9	586.1 ± 112.7	247.4 ± 61.4	44.1 ± 11.3
Quinidine	166.1 ± 18.6	97.0 ± 10.4	48.3 ± 7.7	18.7 ± 3.4
TTX	14.6 ± 1.4	7.5 ± 0.3	2.4 ± 0.3	0.81 ± 0.17

Table 1. IC_{50}s for all compounds tested were determined at two different points in the pulsetrain –part of the voltage protocol, namely the first and last (50th) peaks, corresponding to resting-state block and use-dependent block, respectively. All tests were conducted at a holding potential of either -80 mV, to be close to the true physiological conditions of the cell; or -115 mV to test at a potential where no ion channels are inactivated ($V_{½}$ for $Na_V1.5$ is approximately -60 mV under the same experimental conditions as is used here – data not shown).

Table 2	V_{hold} -115 mV				V_{hold} -80 mV			
IC_{50} (µM)	Conc. (µM)	tau (ms)	Conc. (µM)	tau (ms)	Conc. (µM)	tau (ms)	Conc. (µM)	tau (ms)
Flecainide	40	n/a	20	n/a	4	575	2	560
Lidocaine	2000	128	500	147	250	317	50	425
Quinidine	200	n/a	100	n/a	50	1151	20	795

Table 2. Time constants of recovery from use-dependent block. Recovery was determined after the 30 Hz pulsetrain-induced decay. The extended voltage protocol with small time increments per sweep was used for this determination. The rank order of time constants obtained with the extended voltage protocol correlates well with the rank order of recovery from use-dependent block obtained with the screening protocol (see the upper graphs in Figure 2 (fits/time constants not shown) and compare with the small graphs, where the time constants are calculated from), where lidocaine has the quickest recovery, flecainide intermediate and quinidine the slowest.

Figure 2.
Upper graphs: Decay and recovery of $Na_V1.5$ current in the presence of increasing concentrations of compound. The V_{hold} used in the voltage protocol is indicated above each figure. The datapoints up to zero ms are taken from peak 1, 5, 10, 20, 30, 40 and 50 of the pulsetrain (the decay of the current). The datapoints after zero are the current elicited after the interpulse (the recovery of the current). All peaks are normalized to the first peak in the pulsetrain. (Average of 3-7 experiments.) Lower graphs: Recovery of $Na_V1.5$ current estimated with the extended protocol in the presence of compound at a concentration of approximately the IC_{50} for resting block (left) and use-dependent block (right). Data was fitted with a monoexponential equation and the resulting time constants are shown in Table 2.

Conclusions on QPatch experiments
The QPatch running in multi-hole mode is a versatile system, enabling easy setup of a screening campaign for use- and state-dependent compounds. In these experiments, flecainide exhibits a slow decay of current in the pulsetrain, and a delayed recovery, demonstrating it as a use-dependent blocker. Lidocaine produces a fast decay of current in the pulsetrain, and a fast recovery, demonstrating it as a state-dependent blocker. Finally, Quinidine introduced a slow decay of current in the pulsetrain, and delayed recovery. This data shows clearly that this screening protocol used on the QPatch can provide IC_{50}s as well as indicate modes of action of test compounds. This is confirmed by the fact that the data obtained with the extended protocol for recovery from use-dependent block correlates well with the screening data.

Langendorff heart model

Figure 3.

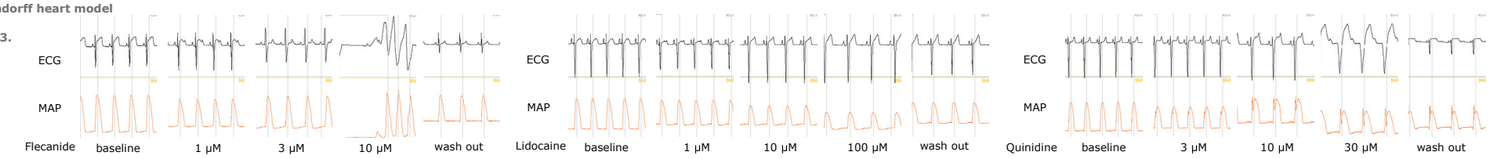

Table 3	Conc. (µM)	HR (%)	PR (%)	QRS (%)	MAP_{90c} (%)
Flecainide	1	-7.5	15.5	9.0	-2.1
	3	-15.8	54.0	80.2	-9.6
	10	Arrhythmia appeared			
Lidocaine	1	-4.0	4,8	0.7	-7.6
	10	-9.4	5.8	3.3	-12.2
	100	-24.4	29.7	21.2	-6.4
Quinidine	3	-11.8	14.5	4.0	10.8
	10	-29.8	31.9	20.2	9.5
	30	Severe morphology change of ECG			

Table 3. Summary of rabbit Langendorff-perfused heart preparations (n=3).

Figure 3.
Flecainide at 1 µM showed only 15% prolongation of PR. At 3 µM, flecainide reduced the heart rate 16% and showed marked prolongation of PR (54%) and QRS (80%) but had almost no effect on $MAP_{90}C$ (reduction of 10%) (see also Table 3). At 10 µM, flecainide induced ventricular tachycardia, ventricular fibrillation or ventricular pause in all the preparations.
Lidocaine showed no effect at concentrations of 1 and 10 µM. At 100 µM, lidocaine reduced the heart rate by 25% and showed a 30% prolongation of PR and a 21% prolongation of QRS. No effect on $MAP_{90}C$ was observed (see also Table 3). Quinidine at 3 µM showed 12% reduction of the heart rate, 15% prolongation of PR and 11% prolongation of $MAP_{90}C$. At 10 µM, the changes in heart rate and PR were enhanced to 30% and 32%, respectively, and QRS was also prolonged 20%. $MAP_{90}C$ was not more enhanced at 10 µM than at 3 µM (about 10%). At 30 µM, ECG could not be analyzed because quinidine induced severe morphological changes of the ECG.

Conclusions on Langendorff model
All the representative three $Na_V1.5$ blockers, flecainide, lidocaine and quinidine, induced decrease in heart rate and prolongation of PR and QRS with minor changes on $MAP_{90}C$. However, there were apparent differences of the degree of PR or QRS prolongation, and arrhythmogenic activity, namely flecainide showed the most potent inhibition on cardiac conduction velocity and arrhythmogenic activity, while lidocaine was the most weak and not arrhythmogenic.

***In vivo* ECG anesthetized rabbit model**

Figure 4.

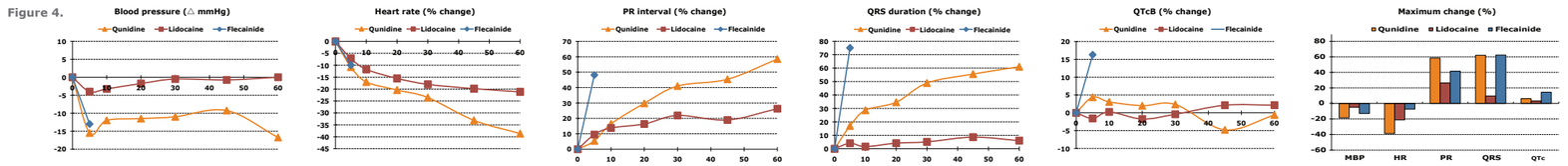

Table 4	MBP	HR	PR	QRS	QTc	Arrhythmia incidence (%)	Onset of arrythmia (min)
Flecainide	-13.0	-7.4	41.4	62.3	14.5	100%	6
Lidocaine	-5.0	-21.2	26.3	9.6	3.1	0%	>60
Quinidine	-18.5	-38.7	58.4	62.1	6.2	0%	>60

Table 4. Mean of individual maximum change (% change; BP: delta mmHg) in anesthetized rabbits (n=3-4).

Figure 4.
Effect of $Na_V1.5$ blockers on cardiovascular systems in ketamine and xylazine anesthetized rabbits (n=3-4). Flecainide at 1 mg/kg/min for 60 min i.v. infusion promptly decreased the blood pressure and heart rate, and prolonged the PR and QRS intervals and QTcB with individual maximum changes of about -15 mmHg, -10%, 40%, 60%, and 15%. All the animals showed arrhythmia (VT followed by Vf), followed by death within 20 min after the start of administration (N=3). Lidocaine at 1 mg/kg/min for 60 min i.v. infusion gradually decreased the heart rate and prolonged the PR interval with individual maximum changes of about -20% and 25%, while it had little or no effect on blood pressure, QRS or QTc. No arrhythmia was observed (N=4). Quinidine at 1 mg/kg/min for 60 min i.v. infusion gradually decreased the heart rate and prolonged PR and QRS intervals with individual maximum changes of about -40%, 60% and 60%, while it introduced a slight decrease in blood pressure (about -20 mmHg at 60 min) with little or no effect on QTc. No arrhythmia was observed but severe morphological changes of ECG were observed in 2 out of 4 animals.

Conclusions on *in vivo* rabbit model
The IC_{50}s of $Na_V1.5$ block of flecainide, quinidine and lidocaine are here reported to be 2 µM, 18 µM, and 44 µM (at the physiologically relevant V_{hold} of -80 mV, and determined at the last peak in the pulsetrain). In the present study, flecainide and quinidine showed stronger changes of ECG and arrhythmogenic activity than lidocaine. These differences are thus expected given that the same test concentration was used in this *in vivo* assay. The other $Na_V1.5$ blocking profile such as an effect on recovery from use-dependent block might be related to their *in vivo* effects.