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## Research Article

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# **Characterization of pharmacological inhibition of the hERG potassium channel by nitazene opioids**

**By**

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## Abstract

Nitazene opioids are 'street' drugs, many of which are substantially more potent than heroin. They are linked to respiratory depression, but other adverse effects have not been widely investigated. Machine learning models have predicted that several nitazenes may interact with a major drug anti-target, the hERG cardiac potassium channel. This study was conducted to evaluate effects on hERG of isotonitazene, protonitazene, etonitazene, metonitazene and the related piperidine benzimidazoline bromophine. hERG-expressing HEK293 cells were studied using automated patch clamp. Using a standard +20 mV command (WT) hERG tail current ( $I_{\text{hERG}}$ ) was acutely inhibited by all 5 compounds with  $\text{IC}_{50}$  values between 0.5  $\mu\text{M}$  (protonitazene) and 1.32  $\mu\text{M}$  (isotonitazene). Inhibition showed voltage- and time-dependence consistent with gating-dependent block. The  $\text{IC}_{50}$  values for  $I_{\text{hERG}}$  inhibition for inactivation-attenuated (N588K) hERG were 3.3-5.1 fold those for WT for protonitazene and isotonitazene. Mutations at S5 and S6 aromatic residues (F557, Y652, F656) affected both compounds, with a particularly marked influence of F656A (13-fold WT  $\text{IC}_{50}$  for isotonitazene and 73-fold for protonitazene). Neither isotonitazene nor protonitazene impaired hERG channel trafficking to the surface membrane. *In silico* docking and molecular dynamics simulations reproduced binding to canonical binding residues. Combining fentanyl with isotonitazene or protonitazene exacerbated block compared to each compound alone. Hypokalemia (1 mM  $[\text{K}^+]_o$ ) increased the potency of isotonitazene but not protonitazene. In conclusion, hERG is a direct target for nitazene opioids.

**Key words:** bromophine; etonitazene; hERG; isotonitazene; metonitazene; nitazene; patch clamp; potassium channel; protonitazene; QT interval.

## 1. Introduction

Potassium channels encoded by the *human ether-à-go-go Related Gene* (*hERG*, alternative nomenclature *KCNH2*) play key roles in cardiac ventricular repolarization (1). *hERG* underpins the rapid delayed rectifier ( $I_{Kr}$ ) channel (2;3); loss- and gain-of-function *hERG* mutations respectively reduce and augment  $I_{Kr}$ , leading to subtypes of long- and short- QT syndromes (LQTS / SQTs) (1;4). Due to unique structural features, *hERG* channels can interact with numerous clinically used drugs: *hERG* is the major culprit in the acquired (drug-induced) form of LQTS and the associated arrhythmia *torsades de pointes* (TdP) (1;5). Consequently, novel drugs are routinely evaluated for any propensity to produce pharmacological inhibition of *hERG* channels (1;5).

Opioid drugs are widely used for the management of moderate to severe pain but, due to their addiction potential, development of opioid tolerance and difficulties in withdrawal, they are linked to opioid use disorder (6). Due to their psychotropic effect, opioids have also long been subject to illicit use; there is currently a global epidemic of opioid overdose and misuse which, in the USA at least, is primarily driven by the use of fentanyl and its relatives (7;8). While respiratory depression is the dominant acute health risk with opioid use, some reviews have noted a link between some opioids and QT interval prolongation/TdP (9;10). Thus, methadone has potential for QT prolongation and TdP at low concentrations, while tramadol and oxycodone carry some risk at high doses (9;10). Additionally, methadone, oxycodone and fentanyl can all inhibit *hERG* channel current (11-13).

In recent years, nitazene opioids have emerged as a new threat in the opioid crisis (14-18). Nitazenes (2-benzylbenzimidazoles) are highly potent synthetic mu ( $\mu$ ) opioid receptor agonists that were originally developed in the 1950s, but have never been deployed clinically because they are strongly linked to respiratory depression (14;15). Nitazenes are relatively easy and cheap to synthesize and are increasingly misrepresented as/contaminate other illicitly sold drugs, which can lead to unintended exposure, with a high risk of severe respiratory depression and death (14;15;19). While it is recognized that nitazene use is linked to fatalities, mortality is likely to be underestimated both because nitazenes are not routinely tested for and because they are unstable in post-mortem blood samples (20;21).

Although pharmacological effects of nitazenes beyond respiratory depression are relatively understudied, cardiac arrest with nitazene consumption has been observed (22) and preclinical experiments on zebrafish have demonstrated direct cardiotoxic effects of etazene (23). To date, no *in vitro* study has investigated whether nitazenes interact with the hERG potassium channel, though a propensity for this has been suggested from deployment of machine-learning-model (MLM) based tools (24). The nitazenes etonitazene, isotonitazene, metonitazene, protonitazene and the piperidine benzimidazolone ('orphine') brorphine have been predicted (though not yet experimentally demonstrated) to inhibit hERG (24). The present study was undertaken to determine experimentally whether or not these drugs inhibit hERG and to investigate the underlying nature and basis of any observed effects. Our findings demonstrate clearly that acute nitazene exposure results in inhibition of hERG channel current ( $I_{hERG}$ ), while chronic exposure does not affect hERG channel trafficking to the cell surface membrane.

## 2. Methods

### 2.1 Cell line maintenance and hERG constructs

Human embryonic kidney 293 (HEK293) cells expressing wild-type (WT) hERG were donated by Dr Craig January, University of Wisconsin (25). The N588K, Y652A, F557L and F656A mutations to hERG1a have been previously used in our laboratory (e.g. (26-28)). N588K and F557L mutants were generated in our laboratory based on a template of wild-type (WT) hERG1a construct in a modified pcDNA3.0 vector (L15) using the QuikChange® II site-directed mutagenesis kit (Agilent Technologies) (29;30).

In brief, a pair of complementary oligonucleotide primers containing the mutation (forward primer sequence 5' CTCATGTGCACCTTAGCGCTCATCG 3' and reverse primer sequence 5' CGATGAGCGCTAAGGTGCACATGAG 3' for F557L and forward primer sequence 5' GCTGGCTGCACAACTGGGCGACC 3' and reverse primer sequence 5' GGTCGCCCAGTTTGTGCAGCCAGC 3' for N588K), which were synthesized by Merck (Dorset, UK), was used in a PCR reaction (95 °C for 1 min, 60 °C for 1 min, 68 °C for 16 min for 18 cycles). A DpnI digest of the PCR mix was then performed for 1 h at 37 °C. Competent DH5α Escherichia coli (Invitrogen, Paisley, UK) were transformed using standard procedures. The mutations were confirmed by sequencing of the entire open reading frame (Eurofins MWG Operon, Ebersberg, Germany) (29).

The full length F557L sequence in L15 was subcloned into an inducible expression vector pcDNA5/FRT/TO™ (V6520-20, Invitrogen, UK) by In Fusion HD Cloning (Takara Bio USA), N588K in L15 was subcloned into pcDNA5/FRT/TO™ using HindIII and NotI restriction enzyme sites. Cell lines stably expressing tetracycline-inducible F557L and N588K hERG channels were then generated by cotransfection of each of plasmid with Flp-recombinase expression vector pOG44 (V6005-20, Thermo Fisher Scientific, UK) into the Flp-In™ T-REx™ 293 Cell Line (R78007, Thermo Fisher Scientific, UK) as described in (31). Hygromycin (H011, TOKU-E 100 µg/ml final concentration) and Blasticidin (ant-bl-1, Invivogen 15 µg/ml) were used for selection and maintenance of the stable cell lines.

## *2.2 Cell culture*

Cells were maintained and passaged as described previously (26;28;29). Cells were cultured in T-75 flasks and used for experiments when they reached approximately 70-80% confluency. Cells were passaged every 3-4 days and used between passages 15-30.

## *2.3 Electrophysiological recordings*

Whole-cell patch-clamp experiments were performed using the QPatch Compact platform (Sophion Bioscience). QPlate 8 recording plates (SB0201) were stored at 4 °C prior to use. Once a recording plate was opened it was kept at room temperature and used within a week. Currents were recorded using standard extracellular and intracellular solutions. The external Tyrode's solution for WT, N588K, Y652A and F557L contained (mM): 140 NaCl, 4 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 glucose, and 5 HEPES, adjusted to pH 7.4 with NaOH. For experiments using F656A and WT control the solution contained 94 mM KCl (with NaCl concentration correspondingly reduced) (27;28). For experiments investigating hypokalaemic conditions, the external solution contained 1 mM KCl (with NaCl concentration correspondingly increased) (32). The intracellular solution contained (mM): 130 KCl, 1 MgCl<sub>2</sub>, 5 EGTA, 5 MgATP, and 10 HEPES, adjusted to pH 7.4 with KOH. Recordings were performed at room temperature, with a sampling rate of 10–25 kHz and filtered at 3.33-8.33 kHz (depending on the protocol used).

For each recording, data were assessed against predefined quality control criteria before inclusion in the analysis. Recordings were accepted if peak tail current exceeded 300 pA, cell capacitance (C<sub>slow</sub>) was greater than 8 pF, leak current at –80 mV was less than –300 pA, seal resistance (R<sub>seal</sub>) exceeded 300 MΩ, and series resistance (R<sub>s</sub>) was below 10 MΩ (Sophion Application Report).

## *2.4 Drug preparation*

The drugs studied (isotonitazene, etonitazene, protonitazene, metonitazene and bupropion) were selected on the basis of prior MLM prediction of hERG interaction (24). Isotonitazene and protonitazene were selected for detailed study as isotonitazene was the first nitazene to be identified as a street drug in Europe (14;15) and protonitazene is a structural isomer of

isotonitazene and was predicted to have the strongest hERG action in MLM evaluation (24). All the compounds studied are controlled substances and were purchased from Cayman Chemical through Cambridge Biosciences, under a UK Home Office Schedule 1 Controlled Drugs licence. Nitazenes were prepared as stock solutions in DMSO and diluted to final concentrations in external solution on the day of the experiment. Drug stock solutions were made to make 1 to 30 mM and stored at -20°C. Quinidine gluconate (Sigma-Aldrich, Gillingham, UK) was dissolved in Milli-Q water to produce a stock solution of 10 mM and was diluted in Tyrode's solution for use. The final solvent concentration in external solutions to which cells were exposed did not exceed 0.1%.

### 2.5 Trafficking assay

Evaluation of the effects of isotonitazene and protonitazene on hERG channel trafficking was conducted as described previously ((26;33); see online supplement for more details).

### 2.6 Data Analysis

Raw traces were processed on Sophion Analyzer 8.0 (Sophion Biosciences, Denmark) and analysed using Microsoft Excel (Microsoft Corporation, USA), GraphPad Prism 10.4.2 (GraphPad Software Inc., USA) and Clampfit 10.7 (Molecular Devices, USA) with results expressed as mean  $\pm$  SEM. Statistical comparisons were made using paired t-test, one-way and two-way repeated-measures ANOVA with Tukey post hoc test as appropriate. A p value  $<0.05$  was considered statistically significant.

All nitazene compounds produced a steady state block after ~3-4 minutes of drug exposure at which point  $I_{\text{hERG}}$  was measured to observe the drugs inhibitory effects. The fractional block of different compounds at varying concentrations was determined by using the equation:

$$\text{Fractional block} = 1 - I_{\text{drug}}/I_{\text{control}} \quad (1)$$

Where 'fractional block' represents the inhibition of  $I_{\text{hERG}}$  at a given concentration,  $I_{\text{control}}$  represents the current before drug application and  $I_{\text{drug}}$  is the current after ~3-4 minutes of drug exposure.

The concentration–response relationship for  $I_{\text{hERG}}$  inhibition was analysed by fitting fractional block data with the Hill equation in order to determine  $IC_{50}$  and Hill slope ( $n_H$ ) values.

$$\text{Fractional block} = 1 / (1 + 10^{(\log IC_{50} - X) n_H}) \quad (2)$$

Where ‘fractional block’ shows the inhibition of  $I_{\text{hERG}}$  at a given concentration,  $x$  represents the logarithm concentration,  $IC_{50}$  is the concentration producing half-maximal inhibition of  $I_{\text{hERG}}$ , and  $n_H$  is the Hill coefficient describing the steepness of the concentration–response curve.

Voltage-dependent activation of  $I_{\text{hERG}}$  was assessed from current–voltage relationships generated from tail currents and fitted with a Boltzmann function.

$$I = I_{\text{max}} / (1 + \exp((V_{0.5} - V_m)/k)) \quad (3)$$

where  $I$  is the tail current amplitude measured following each test potential ( $V_m$ ),  $I_{\text{max}}$  is the maximum tail current obtained during the protocol,  $V_{0.5}$  is the membrane potential at which half-maximal activation occurred, and  $k$  is the slope factor describing the steepness of the activation curve.

Voltage dependence of  $I_{\text{hERG}}$  inactivation was assessed using the equation:

$$(I/I_{\text{max}}) = 1 - 1 / (1 + \exp((V_{0.5} - V_m)/k)) \quad (4)$$

where  $I/I_{\text{max}}$  is the normalised current at a given membrane potential,  $V_m$  is the membrane potential,  $V_{0.5}$  is the voltage at which inactivation is half maximal, and  $k$  is the slope factor describing the voltage dependence of inactivation. For inactivation data plots  $I_{\text{hERG}}$  transients were corrected for potential deactivation as described in (34).

## 2.7 In silico molecular docking and molecular dynamics

Docking poses for isotonitazene and protonitazene at hERG were generated using the GNINA docking platform (35), which employs a convolutional neural network (CNN) scoring function on top of the standard AutoDock Vina (36) methodology. Docking was performed against the Dickson pore model of hERG (37). Based on the 5VA1 structure, this model conserves the canonical pore architecture while representing an MD-derived conformation consistent with experimentally validated hERG blocker binding and mutagenesis data (37). The docking grid box was centred on residue F656, with dimensions of  $x=20 \text{ \AA} \times y=20 \text{ \AA} \times z=20 \text{ \AA}$ . Docking via GNINA yielded nine poses per molecule; the pose selected for simulation was that with the lowest variance among poses which also conserved the overall docking ensemble.

MD simulations were used to assess pore-residue interactions between the nitazene compounds and the stability of the hERG–nitazene complexes. Ligands were parameterised using the CHARMM-GUI Ligand Modeller, with the tertiary amine of each compound modelled in the protonated form, as predicted from pKa analysis (38). Parameterised ligands were aligned to their respective GNINA poses and embedded into a lipid bilayer using CHARMM-GUI Membrane Builder (39;40). Each bilayer comprised a 4:1 ratio of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) to cholesterol, solvated with TIP3P water and neutralised with KCl at 0.15 M. Protein side chains were assigned default protonation states, based on pKa analysis using propKa 3.1 (41).

Systems were minimised and equilibrated locally using GROMACS following the standard CHARMM-GUI protocol (42;43). Following equilibration, production simulations were run with a 2 fs timestep for 5 runs of 500 ns per run, using Parrinello–Rahman pressure coupling (semi-isotropic, reference pressure 1 bar,  $\tau_P = 5.0 \text{ ps}$ , compressibility =  $4.5 \times 10^{-5} \text{ bar}^{-1}$ ) and Nosé–Hoover temperature coupling ( $\tau_T = 1.0 \text{ ps}$ ) maintaining system at 303.15 K.

Binding free energies were estimated using gmx\_MMPBSA for the isotonitazene and protonitazene systems (44). Prior to analysis, gmx trjconv was used to centre trajectories and fit them to the protein and ligand coordinates, removing rotational and translational motion. The protein and ligand groups were then indexed together to define a single complex group required for the MM-PBSA calculation. Gmx\_MMPBSA v1.5.0 requires GROMACS 2019.2-format run input, gmx grompp was used to regenerate tpr files in this version prior to submission.

Analyses were performed over frames 50–550 of the production trajectory at a temperature of 303.15 K. These were chosen to unbiased unstable docking poses. Given that the target is a membrane-embedded ion channel, membrane-specific Poisson-Boltzmann settings were applied (memopt=2), with a membrane dielectric of 4.0, solute dielectric of 10.0, membrane thickness of 40 Å centred at  $z = 62$ , and a single-pore geometry (poretype=1) (45). The CHARMM-specific radii set (PBRadii=7, charmm\_radii) was used in accordance with the CHARMM36m force field used to parameterise the system, and an ionic strength of 0.150 M was applied (istrng=0.150). Per-residue free energy decomposition (idecomp=2) was performed to identify residues contributing to the binding interaction, with output restricted to residues within 5 Å of the receptor–ligand interface. The change in total decomposition contribution (DTDC) was used as a metric to assess residue-ligand interaction.

## Results

### 3.1 Concentration-dependence of inhibition of $I_{hERG}$ by nitazenes

Previous MLM work has predicted  $I_{hERG}$  inhibition by nitazenes (24). *In vitro* evaluation of five compounds presented here provides the first experimental demonstration that this is the case (Figure 1). A standard protocol was employed to elicit  $I_{hERG}$  that was comprised of an activating command from -80 mV to +20 mV followed by a repolarizing command to -40 mV (lower panels of Figures 1C and 1D; (26-28). Figure 1C shows effects of isotonitazene, while Figure 1D shows similar data for protonitazene (upper traces in Figures 1C and 1D respectively). In the example shown, 0.3  $\mu$ M isotonitazene produced modest inhibition of  $I_{hERG}$  during the test pulse and of the tail current,  $I_{tail}$ , on repolarization to -40 mV. Substantially greater inhibition of  $I_{hERG}$  was seen with 1  $\mu$ M isotonitazene, though more than 50% of the  $I_{tail}$  remained (Figure 1C). By contrast, in the example shown, protonitazene inhibition of  $I_{hERG}$  at each of the two concentrations (Figure 1D) was markedly greater than that seen with isotonitazene. For each drug, similar measurements were made (in separate experiments) over a range of concentrations between 0.1 and 30  $\mu$ M. For each concentration tested of each drug, fractional inhibition of  $I_{tails}$  was quantified using equation 1 and pooled values at each concentration were plotted as shown in Figure 1E and fitted with equation 2. The derived half-maximal inhibitory concentration ( $IC_{50}$ ) for isotonitazene was 1.32  $\mu$ M, while that for protonitazene was 0.5  $\mu$ M. For comparative purposes, similar measurements were made for a well-established  $I_{hERG}$  blocker, the Class Ia antiarrhythmic drug quinidine. Under the conditions of this study, quinidine exhibited an  $IC_{50}$  of 1.27  $\mu$ M (Figure 1E). Summary data for  $IC_{50}$  and  $n_H$  values for all three compounds are shown in Table 1.

Experiments were extended to three other related compounds: metonitazene, etonitazene and the related piperidine benzimidazole bromophine. All three compounds exhibited concentration-dependent inhibition of  $I_{hERG}$ , as shown in Figure 1F and summarized in Table 1. The derived  $IC_{50}$  values lay between 0.68  $\mu$ M (metonitazene) and 1.32  $\mu$ M (isotonitazene). Thus, all compounds tested here inhibited  $I_{hERG}$  with  $IC_{50}$  values in the low  $\mu$ M or sub  $\mu$ M range. As highlighted in the Methods (section 2.4), isotonitazene and protonitazene were selected for more detailed investigation.

The effects of isotonitazene and protonitazene on  $I_{hERG}$  deactivation were evaluated using 1  $\mu$ M concentrations with the standard +20 mV protocol. For isotonitazene, deactivation in control solution was described by fast and slow time-constants ( $\tau_f$  and  $\tau_s$  respectively) of  $307 \pm 21$  ms and  $2093 \pm 105$  ms, while in drug the values were  $309 \pm 25$  and  $2092 \pm 156$  ms respectively (n=8; paired t-test: P=0.94 and 0.99, respectively). The proportion of deactivation described by  $\tau_f$  was unchanged ( $0.693 \pm 0.05$  and  $0.625 \pm 0.03$  respectively). For protonitazene,  $\tau_f$  and  $\tau_s$  were  $224 \pm 17$  and  $1527 \pm 73$  ms in control, while in drug the values were  $201 \pm 26$  and  $2788 \pm 181$  ms (n=6; paired t-test: P=0.53 and 0.001). The proportion of deactivation described by  $\tau_f$  was unchanged ( $0.567 \pm 0.01$  and  $0.600 \pm 0.04$  respectively).

Chronic effects of isotonitazene and protonitazene exposure on hERG protein expression are shown in supplemental Figure S1. Neither drug reduced cell surface expression (CSE) of hERG channel protein. Indeed, a small (~13-16%) increase in hERG CSE was seen for both isotonitazene and protonitazene. No significant changes to total cell hERG protein expression were seen with either drug.

### *3.2 Voltage dependence of $I_{hERG}$ inhibition by isotonitazene and protonitazene*

The lower traces of Figure 2A and 2B show the voltage protocol used to elicit  $I_{hERG}$  at test potentials between -40 and +50 mV; drug effects on  $I_{tails}$  elicited on repolarization to -40 mV were evaluated. The upper panels of Figure 2A and 2B show representative traces for the effects of 1  $\mu$ M isotonitazene. Figure 2C shows  $I_{tail}$  density-voltage plots in control and with isotonitazene. These progressively diverged with increasingly positive test commands, with significant divergence of control and drug relationships for commands to 0 mV and more positive. This is also shown in Figure 2D, which shows mean fractional block at -30 mV and +50 mV, with individual experimental values shown overlain. Fractional block was greater at +50 mV than -30 mV. Fits to the tail current data in Figure 2C with equation 3, gave activation  $V_{0.5}$  values of  $-11.64 \pm 3.61$  mV in control and  $-13.20 \pm 3.09$  mV in isotonitazene (p>0.05;  $k$  of  $6.69 \pm 0.34$  mV in control and  $5.75 \pm 0.45$  in drug, p>0.05).

Figures 2E and 2F summarize the results of similar experiments with 1  $\mu$ M protonitazene. This concentration of protonitazene produced a greater inhibition of hERG  $I_{tails}$  than observed for isotonitazene, consistent with the concentration-response data in Figure 1 and Table 1. For

the  $I_{tail}$  density plots (Figure 2E), inhibitory effects of protonitazene were statistically significant for commands to 0 mV and more positive (with  $I_{hERG}/I_{control}$  showing a similar relationship with voltage to that for isotonitazene). Figure 2F shows mean fractional block at -30 mV and +50 mV, with individual experimental values shown overlain. Fractional block at +50 mV was significantly greater than that at -30 mV ( $p < 0.05$ ). Fits to the tail current data in Figure 2E with equation 3, gave activation  $V_{0.5}$  values of  $-6.19 \pm 5.92$  mV in control and  $-12.76 \pm 0.82$  mV in protonitazene ( $p < 0.05$ ;  $k$  of  $5.61 \pm 0.45$  mV in control and  $4.82 \pm 0.31$  mV in drug,  $p > 0.05$ ). Thus, there was a modest but statistically significant leftward shift in  $I_{hERG}$  activation with protonitazene.

### *3.3 Time dependence of $I_{hERG}$ inhibition by isotonitazene and protonitazene*

An envelope of tails protocol (26;46) was used to evaluate development of  $I_{hERG}$  inhibition with time by the two nitazene drugs. It was comprised of progressively longer activating commands to +20 mV (between 20 and 2594 ms in duration). The protocol is shown in the lower panels on Figures 3A and 3B, while the corresponding current records are shown in the upper panels. It is evident from the experimental records shown that currents in the presence of isotonitazene diverged from those in control to a greater extent with progressively longer test pulse durations. Figure 3C shows fits to the tail current magnitudes, following normalization to maximal tail current observed during the protocol, for both control and isotonitazene conditions. These fits were used to derive time constants of activation ( $\tau_{act}$ ) in control and drug. It is clear that the relationship rose more quickly in the presence than in the absence of isotonitazene (with derived  $\tau_{act}$  of  $319.3 \pm 34.29$  ms in control and  $144.8 \pm 52.51$  ms in isotonitazene;  $p < 0.05$ ). Figure 3D shows similar mean data for 1  $\mu$ M protonitazene, with  $\tau_{act}$  of  $310 \pm 90.81$  ms in control and  $142 \pm 53.72$  ms in protonitazene ( $p < 0.05$ ). Thus, both nitazenes produced an apparent acceleration of the time-course of  $I_{hERG}$  activation during the envelope of tails protocol. It is also notable that in the presence of either drug, after reaching a maximal value,  $I_{tail}$  amplitude decreased rather than attaining a steady-state. This is indicative of marked gating-dependence of inhibition. In order to evaluate this further, fractional inhibition of the  $I_{tail}$  was measured for each command pulse duration and was plotted as shown in Figure 3E and 3F.

Due to the apparent acceleration in  $I_{hERG}$  activation (Figures 3C and 3D), for the shortest duration voltage commands, an increase rather than inhibition in  $I_{hERG}$  was observed (resulting in an apparent negative fractional inhibition). From 228 ms onwards for isotonitazene and protonitazene positive fractional inhibition was observed and in each case this increased progressively with longer duration depolarizations. The time-course of inhibition during this protocol was obtained by quantifying the time taken for inhibition to reach half of its maximal value ( $\tau_{1/2}$ ). For isotonitazene, the  $\tau_{1/2}$  of development of inhibition was  $493.4 \pm 98.0$  ms ( $n=5$ ). For protonitazene, the  $\tau_{1/2}$  of development of inhibition was  $535.5 \pm 124.2$  ms ( $n=5$ ). These observations indicate that  $I_{hERG}$  inhibition by both nitazenes was strongly gating-dependent, without any evidence for an appreciable contribution of closed-state inhibition.

### *3.4 Nitazene inhibition and $I_{hERG}$ inactivation.*

$I_{hERG}$  inactivation was evaluated using the three-step protocol shown in Figure 4A, which was applied in the absence and presence of isotonitazene. An initial step to +40 mV was applied to activate and inactivate  $I_{hERG}$  followed by a “ladder” of 10 ms duration repolarising steps (from -140 mV to +40 mV) to relieve inactivation to differing extents. Following this step a depolarization to +40 mV was applied. The magnitude of  $I_{hERG}$  during this third step depended on the extent to which the preceding ‘ladder’ step had relieved inactivation. Figure 4B shows representative control currents (upper panel) elicited during the third step following the ‘ladder’ part of the protocol (voltage shown in lower panel). Maximal current was observed following a ladder step to -140 mV, and was about half of this amplitude at  $\sim -30$  mV. Figure 4C shows currents from the same experiment following application of isotonitazene. Under each condition the amplitude of currents following the different ladder steps was normalized to the maximal current amplitude seen during the protocol. The resulting data from 12 experiments were plotted as shown in Figure 4D and were fitted by equation 4 (Methods). From these plots inactivation  $V_{0.5}$  values were obtained of  $-29.16 \pm 2.05$  in control and  $-29.26 \pm 1.99$  mV in isotonitazene ( $p > 0.05$ ;  $k$  of  $-17.16 \pm 1.38$  mV in control and  $-17.98 \pm 0.81$  mV in drug,  $p > 0.05$ ). Thus, there wasn’t any significant voltage shift in  $I_{hERG}$  inactivation with isotonitazene, as might occur if the drug stabilized the inactivated state.

In order to probe contingency of  $I_{hERG}$  inhibition by isotonitazene on intact inactivation gating, experiments were conducted using the N588K mutation (47). An advantage of this mutation is that the N588 residue is located in the S5-Pore linker region and is remote from the pore and canonical pore binding residues. Figure 4E shows representative traces of N588K  $I_{hERG}$  in the absence and presence of 10  $\mu$ M isotonitazene. Figure 4F shows the concentration response relation for N588K  $I_{hERG}$  inhibition by isotonitazene, with the WT  $I_{hERG}$  relationship shown overlain. The derived half-maximal inhibitory concentration ( $IC_{50}$ ) for isotonitazene inhibition of was 6.68  $\mu$ M: 5.1-fold that of WT  $I_{hERG}$ . This indicates that impaired inactivation had a marked effect on isotonitazene action. Similar experiments were performed with protonitazene (Figure 4G and 4H). The derived half-maximal inhibitory concentration ( $IC_{50}$ ) for protonitazene inhibition of was 1.67  $\mu$ M: 3.3-fold that of WT  $I_{hERG}$ . Thus, impairment of  $I_{hERG}$  inactivation had a notable, though not profound effect on the potency of both isotonitazene and protonitazene block of  $I_{hERG}$ .

### *3.5 Effect of pore mutants on inhibitory effects of isotonitazene and protonitazene*

Aromatic residues in the S5 and S6 transmembrane segments have been implicated in the binding to hERG of a range of drugs (1;48-50). Results of experiments deploying S5 and S6 mutants are summarized in Figure 5 and Table 2. Figure 5A shows effects of the Y652A mutation on isotonitazene and protonitazene (effects of 10  $\mu$ M of each drug are shown). The Y652A mutation markedly reduced drug action. Figure 5B and 5C shows concentration response relations for  $I_{hERG}$  inhibition by isotonitazene (Figure 5B) and protonitazene (Figure 5C), with  $IC_{50}$  values being 7.5-fold and 8.3-fold that for WT  $I_{hERG}$  respectively. Also shown are similar recordings from mutation of the S5 residue F557 (F557L). Again, for both isotonitazene and protonitazene inhibitory actions were reduced. Concentration-response relations for F557L are shown in Figure 5B (isotonitazene) and Figure 5C (protonitazene). For both drugs, the effects of the F557L mutation were similar to those of Y652A, with the  $IC_{50}$  for F557L  $I_{hERG}$  being 6.9-fold and 7.4-fold the WT control, respectively, for isotonitazene and protonitazene.

Further experiments were performed using the S6 F656A mutation, which typically is low expressing and requires to be measured as inward  $I_{hERG}$  in high external potassium ( $[K^+]_o$  of 94 mM) (51;52). Figure 5D shows inward  $I_{hERG}$  tails for WT  $I_{hERG}$  and F656A  $I_{hERG}$ : at 10  $\mu$ M both

compounds largely abolished the WT current. By contrast considerable current remained in the presence of this concentration of both isotonitazene and protonitazene for F656A. Figures 5E and 5F respectively show concentration response relations for isotonitazene and protonitazene for F656A  $I_{hERG}$ . For isotonitazene the F656A  $IC_{50}$  was 12.9-fold that of the WT control; for protonitazene the fold change was higher (73.1-fold WT control). Thus, of the residues studied F557 and Y652 appeared to be significant for hERG inhibition by both drugs and mutations of these residues had similar effects to one another for both drugs. By contrast mutation of F656 had a larger effect and was particularly notable in its effect on protonitazene.

Docking with GNINA generated nine within-pore site poses for both isotonitazene and protonitazene (Supplemental Figure S2). These poses demonstrated that the nitazene ligands could simultaneously contact the canonical aromatic pore residues F557, Y652, and F656, consistent with those identified experimentally (Figure 6). A notable difference between the two ligands was their orientation within the pore: isotonitazene adopted a more pocket-oriented conformation directed towards F557 (ca 3.69 Å), whereas protonitazene predominantly displayed pore-spanning poses. Despite this distinction, the overall pose geometries of the two ligands were broadly similar. For each nitazene, one representative pose was selected for simulation on the basis of low CNN variance (0.007 for isotonitazene, 0.02 for protonitazene) and conservation of the average pose position.

Subsequent trajectory analysis post-simulation revealed that selected poses were stable across the 5 repeats, demonstrating maximum RMSF values of 0.18 nm, shown by tertiary amide groups (Figure 7 Ai, Aii). The variable regions interestingly appeared to show similar peaks in RMSF, showing that the slight structural variation did not result in a significant change in RMSF. Moreover, repeats did not uncover a ligand rotation, proposing that these two nitazenes maintain a pore-spanning horizontal conformation, rather than a vertical insertion pose. Similarly, ligands did not demonstrate significant movement in relation to the hERG channel, as computed via RMSD (Supplemental Figure 2D).

Analysis of trajectories by gmx\_MMPBSA supplemented our basis for simultaneous interactions between F557, Y652 and F656, and confirmed that binding energies between these residues were present within simulation data (Figure 7B). MMPBSA analysis was able

to show that protonitazene demonstrated an increase in DTDC to Y652 and F656, albeit that these were deemed non-significant by Mann-Whitney analysis (0.15 and 0.056 respectively  $p=0.05$ ).

### *3.6 Effects of low $[K^+]_o$ and drug combination on nitazene actions.*

Drug induced QT prolongation tends to be a multi-hit phenomenon with known risk factors including drug combination and electrolyte abnormalities, particularly hypokalemia (53). In the experiments summarized in Figure 8 we investigated the effect of reduced  $[K^+]_o$  on WT  $I_{HERG}$  by isotonitazene and protonitazene. For these experiments, each drug was studied using the same standard protocol as deployed in Figure 1, but using an external solution containing 1 mM  $[K^+]_o$ . Effects of 0.3  $\mu$ M of each drug are shown in Figure 8A (isotonitazene) and Figure 8B (protonitazene). Low  $[K^+]_o$  had a marked effect on isotonitazene, as 0.3  $\mu$ M of this compound produced substantial inhibition (not the case with 4 mM  $[K^+]_o$ ). By contrast, the effect of protonitazene seemed little affected by lowering  $[K^+]_o$ . These observations were confirmed by constructing full concentration response relations for both compounds in low  $[K^+]_o$ . Figure 8C shows a marked left-ward shift of the concentration response relationship for  $I_{HERG}$  block by isotonitazene in reduced  $[K^+]_o$  ( $IC_{50}$  of 0.2  $\mu$ M vs 1.32  $\mu$ M for 4 mM  $[K^+]_o$ ), whereas Figure 8D shows close superimposition of the concentration response relations in reduced and normal  $[K^+]_o$  for protonitazene ( $IC_{50}$  of 0.56  $\mu$ M vs 0.5  $\mu$ M for 4 mM  $[K^+]_o$ ).

Nitazenes may be administered deliberately in combination with other illicitly used drugs, or as contaminants of other preparations. Therefore, in a final series of experiments, we investigated the effects of combining isotonitazene and (separately) protonitazene with fentanyl. Figure 9A shows representative traces in control and presence of 1  $\mu$ M fentanyl, while Figure 9B and 9C show effects of combining each of isotonitazene (1  $\mu$ M, Figure 9B) and protonitazene (0.3  $\mu$ M, Figure 9C) with fentanyl. Mean  $I_{HERG}$  tail fractional block data are shown in Figure 9D and 9E. For each compound, combination with fentanyl led to significantly greater inhibition of  $I_{HERG}$  tails than was the case when either fentanyl or each nitazene was applied on their own. Thus, as might be anticipated, drug combination exacerbated  $I_{HERG}$  block by each nitazene.

## 4. Discussion

### 4.1 Nitazenes as gated state-dependent $I_{hERG}$ inhibitors

While illicit nitazene use is recognized to be a growing threat, these drugs have no therapeutic role in clinical medicine and there is comparatively little information available on adverse effects other than respiratory depression (15) or from wider pre-clinical studies. Our recent work using *in silico* prediction methods highlighted the potential for nitazenes to interact with hERG (24). The present study is the first to provide *definitive* experimental evidence that well known nitazenes do produce  $I_{hERG}$  block. We used the antiarrhythmic drug quinidine as a positive control drug that is known to carry proarrhythmic risk (54;55); it produced an  $IC_{50}$  within the range of published values (Table 1). Isotonitazene and etonitazene were of comparable potency to quinidine as  $I_{hERG}$  inhibitors in the present study and the other drugs had lower  $IC_{50}$  values than that for quinidine.

Both the use of experimental protocols that interrogate voltage- and time- dependence of block and the use of mutations to the canonical drug binding site on hERG demonstrate that the blocking effects of protonitazene and isotonitazene (the two nitazenes that were studied in detail) arise from direct interactions of these compounds with the channel rather than as secondary effects of mu ( $\mu$ ) opioid receptor activation. A positive voltage-dependence of inhibition, accompanied by a modest left-ward voltage shift in activation is consistent with gated-state  $I_{hERG}$  inhibition (eg (27;46;56)). The results with the envelope of tails protocol show that protonitazene and isotonitazene produced little or no closed-state inhibition, requiring channels to undergo gating for inhibition to develop. The progressive development of blockade during applied depolarisation evident in Figure 3 is indicative of strong gating dependence of inhibition, although on its own is insufficient to distinguish between activated- and inactivated- state dependence. A number of high affinity hERG channel inhibitors show strong dependence on intact inactivation gating (e.g. (47;57-60)). For such drugs, potency of  $I_{hERG}$  inhibition can be reduced profoundly by mutations that attenuate or abolish inactivation (47;57-59). Some drugs with  $IC_{50}$  values in the  $\mu$ M range are strongly sensitive to inactivation attenuation: for example, the  $IC_{50}$  for ranolazine inhibition of N588K  $I_{hERG}$  was  $\sim 15$ -fold that of the wild-type channel (27). By contrast, other compounds – including the Class Ia antiarrhythmics quinidine and disopyramide are comparatively little affected by inactivation attenuation (47;58;59;61). Protonitazene and isotonitazene exhibited  $IC_{50}$  values for N588K

that were respectively  $\sim 3.3$  and  $5.1$ -fold that for WT  $I_{\text{hERG}}$ . This compares to  $1.5 - 4.2$  fold N588K/WT  $IC_{50}$  for disopyramide, quinidine, propafenone and amiodarone vs  $11$ -fold for the high affinity methansulphonanilide E-4031 (47). The principal role of inactivation for conventional hERG blockers may be in facilitating optimal orientation of S6 binding residues for drug interaction (62-64). An allosteric effect is particularly likely in the case of the N588K residue, as its location in the S5-Pore linker region is remote from inner cavity binding residues.

#### *4.2 The nitazene binding site*

Although some compounds are comparatively little affected by mutations of S6 aromatic residues and the potential for non-canonical drug binding to hERG is recognized (26;46;51;65), the majority of drugs that have been examined in detail bind within the channel pore and interact with key aromatic residues (1;5;66). Y652 and F656 have long been implicated in hERG block (48;58). Cryo-EM structures of hERG indicate the presence of 4 lateral hydrophobic pockets in the tetrameric channel (67;68). In principle, such pockets may provide access to inner cavity binding sites or provide primary binding regions (26;46;66;69). F557 on S5 lies in the hydrophobic pathway and is close apposition to the side chain of Y652 (49;66;69). Within the last decade the F557L mutation has been reported to attenuate block by a number of compounds (28;46;49;69;70). Its effects on blocking potency are generally comparable to those of Y652A (28;46;49;69;70). In this study, the Y652A and F557L mutations had similar effects to one another in reducing blocking potency of both isotonitazene and protonitazene, which is consistent with prior reports on other drugs (28;46;49;69;70). However, the most striking effects in the present study arose with the F656A mutation, which increased the  $IC_{50}$  values of isotonitazene and protonitazene respectively to  $\sim 13$  and  $\sim 73$  fold their WT controls. This (a) implicates F656 as particularly important in nitazene inhibition of hERG and (b) is consistent with the stronger inhibitory action of protonitazene than isotonitazene. The difference in effect of F656 on the two compounds is remarkable given the close structural similarity of these nitazenes, which differ only in the specific alkyl group attached to the oxygen atom of the benzyl ring (a branched isopropoxy group in isotonitazene and a more linear propoxy group in protonitazene).

Consistent with this, our docking results support interactions between both isotonitazene and protonitazene and the aromatic residues investigated in this study (F557, Y652, and F656). Docking results further suggest that isotonitazene and protonitazene may occupy slightly different conformations within the pore, with protonitazene occupying a more central position within the pore and isotonitazene favouring more of an orientation towards the F557 pocket site. Structurally, the two compounds differ only in the alkyl group attached via the ether oxygen to the benzyl ring, a branched isopropoxy group in isotonitazene vs a more linear propoxy group in protonitazene. We observed a slightly higher RMSF value in this linear region for protonitazene, though this did not clearly contribute to the differing per-residue  $\Delta TDC$  values obtained by gmx\_MMPBSA. The orientation of these pore-lining residues towards the interior of the pore suggests that channel block may require simultaneous contact with multiple residues, and that a compound able to span the pore cavity may therefore have a greater capacity to cause blockade. gmx\_MMPBSA analysis was unable to confidently account for the marked difference in impact of the F656A mutation-associated potency observed experimentally, as this difference in gmx\_MMPBSA did not quite reach conventional statistical significance ( $p=0.055$ ).

#### 4.3 Clinical significance

This study establishes nitazenes as drugs that can produce acute inhibition of hERG channels. Both isotonitazene and protonitazene were evaluated for any propensity to reduce cell surface expression of hERG and neither compound reduced surface hERG levels. Thus, any hERG-related risk with these drugs is likely to result from acute inhibitory actions. Nitazenes are not used in clinical medicine and there are no recommended clinical levels of these drugs (24). In a very recent report of an isotonitazene-linked fatality, cardiac levels of 20 ng/g were reported (roughly equivalent to 50 nM) (71). While such an isotonitazene level would produce comparatively low levels of  $I_{hERG}$  inhibition with normal  $[K^+]_o$ , in principle it could give a substantial inhibition under conditions of hypokalemia (blood  $[K^+]$  was not given in (71)). Current data in the UK National Programme on Substance Use Mortality (NPSUM) show cardiovascular involvement in a number of nitazene-related deaths, but are insufficient to implicate direct cardiac electrophysiological effects (Copeland *et al*, personal observations).

On the other hand, in a fatality linked to protonitazene overdose, the [protonitazene] in cardiac blood was 1400 ng/ml (equivalent to 3.41  $\mu$ M) (72). While post-mortem concentrations should be extrapolated to *in vivo* or experimental concentrations with caution, it is notable that our data indicate that at such a concentration substantial  $I_{hERG}$  block would be observed. Most cases of drug-induced QT interval prolongation are associated with the additional presence of one or more risk factors (53). The actions of quinidine and dofetilide have long been known to be sensitive to external  $[K^+]$  (73). Our data on isotonitazene demonstrate that the drug's action was considerably more potent in the context of simulated hypokalemia, while for protonitazene this was not the case. Extracellular  $[K^+]$  can modulate  $I_{hERG}$  inactivation (2;32). It was notable that high  $[K^+]_o$  did not markedly affect  $IC_{50}$  of either drug, but that low  $[K^+]_o$  did so for isotonitazene. It is notable that the N588K mutation had a greater effect on isotonitazene than on protonitazene. It is possible, therefore, that for isotonitazene (but not protonitazene) S6 positioning during inactivation gating at reduced  $[K^+]_o$  was more favourable for isotonitazene interaction with key binding residues.

Many nitazene users either take nitazenes alongside other drugs or, (unknowingly) consume preparations in which nitazenes are mixed with other illicitly used drugs; thus nitazenes can be taken at the same time as with benzodiazepines, fentanyl, heroin and cocaine (15;16;19;72). Concomitant administration of >1 QT prolonging drug is a known risk in drug-induced QT syndrome (53). Both cocaine and fentanyl are known hERG channel inhibitors (74-76) and ingestion of nitazenes together with these drugs might therefore compound risk. Indeed, our experimental results demonstrate that combining fentanyl with either protonitazene or isotonitazene produced greater  $I_{hERG}$  inhibition than either nitazene or fentanyl on its own. This highlights that nitazene users should be advised that both deliberate and inadvertent combination of nitazenes with fentanyl is likely to increase the potential for cardiac electrophysiological effects, quite aside from compounding risk of respiratory depression. Moreover, it would probably be prudent to avoid combining nitazenes with any known hERG blocking/QT prolonging drug and this is a consideration for clinicians treating individuals known to use nitazenes. Additionally, and consistent with our findings on buprenorphine, a recent preprint ((83), a publication pre-peer review) provides independent evidence that "orphine" opioids can inhibit hERG. Thus, clinicians should also be mindful of

the potential for hERG blockade when treating individuals known to use buprenorphine or other opioid agonists.

### *Limitations and conclusions*

This study has investigated the propensity of nitazene drugs to inhibit hERG potassium channels and the underlying mechanisms by which inhibition occurs. If nitazenes also produce inhibition of other cardiac ion channels at overlapping concentrations, this could exacerbate or offset hERG/ $I_{Kr}$  inhibition and thereby influence overall effects on ventricular repolarisation (77). Therefore, future studies should investigate effects of nitazene drugs against a wider range of cardiac ion channel targets than hERG alone. Although it is important that this limitation is noted, it does not detract from the facts that this study is: (i) the first direct demonstration of hERG channel block by any nitazene and (ii) a demonstration that multiple nitazenes can inhibit  $I_{hERG}$  at micromolar and submicromolar concentration and (iii) to our knowledge, one of the first studies to demonstrate direct inhibition of any ion channel by this class of drug.

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preparation, data collection and analysis were performed by MRC, MB, TO, OH, YZ, HC. The first draft of the manuscript was produced by JCH, MRC, SCH, TO, RC, YZ. All authors reviewed/commented on manuscript drafts prior to submission. All authors read and approved the final manuscript.

#### **Availability of data and materials.**

The data for the study are included in the manuscript and supplementary information. Materials for the study will be made available on reasonable request.

#### **Code availability.**

Docking was performed against the Dickson pore model of hERG (37). Docked poses are available from RC on request.

#### **Declarations**

Ethics approval and consent to participate: N/A.

Consent for publication: N/A.

Competing interests: The authors declare that they have no actual or potential competing interests.

| Compound      | IC <sub>50</sub> (μM)<br>[95% C.I.] | n <sub>H</sub><br>[95% C.I.] | Number of<br>Cells | Published<br>IC <sub>50</sub> range | Example<br>References |
|---------------|-------------------------------------|------------------------------|--------------------|-------------------------------------|-----------------------|
| Isotonitazene | 1.32<br>[1.16-1.51]                 | 1.38<br>[1.16-1.67]          | 7-12               | N/A                                 |                       |
| Protonitazene | 0.50<br>[0.41–0.62]                 | 1.16<br>[0.93–1.47]          | 6                  | N/A                                 |                       |
| Metonitazene  | 0.68<br>[0.52–0.90]                 | 0.98<br>[0.77–1.27]          | 8                  | N/A                                 |                       |
| Etonitazene   | 1.28<br>[1.16–1.41]                 | 1.30<br>[1.16-1.47]          | 9                  | N/A                                 |                       |
| Brorphine     | 0.93<br>[0.77- 1.13]                | 1.15<br>[0.95-1.42]          | 5-6                | N/A                                 |                       |
| Quinidine     | 1.27<br>[1.06–1.51]                 | 1.02<br>[0.87-1.22]          | 5                  | 0.3-8.6                             | (78-82)               |

**Table 1** Concentration response summary data for nitazene opioids

Summary data derived from fits to concentration response relations shown in Figure 1, using equation 2 (Methods). For both IC<sub>50</sub> and n<sub>H</sub> values means and 95% confidence intervals (C.I.) are shown. ‘n’ values refer to number of replicates used for concentration-response data. Quinidine was used as a known reference compound, with the observed IC<sub>50</sub> value from this study lying within the range of published IC<sub>50</sub> values.

| Drug          | Construct | n    | IC <sub>50</sub> (μM) | 95% C.I. (μM) | n <sub>H</sub> | 95% C.I.  | Fold of WT IC <sub>50</sub> |
|---------------|-----------|------|-----------------------|---------------|----------------|-----------|-----------------------------|
| Isotonitazene | WT        | 7-12 | 1.32                  | 1.16-1.51     | 1.38           | 1.16-1.67 | 1                           |
| Isotonitazene | Y652A     | 6    | 9.89                  | 7.24-13.91    | 0.72           | 0.54-0.99 | 7.5                         |
| Isotonitazene | F557L     | 5    | 9.11                  | 5.94-13.79    | 0.68           | 0.52-0.91 | 6.9                         |
| Protonitazene | WT        | 6    | 0.5                   | 0.41-0.62     | 1.16           | 0.93-1.47 | 1                           |
| Protonitazene | Y652A     | 7    | 4.17                  | 3.33-5.21     | 0.85           | 0.71-1.03 | 8.3                         |
| Protonitazene | F557L     | 5-6  | 3.69                  | 2.97-4.60     | 0.97           | 0.8-1.19  | 7.4                         |
| Isotonitazene | WT inward | 5    | 1.02                  | 0.97-1.15     | 1.09           | 0.97-1.24 | 1                           |
| Isotonitazene | F656A     | 5-9  | 13.18                 | 8.42-21.21    | 0.44           | 0.35-0.55 | 12.9                        |
| Protonitazene | WT inward | 6    | 0.44                  | 0.37-0.54     | 0.94           | 0.79-1.14 | 1                           |
| Protonitazene | F656A     | 5    | 32.15                 | 14.62-87.71   | 0.34           | 0.22-0.47 | 73.1                        |

**Table 2 Concentration-response summary data for nitazene inhibition of WT and mutant hERG channels**

Summary data were derived from fits to concentration-response relationships shown in Figure 5, using equation 2 (Methods). For both IC<sub>50</sub> and n<sub>H</sub> values, 95% confidence intervals (C.I.) are shown with 'n' values referring to the number of replicates used for concentration-response data. Fold-shift values were calculated relative to the matched WT condition.

## Figure Legends

**Figure 1. hERG inhibition by nitazenes.** (A, B) Chemical structures of isotonitazene (A) and protonitazene (B). (C,D)  $I_{\text{hERG}}$  current traces (upper) recorded using a standard +20 mV command protocol (lower) under control conditions and in the presence of isotonitazene (C) and protonitazene (D). Two concentrations (0.3 and 1  $\mu\text{M}$ ) are shown to illustrate concentration-dependent inhibition. (E) Six concentrations of protonitazene, isotonitazene, and quinidine (used as a reference compound), ranging from 100 nM to 30  $\mu\text{M}$ , were tested to generate a concentration-response plot (fitted with equation 2 methods) ( $n=5-12$ ). (F) Concentration-response relationships for etonitazene, metonitazene, and bromophine, plotted and fitted as in (E) ( $n=5-9$ ).

**Figure 2. Voltage dependence of  $I_{\text{hERG}}$  inhibition by isotonitazene and protonitazene.** (A, B) Representative  $I_{\text{hERG}}$  traces (upper) recorded using a current-voltage (I-V) protocol (lower) under control conditions (A) and 1  $\mu\text{M}$  isotonitazene (B). (C) Mean tail current density-voltage relationships for  $I_{\text{hERG}}$  in control and in the presence of 1  $\mu\text{M}$  isotonitazene. Tail current density was measured on repolarisation to -40 mV following test pulses between -40 and +50 mV fitted with equation 3 gave  $V_{0.5}$  values of  $-11.64 \pm 3.61$  mV in control and  $-13.20 \pm 3.09$  mV in isotonitazene, with  $k$  values of  $6.69 \pm 0.34$  mV and  $5.75 \pm 0.45$  mV, respectively ( $n=8$ ). (D) Fractional block of  $I_{\text{hERG}}$  by isotonitazene at -30 mV and +50 mV compared ( $n=7$ ). (E) Mean tail current density-voltage relationships for  $I_{\text{hERG}}$  in control and in the presence of 1  $\mu\text{M}$  protonitazene, plotted and analysed as in (C). Fits to the tail current data gave  $V_{0.5}$  values of  $-6.19 \pm 5.92$  mV in control and  $-12.76 \pm 0.82$  mV in protonitazene, with  $k$  values of  $5.61 \pm 0.45$  mV and  $4.82 \pm 0.31$  mV, respectively ( $n=6$ ). (F) Fractional block of  $I_{\text{hERG}}$  by protonitazene at -30 mV and +50 mV, compared as in (D) ( $n=7$ ). Statistical comparisons were made using repeated-measures two-way ANOVA with post hoc comparisons for current density-voltage relationships and paired t-tests for fractional block comparisons. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ .

**Figure 3. Time-dependence of  $I_{\text{hERG}}$  inhibition by isotonitazene and protonitazene.**

(A, B) Representative  $I_{\text{hERG}}$  currents recorded using the envelope-of-tails protocol under control conditions (A) and following application of 1  $\mu\text{M}$  isotonitazene (B). The corresponding voltage command protocol is shown below. Cells were depolarised to +20 mV for progressively increasing pulse durations before repolarisation to -40 mV, at which peak tail current amplitude was measured. (C) Normalised tail current amplitudes plotted against pulse duration under control conditions and in the presence of 1  $\mu\text{M}$  isotonitazene. Tail current amplitudes were normalised to the maximal tail current observed and fitted with a one-phase association function to give the  $\tau$  values shown in the "Results" text ( $n=5$ ). (D) Normalised tail current amplitudes plotted against pulse duration under control conditions and in the presence of 1  $\mu\text{M}$  protonitazene, analysed as in (C) ( $n=5$ ). (Ei, Fi) Fractional block of  $I_{\text{hERG}}$  plotted against depolarising pulse duration for 1  $\mu\text{M}$  isotonitazene ( $n=5$ ) (Ei) and 1  $\mu\text{M}$  protonitazene ( $n=5$ ) (Fi). Fractional block values were fitted with a bi-exponential association

function to describe the time-course of development of inhibition. (Eii, Fii) Expanded view of the early pulse durations from the fractional block plots shown in Ei and Fi (n=5).

**Figure 4. Effects of isotonitazene on  $I_{hERG}$  inactivation.** (A) Voltage protocol used to assess the voltage dependence of  $I_{hERG}$  inactivation.  $I_{hERG}$  was first activated and inactivated by a depolarising step to +40 mV, followed by 10 ms repolarising steps from -140 mV to +40 mV in 20 mV increments, to relieve inactivation. The membrane potential was then stepped back to +40 mV to elicit transient currents. The inset shows the “ladder portion” of the protocol. (B, C) Representative  $I_{hERG}$  current traces under control conditions (B) and following application of 1  $\mu$ M isotonitazene (C). The corresponding voltages are shown beneath the current traces. (D) Normalised inactivation relationships for control and 1  $\mu$ M isotonitazene. Current amplitudes were normalised to the maximal current recorded during the protocol and fitted with a Boltzmann function.  $V_{0.5}$  values were  $-29.16 \pm 2.05$  mV in control and  $-29.26 \pm 1.99$  mV in 1  $\mu$ M isotonitazene;  $k$  values were  $-17.16 \pm 1.38$  mV and  $-17.98 \pm 0.81$  mV, respectively (n=12;  $p>0.05$ ). (E) Representative N588K  $I_{hERG}$  traces recorded under control conditions (black) and 10  $\mu$ M isotonitazene (green). (F) Concentration-response relationship for isotonitazene inhibition of N588K  $I_{hERG}$  and WT  $I_{hERG}$  (n=5). (G) Representative N588K  $I_{hERG}$  traces recorded under control conditions (black) and 10  $\mu$ M protonitazene (green). (H) Concentration-response relationship for protonitazene inhibition of N588K  $I_{hERG}$  and WT  $I_{hERG}$  (n=6). Concentration-response relationships were fitted with the Hill equation with data shown as mean  $\pm$  SEM.

**Figure 5. Block of  $I_{hERG}$  for wild-type and mutant channels.** (A) Representative current traces showing the effects of 10  $\mu$ M isotonitazene and 10  $\mu$ M protonitazene on WT, Y652A and F557L hERG channels. Traces were recorded using the standard voltage protocol as shown in the panel. (B, C) Concentration-response relationships for inhibition of WT (n=7-12), Y652A (n=6) and F557L (n=5)  $I_{hERG}$  by isotonitazene (B) and inhibition of WT (n=6), Y652A (n=7) and F557L (n=5-6)  $I_{hERG}$  by protonitazene (C). Fractional block was calculated and data were fitted with the Hill equation. WT relationships are shown superimposed for comparison with mutant channel responses. (D) Representative inward current traces recorded from WT and F656A under control conditions and in the presence of 10  $\mu$ M isotonitazene or 10  $\mu$ M protonitazene. (E, F) Concentration-response relationships for inhibition of WT and F656A by isotonitazene (E) (n=5-9) and protonitazene (F) (n=5-6) analysed as in (B, C).

### Figure 6. Molecular docking of isotonitazene and protonitazene

(A) and (B) respectively show selected poses for isotonitazene (top, cyan) and protonitazene (bottom, purple). Both drugs show poses that simultaneously contact the three aromatic proposed pore residues F557 (green), Y652 (yellow) and F656 (red). Noticeably, protonitazene occupies a more central orientation within the pore; however, both drugs show a flat horizontal orientation rather than a vertical insertion into the pore. Distances to proximal residues of interest are included in Angstroms.

### Figure 7. Further in silico analysis of isotonitazene and protonitazene binding interactions

(A) show RMSF by B-Factor and mean per atom RMSF of isotonitazene and protonitazene during MD simulation. The RMSF (nm) of ligand heavy atoms is shown across five independent simulation repeats, for (Ai) isotonitazene and (Aii) protonitazene. Atoms are grouped and labelled according to their constituent functional group: amide (AMI), benzyl (BZ), variable region (VR), benzimidazole (BZIM), and nitro (NIT).

(B) Summed residue enthalpy by  $\Delta$ TDC across interacting chain. Total per-residue  $\Delta$ TDC, kcal/mol was summed across each ligand-interacting chain. Per-residue values were summed within each chain, then averaged across 5 independent replicate simulations. Error bars represent SEM.

### Figure 8. Effect of reduced extracellular $K^+$ on nitazene inhibition of $I_{hERG}$ .

(A, B) Representative  $I_{hERG}$  traces recorded using standard voltage protocol under low extracellular  $K^+$  conditions, showing the effects of 0.3  $\mu$ M isotonitazene (A) and 0.3  $\mu$ M protonitazene (B). The corresponding voltage protocol is shown below each set of current traces. Currents were elicited by depolarisation to +20 mV followed by repolarisation to -40 mV, at which peak tail current amplitude was measured. (C) Concentration-response relationship for isotonitazene inhibition of  $I_{hERG}$  recorded in standard 4 mM extracellular  $K^+$  (solid black line) inhibited  $I_{hERG}$  with an  $IC_{50}$  of 1.32  $\mu$ M and  $n_H$  1.38 ( $n=7-12$ ) and reduced 1 mM extracellular  $K^+$  inhibited  $I_{hERG}$  with an  $IC_{50}$  of 0.2  $\mu$ M with  $n_H$  1.19 (dashed black line) ( $n=5$ ). (D) Concentration-response relationship for protonitazene inhibition of  $I_{hERG}$  recorded in 4 mM inhibited  $I_{hERG}$  with an  $IC_{50}$  of 0.5  $\mu$ M and  $n_H$  1.16 ( $n=6$ ) and 1 mM extracellular  $K^+$  inhibited  $I_{hERG}$  with an  $IC_{50}$  of 0.56  $\mu$ M with  $n_H$  1.08 ( $n=6$ ).

### Figure 9. Effects of drug combinations on the inhibition of $I_{hERG}$ .

(A–C) Representative  $I_{hERG}$  traces recorded using the standard +20 mV protocol under control conditions and following application of 1  $\mu$ M fentanyl alone (A), a combined mixture of 1  $\mu$ M

isotonitazene and 1  $\mu$ M fentanyl (**B**), or a combined mixture of 0.3  $\mu$ M protonitazene and 1  $\mu$ M fentanyl (**C**). Tail current amplitude was measured on repolarisation to -40 mV. (**D**) Summary of percentage  $I_{hERG}$  block produced by 1  $\mu$ M fentanyl, 1  $\mu$ M isotonitazene and 1  $\mu$ M isotonitazene + 1  $\mu$ M fentanyl combination. (**E**) Summary of the percentage  $I_{hERG}$  block produced by 1  $\mu$ M fentanyl, 0.3  $\mu$ M protonitazene, and 0.3  $\mu$ M protonitazene + 1  $\mu$ M fentanyl combination. Block was calculated from peak tail current amplitudes as  $1 - I_{drug}/I_{control}$  and expressed as percentage block. Statistical comparisons were made using ordinary one-way ANOVA with Tukey's multiple comparisons test. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ .

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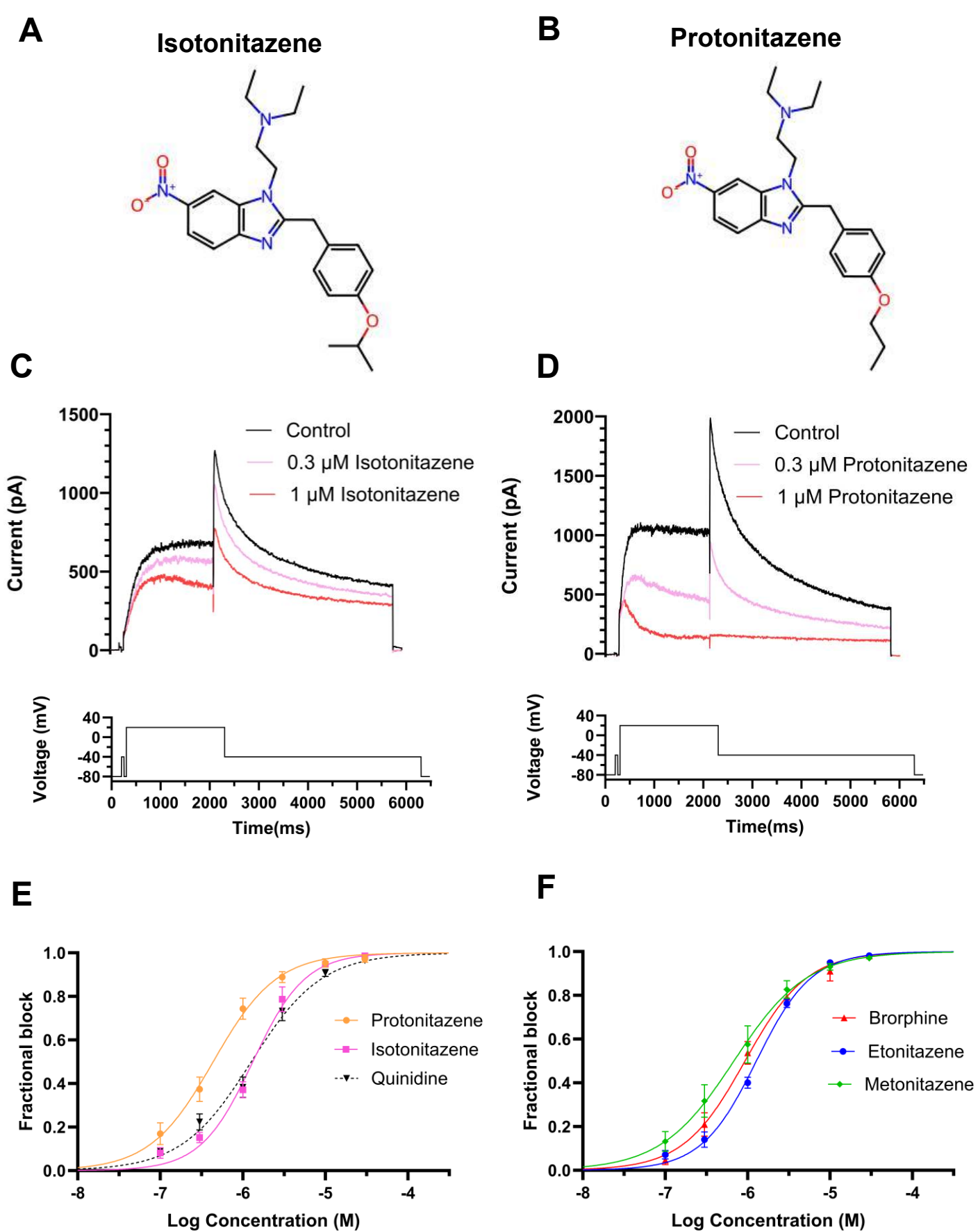


**Figure 1**

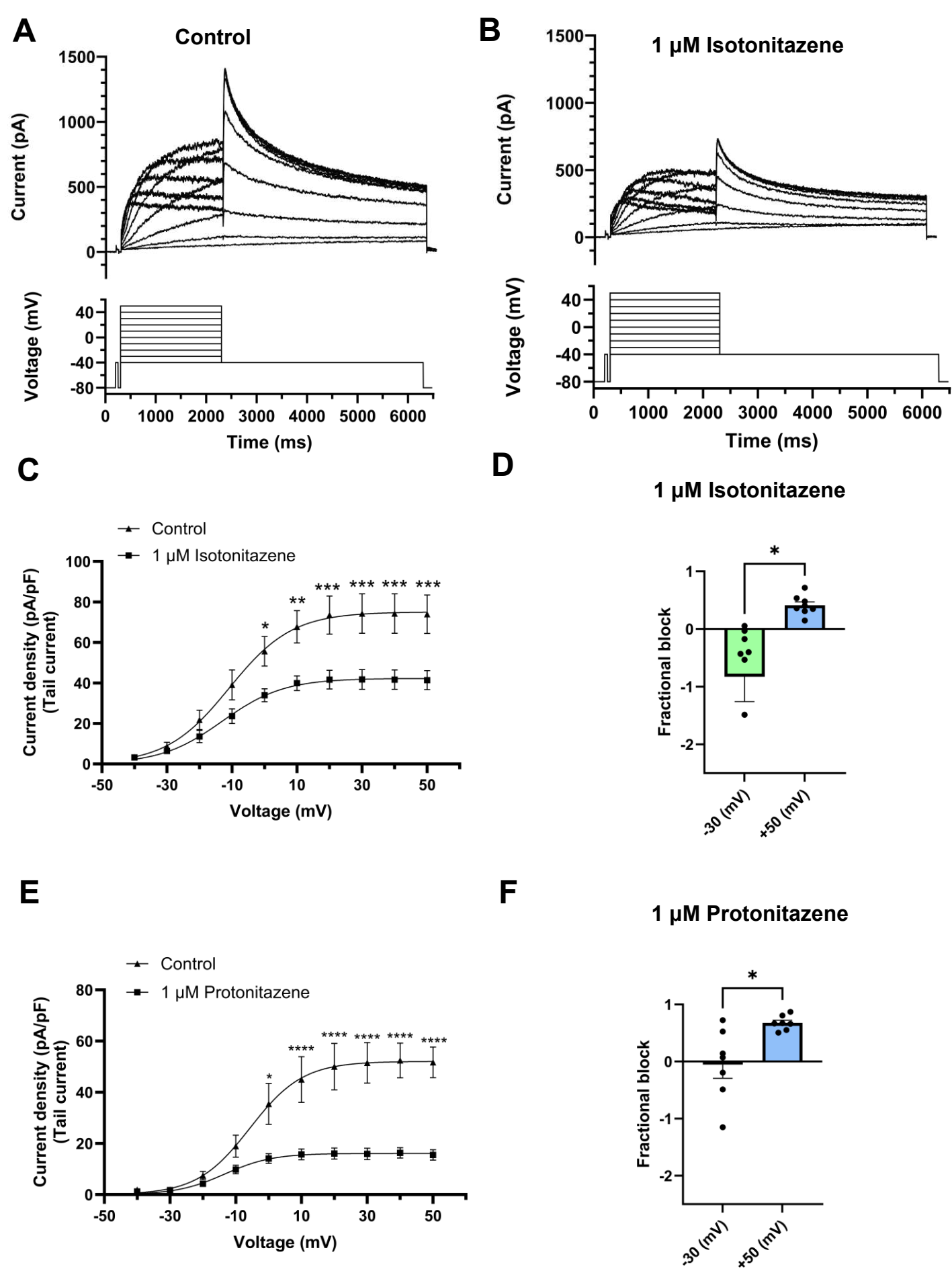


**Figure 2**

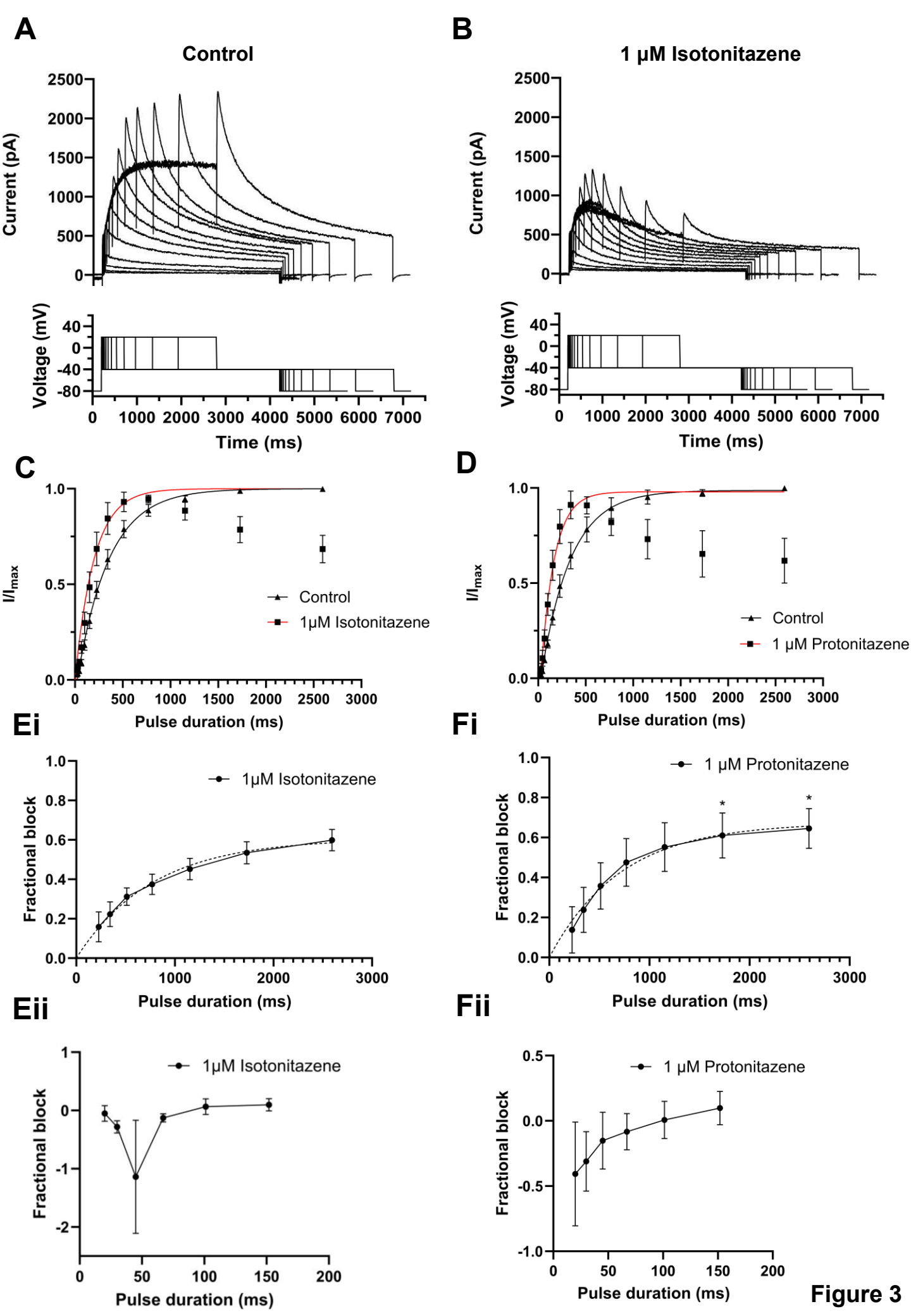


**Figure 3**

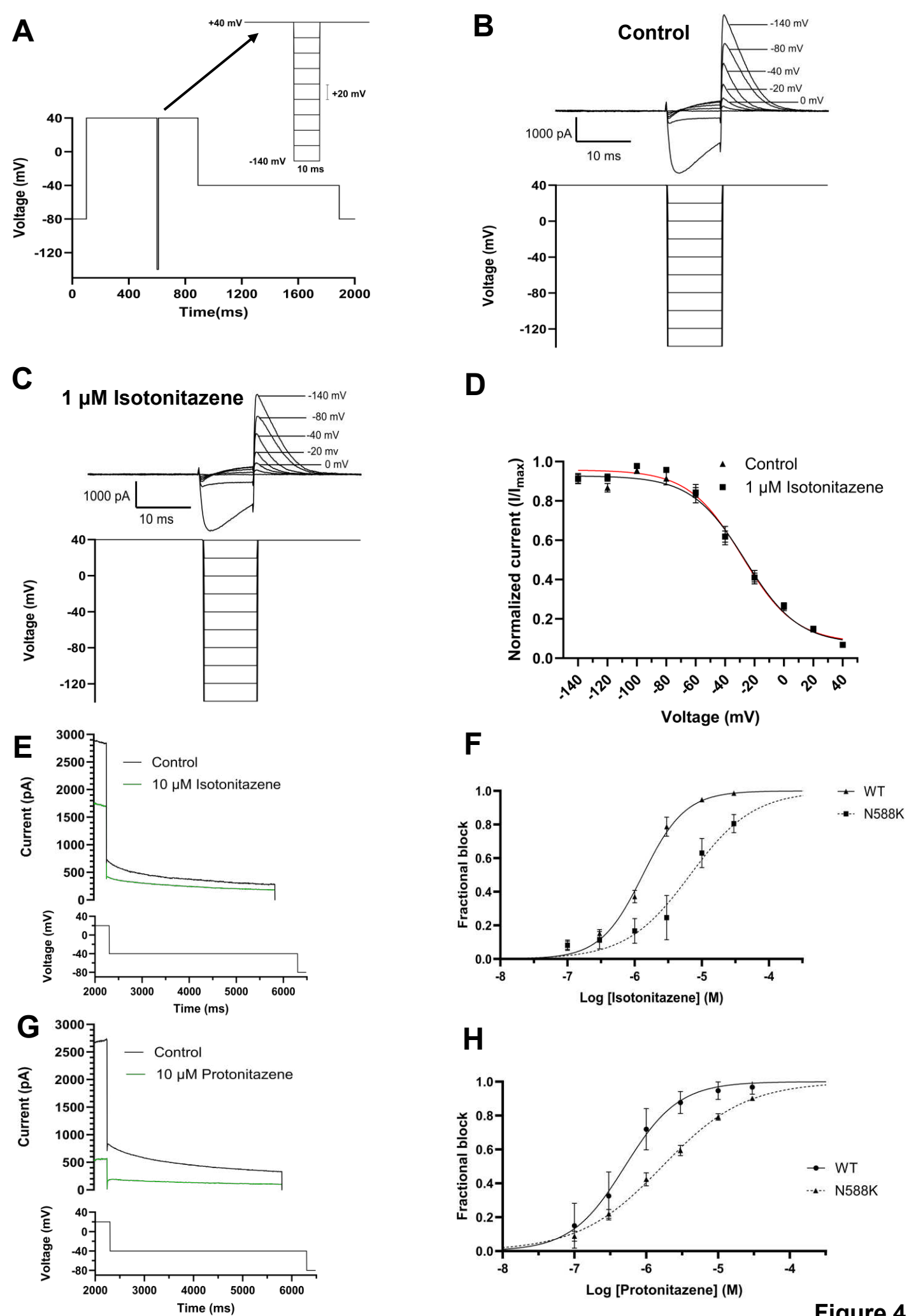
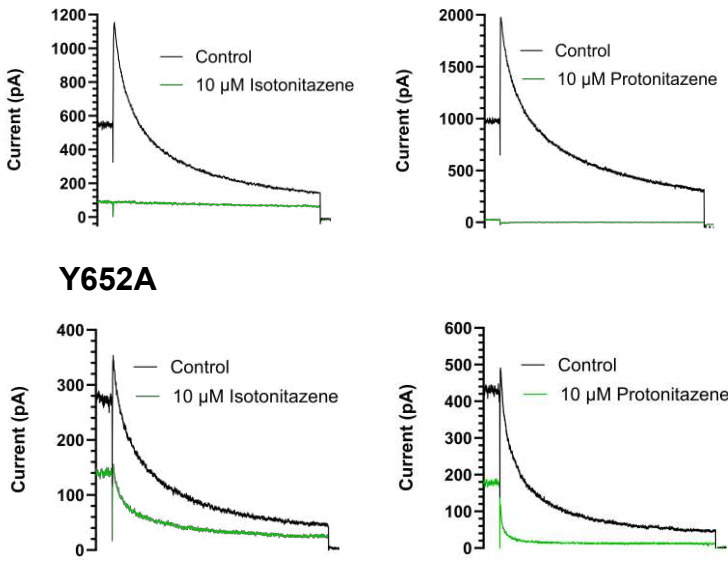
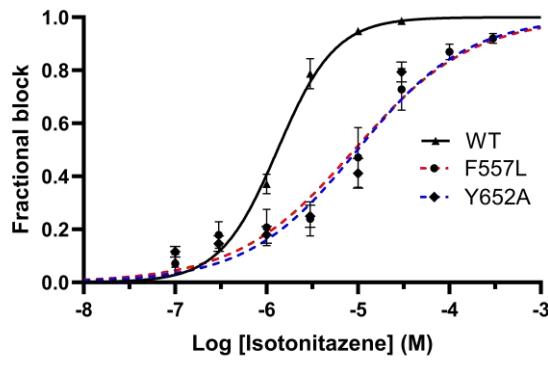
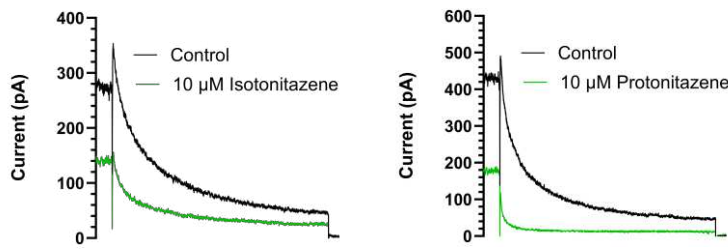
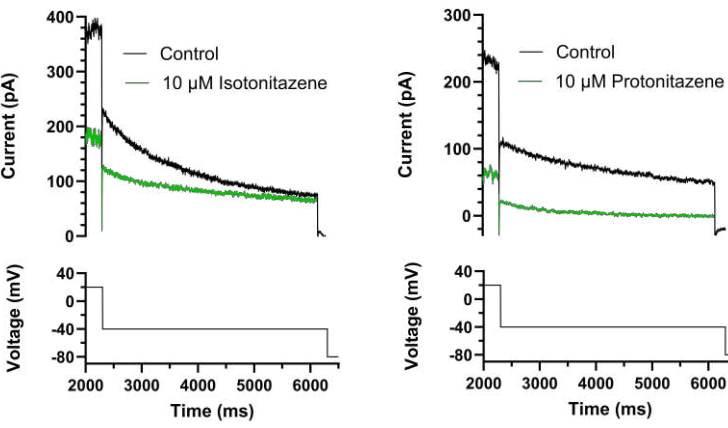
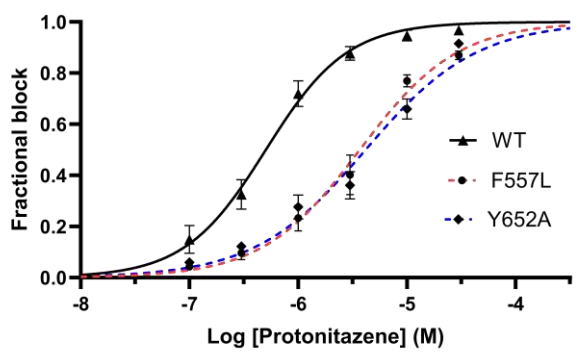
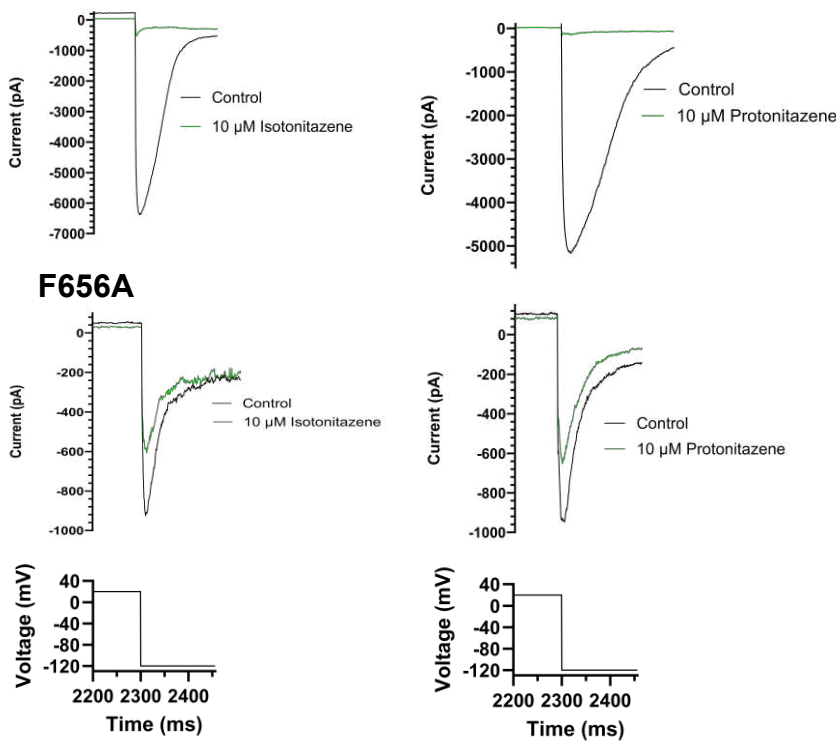
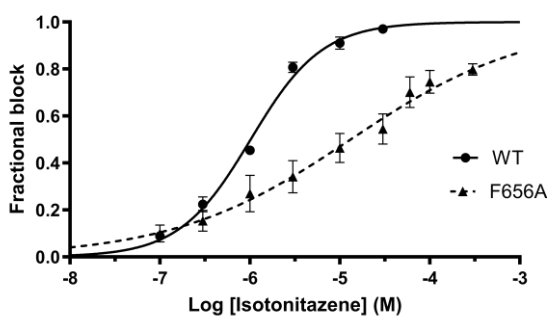
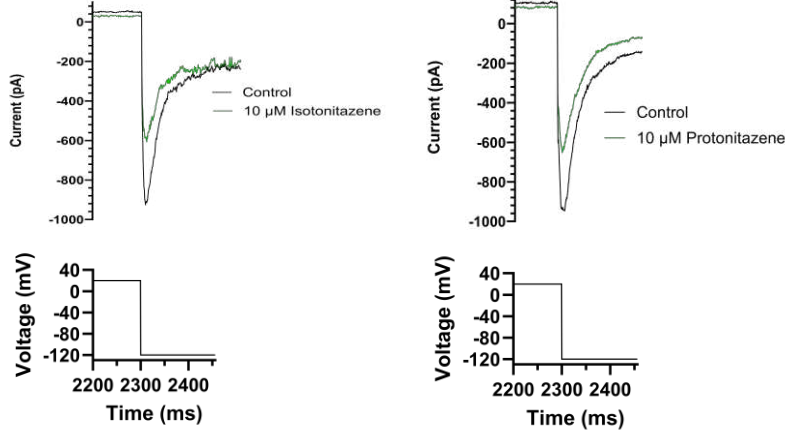
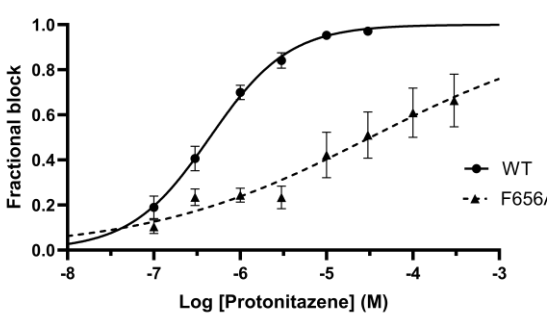
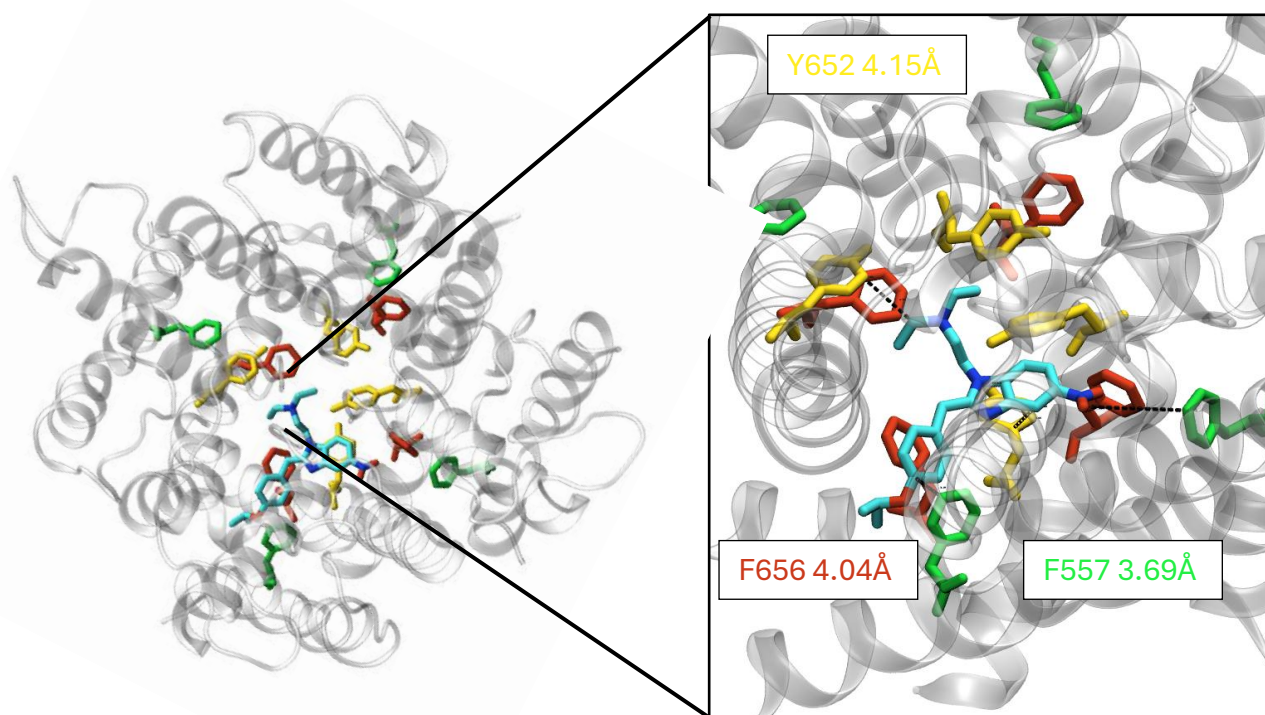
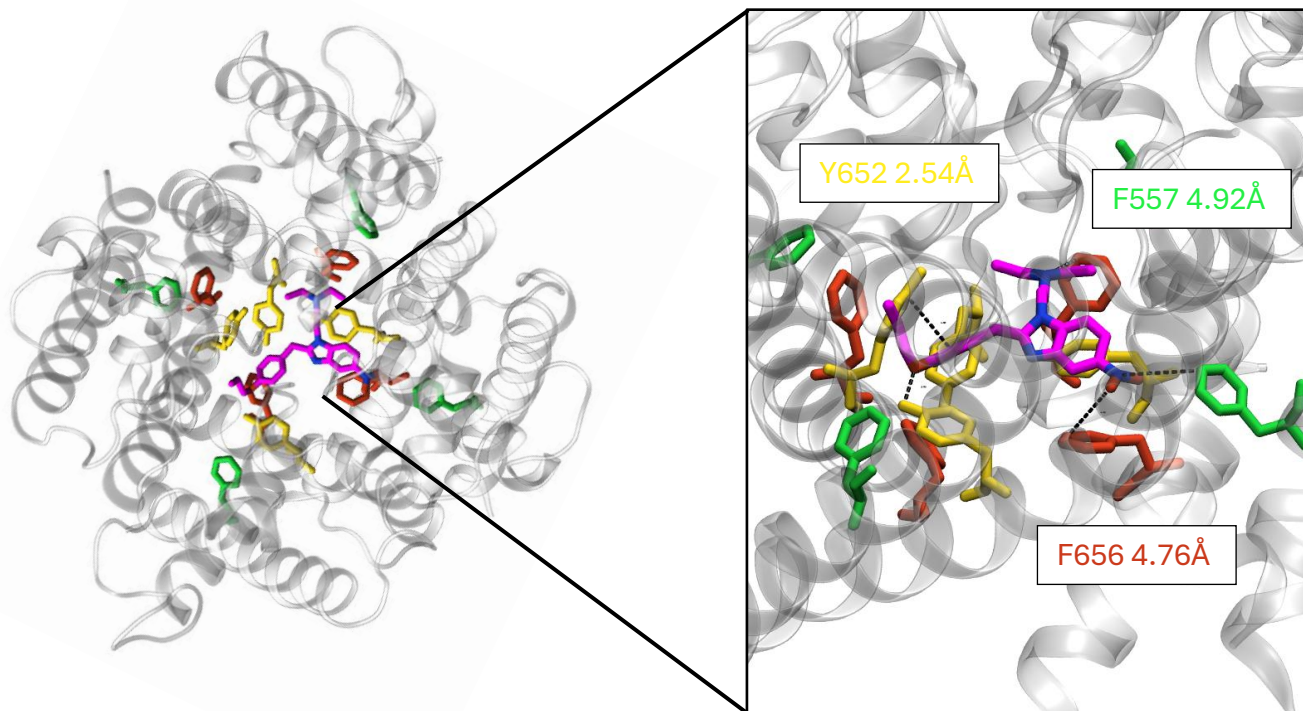
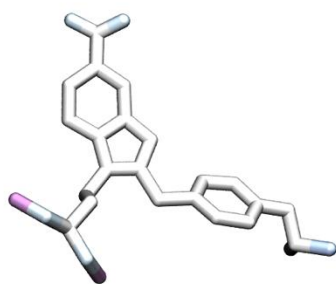
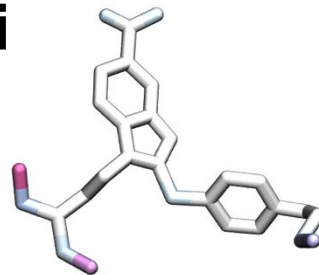
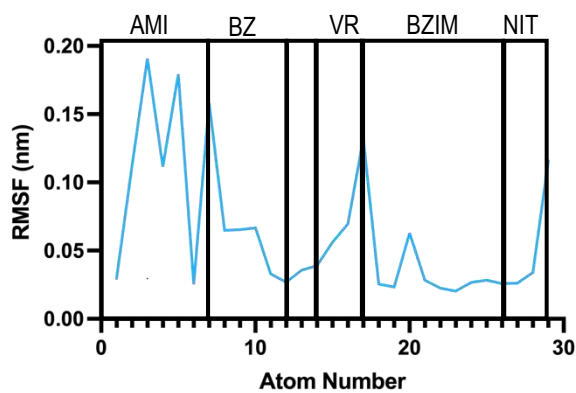
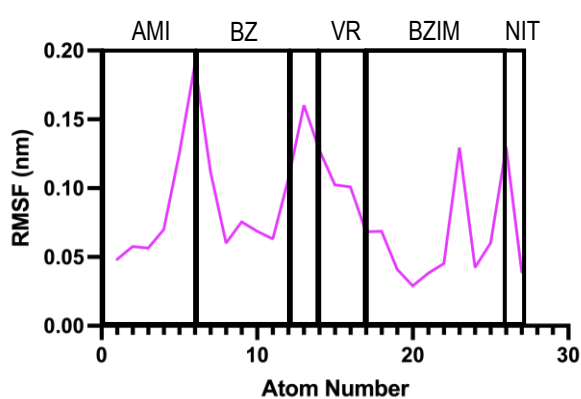
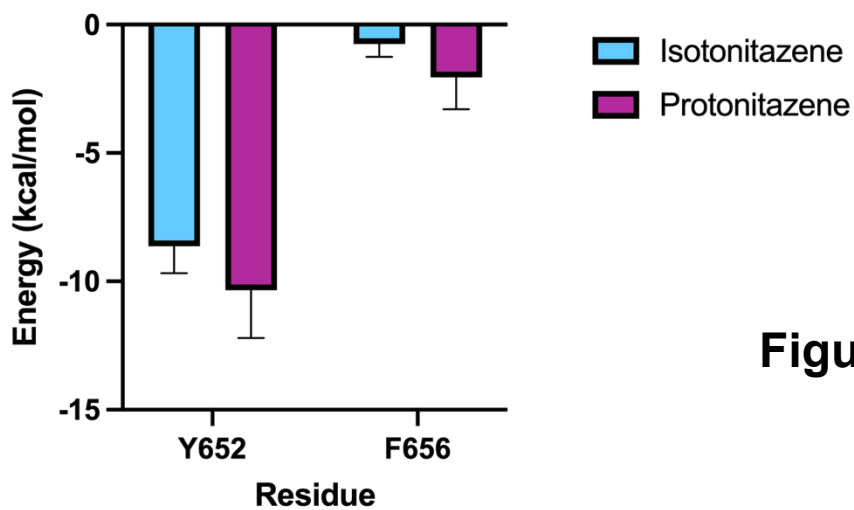


Figure 4

**A****hERG WT****B****Y652A****F557L****C****D****hERG WT****E****F656A****F**

**A****B****Figure 6**

**Ai****Aii****Isotonitazene RMSF****Protonitazene RMSF****B** **$\Delta$ TDC summed per pore residue****Figure 7**

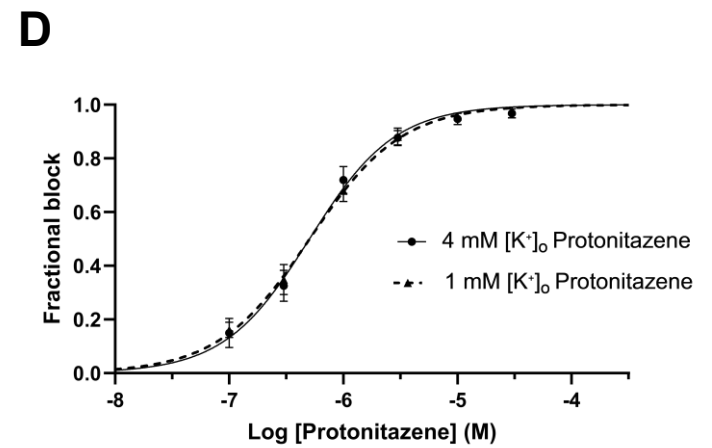
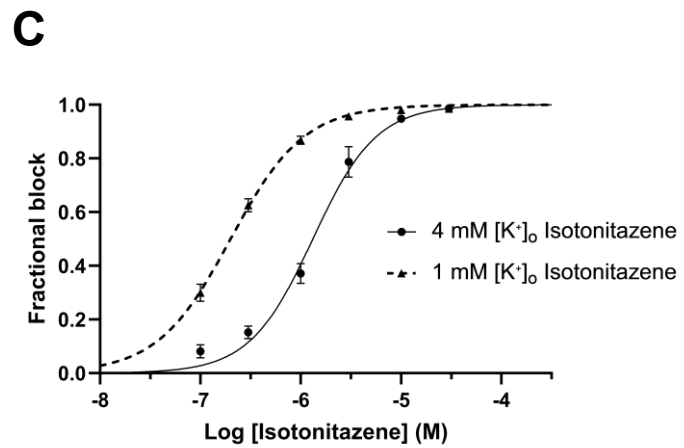
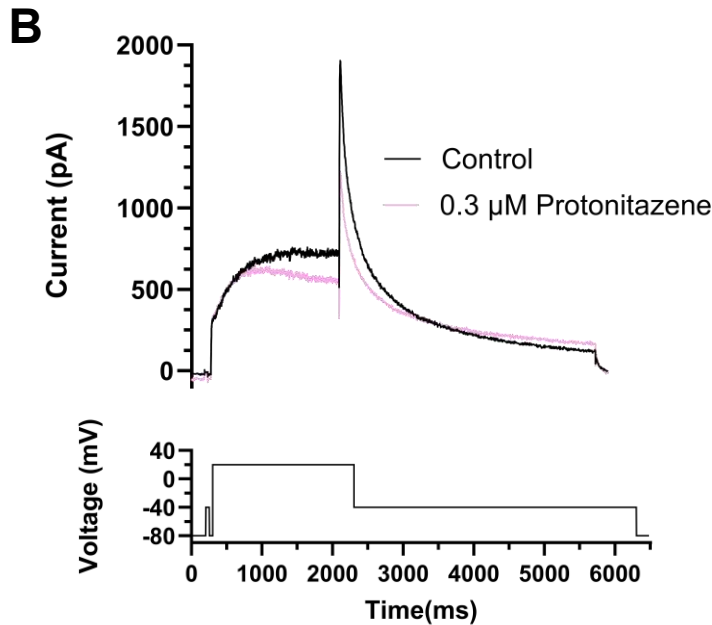
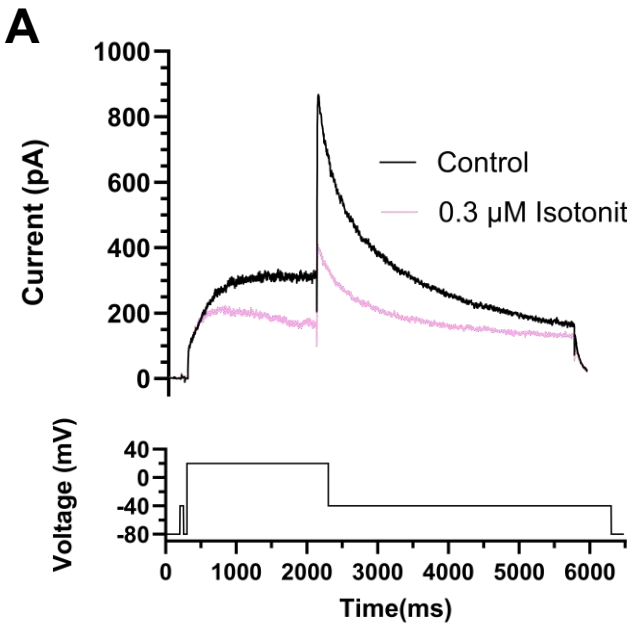


**Figure 8**

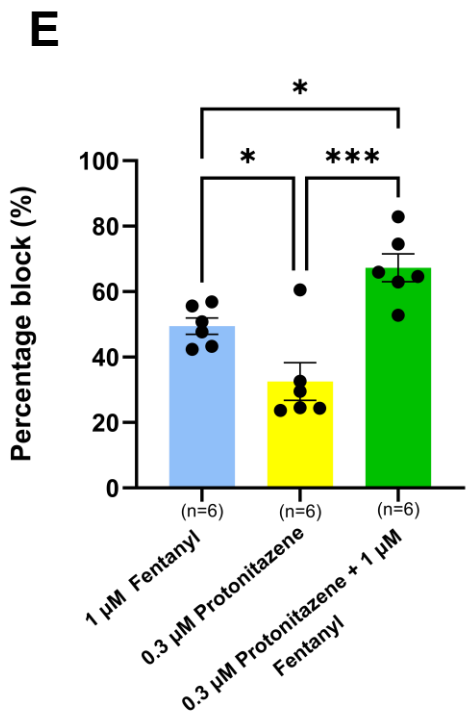
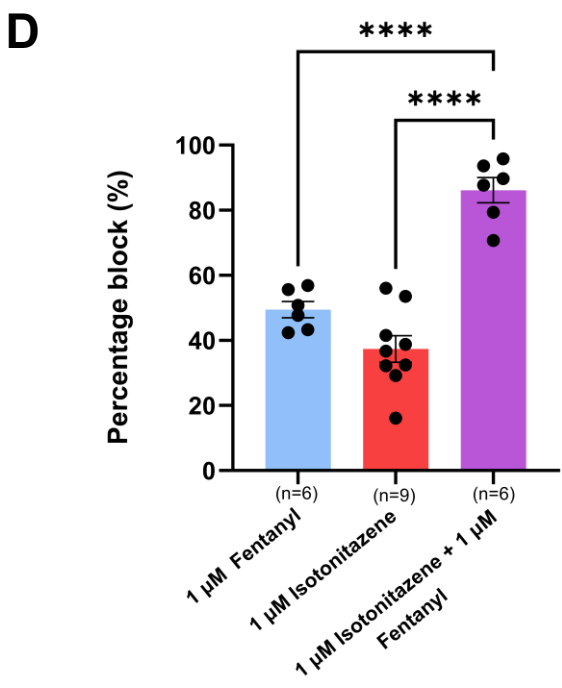
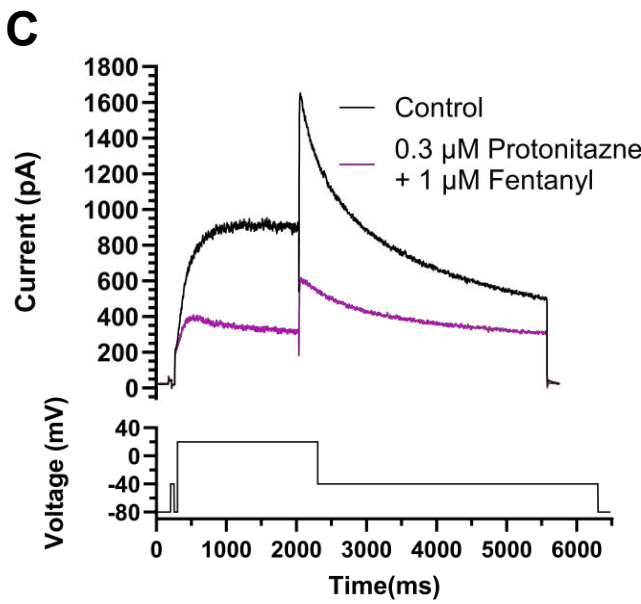
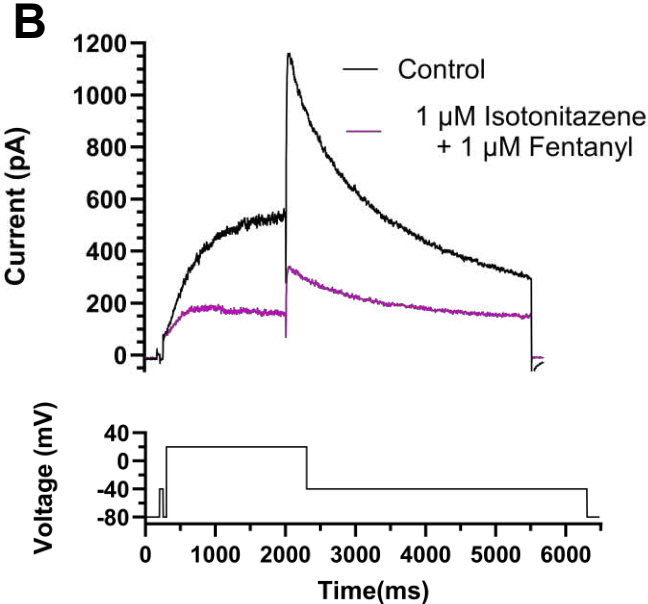
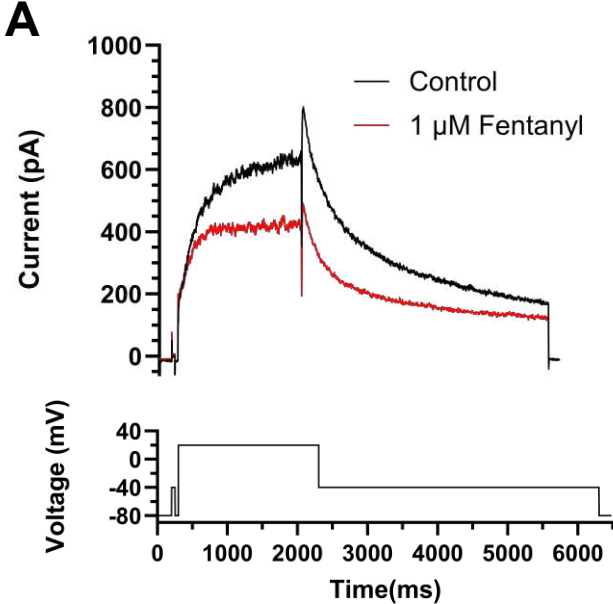


**Figure 9**

## Supplementary Files

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- [NitazenehERGpapersupplementFinal.docx](#)