



## **A New Negative Allosteric Modulator AP14145 for the Study of Small Conductance Calcium-Activated Potassium Channels**

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*Published in:*  
British Journal of Pharmacology

*DOI:*  
[10.1111/bph.14043](https://doi.org/10.1111/bph.14043)

*Publication date:*  
2017

*Document version*  
Peer reviewed version

*Citation for published version (APA):*  
Simo Vicens, R., Kirchhoff, J. E., Dolce , B., Abildgaard, L., Speerschneider, T., Sørensen, U. S., ... Bentzen, B. H. (2017). A New Negative Allosteric Modulator AP14145 for the Study of Small Conductance Calcium-Activated Potassium Channels. *British Journal of Pharmacology*, 174(23), 4396-4408. <https://doi.org/10.1111/bph.14043>

1 **A New Negative Allosteric Modulator AP14145 for the Study of Small**  
2 **Conductance Calcium-Activated Potassium Channels**

3 **Running title: Novel K<sub>Ca</sub>2 Channel Inhibitor**

4 Rafel Simó-Vicens<sup>1,2</sup>, Jeppe E. Kirchhoff<sup>2</sup>, Bernardo Dolce<sup>3</sup>, Lea Abildgaard Jensen<sup>2</sup>,  
5 Tobias Speerschneider<sup>2</sup>, Ulrik S. Sørensen<sup>2</sup>, Morten Grønnet<sup>2</sup>, Jonas G. Diness<sup>2</sup>, Bo H.  
6 Bentzen<sup>1,2</sup>

7 *<sup>1</sup>Biomedical Institute, University of Copenhagen, Blegdamsvej 3B, DK-2200,*  
8 *Copenhagen, Denmark*

9 *<sup>2</sup>Acesion Pharma, Ole Maaløes Vej 3, DK-2200, Copenhagen, Denmark.*

10 *<sup>3</sup>Department of Experimental Pharmacology and Toxicology, University Medical*  
11 *Center Hamburg-Eppendorf, Germany & DZHK (German Center for Cardiovascular*  
12 *Research), partner site Hamburg/Kiel/Lübeck, Hamburg*

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15 Corresponding author: Bo H. Bentzen, [bobe@sund.ku.dk](mailto:bobe@sund.ku.dk), Blegdamsvej 3B, DK-2200,  
16 Copenhagen, Denmark

17

18 **Abbreviations:**

19 AERP: atrial effective refractory period

20 AF: atrial fibrillation

21 K<sub>Ca</sub>1.1 big conductance calcium-activated potassium channel

22 K<sub>Ca</sub>2: small conductance calcium-activated potassium channel

23 K<sub>Ca</sub>3.1: intermediate conductance calcium-activated potassium channel

24 PEG: polyethylene glycol

25 WT: wild type

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29 **Abstract**

30 **Background and purpose:** Small conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  ( $\text{K}_{\text{Ca}2}$ ) channels  
31 represent a promising atrial-selective target for treatment of atrial fibrillation (AF).  
32 Here, we establish the mechanism of  $\text{K}_{\text{Ca}2}$  inhibition by the new compound AP14145.

33 **Experimental approach:** Using site directed mutagenesis binding determinants for  
34 AP14145 inhibition were explored. AP14145 selectivity and mechanism of action were  
35 investigated by patch clamp recordings of heterologously expressed  $\text{K}_{\text{Ca}2}$  channels. The  
36 biological efficacy of AP14145 was assessed by measuring atrial effective refractory  
37 period (AERP) prolongation in anaesthetised rats and a beam walk test was performed  
38 in mice to determine acute CNS related effects of the drug.

39 **Key results:** AP14145 was found to be an equipotent negative allosteric modulator of  
40  $\text{K}_{\text{Ca}2.2}$  and  $\text{K}_{\text{Ca}2.3}$  channels ( $\text{IC}_{50} = 1.1 \pm 0.3 \mu\text{M L}^{-1}$ ). The presence of AP14145 ( $10$   
41  $\mu\text{M L}^{-1}$ ) increased the  $\text{EC}_{50}$  of  $\text{Ca}^{2+}$  on  $\text{K}_{\text{Ca}2.3}$  from  $0.36 \pm 0.02 \mu\text{M L}^{-1}$  to  $1.2 \pm 0.1 \mu\text{M}$   
42  $\text{L}^{-1}$ . The inhibitory effect strongly depended on two amino acids, S508 and A533.  
43 AP14145 concentration-dependently prolonged AERP in rats. Moreover, AP14145 ( $10$   
44  $\text{mg kg}^{-1}$ ) did not trigger any apparent CNS effects in mice.

45 **Conclusion and implications:** AP14145 is a negative allosteric modulator of  $\text{K}_{\text{Ca}2.2}$   
46 and  $\text{K}_{\text{Ca}2.3}$  that shifts the calcium dependence of channel activation, an effect strongly  
47 dependent on two identified amino acids. AP14145 prolongs AERP in rats and does not  
48 trigger any acute CNS effects in mice. The understanding of how  $\text{K}_{\text{Ca}2}$  inhibition is  
49 accomplished at the molecular level will help future development of drugs targeting  
50  $\text{K}_{\text{Ca}2}$  channels.

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## 58 **Introduction**

59 Small conductance calcium-activated potassium channels ([K<sub>Ca</sub>2.1](#), [K<sub>Ca</sub>2.2](#) and [K<sub>Ca</sub>2.3](#)  
60 channels) are widely distributed in humans (Chen et al., 2004), where they serve  
61 important roles such as contributing to the afterhyperpolarization in neurons (Pedarzani  
62 et al., 2005), the endothelium-derived hyperpolarization (Milkau et al., 2010) or the late  
63 repolarization phase in cardiomyocytes (Li et al., 2009). These channels are  
64 constitutively associated to calmodulin, which binds intracellular calcium and activates  
65 K<sub>Ca</sub>2 (Adelman, 2016). Since cloning of the channels 20 years ago (Köhler et al., 1996),  
66 they have piqued the interest of pharmacologists in different therapeutic areas. Although  
67 at the beginning most of the efforts were focused on the CNS and the treatment of  
68 neurodegenerative and psychiatric diseases (Lam et al., 2013), the therapeutic potential  
69 of K<sub>Ca</sub>2 channels rapidly spread to other areas (Wulff et al., 2007). Currently, one of the  
70 most promising therapeutic opportunities for K<sub>Ca</sub>2 channel modulation seems to be in  
71 cardiovascular diseases, more specifically in atrial fibrillation (AF).

72 AF is the most common type of cardiac arrhythmia and it is considered one of the  
73 largest public health problems in developed countries (Zoni-Berisso et al., 2014). The  
74 disease is characterized by rapid uncoordinated activation of the atria, resulting in  
75 reduced ventricular filling and blood stasis in atria, which predisposes to heart failure  
76 and thromboembolic stroke (Nattel, 2002). Unfortunately, current rhythm therapy is  
77 only moderately effective and may trigger serious non cardiac as well as ventricular  
78 adverse effects (Waks and Zimetbaum, 2017).

79 K<sub>Ca</sub>2 channels are considered a promising new target for AF treatment for several  
80 reasons. First, the functional role of K<sub>Ca</sub>2.2 and K<sub>Ca</sub>2.3 channels appears to be greater in  
81 the atria as compared to ventricles which may help avoiding undesired ventricular  
82 adverse effects (Diness et al., 2015). Second, K<sub>Ca</sub>2 channel inhibition prolongs the atrial  
83 effective refractory period (AERP, Diness *et al.*, 2010), a pharmacological strategy that  
84 has successfully been used in the development of other class III antiarrhythmic drugs  
85 (Schmitt et al., 2014). Furthermore, common variants of the genes that encode K<sub>Ca</sub>2.2  
86 and K<sub>Ca</sub>2.3 have been associated with atrial fibrillation (Ellinor et al., 2010;  
87 Christophersen et al., 2017).

88 The first described negative allosteric modulator was [NS8593](#) (Fig 1), a chiral 2-  
89 aminobenzimidazole derivative able to inhibit K<sub>Ca</sub>2 channels at nanomolar

90 concentrations with no subtype selectivity (Strøbæk et al., 2006; Sørensen et al., 2008).  
91 The negative modulation of NS8593 relies in the compound's ability to increase  $\text{Ca}^{2+}$   
92  $\text{EC}_{50}$  for  $\text{K}_{\text{Ca}2}$  activation and its binding site has been found to be in the inner pore  
93 vestibule of the channel (Jenkins et al., 2011).

94  $\text{K}_{\text{Ca}2}$  channels are widely expressed in the CNS (Stocker and Pedarzani, 2000) and  
95 therefore one of the challenges encountered during the development of compounds  
96 targeting peripheral  $\text{K}_{\text{Ca}2}$  is potential CNS mediated adverse effects (Habermann, 1984).  
97 It is thus important to develop compounds with reduced blood brain barrier penetrance  
98 in order to avoid possible adverse effects. In this work, we present a novel  $\text{K}_{\text{Ca}2}$   
99 negative allosteric modulator, AP14145 (Fig 1), a structurally close analogue of  
100 NS8593 designed to inhibit specifically peripheral  $\text{K}_{\text{Ca}2}$  channels by preventing its entry  
101 in the CNS. In contrast to NS8593, it does not appear to have any immediate CNS  
102 effects when dosed to rodents and therefore represents a new and improved tool  
103 compound for studying  $\text{K}_{\text{Ca}2}$  channel inhibition in rodents *in vivo*.

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## 105 **Methods**

### 106 *Molecular biology*

107 r $\text{K}_{\text{Ca}2.3}$  WT (wild type) and r $\text{K}_{\text{Ca}2.3}$  S508T A533V were inserted in pXOON plasmids.  
108 The r $\text{K}_{\text{Ca}2.3}$  NS8593 insensitive mutant was obtained by introducing the double point  
109 mutation to the WT r $\text{K}_{\text{Ca}2.3}$  with the oligonucleotides  
110 CCATAGCCAATggtAAGGAACGTGATG for S508T and  
111 CATCATGGGTgtaGGCTGCACTGCCCTC for A533V, using PfuUltra II Fusion  
112 polymerase (Agilent, USA) and T4 ligase (New England Biolabs, USA). Note that S508  
113 and A533 on the r $\text{K}_{\text{Ca}2.3}$  are the equivalent positions of S507 and A532 on the h $\text{K}_{\text{Ca}2.3}$   
114 channel. Competent *E. coli* were transformed using an aliquot of the mutagenesis  
115 product by thermic shock and the plasmid DNA was purified using standard methods.  
116 The h $\text{K}_{\text{Ca}3.1}$  T250S V275A mutant was kindly donated by Dorte Strøbæk. All  
117 constructs were verified by sequencing.

### 118 *Cell culture and cell preparation*

119 To study the effect of AP14145 on the h $\text{K}_{\text{Ca}1.1}$  and h $\text{K}_{\text{Ca}2.x}$  channels we used four  
120 different stable HEK293 cell lines expressing h $\text{K}_{\text{Ca}1.1}$ , h $\text{K}_{\text{Ca}2.1}$ , h $\text{K}_{\text{Ca}2.2}$  or h $\text{K}_{\text{Ca}2.3}$

121 channels obtained from NeuroSearch A/S (Ballerup, Denmark). The cell lines were  
122 established as described in Strøbæk et al., 2004. For the identification of the binding  
123 determinants of AP14145, wild-type HEK293 cells were transiently co-transfected with  
124 rK<sub>Ca</sub>2.3 WT, rK<sub>Ca</sub>2.3 S508T A533V, hK<sub>Ca</sub>3.1 WT or hK<sub>Ca</sub>3.1 T250S V275A and 0.1 µg  
125 of eGFP plasmid DNA using standard Lipofectamine™ (Thermo Fisher, USA)  
126 protocols. Between one or two days after the transfection, patch clamp experiments  
127 were conducted. The cells were cultured in Dulbecco's modified Eagle's medium  
128 (DMEM1965, Thermo Fisher, USA) supplemented with 26.2 mM L<sup>-1</sup> NaHCO<sub>3</sub>, 25 mM  
129 L<sup>-1</sup> HEPES, 10 ml L<sup>-1</sup> Glutamax (Gibco, USA), 10 % foetal bovine serum (Biowest,  
130 France) and 100 U ml<sup>-1</sup> of penicillin/streptomycin (Sigma, Germany). In the case of the  
131 stable cell lines, 100 µg ml<sup>-1</sup> geneticin (Gibco, USA) were added to the medium. On the  
132 day of the experiment, cells were detached from the flask using 1 ml of Detachin™  
133 (Amsbio, United Kingdom). After being washed with free calcium and magnesium PBS  
134 the cells were plated on 0.5 mm Ø coverslips. In the case of inside-out patch clamp, the  
135 coverslips were treated overnight at 37°C with 50 mg ml<sup>-1</sup> poly-L-lysine (Sigma,  
136 Germany) to get firmer cell attachment.

### 137 *Solutions and drugs*

138 K<sub>Ca</sub>2 and K<sub>Ca</sub>3.1 patch-clamp experiments were conducted using symmetrical K<sup>+</sup>  
139 solutions. The extracellular solution contained 0.1 mM L<sup>-1</sup> CaCl<sub>2</sub>, 3 mM L<sup>-1</sup> MgCl<sub>2</sub>, 154  
140 mM L<sup>-1</sup> KCl, 10 mM L<sup>-1</sup> HEPES and 10 mM L<sup>-1</sup> glucose (pH = 7.4 and 285 - 295  
141 mOsm). The intracellular solution contained 8.106 mM L<sup>-1</sup> CaCl<sub>2</sub> (final free Ca<sup>2+</sup>  
142 concentration of 400 nM L<sup>-1</sup>), 1.167 mM L<sup>-1</sup> MgCl<sub>2</sub>, 10 mM L<sup>-1</sup> EGTA, 154 mM L<sup>-1</sup>  
143 KCl, 10 mM L<sup>-1</sup> HEPES, 31.25/10 mM L<sup>-1</sup> KOH/EGTA and 15 mM L<sup>-1</sup> KOH (pH =  
144 7.2). In addition, to study the activation of the channel with or without the presence of  
145 AP14145 we used a range of intracellular solutions containing different free Ca<sup>2+</sup>  
146 concentrations (0.01 - 30 µM L<sup>-1</sup>). The composition of these intracellular solutions was  
147 determined as described in Strobaek *et al.*, 2006.

148 For K<sub>Ca</sub>1.1 the extracellular solution contained 2 mM L<sup>-1</sup> CaCl<sub>2</sub>, 1 mM L<sup>-1</sup> MgCl<sub>2</sub>, 145  
149 mM L<sup>-1</sup> NaCl, 4 mM L<sup>-1</sup> KCl, 10 mM L<sup>-1</sup> HEPES and 10 mM L<sup>-1</sup> glucose (pH = 7.4 and  
150 285 - 295 mOsm). The intracellular solution contained 5.374 mM L<sup>-1</sup> CaCl<sub>2</sub> (final free  
151 Ca<sup>2+</sup> concentration of 100 nM L<sup>-1</sup>), 1.75 mM L<sup>-1</sup> MgCl<sub>2</sub>, 120 mM L<sup>-1</sup> KCl, 10 mM L<sup>-1</sup>  
152 HEPES, 31.25/10 mM L<sup>-1</sup> KOH/EGTA (pH = 7.2).

153 The osmolarity of the intracellular solutions was adjusted using sucrose (Sigma,  
154 Germany) to match the extracellular solutions.

155 AP14145 (*N*-(2-[[ $(1R)^{-1}$ ]-[3-(trifluoromethyl)phenyl]ethyl]amino) $^{-1}H^1$ ,3-benzodiazol-4-  
156 yl)acetamide) was synthesized by Syngene (India) as described in WO 2013104577 A1.  
157 For *in vitro* experiments, AP14145 was solubilized in pure DMSO (Sigma-Aldrich,  
158 Germany) at 10 mM L $^{-1}$  stock solutions. These stock solutions were stored at -20°C and  
159 aliquots were solubilized at the desired concentration on the day of the experiment. For  
160 *in vivo* experiments, 5 mg ml $^{-1}$  AP14145 were dissolved in a vehicle consisting of 50%  
161 polyethylene glycol (PEG) 400 (Merck, Germany) and 50% sterile saline (PanReac  
162 AppliChem, Germany) for infusion and the solution was sterile filtered (Nalgene, Rapid  
163 flow 90 mM L $^{-1}$  filter unit, Thermo Scientific, USA) before use. The K<sub>Ca</sub>1.1 selective  
164 inhibitor [paxilline](#) was purchased from Sigma-Aldrich (Germany).

#### 165 *Electrophysiology*

166 Patch clamp recordings were made using a HEKA EPC9 amplifier and the Patchmaster  
167 software (HEKA Elektronik, Germany) at room temperature. Patch pipettes were pulled  
168 using a horizontal DMZ Universal Puller (Zeitz, Germany) with resistances of  $2.5 \pm 0.1$   
169 M $\Omega$  for whole-cell patch clamp and  $2.2 \pm 0.6$  M $\Omega$  for inside-out patch clamp. K<sub>Ca</sub>2 and  
170 K<sub>Ca</sub>3.1 currents were elicited every 2 seconds using a 200 ms voltage ramps ranging -80  
171 mV to +80 mV from a holding potential of 0 mV. K<sub>Ca</sub>1.1 currents were elicited every 2  
172 seconds using a 200 ms voltage ramps ranging -80 mV to +50 mV from a holding  
173 potential of 0 mV. Data were sampled at 10 kHz. Series resistance values were  $5.4 \pm 0.6$   
174 M $\Omega$  with 80% of compensation. Two Bessel filters of 10 kHz and 2.9 kHz were used to  
175 avoid background noise.

#### 176 *Plasma Protein Binding*

177 Plasma protein binding (PPB) was experimentally determined by Syngene International  
178 (India) in rat plasma using rapid equilibrium dialysis.

#### 179 *Ex vivo experiments*

180 *Ex vivo* and *in vivo* experiments were performed under a license from the Danish  
181 Ministry of justice (license No 2013 $^{-1}5$ -2934/00964) and in accordance with the Danish  
182 guidelines for animal experiments according to the European Commission Directive  
183 86/609/EEC. The animals were housed in groups of 2-4 in high-top cages with bedding

184 (wood shavings) and under constant climatic conditions (22°C) at the Department of  
185 Experimental Medicine, University of Copenhagen. The animals were kept at a 12 hour  
186 light-dark cycle with ad libitum access to clean water and standard laboratory rodent  
187 diet.

### 188 *Isolated perfused heart preparation*

189 Rats express  $K_{Ca2}$  channels in the atria, and have previously been used to study the  
190 effect of  $K_{Ca2}$  inhibition on atrial refractoriness. Male Sprague-Dawley rats (250 - 350  
191 g, 1-3 months old, Janvier Labs, France) were anaesthetized with fentanyl-midazolam  
192 mixture, 5 mg ml<sup>-1</sup> dose 0.3 mL/100 g BW, s.c. A tracheotomy was performed in the  
193 ventilated rat. The aorta was cannulated and the heart was excised, and connected to a  
194 Langendorff retrograde perfusion setup (Hugo Sachs Elektronik, Harvard Apparatus  
195 GmbH, Germany). The heart was retrogradely perfused with Krebs–Henseleit buffer (in  
196 mM L<sup>-1</sup>: NaCl 120.0, NaHCO<sub>3</sub> 25.0, KCl 4.0, MgSO<sub>4</sub> 0.6, NaH<sub>2</sub>PO<sub>4</sub> 0.6, CaCl<sub>2</sub> 2.5,  
197 Glucose 11.0, saturated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, 37°C, pH 7.4) at a constant  
198 perfusion pressure of 80 mmHg. The electrical activity of the heart was measured by  
199 volume conducted electrocardiograms (ECGs) and the atrial epicardial monophasic  
200 action potentials by an electrode on the right atrium. The signal was sampled at 1 kHz  
201 (PowerLab systems, ADInstruments, UK) and monitored by using LabChart 7 software  
202 (ADInstruments, UK). The hearts were immersed into a temperature-controlled and  
203 carbonated bath containing Krebs–Henseleit buffer. A bipolar pacing electrode was  
204 placed on the right atria in order to stimulate the heart and measure the AERP, which  
205 was defined as the longest S1-S2 interval failing to elicit an action potential. The AERP  
206 was measured every five minutes by applying electrical stimulation (2 times rheobase)  
207 with a fixed interval of 133 ms (S1 stimulation) and for every 10<sup>th</sup> beat an extra stimulus  
208 (S2 stimulation) was applied with 1 ms increments.

209 Baseline recordings were made for at least 20 minutes and continued until the ECG  
210 morphology and AERP recording were stable. After the baseline recording, four 20-  
211 minute episodes followed in which, the heart, was perfused with: 1) 1 μM L<sup>-1</sup> paxilline  
212 2) 3 μM L<sup>-1</sup> paxilline 3) washout 4) 10 μM L<sup>-1</sup> AP14145 and AERP measurements were  
213 performed every fifth minute. Measurements after 20 minutes of drug perfusion or  
214 washout were used for statistical analysis.

### 215 *In vivo experiments*



216 *Closed chest recording of atrial refractoriness in rats*

217 A total of 18 1-3 months old male Sprague-Dawley rats (Janvier, France) weighing 400-  
218 550 g were anesthetized and randomly divided in three groups: one group receiving  
219 AP14145 as bolus injections (n=6), a time matched control group receiving vehicle as  
220 bolus injections (n=6), and a group receiving AP14145 as a constant-rate infusion  
221 (n=6). The rats were anaesthetized with 3 % isofluran/oxygen and an intravenous  
222 catheter was placed in the femoral vein for drug injection. Needle ECG electrodes were  
223 placed in each limb for ECG recordings (ADInstruments, UK). The temperature of the  
224 rats was monitored and kept stable during the experiment with a heating lamp (37 °C).  
225 A catheter with eight electrodes (Millar Inc., US) was placed in the right atrium of an  
226 anaesthetized rat via the jugular vein. Two of the electrodes were used to pace the  
227 atrium and six electrodes were used to measure the electrical activity in the atrium. This  
228 combination allows measurements of the AERP and the changes in AERP as a  
229 consequence of injection of the test compound. Once the experiment was completed,  
230 rats were euthanized by a mixture of 200 mg ml<sup>-1</sup> pentobarbital and 20 mg ml<sup>-1</sup>  
231 lidocaine hydrochloride (Glostrup Apotek, Denmark) i.v. injection. Since the risk of  
232 placebo effect and subjective interpretation of the results was inexistent or minimal, no  
233 blinding was used.

234 *Measurement of AERP*

235 Each experiment lasted for at least 60 minutes and was divided into three 20-minutes  
236 episodes. During the entire experiment the ECG was monitored. The AERP was  
237 measured by applying electrical stimulation (5 times rheobase) with a fixed interval of  
238 120 ms (S1 stimulation) and for every 10th beat an extra stimulus (S2 stimulation) was  
239 applied with 1 ms increments. The AERP was defined as the longest S1-S2 interval  
240 failing to elicit an action potential. Between the AERP recordings, the heart remained  
241 unpaced. Baseline AERP recordings with no compound present were made every 5th  
242 minute for 20 minutes before adding test compound.

243 *Increasing bolus dosing*

244 After the baseline recording, two 20-minute episodes followed in which two groups of  
245 rats (n=6 each) were injected with increasing doses of AP14145 (2.5 mg kg<sup>-1</sup> and 5.0  
246 mg kg<sup>-1</sup>) or equivalent volumes of vehicle (50% PEG-400 and 50% saline). The

247 injection time was 30 seconds. AERP was measured 1, 5, 10 and 15 minutes after the  
248 start of each injection.

#### 249 *Constant rate infusion of AP14145*

250 After the baseline recording, a third group of rats received a constant rate infusion of 40  
251 mg kg<sup>-1</sup> h<sup>-1</sup> AP14145 over 20 minutes followed by a 20-minute post-infusion period. In  
252 these animals the AERP was measured every 2 minutes during and after infusion.

#### 253 *Beam walk test - assessment of motor balance and coordination in mice*

254 A beam walk test was performed in mice in order to assess CNS exposure of K<sub>Ca</sub>2  
255 inhibitors in vivo. The mouse beam walk test is a validated test for addressing motor  
256 function (Brooks and Dunnett, 2009). Three groups of 1-2 months old male NMRI mice  
257 (Taconic Biosciences, USA) weighing 23-49 g were used. The mice were randomly  
258 assigned to either the vehicle control group, a 10 mg kg<sup>-1</sup> NS8593 or a 10 mg kg<sup>-1</sup>  
259 AP14145 group. The mice were placed on a 1 meter wooden beam (ø 12 mm) and  
260 briefly trained in crossing the beam. After training, a 1 minute baseline recording was  
261 initiated and the number of falls and slips were noted. Hereafter the mice were  
262 randomly assigned to receive K<sub>Ca</sub>2 inhibitors or vehicle (50% PEG-400 and 50% saline)  
263 by i.v. bolus injection in the tail vein. The mouse was observed immediately after  
264 injection for any behavioural changes. If any adverse effects occurred, the mouse was  
265 euthanized. Otherwise the mice were observed for behavioural changes and challenged  
266 with the beam walk 12 minutes post injection. All experiments were documented by  
267 video recordings.

#### 268 *Data analysis*

269 Data was extracted from PatchMaster and analysed using GraphPad Prism 7.

270 To calculate the IC<sub>50</sub> value of AP14145, the measured currents were first normalized.  
271 Recorded currents without the presence of the drug were used as baseline and currents  
272 recorded at the highest tested concentration of AP14145 (30 µM L<sup>-1</sup>) were used as total  
273 inhibition of the channel. Individual IC<sub>50</sub> values for each experiment were calculated  
274 using the equation:

$$Y = Y_{min} + \frac{(Y_{max} - Y_{min})}{1 + 10^{X - \log IC_{50}}}$$

275 where X is the log of dose of AP14145 and Y is the normalized measured current. In all  
276 cases a Hill slope of -1.0 was considered. Individual IC<sub>50</sub> values were later used to  
277 obtain the final  $\bar{x} \pm \text{SEM}$ . IC<sub>50</sub>. The individual IC<sub>50</sub> values were then used in student's t-  
278 tests to determine subtype selectivity.

279 To calculate the EC<sub>50</sub> of calcium, the values were normalized using the currents  
280 recorded at the lowest calcium concentration (0.01  $\mu\text{M L}^{-1}$ ) for total inactivation and at  
281 the highest calcium concentration (30  $\mu\text{M L}^{-1}$ ) for maximum activation of the channel.  
282 Individual EC<sub>50</sub> values for each experiment were calculated using the equation:

$$Y = Y_{min} + \frac{(Y_{max} - Y_{min})}{1 + 10^{(\log EC_{50} - X) \times HillSlope}}$$

283 where X is the log of dose of calcium and Y is the normalized measured current with  
284 variable Hill slope. Individual EC<sub>50</sub> values were used to determine the final  $\bar{x} \pm \text{SEM}$   
285 EC<sub>50</sub>.

286 Currents were also normalized to assess and compare the inhibitory effect of 10  $\mu\text{M L}^{-1}$   
287 AP14145 on HEK cells expressing K<sub>Ca</sub>1.1, K<sub>Ca</sub>2.1, K<sub>Ca</sub>2.2, K<sub>Ca</sub>2.3, K<sub>Ca</sub>2.3 S508T  
288 A532V, K<sub>Ca</sub>3.1 or K<sub>Ca</sub>3.1 T250S V275A. For each individual cell, 0% current was  
289 defined as 0 nA and 100% current was defined as the current recorded in the absence of  
290 the compound. The final results are summarized as  $\bar{x} \pm \text{SEM}$  of the individual values.

291 To quantify and compare the activation and inhibition effects of 10  $\mu\text{M L}^{-1}$  NS309 and  
292 10  $\mu\text{M L}^{-1}$  AP14145 on the K<sub>Ca</sub>2.3 channel, values were normalized for each individual  
293 cell. In this case, 0 was defined as 0 nA and currents recorded in the absence of both  
294 compounds were defined as 1. These individual values were used to calculate the final  $\bar{x}$   
295  $\pm \text{SEM}$ .

296 The last ten data points obtained after the application of a new compound or solution  
297 and corresponding to the steady state were used to create every single value.

298 Student's t-test was performed to assess statistical significance of the effect of  
299 AP14145. P values < 0.05 were considered significant.

300 Physicochemical properties of AP14145 and NS8593 were calculated using the Instant  
301 JChem (ChemAxon) software.

302 Perfused rat heart data and closed chest continuous data are summarized using the  $\bar{x} \pm$   
303 SEM. A one-way ANOVA with Tukey's comparison post-test was used to compare the

304 effect of paxilline and AP14145 on the isolated rat heart. Multiple t tests with Holm-  
305 Sidak's correction for multiple comparisons were used to compare AERP differences  
306 between the group of rats that received AP14145 as increasing bolus doses and the time  
307 matched control group at matching time points. Multiple t tests with Holm-Sidak's  
308 correction for multiple comparisons were used to compare each AERP-value during and  
309 after infusion to the mean baseline AERP values. P values < 0.05 were considered  
310 significant.

### 311 *Nomenclature of Targets and Ligands*

312 Key protein targets and ligands in this article are hyperlinked to corresponding entries in  
313 <http://www.guidetopharmacology.org>, the common portal for data from the  
314 IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016), and are  
315 permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander  
316 et al., 2015).

317

## 318 **Results**

### 319 *AP14145 inhibits both hK<sub>Ca</sub>2.2 and hK<sub>Ca</sub>2.3 currents with equal potency*

320 Using inside-out manual patch clamp we tested AP14145 on both the hK<sub>Ca</sub>2.2 and  
321 hK<sub>Ca</sub>2.3 channels. Once the patch was excised, the channels were exposed to the bath's  
322 intracellular solution, containing 400 nM L<sup>-1</sup> free [Ca<sup>2+</sup>]. In symmetrical intra- and  
323 extra-cellular K<sup>+</sup> solutions, hK<sub>Ca</sub>2.3 currents displayed a characteristic inwardly  
324 rectifying current-voltage relationship (Fig 2a). Currents were elicited using voltage  
325 ramps from -80 mV to +80 mV applied every 2 seconds. Once the hK<sub>Ca</sub>2 current was  
326 stable, up to 8 increasing concentrations of AP14145 between 0.01 - 30 μM L<sup>-1</sup> were  
327 applied and perfused by gravity flow on the patch (Fig 2b). For each concentration the  
328 drug was applied until steady state was reached.

329 AP14145 was able to inhibit both hK<sub>Ca</sub>2.2 and hK<sub>Ca</sub>2.3 in a concentration-dependent  
330 fashion (Fig 2b, data not shown for hK<sub>Ca</sub>2.2). The effect started at nanomolar  
331 concentrations and total inhibition was reached at 30 μM L<sup>-1</sup> (Fig 2b). Calculated IC<sub>50</sub>  
332 for AP14145 on both the hK<sub>Ca</sub>2.2 and hK<sub>Ca</sub>2.3 was 1.1 ± 0.3 μM L<sup>-1</sup> (n = 7 each, Fig  
333 2c), with a fixed Hill slope of -1.0. The drug consequently did not display any subtype  
334 selectivity between hK<sub>Ca</sub>2.2 and hK<sub>Ca</sub>2.3.

335 Additionally, the inhibitory effect of AP14145 was also tested on the hK<sub>Ca</sub>1.1, hK<sub>Ca</sub>2.1  
336 and hK<sub>Ca</sub>3.1 channels using whole cell patch clamp for further selectivity assessment  
337 (Fig 3). The application of 10 μM L<sup>-1</sup> AP14145 inhibited 50 ± 10% of the hK<sub>Ca</sub>1.1  
338 current (n = 6) and 90 ± 4% of the hK<sub>Ca</sub>2.1 current (n = 7). In contrast, hK<sub>Ca</sub>3.1 currents  
339 were not significantly affected by the application of 10 μM L<sup>-1</sup> AP14145 (n = 8).

#### 340 *AP14145 modifies hK<sub>Ca</sub>2.3 calcium sensitivity*

341 To establish how AP14145 inhibits the channel, we performed inside out patch clamp  
342 recordings to assess the effect of the drug on the calcium sensitivity of the hK<sub>Ca</sub>2.3  
343 channel. Patches were excised from HEK cells stably expressing the hK<sub>Ca</sub>2.3 channel  
344 and currents elicited using voltage ramps. We exposed the patches to eight different free  
345 Ca<sup>2+</sup> concentrations and calculated the EC<sub>50</sub> for calcium activation of the channel in the  
346 absence and presence of 10 μM L<sup>-1</sup> AP14145 (Fig 4a and b). Free calcium  
347 concentrations ranged from 0.01 - 30 μM L<sup>-1</sup> and were perfused using gravity flow.  
348 Solutions were applied until steady state was reached.

349 In the absence of AP14145, hK<sub>Ca</sub>2.3 channels were fully activated at 3 μM L<sup>-1</sup> of  
350 intracellular Ca<sup>2+</sup> (Fig 4a), but in the presence of 10 μM L<sup>-1</sup> AP14145, up to 10 μM L<sup>-1</sup>  
351 were needed to reach total activation of the channel (Fig 4b). At a 10 μM L<sup>-1</sup>  
352 concentration, the drug shifted the calcium-activation curve of the K<sub>Ca</sub>2.3 channel to the  
353 right (Fig 4c), so higher calcium concentrations were needed to activate the channels.  
354 This was also shown by the significantly increased EC<sub>50</sub> of Ca<sup>2+</sup> from 0.36 ± 0.02 μM L<sup>-1</sup>  
355 (n = 9) to 1.3 ± 0.2 μM L<sup>-1</sup> (n = 7). Most prominently, the Hill coefficients were also  
356 significantly modified by the presence of AP14145 from 5.2 ± 0.3 to 1.2 ± 0.1 (absence  
357 of AP14145 vs. 10 μM L<sup>-1</sup> AP14145).

#### 358 *AP14145 reverses the effect of the positive K<sub>Ca</sub>2 gating modulator [NS309](#)*

359 The inhibitory effect of AP14145 was also studied in the presence of a high  
360 concentration of the K<sub>Ca</sub>2 positive gating modulator NS309. Patches excised from HEK  
361 cells stably expressing the hK<sub>Ca</sub>2.3 channel were exposed to 400 nM L<sup>-1</sup> free [Ca<sup>2+</sup>]  
362 intracellular solution. After stabilisation of the baseline, 10 μM L<sup>-1</sup> NS309 were applied  
363 on the patch, further activating the channel and increasing hK<sub>Ca</sub>2.3 current by 4 ± 1 fold  
364 (n = 7, Fig 5). When steady state was reached, 10 μM L<sup>-1</sup> AP14145 were added to the  
365 bath, in the continued presence of NS309. The application of AP14145 reduced hK<sub>Ca</sub>2.3  
366 current to values close to the control baseline, reversing the positive gating effect of

367 NS309 (Figure 5). Furthermore, in the presence of  $10 \mu\text{M L}^{-1}$  NS309, total current  
368 inhibition by  $10 \mu\text{M L}^{-1}$  AP14145 was significantly diminished from  $80 \pm 3\%$  ( $n = 7$ ) to  
369  $61 \pm 4\%$  ( $n = 7$ , Fig 5).

370 *AP14145 inhibition strongly depends on two amino acids, S508 and A533, located in*  
371 *the inner pore of the channel*

372 To establish possible molecular determinants of  $\text{rK}_{\text{Ca}2.3}$  inhibition by AP14145 we  
373 mutated two amino acids, S508 and A533 (corresponding to S507 and A532 in  
374  $\text{hK}_{\text{Ca}2.3}$ ), located in the inner pore of the channel and known to confer sensitivity to the  
375 negative allosteric modulator of  $\text{K}_{\text{Ca}2}$  channels, NS8593 (Jenkins et al., 2011).

376 Whole-cell patch clamp experiments were conducted on transiently transfected HEK  
377 cells with either the WT  $\text{rK}_{\text{Ca}2.3}$  channel or the  $\text{rK}_{\text{Ca}2.3}$  S508T A533V mutant. The  
378 channels were activated by  $400 \text{ nM L}^{-1}$  free  $[\text{Ca}^{2+}]$  intracellular solution and currents  
379 were elicited using voltage ramps. Again, in symmetrical intra- and extra-cellular  $\text{K}^+$   
380 solutions, WT  $\text{rK}_{\text{Ca}2.3}$  currents displayed a characteristic inwardly rectifying current-  
381 voltage relationship (Fig 6a). In contrast to the WT  $\text{rK}_{\text{Ca}2.3}$  channel, the maximum  
382 current normalized to cell capacitance of the mutant  $\text{rK}_{\text{Ca}2.3}$  was significantly reduced  
383 (WT:  $650 \pm 96 \text{ pA/pF}$  vs. mutant:  $87 \pm 23 \text{ pA/pF}$ ,  $n = 7$  each, Fig 6b). Rectification,  
384 determined as the ratio of the current amplitude at  $-80$  and  $+80 \text{ mV}$ , was also changed  
385 by the mutation, from  $9 \pm 1 I_{-80}/I_{+80}$  in the WT to  $2.9 \pm 0.4 I_{-80}/I_{+80}$  in the mutant. These  
386 observations are in agreement with what was previously described by Jenkins *et al.*,  
387 2011. After current stabilisation,  $10 \mu\text{M L}^{-1}$  of AP14145 were applied on the cell  
388 transfected with the WT or the mutant protein for 1 - 2 min or until a steady state drug  
389 effect was reached (Fig 6a and b).

390 The experiments showed that, while  $\text{rK}_{\text{Ca}2.3}$  currents recorded from cells transfected  
391 with the WT channel were strongly inhibited by application of  $10 \mu\text{M L}^{-1}$  AP14145 (Fig  
392 6a), currents recorded from cells transfected with the mutant  $\text{rK}_{\text{Ca}2.3}$  were only  
393 partially affected by the presence of the compound (Fig 6b). The inhibitory effect of  
394 AP14145 was statistically different between the WT and the mutant when analysed as  
395 the relative current inhibition after application of AP14145 ( $95 \pm 1\%$  and  $22 \pm 6\%$   
396 inhibition, WT  $\text{rK}_{\text{Ca}2.3}$  vs  $\text{rK}_{\text{Ca}2.3}$  S508T A533V,  $n = 7$  each, Fig 6e).

397 To ensure that S508 and A533 are important for AP14145 sensitivity, we introduced  
398 these amino acids in their homologous positions in the AP14145 insensitive  $\text{K}_{\text{Ca}3.1}$

399 channel, T250 and V275, respectively. The  $K_{Ca3.1}$  T250S V275A mutant was tested  
400 using whole cell patch clamp to determine its sensitivity to AP14145. After activation  
401 of the channel with  $400 \text{ nM L}^{-1}$  free  $[Ca^{2+}]$  intracellular solution and current  
402 stabilization,  $10 \text{ }\mu\text{M L}^{-1}$  AP14145 was applied on the cell. In contrast to the  $K_{Ca3.1}$  WT  
403 channel (Fig 6c), the mutant  $K_{Ca3.1}$  current was inhibited by  $92 \pm 1 \%$  ( $n=7$ , Fig 6d),  
404 comparable to the inhibitory effect observed on  $rK_{Ca2.3}$ .

405 *AP14145 increases the duration of the atrial effective refractory period in isolated*  
406 *perfused rat hearts*

407 Excised rat hearts were connected to a retrograde perfusion Langendorff setup to  
408 measure the effect of AP14145 on the AERP and discard any  $K_{Ca1.1}$  mediated effects.

409 Once a stable baseline was achieved, the hearts were perfused first with  $1 \text{ }\mu\text{M L}^{-1}$  of the  
410  $K_{Ca1.1}$  inhibitor paxilline. Twenty minutes later, the dose of paxilline was increased to  $3$   
411  $\text{ }\mu\text{M L}^{-1}$  for 20 more minutes. Finally, after a 20 minute wash period,  $10 \text{ }\mu\text{M L}^{-1}$  of  
412 AP14145 were perfused into the heart.

413 Paxilline did not affect the AERP in any of the tested doses, but AP14145 was able to  
414 prolong the AERP significantly, from  $19 \pm 3 \text{ ms}$  to  $57 \pm 12 \text{ ms}$  ( $n = 5$ , Fig. 7).

415 *AP14145 increases the duration of the atrial effective refractory period in rats*

416 To investigate the *in vivo* effects of AP14145, 6 male rats received AP14145 in  
417 increasing doses ( $2.5 \text{ mg kg}^{-1}$  and  $5 \text{ mg kg}^{-1}$ ) and 6 time matched control rats received  
418 corresponding volumes of vehicle ( $0.5 \text{ ml kg}^{-1}$  and  $1 \text{ ml kg}^{-1}$ , respectively).

419 One minute after injection of  $2.5 \text{ mg kg}^{-1}$  AP14145 the AERP was significantly  
420 increased from  $37 \pm 2 \text{ ms}$  in the time matched control group to  $53 \pm 6 \text{ ms}$  (Fig. 8a). The  
421 AERP returned towards baseline values, and five minutes after the injection of  $2.5 \text{ mg}$   
422  $\text{ kg}^{-1}$  the AERP was no longer significantly different from that of the time matched  
423 controls. One minute after injection of  $5 \text{ mg kg}^{-1}$  AP14145 the AERP was significantly  
424 increased from  $31 \pm 2 \text{ ms}$  in the time matched control group to  $58 \pm 8 \text{ ms}$  (Fig. 8a).  
425 Again, the AERP returned towards baseline values, and ten minutes after the injection  
426 of  $5 \text{ mg kg}^{-1}$  the AERP was no longer significantly different from that of the time  
427 matched controls.

428 A third group of rats ( $n = 6$ ) received a constant rate infusion of  $40 \text{ mg kg}^{-1} \text{ h}^{-1}$  over 20  
429 minutes and were monitored for an additional 20 minutes after infusion (Fig 8b). In

430 these rats the AERP was significantly increased compared to baseline values from 4  
431 minutes after the infusion started (i.e. after a cumulative dose of 2.7 mg kg<sup>-1</sup>). The  
432 AERP continued to increase during the rest of the infusion and returned towards  
433 baseline values after infusion.

#### 434 *AP14145 does not impair motor coordination in mice*

435 A beam walk test was performed in mice in order to assess CNS exposure of K<sub>Ca</sub>2  
436 inhibitors *in vivo*. Three groups of male NMRI mice were used, a vehicle control group  
437 (30.5 ± 0.3 g, n = 6), a 10 mg kg<sup>-1</sup> NS8593 (26 ± 1 g, n = 3) and a 10 mg kg<sup>-1</sup> AP14145  
438 group (36 ± 5 g, n = 6).

439 Shortly after the injection of 10 mg kg<sup>-1</sup> NS8593, all mice showed severe convulsions  
440 making them unable to walk on the beam (Fig 9). Therefore, the mice were euthanized  
441 by cervical dislocation and the experiment was terminated.

442 In contrast, when mice were injected with AP14145, no acute effects were observed and  
443 the beam walk test was performed 12 minutes after dosing. The mice did not slip or fall  
444 from the beam in either of the two tests and no behavioural changes were observed.  
445 These observations were not different to the ones from the vehicle control group (Fig 9).

446

#### 447 **Discussion**

448 K<sub>Ca</sub>2 channels are inwardly rectifying potassium channels (Köhler et al., 1996) widely  
449 distributed in humans, both in the CNS and peripheral tissues (Chen et al., 2004). In the  
450 heart, they play an important role in the late repolarization phase of the atria (Li et al.,  
451 2009). Moreover, it has been demonstrated that inhibition of K<sub>Ca</sub>2 channels prolongs the  
452 AERP (Diness et al., 2010; Skibsbye et al., 2011; Qi et al., 2014; Haugaard et al.,  
453 2015). Therefore, the K<sub>Ca</sub>2 channel is considered a promising new target to treat AF.  
454 Here we present a new K<sub>Ca</sub>2 inhibitor, AP14145, which could constitute an important  
455 tool in rodents, to target and study the inhibition of peripheral K<sub>Ca</sub>2.x channels *in vivo*.

456 In initial experiments it was established that AP14145 inhibits hK<sub>Ca</sub>2.2 and hK<sub>Ca</sub>2.3 in  
457 an equipotent manner with IC<sub>50</sub> values of 1.1 ± 0.3 μM L<sup>-1</sup>. To determine the  
458 mechanism of inhibition of AP14145, we conducted inside-out patch clamp  
459 experiments. Patches were excised from HEK 293 cells stably expressing the hK<sub>Ca</sub>2.3  
460 channel and exposed to a range of intracellular solutions with different free calcium



461 concentrations. Calcium activation was assessed in the absence and presence of  
462 AP14145. The compound significantly increased the  $EC_{50}$  of  $Ca^{2+}$  from  $0.36 \pm 0.02 \mu M$   
463  $L^{-1}$  to  $1.3 \pm 0.2 \mu M L^{-1}$ , thereby shifting the  $Ca^{2+}$  activation curve of  $K_{Ca2}$  channel  
464 activation to higher values. The Hill coefficients were also modified by the presence of  
465 AP14145 from  $5.2 \pm 0.3$  to  $1.2 \pm 0.1$  (absence of AP14145 vs.  $10 \mu M L^{-1}$  AP14145).  
466 These results suggest that the drug modifies the channel's calcium sensitivity and acts  
467 as a negative allosteric modulator, very similar to the previously reported NS8593. The  
468 change of the Hill coefficient may also indicate a loss of calcium cooperativity which  
469 may impede calcium binding. Moreover, the inhibitory effect of AP14145 was studied  
470 in the presence of the  $K_{Ca2.x}$  positive allosteric modulator NS309, which is known to  
471 increase the calcium sensitivity of the channel (Strøbæk et al., 2004). In these  
472 experiments, AP14145 was able to reverse NS309-mediated  $K_{Ca2.3}$  channel activation,  
473 suggesting a functional competition between the two compounds and further supporting  
474 the negative allosteric mechanism of AP14145.

475 In the study by Jenkins *et al.* in 2011, the binding site of NS8593, another  $K_{Ca2}$  negative  
476 allosteric modulator, was found to be located at the inner pore of the channel. This was  
477 an interesting finding since the drug had previously been found to decrease calcium  
478 sensitivity of the channel and could be speculated to locate the binding site at the C-  
479 terminal domain, close to the calmodulin binding domain. Instead, binding interacts  
480 with two specific amino acids S507 and A532, located on helices S5 and S6,  
481 respectively. When these two amino acids were mutated to the corresponding residues  
482 found on the closely related  $K_{Ca3.1}$  channel, which is not inhibited by NS8593, they  
483 obtained a  $K_{Ca2.3}$  mutant resistant to the effect of the drug, with preserved channel  
484 confirmation and calcium sensitivity. In order to find out if AP14145 and NS8593  
485 shared the same binding site, we conducted whole cell patch clamp experiments on  
486 transiently transfected HEK cells with r $K_{Ca2.3}$  WT or r $K_{Ca2.3}$  S508T A533V,  
487 corresponding to S507 and A532 in the h $K_{Ca2.3}$  channel. While the WT current was  
488 strongly inhibited using  $10 \mu M L^{-1}$  AP14145, the NS8593 resistant mutant was only  
489 partially affected by the presence of the drug, suggesting that S508 and A533 are  
490 important also for AP14145 inhibition. The loss of AP14145 sensitivity in r $K_{Ca2.3}$   
491 S508T A533V cannot be explained by differences in calcium-activation or channel  
492 conformation as these characteristics are preserved in the mutant (Jenkins et al., 2011).  
493 AP14145 and NS8593 are structurally close analogues (Fig 1) and these experiments

494 demonstrate that AP14145 and NS8593 appear to share the same inhibition mechanism  
495 as well as some of their binding determinants. Finally, to confirm that S507 and A532  
496 are important for the inhibitory effect of AP14145 on  $K_{Ca2.3}$  we introduced these amino  
497 acids in their homologous positions on the insensitive  $K_{Ca3.1}$  channel. With the addition  
498 of these two amino acids, AP14145 sensitivity was fully restored in the  $K_{Ca3.1}$  channel  
499 (Fig 6), further demonstrating that S507 and A532 are important for AP14145  
500 inhibition.  $K_{Ca2}$  channels are widely expressed in the brain, including the cerebellum  
501 (Stocker and Pedarzani, 2000), where they contribute to the action potential  
502 afterhyperpolarization (Hosy et al., 2011). It has further been demonstrated that  
503 inhibition of  $K_{Ca2.2}$  channels in cerebellum disturbs the motor output which is revealed  
504 as ataxia or convulsion like phenotype especially apparent in the hind legs (Alvina and  
505 Khodakhah, 2010). As an indication of CNS exposure and inhibition of central  $K_{Ca2}$   
506 channels a beam walk test was performed. The test is designed to assess impaired motor  
507 coordination and balance in mice.

508 Mice injected intravenously with  $10 \text{ mg kg}^{-1}$  NS8593 immediately showed acute CNS  
509 effects in the form of convulsions, and consequently were euthanized. In contrast i.v.  
510 injection of  $10 \text{ mg kg}^{-1}$  AP14145 had no apparent CNS effects, and the mice were able  
511 to cross the beam without slipping or falling from the beam, similarly to the vehicle  
512 control mice. Importantly, NS8593 plasma protein binding is higher than AP14145  
513 (95.38% vs. 91.35% bound, respectively, Table 1), meaning that a higher amount of  
514 AP14145 is freely available in plasma compared to NS8593 when the same dose of both  
515 compounds is injected. Moreover, we found that i.v. injection of  $2.5 \text{ mg kg}^{-1}$  AP14145  
516 significantly increases the atrial refractoriness in rats within 1 min of injection,  
517 suggesting that  $10 \text{ mg kg}^{-1}$  is sufficient to peripheral  $K_{Ca2}$  target engagement.

518 A possible explanation for the apparent difference in CNS penetration of the two  
519 compounds lies in their structure (Fig 1) and physicochemical properties (Table 1). In  
520 particular, AP14145 contains a carboxamide moiety on the bicyclic benzimidazole ring,  
521 a chemical moiety that adds polarity to the molecule and is known to be a common  
522 substrate for P-glycoprotein transporter mediated efflux. The calculated polar surface  
523 area (PSA) of AP14145 is 70, which is significantly higher than NS8593 (Table 1),  
524 indicating a lower likelihood of penetrating the blood-brain barrier. It can thus be  
525 hypothesised that the structural features in AP14145 make the compound less likely to  
526 penetrate to induce CNS mediated convulsions when compared to NS8593. Although

527 this difference in profile could in principle be caused by differences in the  
528 pharmacokinetic profile of the two compounds, this seems an unlikely explanation,  
529 since, as has been shown in Diness et al. (2010), 5 mg kg<sup>-1</sup> of NS8593 cause similar  
530 increases of the AERP in rats when compared to the effect of 5 mg kg<sup>-1</sup> of AP14145  
531 (Fig 8a).

532 High concentrations of AP14145 (10 μM L<sup>-1</sup>) significantly inhibited the K<sub>Ca</sub>1.1  
533 channel. However, as paxilline, which is a well-known specific inhibitor of K<sub>Ca</sub>1.1  
534 channels (Nardi and Olesen, 2008), did not have any effect on the atrial refractoriness of  
535 isolated perfused rat hearts, we conclude that the inhibition of K<sub>Ca</sub>1.1 by AP14145 does  
536 not contribute to the AERP prolonging effects of AP14145. This is in accordance with  
537 studies demonstrating the lack of K<sub>Ca</sub>1.1 channels and currents in the plasma membrane  
538 of cardiomyocytes (Bautista et al., 2009; Singh et al., 2013).

539 The apparent reduced CNS exposure of the drug makes AP14145 a unique and useful  
540 new tool compound that allows the study of peripheral K<sub>Ca</sub>2 inhibition without  
541 apparently interfering with CNS function in awake rodents. This might help further  
542 development and understanding of the cardiac and endothelial role of K<sub>Ca</sub>2 in a number  
543 of physiological and pathological settings.

544

## 545 **Conclusions**

546 In this work we present the novel K<sub>Ca</sub>2 negative gating modulator AP14145. This new  
547 drug inhibits both the hK<sub>Ca</sub>2.2 and hK<sub>Ca</sub>2.3 with equal potency (IC<sub>50</sub> = 1.1 ± 0.3 μM L<sup>-1</sup>  
548 with 400 nM L<sup>-1</sup> intracellular Ca<sup>2+</sup>) by decreasing the calcium sensitivity of the channel.  
549 The inhibitory effect of AP14145 effect is strongly dependent on two amino acids, S508  
550 and A533 in the rK<sub>Ca</sub>2.3, located in the inner pore of the channel. *In vivo*, AP14145  
551 significantly increases the atrial refractoriness in rats shortly after a 2.5 mg kg<sup>-1</sup> or 5.0  
552 mg kg<sup>-1</sup> bolus injection. In contrast to NS8593, a dose of 10 mg kg<sup>-1</sup> of AP14145 did not  
553 trigger any apparent acute CNS mediated effects in mice, suggesting that the compound  
554 does not penetrate the blood brain barrier to the same degree as NS8593 in rodents. This  
555 key difference could for the first time allow for the use of a K<sub>Ca</sub>2 negative modulator *in*  
556 *vivo* without interfering with CNS function. We expect this feature might help further  
557 development and understanding of the cardiac and endothelial role of K<sub>Ca</sub>2 channels.

558

559 **Author contributions**

560 *Rafel Simó-Vicens*: conception and design of the study, data acquisition  
561 (electrophysiology and molecular biology) and analysis, drafting and critical revision of  
562 the work.

563 *Jeppe E. Kirchhoff*: acquisition of data (closed chest recordings), data analysis, drafting  
564 and critical revision of the work.

565 *Lea Abildgaard Jensen*: acquisition of data (beam walk test and perfused heart  
566 preparation), data analysis, drafting and critical revision of the work.

567 *Bernardo Dolce*: data acquisition (electrophysiology and molecular biology) and  
568 analysis.

569 *Tobias Speerschneider*: acquisition of data (closed chest recordings), data analysis,  
570 drafting and critical revision of the work.

571 *Ulrik S. Sørensen*: conception and design of AP14145, conception and design of the  
572 study, drafting and critical revision of the work.

573 *Morten Grunnet*: conception and design of the study, critical revision of the work.

574 *Jonas G. Diness*: conception and design of the study, data analysis, drafting and critical  
575 revision of the work.

576 *Bo H. Bentzen*: conception and design of the study, drafting and critical revision of the  
577 work.

578

579 **Acknowledgements**

580 The study was supported by Innovation Fund Denmark, the Carlsberg Foundation, and  
581 the European Union's Horizon 2020 research and innovation programme under the  
582 Marie Skłodowska-Curie grant agreement No. 675351. We would also like to thank  
583 Dorte Strøbæk for kindly donating the hK<sub>Ca</sub>3.1 T250S V275A mutant.

584

585 **Conflicts of interest statement**

586 All authors of this study are or have been employed by Acesion Pharma.

587

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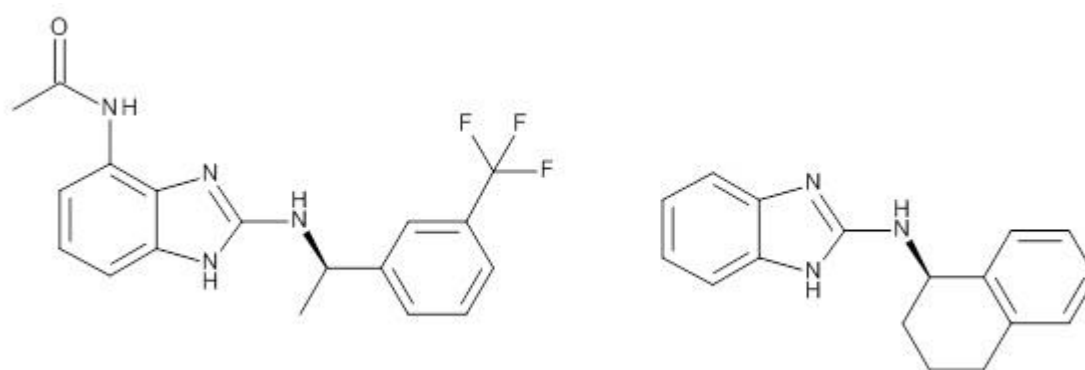
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### 688 **Supporting information**

689 None supplied.

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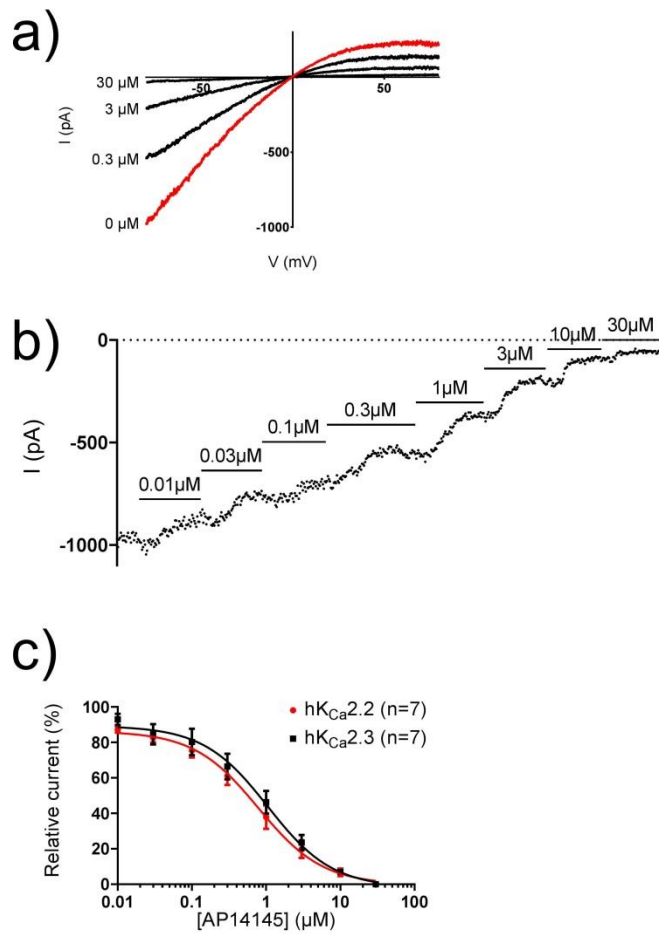
### 691 **Figures and figure legends**



692

693 Fig. 1. Chemical structures of AP14145 (left) and NS8593 (right).



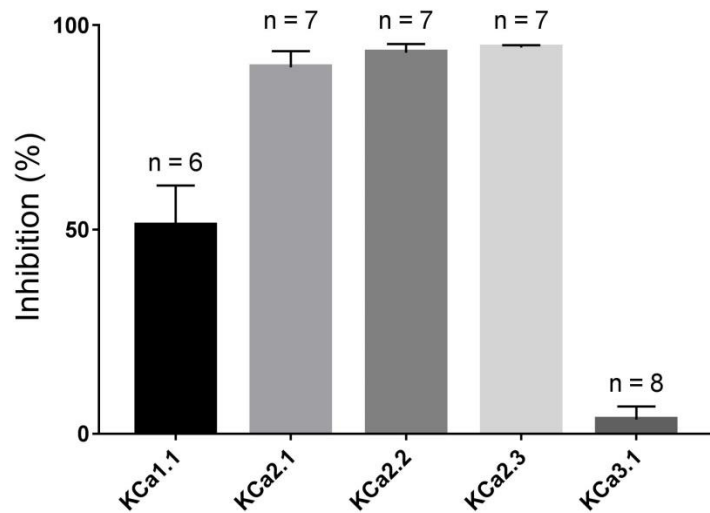


694

695 Fig. 2. a) Representative current-voltage recordings of the inhibition of hKCa2.3 by  
 696 increasing concentrations of the drug AP14145 and b) its current-time plot obtained by  
 697 inside-out patch clamp on HEK cells stably expressing the channel. c) Inhibition curves  
 698 of AP14145 on both the hKCa2.2 (n = 7) and hKCa2.3 (n = 7) channel.

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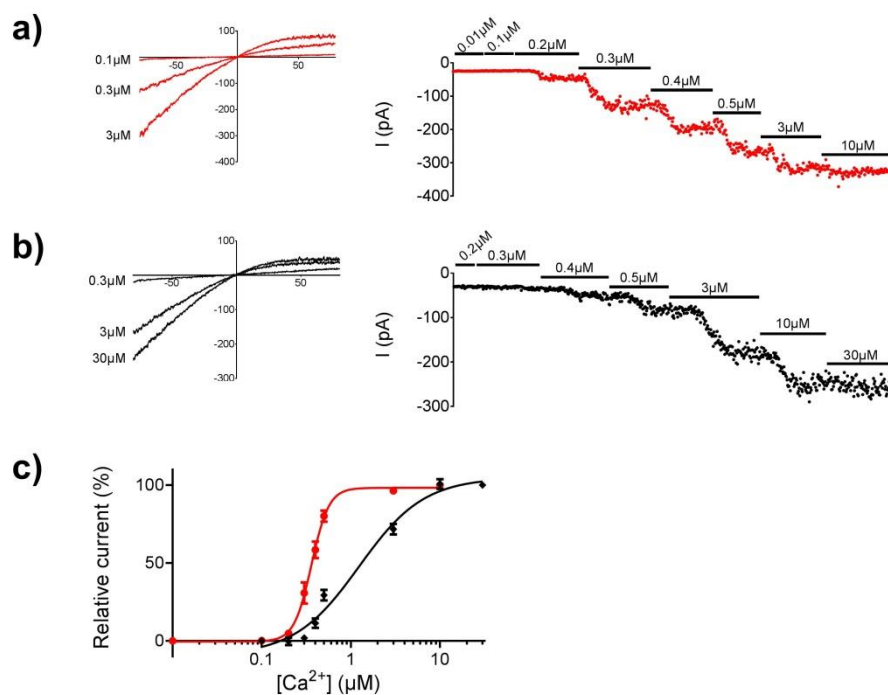


701

702 Fig. 3. Inhibitory effect of  $10 \mu\text{M L}^{-1}$  AP14145 on  $\text{KCa}1.1$ ,  $\text{KCa}2.1$ ,  $\text{KCa}2.2$ ,  $\text{KCa}2.3$  and

703  $\text{KCa}3.1$  channels. All measurements were obtained by whole cell patch clamp except

704  $\text{KCa}2.2$ , which were obtained by inside-out patch clamp.



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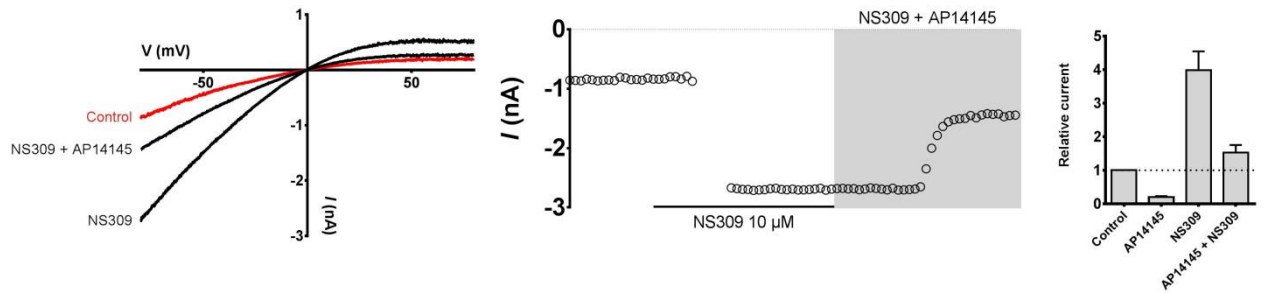
706 Fig. 4. Representative current-voltage plots (left) and their corresponding current-time

707 plots (right) of  $\text{hKCa}2.3$  calcium activation a) in the absence of AP14145 and b) in the

708 presence of  $10 \mu\text{M L}^{-1}$  AP14145. c) Calcium activation curves for the  $\text{hKCa}2.3$  channel

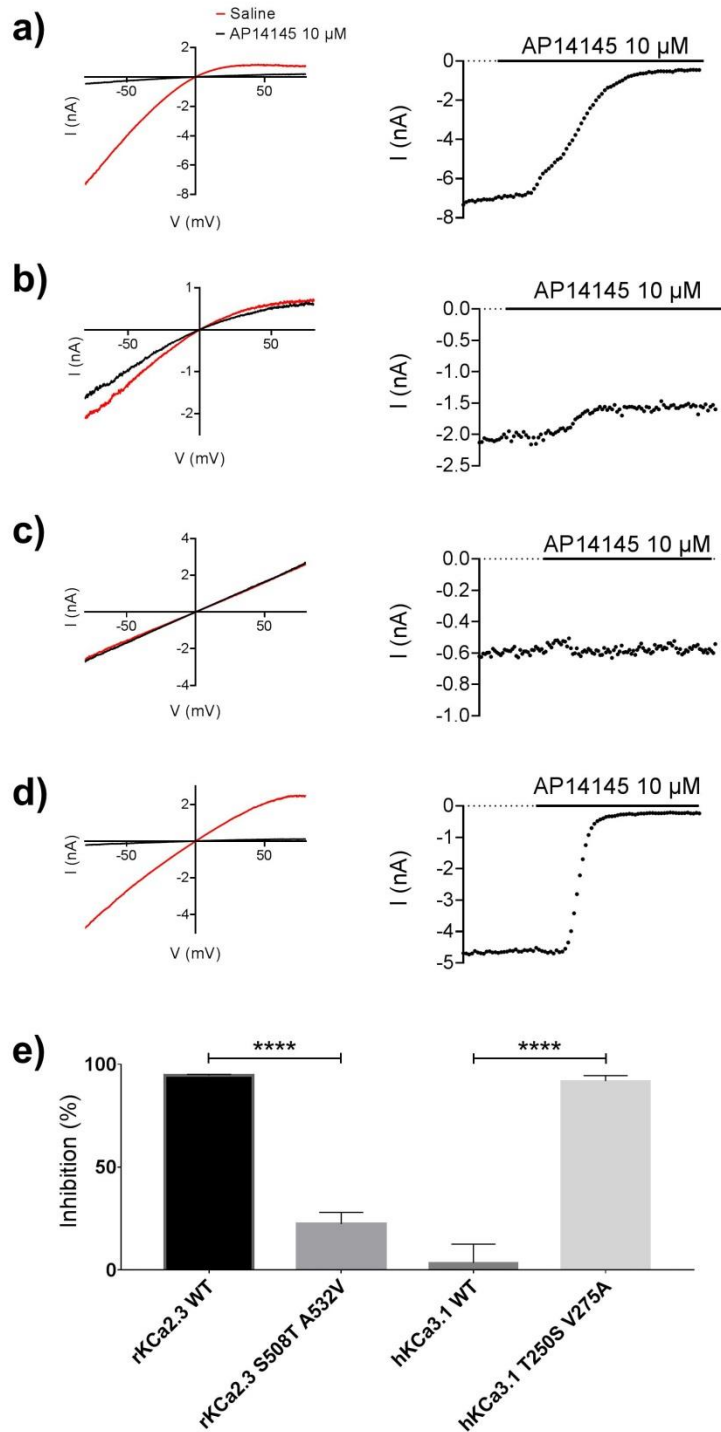
709 in the absence of AP14145 (red curve,  $n = 9$ ) and in the presence of  $10 \mu\text{M L}^{-1}$

710 AP14145 (black curve,  $n = 7$ ).



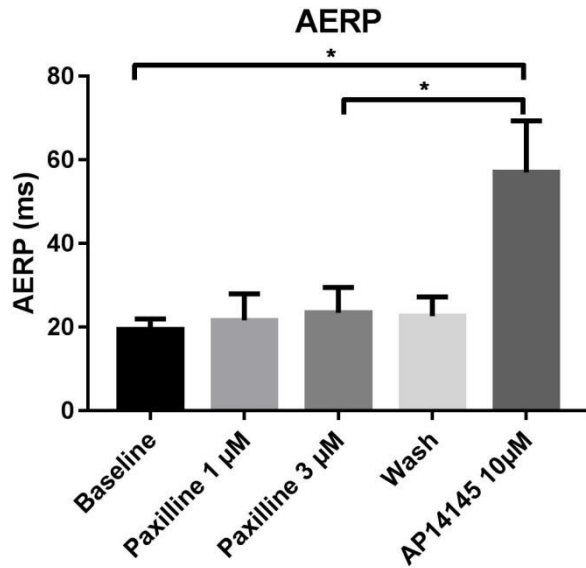
711

712 Fig. 5. Representative current-voltage plot (left) and its corresponding current-time plot  
 713 (centre) of the effect of  $10 \mu\text{M L}^{-1}$  NS309 in the absence and in the presence of  $10 \mu\text{M}$   
 714  $\text{L}^{-1}$  AP14145 on excised  $\text{hK}_{\text{Ca}2.3}$  patches. On the right, bar graph comparing the effects  
 715 of  $10 \mu\text{M L}^{-1}$  AP14145 ( $n = 7$ ),  $10 \mu\text{M L}^{-1}$  NS309 ( $n = 7$ ), and  $10 \mu\text{M L}^{-1}$  AP14145 +  $10$   
 716  $\mu\text{M L}^{-1}$  NS309 ( $n = 7$ ) on excised  $\text{hK}_{\text{Ca}2.3}$  patches.



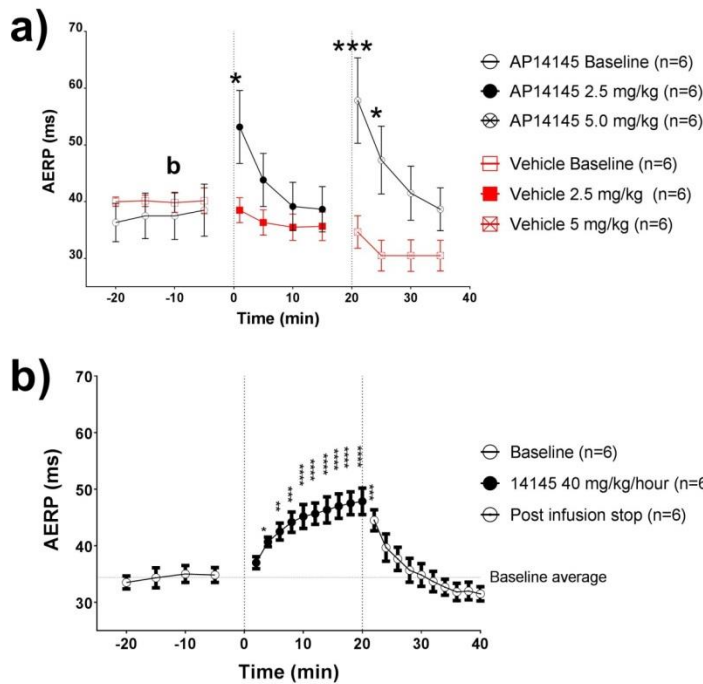
717

718 Fig. 6. Representative current-voltage plots (left) and current-time plots (right)  
 719 depicting the effect of AP14145 10 μM L<sup>-1</sup> on HEK cells transiently transfected with a)  
 720 rKCa<sub>2.3</sub> WT, b) rKCa<sub>2.3</sub> S508T A533V, c) hKCa<sub>3.1</sub> WT and d) hKCa<sub>3.1</sub> T250S V275A  
 721 recorded using whole cell patch clamp. e) Bar graph comparing the inhibitory effect of  
 722 10 μM L<sup>-1</sup> AP14145 on rKCa<sub>2.3</sub> WT (n = 7), rKCa<sub>2.3</sub> S508T A533V (n = 7), hKCa<sub>3.1</sub>  
 723 WT channel (n = 8) and hKCa<sub>3.1</sub> T250S V275A (n = 7).



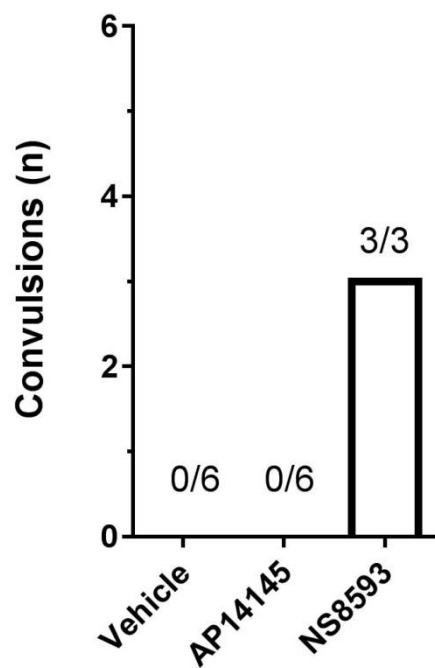
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725 Fig 7. Effect of 1  $\mu\text{M L}^{-1}$  and 3  $\mu\text{M L}^{-1}$  of the  $\text{K}_{\text{Ca}1.1}$  inhibitor paxilline and 10  $\mu\text{M L}^{-1}$   
 726 AP14145 on the AERP of isolated perfused rat hearts (n = 5).



727

728 Fig. 8. Effects on AERP in closed chest in vivo rats: a) Bolus doses of 2.5  $\text{mg kg}^{-1}$  and 5  
 729  $\text{mg kg}^{-1}$  AP14145 significantly increased the AERP compared to time matched controls  
 730 receiving corresponding volumes of vehicle. b) AP14145 given as a constant rate  
 731 infusion of 40  $\text{mg kg}^{-1} \text{h}^{-1}$  over 20 minutes increased AERP compared to the baseline  
 732 average and returned towards baseline values post-infusion.



733

734 Fig. 9. Bar graph depicting the amount of convulsions triggered by the administration of  
 735 the vehicle, 10 mg kg<sup>-1</sup> AP14145 and 10 mg kg<sup>-1</sup> NS8593.

736

737 Table 1. Calculated physicochemical properties and plasma protein binding (PPB) of  
 738 NS8593 and AP14145.

	<b>log D (pH 7.4)</b>	<b>log P</b>	<b>PSA (Å<sup>2</sup>)</b>	<b>PPB (% bound)</b>
<b>NS8593</b>	4.0	4.1	41	95.38
<b>AP14145</b>	3.6	3.7	70	91.35

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