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2   **LUF7244 plus dofetilide rescues aberrant Kv11.1 trafficking and**

3   **produces functional IKv11.1**

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21   Running title: Dofetilide+LUF7244 rescues $K_v11.1$ trafficking and restores

22    $I_{Kv11.1}$

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38

39   Abbreviations:

40   APD: action potential duration; cAVB: chronic atrio-ventricular block; hiPSC,

41   human-induced Pluripotent Stem Cell; MD: molecular dynamics; NRVM:

42   neonatal rat ventricular myocytes; SR: sinus rhythm; WT: wild type.

2

Abstract

$K_v11.1$ (hERG) channels play a critical role in repolarization of cardiomyocytes

during the cardiac action potential (AP). Drug mediated $K_v11.1$ blockade results

in AP prolongation, which poses an increased risk of sudden cardiac death.

Many drugs, like pentamidine, interfere with normal $K_v11.1$ forward trafficking

and thus reduce functional $K_v11.1$ channel densities. Although class III

antiarrhythmics, e.g. dofetilide, rescue congenital and acquired forward

trafficking defects, this is of little use due to their simultaneous acute channel

blocking effect. We aimed to test the ability of a combination of dofetilide plus

LUF7244, a $K_v11.1$ allosteric modulator/activator, to rescue $K_v11.1$ trafficking

and produce functional $K_v11.1$ current. LUF7244 treatment by itself did not

disturb or rescue WT or G601S $K_v11.1$ trafficking as shown by western blot and

immunofluorescence microcopy analysis. Pentamidine-decreased maturation

of WT $K_v11.1$ levels was rescued by 10 μM dofetilide or 10 μM dofetilide + 5

μM LUF7244. In trafficking defective G601S $K_v11.1$ cells, dofetilide (10 μM) or

dofetilide+LUF7244 (10+5 μM) restored $K_v11.1$ trafficking also, as

demonstrated by western blot and immunofluorescence microscopy. LUF7244

(10 μM) increased $I_{Kv11.1}$ despite the presence of dofetilide (1 μM) in WT $K_v11.1$

cells. In G601S expressing cells, long-term treatment (24-48 h) with LUF7244

(10 μM) and dofetilide (1 μM) increased $I_{Kv11.1}$ compared to non-treated, or

acutely treated cells. We conclude that dofetilide plus LUF7244 rescues $K_v11.1$

trafficking and produces functional $I_{Kv11.1}$. Thus, combined administration of

3

65   LUF7244 and an $I_{Kv11.1}$ trafficking corrector could serve as a new

66   pharmacological therapy of both congenital and drug-induced $K_v11.1$ trafficking

67   defects.

68

69   Key words: trafficking; $K_v11.1$; LUF7244; dofetilide; E4031; electrophysiology;

70   molecular dynamics

71 **Significance Statement**

72 LUF7244, a negative allosteric modulator/activator, in combination with

73 dofetilide corrected both congenital and acquired $K_v11.1$ trafficking defects

74 resulting in functional $K_v11.1$ current.

Introduction

75

76 Human $K_v11.1$ potassium ion channels (also known as hERG channels) stand

77 at the basis of the rapidly activating delayed rectifier current (I_{Kr}) which is

78 involved in phase three repolarization of the action potential (AP) in working

79 cardiomyocytes (Vandenberg et al., 2012). Interference with normal I_{Kr} function

80 can either shorten (gain-of-function) or prolong (loss-of-function) the process of

81 ventricular repolarization as evident from QT-shortening or lengthening

82 respectively, on the electrocardiogram (ECG) (Vandenberg et al., 2012). I_{Kr}

83 inhibition in humans, e.g. by the class III agent dofetilide, is associated with life

84 threatening ventricular arrhythmias (Torp-Pedersen et al., 1999).

85 Fundamentally different mechanisms of I_{Kr} inhibition have been identified: 1)

86 direct inhibition of potassium flow through the channel and 2) decreased plasma

87 membrane expression of channel proteins, which both can result from

88 mutations (*de novo* or congenital) or environmental factors (acquired, mostly

89 drug-induced) (Sanguinetti and Tristani-Firouzi, 2006; De Git et al., 2013). For

90 these reasons, cardiac safety assessment of new chemical entities and

91 preclinical drugs still has a strong focus on $K_v11.1$ channel function (Bossu et

92 al., 2016) and mainly aims at detection of (semi)acute pore block. Interestingly,

93 the far majority (approximately 90% of 167 tested) of congenital $K_v11.1$ loss-of-

94 function missense mutations result in trafficking defects as a cause of I_{Kr}

95 impairment (Anderson et al., 2006; 2016). For example, the G601S missense

96 mutation in $K_v11.1$, located in the S5-pore helix linker, results in reduced

97   expression of functional I_{Kr} leading to hypomorphic LQT2 (Ficker et al., 2002).

98   Also a number of drugs (>40% of 100 tested) limit expression of $K_v11.1$ at the

99   plasma membrane by inhibiting its forward trafficking, with or without

100   concomitant pore block (Wible et al., 2005). The anti-

101   trypanosomiasis/leishmanias drug pentamidine is currently used as a $K_v11.1$

102   trafficking inhibitor without acute channel inhibition (Cordes et al., 2005;

103   Kuryshev et al., 2005; Nalos et al., 2011; Himmel 2013; Varkevisser et al.,

104   2013a, b; Obergrussberger et al., 2016). Pentamidine inhibits $K_v11.1$ forward

105   trafficking at the level of endoplasmic reticulum exit in a process that involves

106   the high affinity drug binding site F656 (Dennis et al., 2012). As a result, cells

107   mainly express intracellularly localized core-glycosylated $K_v11.1$ with an

108   apparent M_w of 135 kDa.

109   High affinity $K_v11.1$ pore blockers such as E4031, dofetilide, cisapride and

110   astemizole are able to rescue forward trafficking defects caused by either

111   mutations or drugs (Zhou et al., 1999; Ficker et al., 2002; Varkevisser et al.,

112   2013a; Yan et al., 2015). This will result in $K_v11.1$ maturation seen as a fully

113   glycosylated protein with an apparent molecular weight of 155 kDa. The

114   underlying mechanisms have not been clarified completely thus far, although it

115   is found that channel inhibition potency correlates with rescue efficacy and that

116   drug-channel interactions via high affinity binding sites are essential (Rajamani

117   et al., 2002; Ficker et al., 2002; Dennis et al., 2012; Yan et al., 2015).

118   Furthermore, EC_{50} values for rescue are generally much higher than IC_{50} values

119   for acute pore block (eg. Astemizole, IC_{50}=6-13 nM; EC_{50} for rescue=335±33

120   nM with 10 µM pentamidine) (Ficker et al., 2002; Dennis et al., 2012).

121   Therefore, this strategy will not result in rescue of I_{Kr} function as long as the

122   high affinity blocker remains present whereas its withdrawal will not resolve the

123   underlying trafficking defects. Activators and negative allosteric modulators of

124    $K_v11.1$ have been developed as a strategy to counteract undesired I_{Kr} blockade

125   and thus potentially could "save" numerous (pre)clinical drugs with proven I_{Kr}

126   liability (Yu et al., 2014 and 2016; Sala et al., 2016; Qile et al., 2019). Allosteric

127   modulators interact with the $K_v11.1$ channel at a site different than that used by

128   the high affinity inhibitors, and thereby modulate binding affinities for the

129   canonical binding site of the latter. Activators interact with $K_v11.1$ at various

130   sites (Sanguinetti, 2014) and some share overlapping high affinity molecular

131   determinants (Casis et al, 2006; Perry et al, 2007; Garg et al, 2011) with

132   classical pore blockers. We have developed the negative allosteric

133   modulator/activator LUF7244 which indeed is able to counteract drug-induced

134   AP prolongation and proarrhythmia *in vitro* (Yu et al., 2015 and 2016) and drug-

135   induced ventricular arrhythmia *in vivo* (Qile et al., 2019). Specifically,

136   application of 10 µM LUF7244 decreased the affinity of $K_v11.1$ for cisapride,

137   astemizole, dofetilide and sertindole by 4.0-, 3.8-, 3.2-, and 2.2-fold,

138   respectively (Yu et al., 2016). We hypothesized that LUF7244 would not

139   interfere in $K_v11.1$ trafficking by itself, and would also not interfere in dofetilide-

140   mediated rescue of defective forward trafficking, but maintains its ability to

141   increase I_{Kr} in the presence of dofetilide.

142   **Materials and Methods**

143

144   **Chemicals**

145   LUF7244 was custom synthesized at the Division of Drug Discovery and Safety,

146   Leiden Academic Centre for Drug Research, Leiden University, The

147   Netherlands as described earlier (Yu Z et al., 2016), dissolved in DMSO at 100

148   mM. Dofetilide was purchased from Sigma-Aldrich (Zwijndrecht, The

149   Netherlands) and dissolved at 10 mM in DMSO. E4031 was dissolved in DMSO

150   at 1 mM. Pentamidine-isethionate (Pentacarinat® 300, Sanofi Aventis, Gouda,

151   The Netherlands) was dissolved in water to provide a stock solution of 100 mM.

152   All compounds were sterilized by filtration (0.22 µm), aliquoted and stored at -

153   20°C until use.

154

155   **Molecular Modeling**

156   Compounds LUF7244 and dofetilide were docked into the hERG cryo-EM

157   structure (pdb code: 5va1, Wang and Mackinnon, 2017) using the GOLD

158   software v.5.6.2. (Jones et al., 1997) essentially as described before (Qile et

159   al., 2019). Two scoring functions ChemPLP and Goldscore were used and 100

160   poses collected per run with 125.000 Gold algorithm operations. The top 15

161   scoring poses each were analysed using PyMol 1.7.2 (Schrödinger, 2015).

162

163   **Molecular dynamics Simulations**

164 Simulations were essentially performed as described earlier (Qile et al., 2019)

165 with few small modifications. The K_v11.1 hERG structure was embedded in a

166 POPC bilayer and solvated with TIP3P water using the CHARMM-GUI (Jo et

167 al., 2008). KCl (150 mM) was added to the system and potassium ions in the

168 selectivity filter where placed at sites S0, S2 and S4, with water molecules at

169 sites S1 and S3. Steepest descent energy minimization, followed by 2 ns

170 equilibration and 50 ns production runs were performed using GROMACS

171 v.5.1.2 (Abraham et al., 2015) and the charmm36 forcefield (Vanommeslaeghe

172 et al., 2010). Electrostatics were modeled using Particle Mesh Ewald (Darden

173 et al., 1993), and LINCS was used to constrain H-bonds (Hess et al., 1997). V-

174 rescale (Bussi et al., 2007) was used to keep the temperature at 310 K, and

175 semi-isotropic pressure coupling was done using the Parrinello-Rahman

176 barostat (Parrinello and Rahman, 1981).

177

178 **Cells**

179 HEK-hERG cells which is the HEK293T cell line stably expressing human

180 K_v11.1 protein, and hERG-G601S cells (HEK293T cell line stably expressing

181 forward trafficking deficient K_v11.1 protein) were cultured in DMEM (Gibco-

182 FisherScientific, Landsmeer, The Netherlands) supplemented with 10% fetal

183 calf serum (Sigma-Aldrich, Zwijndrecht, the Netherlands), 2 mM L-glutamine,

184 50 U·mL^{-1} penicillin, and 50 µg·mL^{-1} streptomycin (all three Lonza, Breda, the

185 Netherlands) as described before (Varkevisser et al., 2013a).

186 **Patch clamp electrophysiology**

187 HEK-hERG and hERG-G601S cells were grown on Ø12 mm glass cover slips

188 coated with poly-L-lysine (Sigma-Aldrich, German) and placed in a perfusion

189 chamber (Cell Microcontrols, Norfolk, VA, USA). Functional analyses were

190 done by using standard whole-cell patch clamp technique on HEK293T cells

191 stably expressing WT- or G601S-K_v11.1 channels. The external solution

192 contained (mM): 137 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10

193 HEPES (pH 7.4 adjusted with NaOH), and the internal pipette solution

194 contained (mM): 130 KCl, 1 $MgCl_2$, 5 EGTA, 5 MgATP, 10 HEPES (pH 7.2

195 adjusted with KOH). An Axopatch-200B patch clamp amplifier (Axon

196 Instruments, Union City, CA, USA) was used to measure macroscopic currents

197 and cell capacitance. The pipette resistances were 1-3 MΩ and series

198 resistance was compensated up to 90%. The pCLAMP 10 software (Axon

199 Instruments) was used to generate the different voltage protocols, acquire

200 current signals, and for data analyses. We determined the impact that dofetilide

201 and LUF7244 had on K_v11.1 currents compared to control by applying step-like

202 pulses from -80 mV to +60 mV in +10 mV increments for 5 s, followed by a "tail"

203 pulse to -50 mV for 5 s. The maximal $I_{Kv11.1}$ measured during the tail pulse was

204 plotted as a function of the step-pulse potential to generate the corresponding

205 I-V relations.

206

207 **Western blot**

208   Cell lysates were prepared in buffer D (20 mM HEPES, 125 mM NaCl, 10%

209   glycerol, 1 mM EGTA, 1 mM dithiothreitol, 1 mM EDTA and 1% Triton X-100,

210   pH 7.6) supplemented with 1 mM PMSF and 10 µg·mL^{-1} aprotinin. Twenty-five

211   µg protein lysate was mixed with Laemmli sample buffer, separated by 7%

212   SDS-PAGE and blotted onto a nitrocellulose membrane. Ponceau staining was

213   used to reveal equal protein loading and subsequent quantification. Blots were

214   blocked with 5% Protifar dissolved in TBST (20 mM Tris-HCl; pH 8.0, 150 mM

215   NaCl, 0.05% Tween-20 (v/v)) for 1h at room temperature. $K_v11.1$ protein was

216   detected by polyclonal anti-h$K_v11.1$ primary antibody (cat. no. APC-062;

217   Alomone Labs, Jerusalem, Israel, 1:3000) and peroxidase-conjugated anti-

218   rabbit secondary antibody (Jackson ImmunResearch Laboratories Inc., West

219   Baltimore Pike West Grove, PA, USA; 1:10000). Final detection was performed

220   using a standard ECL procedure (GE Lifescience, Marlborough, MA, USA) with

221   ChemiDocXRS system (BioRad Laboratories, Veenendaal, The Netherlands).

222   Quantification analysis was performed using Image J 1.48V software (National

223   Institute of Health USA).

224

225   **Immunofluorescence microscopy**

226   HERG-G601S cells were grown on Ø 15 mm cover slips, coated with poly-L-

227   Lysine, fixated with 3% paraformaldehyde dissolved in PBS containing 1 mM

228   Ca^{2+} and 1 mM Mg^{2+}, pH 7.4 for 20 minutes. Permeabilization was performed

229   with 0.5% Triton X-100 in PBS for 3 minutes and 50 mM glycine-PBS was used

230   as quenching agent, cells were subsequently blocked with NET-gel (150 mM

231   NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, 0.05% Igepal, 0.25% gelatin,

232   0.02% NaN_3). Then the cells were incubated with polyclonal anti-hK_v11.1

233   (1:3000, APC-062, Alomone Labs, Jerusalem, Israel) and anti-Pan-cadherin

234   (1:800, Sigma-Aldrich, St Louis, MO, USA) primary antibody overnight in NET-

235   gel, followed by incubation with secondary antibody of anti-mouse Alexa488

236   (Thermofisher Scientific) and anti-rabbit Alexa568 (Thermofisher Scientific,

237   Landsmeer, The Netherlands) for 2h.

238   The cover slips were mounted with Vectashield (Vector Laboratories Inc.

239   Burlingame, CA, USA), and fluorescent microscopy images were obtained

240   using a Nikon ECLIPSE Ti2-E inverted microscope equipped with a ×60 oil

241   immersion objective (NA 1.49) (CAIRN research, Faversham, UK). Excitation

242   was performed with diode lasers (Omicron LuxX 488 nm 200mW for Alexa488

243   and an OBIS 561 nm 100mW for Alexa561). Colocalization between K_v11.1 and

244   Cadherin in cell-extensions was quantified by determining Pearson's coefficient

245   (r) with the Costes automated threshold method provided by the Coloc_2 plugin

246   for the ImageJ software (1.52p) using Fiji.

247

248   **Statistics**

249   All averages values are expressed as mean ± standard deviation (SD), unless

250   indicated otherwise. All statistical analyses were carried out by using SPSS

251   version 21 and Graphpad Prism version 5. A difference was considered

14

252   significant with P < 0.05. Differences among groups were evaluated using either

253   one-way ANOVA with Dunnett's test for western blot and immunofluorescence

254   microscopy data or two-way ANOVA with Tukey's test for electrophysiology

255   data. Post hoc tests were carried out only if F was significant and there was no

256   variance in homogeneity.

257   **Results**

258

259   **Docking and Molecular dynamics simulation-based prediction of binding**

260   **mode of dofetilide and its interaction with LUF7244**

261   Overview representations of $K_v11.1$ channel interaction with dofetilide and

262   LUF7244 at the structural level, as viewed from the extracellular side, are

263   shown in Figure 1A and Figure 1B. To investigate how LUF7244 might lower

264   the channels affinities for dofetilide (Yu et al, 2016), we compared two binding

265   modes of dofetilide and LUF7244. Figure 1C and D displays two alternative

266   binding modes of dofetilide in the near-atomic resolution cryo-EM structure (3.4

267   Å) of the $K_v11.1$ K^+ channel in agreement with experimental data: in the cavity,

268   (Figure 1D), which is the classical assumed binding mode for blockers (Kamiya

269   et al., 2006; Imai et al., 2009), or in the fenestrations, sticking into the cavity

270   (Figure 1C), as suggested recently, based on hERG homology models (Saxena

271   et al., 2016). Both binding modes can be recapitulated in docking studies to the

272   cryo-EM structure and the drug is stable in 50 ns of Molecular dynamics (Figure

273   1G and H) in both sites. We have recently reported that LUF7244 might bind

274   between the pore helices of two adjacent subunits, thereby stabilizing the

275   conductive state of the channel (Qile et al, 2019). Comparison of the proposed

276   binding modes of dofetilide with that of LUF7244 (Figure 1E and F) suggests

277   that the allosteric negative inhibitor/activator could prevent the inhibitor from

16

278   binding to the fenestration site (Figure 1E), while there is almost no overlap,

279   when dofetilide is bound to the central cavity (Figure 1F).

280

281   **LUF7244 has no effect on $K_v11.1$ forward trafficking and does not interfere**

282   **in dofetilide mediated rescue of pentamidine-induced trafficking defects**

283   Application of LUF7244 (0.05, 0.1, 1, 3 and 5μM) for 48 h did not obviously

284   affect $K_v11.1$ ratio of mature/immature protein as shown in Figure 2A. We

285   demonstrated earlier that dofetilide rescues pentamidine-induced $K_v11.1$

286   forward trafficking defects (Varkevisser et al., 2013a). To determine if LUF7244

287   can restore mature $K_v11.1$ expression, different concentrations of LUF7244

288   were applied to HEK-hERG cells in the continuous presence of 10 μM

289   pentamidine. However, treatment with LUF7244 up to 5 μM did not restore

290   mature $K_v11.1$ protein levels (Figure 2B).

291   Since LUF7244 by itself could not restore normal forward $K_v11.1$ channel

292   trafficking, did not affect trafficking by itself, and was found to counteract

293   dofetilide-mediated I_{Kr} blockade (Qile et al., 2019), we questioned whether

294   LUF7244 would influence dofetilide-mediated rescue of pentamidine induced

295   trafficking defects. To test this hypothesis, the same dose range of LUF7244

296   was applied in combination with 1 μM dofetilide and 10 μM pentamidine for 48h.

297   Interestingly, pentamidine decreased mature WT $K_v11.1$ protein (0.24±0.07 vs.

298   0.54±0.11 (control)), which was rescued by 1 μM dofetilide (0.44±0.09 vs.

299   0.24±0.07) but also by the combination of 1 μM dofetilide and 5 μM LUF7244

17

300   (0.45±0.10 vs. 0.24±0.07) (Figure 2C). All the separate mature or immature

301     $K_v11.1$ protein expression level, quantified from same blots, are shown in the

302   right panel.

303

304   **LUF7244 and dofetilide/E4031 rescue G601S-$K_v11.1$ maturation**

305   In order to test the effects of LUF7244 on a congenital $K_v11.1$ loss-of-function

306   missense mutation, which results in defective forward trafficking, we co-applied

307   five different concentrations of LUF7244 and 1 µM dofetilide on hERG-G601S

308   cells. According to western blot result, trafficking deficiency was observed

309   which means only 135 kDa core-glycosylated immature protein was detected

310   (Figure 3A). After 48 h administration of LUF7244 (0.05, 0.1, 1, 3 and 5 µM),

311   mature $K_v11.1$ protein level was not changed compared with control (Figure 3A),

312   whereas application of 1 µM dofetilide greatly increased mature protein

313   expression. Furthermore, 1 µM dofetilide combined with LUF7244 (0.05-5 µM)

314   also resulted in expression of the fully glycosylated mature protein (Figure 3B).

315   To expand our findings to other I_{Kr} blockers, we used E4031. We applied 5 µM

316   E4031 without or with LUF7244 (0.05, 0.1, 1, 3 and 5 µM) for 48h. Under all

317   rescue conditions, the level of the 155 kDa fully glycosylated mature protein

318   was increased (Figure 3C), although not to the same extent as seen with

319   dofetilide. The separate mature or immature $K_v11.1$ protein expression level

320   can be found in the right panel.

18

321   Immunofluorescence staining was used to determine the subcellular

322   localization of the G601S-K_v11.1 protein. In untreated control hERG-G601S

323   cells, no K_v11.1 protein was detected at the cell membrane structures such like

324   membrane ruffles, in contrast to the transmembrane protein Cadherin (Figure

325   4) (Pearson coefficient of co-localization r=0.20±0.35, n=6). Following 24 h

326   treatment with 10 µM dofetilide or 10 µM dofetilide+3 µM LUF7244, normal

327   trafficking was partly restored as evidenced by plasma membrane expression

328   of K_v11.1 in membrane ruffles, where it colocalized with Cadherin (Figure 4)

329   (r=0.85±0.08 (n=6) and 0.86±0.06 (n=6), respectively, both p<0.01 vs. control

330   or LUF7244). LUF7244 only treatment yielded no rescue of forward trafficking

331   (r=0.13±0.31, n.s. vs. control). A lower concentration of LUF7244 was used to

332   maintain intact cell structure in these experiments.

333

334   **LUF7244 increases $I_{Kv11.1}$ in the presence of dofetilide**

335   Lastly the impact of dofetilide and LUF7244 on $I_{Kv11.1}$ was determined (Figure

336   5). The use of 10 µM LUF7244 in electrophysiology experiment is based on

337   strong $I_{Kv11.1}$ blockade effect of dofetilide. To counteract dofetilide's effect, we

338   needed to use relatively higher concentration than what we used in western blot

339   experiments. On the other hand, 10 µM LUF7244 was used in our previous

340   work (Qile et al., 2019), which was based on the concentration that was used

341   for its structural similar compound ICA-105574.

19

342   Figure 5A shows representative current traces measured from cells expressing

343   WT-K_v11.1 channel proteins in control conditions or with application of dofetilide

344   (1 μM) or LUF7244 (10 μM) or combination to the extracellular recording

345   solution as acute treatment. Cells are voltage-clamped at a holding potential of

346   −80 mV and depolarized to voltages between −80 and 60 mV for 5 s to activate

347     $I_{Kv11.1}$ (pre-pulse). The cells are then clamped to −50 mV for 5 s to record a tail

348   current (test-pules). As shown for control cells in Figure 5A, during depolarizing

349   and tail pulses, an outward current was activated at voltages positive to −40

350   mV, and the current amplitude of the $I_{Kv11.1}$ measured during the tail pulse

351   increases with a maximum $I_{Kv11.1}$ current following depolarizing pulses to >10

352   mV. The peak tail $I_{Kv11.1}$ amplitude following repolarization was used to

353   construct the activation curve shown in Figure 5B. The activation curve

354   measured for control cells shows that the threshold voltage for $I_{Kv11.1}$ activation

355   is about −40 mV and that it is fully activated following voltage pulses to 10 mV.

356   Dofetilide and LUF7244 dramatically alter the activation and kinetic properties

357   of $I_{Kv11.1}$. The outward $I_{Kv11.1}$ measured during the depolarization and tail pulses

358   is larger than control from -70 mV to 10 mV; there is a negative shift in the

359   corresponding I-V relation; but $I_{Kv11.1}$ gets smaller following depolarizing pulses

360   > 0 mV. The changes in the $I_{Kv11.1}$ measured using this protocol in the presence

361   of these drugs show complex changes consistent with both the activating

362   properties of LUF7244 and blocking properties of dofetilide. The relevant

363   LUF7244/dofetilide alone control study are shown in Figure 5.

364 Figure 6A shows representative current traces measured from cells expressing

365 G601S-K_v11.1 channel proteins in control conditions and with acute or long-

366 term application of dofetilide+LUF7244, or LUF7244 only to the extracellular

367 recording solution. There was no statistical difference in $I_{Kv11.1}$ between the

368 control conditions and acute application of dofetilide + LUF7244. Therefore, we

369 tested the long-term effects of these drugs on $I_{Kv11.1}$. We incubated cells in

370 dofetilide + LUF7244 for 24-48 h and then recorded $I_{Kv11.1}$ from cells with these

371 drugs in the extracellular recording solution. The mean I-V relations, based on

372 peak tail $I_{Kv11.1}$ amplitude following repolarization, for cells expressing G601S-

373 K_v11.1 channel proteins in the different conditions indicate that long-term

374 dofetilide + LUF7244 treatment increased $I_{Kv11.1}$. Dofetilide only treatment had

375 a minimal effect (Figure 6B) indicating the presence of only few functional

376 channels at the plasma membrane without prior pharmacological correction of

377 trafficking. Interestingly however, LUF7244 only treatment produced $I_{Kv11.1}$

378 under such condition (Figure 6A, B). Compared to control cells: cells cultured

379 and recorded in dofetilide + LUF7244 increased $I_{Kv11.1}$ following pre-pulses to -

380 40mV to 60 mV (p<0.05), and cells treated with LUF7244 increased $I_{Kv11.1}$

381 following pre-pulses from -80mV to 60mV (p<0.05). Compared to cells recorded

382 in dofetilide: cells cultured and recorded in dofetilide + LUF7244 increased

383 $I_{Kv11.1}$ following pre-pulses to -40mV to 60 mV (p<0.05), and cells recorded in

384 LUF7244 increased $I_{Kv11.1}$ following pre-pulses from -80mV to 60mV (p<0.05).

385 Compared to cells recorded in dofetilide + LUF7244: cells cultured and

21

386   recorded in dofetilide + LUF7244 increased $I_{Kv11.1}$ following pre-pulses to -30mV

387   to 30 mV ($p<0.05$), and cells recorded in LUF7244 increased $I_{Kv11.1}$ following

388   pre-pulses from -80mV to 60mV ($p<0.05$). Compared to cells cultured and

389   recorded in LUF7244 cells, cells recorded in LUF7244 increased $I_{Kv11.1}$ following

390   pre-pulses from -80mV to 60mV ($p<0.05$).

22

391   **Discussion**

392    $K_v11.1$ activators and negative allosteric modulators use mechanistically

393   different ways to increase or maintain normal I_{Kr} levels in the presence of a

394    $K_v11.1$ channel inhibitor. A number of compounds have been demonstrated as

395    $K_v11.1$ activators (Perry et al., 2010). Activators influence gating kinetics and

396   can for example slow down or remove inactivation and/or facilitate activation

397   (Sanguinetti, 2014). $K_v11.1$ activators normally interact with a region distant

398   from the inner cavity (Perry et al., 2010) but they can bind to several distinct

399   sites of the channel (Guo et al, 2015; Perry et al, 2007; Gardner et al 2017).

400   Negative allosteric modulators decrease the binding affinity of I_{Kr} blockers,

401   either by increasing dissociation rates, lowering association rates or both

402   (Christopoulos et al., 2014). In our previous study, and also shown here,

403   LUF7244 alone can dose-dependently increase $K_v11.1$ current and reduce

404   inactivation of $K_v11.1$ at higher concentration (Qile et al., 2019). In the current

405   study, instead of the blockade effect of dofetilide, dofetilide+LUF7244 treatment

406   statistically significant increased $I_{Kv11.1}$ level in HEK-hERG cell. In G601S cells,

407   dofetilide+LUF7244 treatment increased (not statistically significantly) steady

408   state current as well. Furthermore, long term exposure increased $I_{Kv11.1}$

409   continuously. It indicates that I_{Kr} inhibitors acute channel blockade could be

410   reversed by LUF7244. And its trafficking rescue characteristic might further

411   functionally benefits $K_v11.1$ for long term administration.

412 We observed a stronger $I_{Kv11.1}$ increase for G601S channels than for WT

413 channels (Figure 5, 6) which could not be explained by methodological means.

414 Although speculative, we can envision that besides an effect on trafficking, the

415 G601S mutation may result in increased binding affinity for LUF7244, or show

416 subtle differences channel kinetics in response to LUF7244 compared to WT.

417 Whether these effects are mutation specific are points for further investigation.

418 Furthermore, the finding that LUF7244 can strongly activate the low amount of

419 G601S channels that do reach the plasma membrane in cells not treated with

420 dofetilide, may shortcut the need for complete restoration of trafficking.

421 Modeling suggests that LUF7244 disrupts drug block at the fenestration, via

422 binding close to the protein-lipid interface (Figure 1F). Drug binding to this site

423 has recently been reported for Ivabradine, a low µmolar affinity $K_v11.1$ blocker

424 (Perissinotti et al., 2019). It has been reported that this drug interacts with lipid-

425 facing residues in the fenestration, including F557 and F656, in a state-

426 dependent manner. Even though, dofetilide is unlikely to access the $K_v11.1$

427 cavity, via this fenestration, as has been shown for the more lipophilic drug

428 Ivabradine, residue F557 has been shown to reduce binding affinity > 50-fold,

429 when mutated to a leucine (Saxena et al., 2016). This suggests that this lipid-

430 facing residue is critical for high affinity block of different hERG inhibitors. Our

431 modeling proposes that LUF7244 could disrupt coupling between state-

432 dependent dynamics of F557 and F656 and interfere with dofetilide binding to

433 the fenestration (Figure 1E). Given the impact of LUF7244 on inactivation, one

24

434   plausible scenario would be that the negative allosteric inhibitor prevents

435   dofetilide from binding or accessing the "high affinity" inactivated state in the

436   fenestration, but this will require further modeling of the inactivated state(s).

437   However, based on the current simulations of 50 ns, we cannot exclude that

438   K_v11.1 has additional LUF7244 binding sites. Additional binding sites may

439   explain the dual character of LUF7244 as a negative allosteric modulator (Yu

440   et al., 2016) and activator (Qile et al., 2019, this study). Additional experimental

441   analyses that may require in depth NMR studies on drug-channel interaction

442   deem necessary to resolve this issue. The I_{Kr} activator ICA-105574 has

443   structural similarities with LUF7244, and has also similar functional

444   characteristics (Gerlach et al., 2010). It was shown that ICA-105574 enhanced

445   K_v11.1 currents via a mechanism that seems to prevent or limit the inactivation

446   gating process. Additionally, ICA-105574 dose dependently shortened the AP

447   duration in isolated guinea pig ventricular cardiomyocytes. It also remarkably

448   suppressed the K_v11.1 channel inhibitor E4031-induced AP lengthening. In this

449   perspective, it would be interesting to compare these two compounds with

450   respect to the mechanism of action on K_v11.1 channels.

451   Besides, in view of its predicted binding site, LUF7244 by itself had no effects

452   on K_v11.1 channel trafficking, neither did it inhibit pentamidine-associated

453   trafficking defects nor did it affect dofetilide-mediated rescue. Previously, we

454   demonstrated that pentamidine-induced K_v11.1 forward trafficking defects

455   could be rescued by dofetilide and both compounds may compete for the same

456   binding site within the K_v11.1 channel (Varkevisser et al., 2013a). Defective

457   K_v11.1 forward trafficking can be restored by a number of K_v11.1 inhibitors that

458   stabilize the channel via binding to the inner pore, close to the selectivity filter

459   (e.g. Varkevisser et al., 2013a; Perry et al., 2010). We demonstrated that

460   dofetilide analogues with higher affinity tended to provide better rescue in

461   K_v11.1 trafficking defects, while LUF7244 reduced the K_v11.1 channel affinity

462   for dofetilide (Yu et al., 2016). Interestingly, in our current study, the

463   combination of dofetilide and LUF7244 still rescued pentamidine induced

464   K_v11.1 trafficking defects. One possible reason may be that LUF7244 can not

465   completely reduce the binding of dofetilide to the trafficking inhibited channels

466   by which the capacity of dofetilide to rescue K_v11.1 trafficking remained.

467   Another possibility is the absence of LUF7244 binding to intracellularly localized,

468   immature and only core-glycosylated K_v11.1 channels. This may also explain 1)

469   the absence of effects of LUF7244 on defective G601S trafficking, 2) lack of

470   interference of pentamidine-mediated trafficking defects by LUF7244, and 3)

471   permitting dofetilide- and E4031-mediated rescue of K_v11.1 trafficking. Recent

472   preliminary data indicate that dofetilide specifically binds to membrane

473   preparations of G601S cells, which is in line with the observed trafficking rescue

474   effect. It now has to be determined to which extent this binding is sensitive to

475   LUF7244. We hypothesize that LUF7244 will certainly not completely inhibit

476   dofetilide   binding   to   intracellularly   localized K_v11.1,   otherwise

477 dofetilide+LUF7244 would not provide any rescue of maturation, membrane

478 staining and $I_{Kv11.1}$ as shown in the current manuscript.

479

480

481 Negative allosteric modulators and activators can be considered as therapeutic

482 options to prevent drug-induced arrhythmia (Sanguinetti 2014; Yu et al, 2016).

483 Recently, we have shown that LUF7244 suppressed astemizole-induced early

484 after depolarizations (EADs) and AP prolongation in neonatal rat ventricular

485 myocytes (NRVM) (Yu et al., 2016). Additionally, LUF7244 pretreatment

486 prevented the occurrence of astemizole induced EADs, while LUF7244 per se

487 did not shorten AP duration or strongly affect dispersion of APD_{40} in NRVM at

488 10 µM (Yu Z et al., 2016). In contrast, in isolated canine ventricular

489 cardiomyocytes and human iPS-derived cardiomyocytes, LUF7244 remarkably

490 shortened the APD_{90}, which is in line with its activator characteristics (Qile et

491 al., 2019). Moreover, we demonstrated that LUF7244 suppressed EADs in

492 isolated canine ventricular myocytes and prevented dofetilide-induced

493 ventricular arrhythmias in the dog with chronic AV block (Qile et al., 2019).

494 In conclusion, the current study demonstrates that LUF7244, and possibly also

495 other negative allosteric modulators and activators, might also have a role in

496 suppression or preventing arrhythmia caused by defective forward trafficking.

497 Thus, the negative allosteric modulator/activator LUF7244 in combination with

498   a genuine $K_v11.1$ inhibitor could provide a new pharmacological treatment to

499   functionally correct both congenital and acquired $K_v11.1$ trafficking defects.

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29

Authorship contributions

503

504 Participated in research design: Heyden, Stary, Delisle

505 Conducted experiments: Qile, Ji, Golden, Houtman, Romunde, Fransen, Ham

506 Contributed to reagents or analytic tools: IJzerman, January, Heitman, Stary,

507 Delisle

508 Performed data analysis: Qile, Ji, Golden, Houtman, Ham

509 Wrote or contributed to the writing of the manuscript: Heyden, Ji, Qile, Stary,

510 Delisle

511 Critical review and approval of final manuscript: all authors

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707

708 **Footnotes**

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713 Austria

714 **Figure legends**

715

716 **Figure 1**

717 Molecular dynamics simulation-based prediction of binding mode of

718 dofetilide/LUF7244 and $K_v11.1$. (A) Overview of the hERG structure (top view),

719 with bound dofetilide at the fenestration, shown as orange spheres. (B)

720 Overview of the hERG structure (top view), with bound LUF7244. (C) Predicted

721 binding modes for dofetilide at the fenestration. (D) Predicted 'classical' binding

722 mode for dofetilide in the inner cavity of hERG. Yellow dotted lines indicate

723 putative π-π interactions; red dotted lines indicate H-bonds. (E) and (F)

724 superposition of dofetilide binding modes, with predicted LUF7244 binding

725 mode. (G) and (H) Root mean square deviation (RMSD) of $K_v11.1$ and dofetilide

726 docked into the fenestration or cavity, respectively.

727

728 **Figure 2**

729 LUF7244 alone has no effect on $K_v11.1$ trafficking and does not disturb

730 dofetilide-mediated rescue of forward trafficking. (A) Western blot showing that

731 treatment of pentamidine-exposed (10 µM, 48 h) HEK-hERG cells with 1 µM

732 dofetilide restored mature $K_v11.1$ expression. LUF7244 alone has no effect on

733 $K_v11.1$ expression (n=3). Mature (plasma membrane expressed) and immature

734 (intracellular) $K_v11.1$ western blot signals are displayed in left panel. Bar graphs

735 in the middle depict ratio of mature/immature $K_v11.1$ at different conditions. The

42

736   right panel displays separate values for mature and immature K$_v$11.1. (B)

737   LUF7244 does not rescue pentamidine-induced K$_v$11.1 trafficking defects (n=3).

738   (C) Combination of pentamidine, dofetilide and LUF7244 restores K$_v$11.1

739   mature protein after 48 h (n=6). Total protein staining (Ponceau) was used as

740   a loading control. *** P < 0.001, **** P < 0.0001 vs. control. Values are shown

741   as mean ± SD. One-way ANOVA with Dunnett's test was applied for group

742   comparison.

743

744   **Figure 3**

745   LUF7244 combined with dofetilide or E4031 rescues trafficking defective

746   K$_v$11.1-G601S maturation. (A) Western blot analysis of equal amounts (25 µg)

747   of total protein from K$_v$11.1-G601S cells. G601S cells only present a core-

748   glycosylated immature protein of 135 kDa. Dofetilide restores expression of the

749   full-glycosylated mature protein after 48 h. LUF7244 does not change the

750   mature K$_v$11.1 protein levels compared with control (n=3). (B) The combination

751   of dofetilide and LUF7244 rescues mature K$_v$11.1 protein in G601S cells (n=3).

752   (C) Increased mature K$_v$11.1 levels in G601S cells treated with E4031 or

753   E4031+LUF7244 (48h) (n=6). The right panel displays separate values for

754   mature and immature K$_v$11.1. Total protein staining (Ponceau) was used as a

755   loading control. * P < 0.05, ** P < 0.01, *** P < 0.001 vs. control. Values are

756   shown as mean ± SD. One-way ANOVA with Dunnett's test was applied for

757   group comparison.

758

759 **Figure 4**

760 G601S cells were either non-treated (control), treated with 10 μM Dofetilide, 3

761 μM LUF7244, or 10 μM Dof+3 μM LUF for 24 hours. $K_v11.1$ channels were

762 labelled (left column) along with Cadherin as a pseudo-membrane marker

763 (Cadherin). Linescans of selected regions at cell extensions (containing

764 membrane ruffles) are indicated in the merged pictures by boxes. Results of

765 the linescan recordings are given on the right panels. Scale bar represents 10

766 μm.

767

768 **Figure 5**

769 LUF7244 combined with dofetilide acutely rescued $I_{Kv11.1}$ in HEK-hERG cells.

770 (A) Shown are representative currents from cells expressing WT-$K_v11.1$

771 channel proteins using the voltage protocol in the inset. Cells were recorded in

772 control conditions or in dofetilide + LUF7244 (acute application). In B shown are

773 the mean I-V relations, based on peak tail $I_{Kv11.1}$ amplitude following

774 repolarization, recorded from cells expressing WT-$K_v11.1$ channel proteins.

775 Cells were recorded in control conditions (n = 10) or in dofetilide + LUF7244

776 (acute) (n = 10). Data are shown as mean ± SEM. Compared to control cells,

777 cells recorded in dofetilide + LUF7244 increased $I_{Kv11.1}$ following pre-pulses

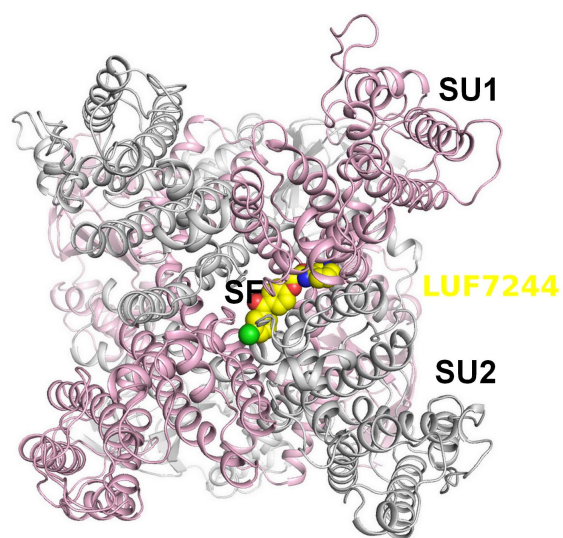778 from -70mV to 10 mV (p<0.05). Two-way ANOVA with Tukey's test was applied.

779

780 **Figure 6**

781 Long-term (24-48h) exposure of LUF7244 combined with dofetilide rescued

782 $I_{Kv11.1}$ in G601S cells. (A) Shown are representative currents from cells

783 expressing G601S-K_v11.1 channel proteins using the voltage protocol in the

784 inset. Cells were recorded in control conditions, in dofetilide + LUF7244 (acute),

785 in dofetilide + LUF7244 after being cultured in dofetilide + LUF7244 for long-

786 term (LT), or in LUF7244 (acute). (B) Shown are the mean I-V relations, based

787 on peak tail $I_{Kv11.1}$ amplitude following repolarization, recorded from cells

788 expressing G601S-K_v11.1 channel proteins. Cells were recorded in control

789 conditions (n = 10), in dofetilide (n = 8), dofetilide + LUF7244 (acute) (n = 10),

790 in dofetilide + LUF7244 after being cultured in dofetilide + LUF7244 for 24-48

791 hours (LT) (n =10), or in LUF7244 (n =7). Data are shown as mean ± SEM.
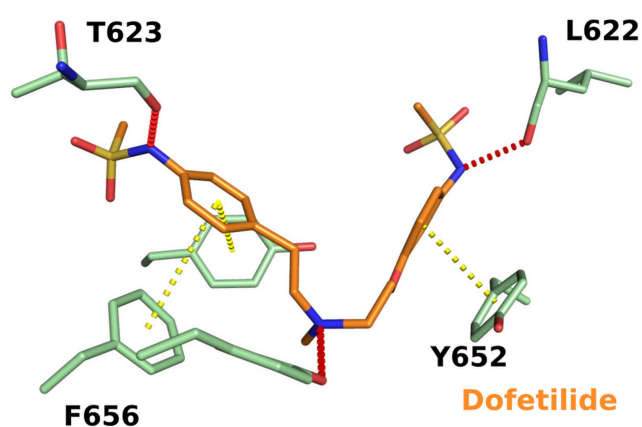
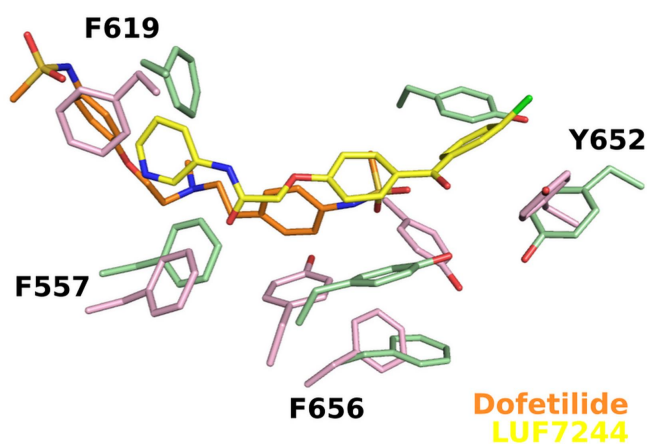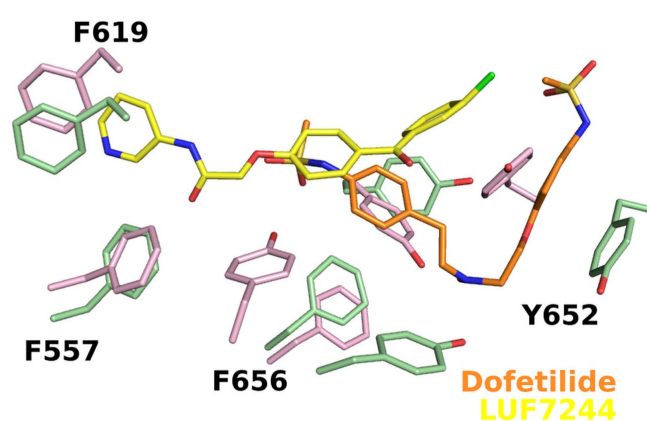792 Two-way ANOVA with Tukey's test was applied.

793

Figure 2

A

Dofetilide (1 μM)
LUF7244 (μM)

Mature K$_v$11.1
Immature K$_v$11.1

B

Dofetilide (1 μM)
LUF7244 (μM)

Mature K$_v$11.1
Immature K$_v$11.1

C

E4031 (5 μM)
LUF7244 (μM)

Mature K$_v$11.1
Immature K$_v$11.1

Mature protein
Immature protein

Figure 3

Kv11.1 Cadherin merge linescan

control

Dofetilide

LUF7244

Dofetilide+LUF7244

linescan control

Gray Level (Avg)
200
175
150
125
100

linescan dofetilide

Gray Level (Avg)
200
175
150
125
100

linescan LUF7244

Gray Level (Avg)
200
175
150
125
100

linescan dofetilide+LUF7244

Gray Level (Avg)
200
175
150
125
100

A.

Control   Dofetilide+LUF7244 (acute)   Dofetilide   LUF7244

B.

Figure 5

A.

Control   Dofetilide+LUF7244 (acute)   Dofetilide+LUF7244 (LT)   LUF7244

B.

Current Density (pA/pF) vs Pre-pulse (mV)

- Control
- Dofetilide
- Dofetilide + LUF7244 (acute)
- Dofetilide + LUF7244 (LT)
- LUF7244

Figure 6