PA-6 inhibits inward rectifier currents carried by V93I and D172N gain-of-function K_{IR}2.1 channels, but increases channel protein expression

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Abstract

Background: The inward rectifier potassium current I_{K1} contributes to a stable resting membrane potential and phase 3 repolarization of the cardiac action potential. KCNJ2 gain-of-function mutations V93I and D172N associate with increased I_{K1}, short QT syndrome type 3 and congenital atrial fibrillation. Pentamidine-Analogue 6 (PA-6) is an efficient (IC_{50} = 14 nM with inside-out patch clamp methodology) and specific I_{K1} inhibitor that interacts with the cytoplasmic pore region of the K_{IR}2.1 ion channel, encoded by KCNJ2. At 10 μM, PA-6 increases wild-type (WT) K_{IR}2.1 expression in HEK293T cells upon chronic treatment. We hypothesized that PA-6 will interact with and inhibit V93I and D172N K_{IR}2.1 channels, whereas impact on channel expression at the plasma membrane requires higher concentrations.

Methods: Molecular modelling was performed with the human K_{IR}2.1 closed state homology model using FlexX. WT and mutant K_{IR}2.1 channels were expressed in HEK293 cells. Patch-clamp single cell electrophysiology measurements were performed in the whole cell and inside-out mode of the patch clamp method. K_{IR}2.1 expression level and localization were determined by western blot analysis and immunofluorescence microscopy, respectively.

Results: PA-6 docking in the V93I/D172N double mutant homology model of K_{IR}2.1 demonstrated that mutations and drug-binding site are >30 Å apart. PA-6 inhibited WT and V93I outward currents with similar potency (IC_{50} = 35.5 and 43.6 nM at +50 mV for WT and V93I), whereas D172N currents were less sensitive (IC_{50} = 128.9 nM at +50 mV) using inside-out patch-clamp electrophysiology. In whole cell mode, 1 μM of PA-6 inhibited outward I_{K1} at −50 mV by 28 ± 36%, 18 ± 20% and 10 ± 6%, for WT, V93I and D172N channels respectively. Western blot analysis demonstrated that PA-6 (5 μM, 24 h) increased K_{IR}2.1 expression levels of WT (6.3 ± 1.5 fold), and V93I (3.9 ± 0.9) and D172N (4.8 ± 2.0) mutants. Immunofluorescent microscopy demonstrated dose-dependent intracellular K_{IR}2.1 accumulation following chronic PA-6 application (24 h, 1 and 5 μM).

Conclusions: 1) KCNJ2 gain-of-function mutations V93I and D172N in the K_{IR}2.1 ion channel do not impair PA-6 mediated inhibition of I_{K1}, 2) PA-6 elevates K_{IR}2.1 protein expression and induces intracellular K_{IR}2.1 accumulation, 3) PA-6 is a strong candidate for further preclinical evaluation in treatment of congenital SQT3 and AF.

Keywords: I_{K1}, K_{IR}2.1, Atrial fibrillation, Short QT syndrome, Drugs, PA-6, Trafficking
Background
In the heart, inward rectifier potassium currents (I\textsubscript{K\textsubscript{IR}}) contribute to stabilization of the resting membrane potential of contractile cardiomyocytes and participate in the final phase of repolarization of the action potential [1]. Gain-of-function mutations in the KCNJ2 gene, that encodes K\textsubscript{IR}2.1 protein underlying I\textsubscript{K\textsubscript{IR}}, associate with ventricular (short QT syndrome type 3 (SQT3)) and atrial (congenital atrial fibrillation (AF)) phenotypes. D172N and K346T are linked to SQT3, whereas V93I associates with congenital AF [2–4]. E299V and M301K have been linked to both SQT3 and AF [5, 6].

Congenital SQT syndrome is diagnosed in the presence of a QTc interval equal or less than 330 ms, and may be diagnosed at a QTc of less than 360 ms when other conditions apply, like a pathologic mutation or a family history of SQT [7]. Congenital SQT can either be caused to excessive repolarization capacity (SQT1-3), or due to decreased depolarization capacity (SQT4-7), and is associated with high risk for sudden cardiac death and therefore implantable cardioverter-defibrillator (ICD) implantation is indicated [8, 9]. However, pharmacotherapy may be beneficial in patients that are unsuitable for ICD therapy (e.g. young children), those that refuse ICD implantation or for bridging the time to ICD implantation [10]. Some drugs are indeed able to inhibit currents produced by K\textsubscript{IR}11.1, K\textsubscript{IR}7.1 and K\textsubscript{IR}2.1 channels bearing gain-of-function mutations associated with SQT1, SQT2 and SQT3, respectively [11–14].

AF is associated with increased risk for stroke and heart failure [15]. Action potential lengthening drugs, e.g. targeting the delayed rectifier (I\textsubscript{K\textsubscript{d}}), or drugs increasing atrial fibrillation cycle length (sodium current (I\textsubscript{Na})) blockers, have the potential to counteract AF [16]. Inhibition of the acetylcholine activated inward rectifier potassium current (I\textsubscript{K\textsubscript{Acch}}) channel, closely related to the I\textsubscript{K\textsubscript{IR}} channel, has been proposed as an effective treatment for AF [17]. Also I\textsubscript{K\textsubscript{IR}} inhibiting compounds, like chloroquine, display anti-AF activity in animal models [18, 19].

We hypothesized that PA-6 inhibits I\textsubscript{K\textsubscript{IR}} channels that are formed by gain-of-function K\textsubscript{IR}2.1 channel proteins and thus can be considered as a candidate drug in treating SQT3 and congenital AF.

Methods
Molecular modelling
Docking of compound PA-6 was conducted using the previously constructed closed state homology model of the human K\textsubscript{IR}2.1 channel [20]. In silico mutations of residues V93I and D172N were generated with SwissPdbViewer [26]. Compound PA-6 was generated as described previously [20]. The docking program FlexX (part of the LeadIT software package version 2.0.1 (BioSolveIT GmbH, St Augustin, Germany) was used for docking. The binding site was specified selecting the carboxylic acids of the Glu224 residues from all four subunits. The radius of the binding site was set to 20 Å. Default settings of FlexX were applied for protonation and torsion angles. The ChemScore scoring function of FlexX was applied and the top 10 docking solutions were saved for analysis.

KCNJ2 constructs
Mutations V93I and D172N were engineered into a human KCNJ2-pcDNA3 expression construct [27], using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and custom designed primers. The presence of the introduced mutations was confirmed by Sanger sequencing.

Patch-clamp electrophysiology
HEK293T cells were transfected with WT, V93I or D172N constructs together with a GFP expression construct to enable detection of transfected cells. Inside-out patch clamp measurements were made using a HEKA EPC-10 Double Plus amplifier controlled by PatchMaster 2.10 software (HEKA, Lambrecht/Pfalz, Germany) at 21 °C. To record WT, V93I and D172N K\textsubscript{IR}2.1 currents, inside-out patch-clamp measurements were performed using a ramp protocol ranging from −100 to +100 mV in 5 s from a holding potential of −40 mV. Excised patches were placed in close proximity to the inflow region of the perfusion chamber. Bath solution contained 125 mM KCl, 4 mM EDTA (2K), 72 mM K\textsubscript{2}HPO\textsubscript{4}, 2.8 mM KH\textsubscript{2}PO\textsubscript{4}, pH 7.20/KOH. Pipette solution contained 145 mM KCl, 1 mM CaCl\textsubscript{2}, 5 mM HEPES, pH 7.40/KOH. Measurements were started following washout of polyamines/Mg\textsuperscript{2+} from the channel pore observed by the disappearance of current rectification. For IC\textsubscript{50} curves, fractional block at −80 and +50 mV was determined by dividing current levels
obtained with PA-6 containing solutions by current levels of control traces recorded in the absence of PA-6. Whole cell patch clamp measurements were done as described before [28] using an AxoPatch 200B amplifier controlled by pClamp9 software (Molecular devices, Sunnyvale, CA, USA) at 21 °C. Whole cell I_{KIR2.1} measurements were performed by applying 1 s test pulses ranging between −120 and +30 mV, in 10 mV increments, from a holding potential of −40 mV, and with series resistance compensation of at least 70%. Steady state current at the end of the pulse was normalized to cell capacitance and plotted versus test potential (corrected for liquid junction potential). Extracellular solution contained 140 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 6 mM glucose, 17.5 mM NaHCO$_3$, 15 mM HEPES, pH 7.4/NaOH. Pipette solution contained 125 mM potassium gluconate, 10 mM KCl, 5 mM HEPES, 5 mM EGTA, 2 mM MgCl$_2$, 0.6 mM CaCl$_2$, 4 mM Na$_2$ATP, pH 7.20/KOH.

PA-6 (Fig. 1a) was custom synthesized by Endotherm GmbH (Saarbrücken, Germany).

Western blot
HEK293T cells were grown in 60 mm culture dishes containing 3 mL DMEM supplemented with 10% FCS, L-Glutamine and Pen-Strep, and transfected using linear polyethylenimine (PEI) (Polysciences Inc, Eppelheim, Germany) as described earlier [29]. Cell lysates were prepared in buffer D (20 mM HEPES, 125 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 1% Triton X-100, pH 7.6, supplemented with 1 mM PMSF and 10 μg/mL aprotinin). 20 μg protein lysate was separated by 10% SDS-PAGE and blotted onto nitrocellulose membrane. Ponceau staining was used to reveal equal protein loading and subsequent quantification. Blots were blocked with 5% (v/v) chicken egg yolk in TBST (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% (v/v) Tween-20) for 1 h at room temperature. K$_{IR}2.1$ WT and mutants were detected by N-terminal K$_{IR}$2.1 antibody (Santa Cruz Biotechnology, Dallas TX, USA, cat. No. sc-18708) and peroxidase-conjugated donkey anti-goat secondary antibody (Jackson ImmunoResearch, West Grove, PA, cat. No. 705-035-003) followed by ECL detection procedure (GE Healthcare, Hoekelaken, The Netherlands). For quantification purposes, untransfected HEK293T cells were used as blank and protein levels were normalized to ponceau staining levels. Differences between group averages were tested by using a one-way ANOVA with a Bonferroni’s post-hoc test.

Immunofluorescence microscopy
HEK293T and MES-1 cells were cultured on pre-coated (0.1% gelatin) glass coverslips and transfected using Lipofectamin (Invitrogen, Breda, The Netherlands). Cell fixation, immunolabeling and imaging were performed exactly as described earlier [29]. Antibodies used were anti-K$_{IR}2.1$ (1:250; Santa Cruz Biotechnology, cat. no. sc-18708) and anti-Pan Cadherin (1:800, Sigma-Aldrich, St. Louis MO, USA, cat. No. C1821). HEK293T cells were imaged by confocal microscopy using a Zeiss LSM 700 confocal microscope (Carl Zeiss Microscopy GmbH, Germany) equipped with a 63× oil immersion objective (NA 1.4). Excitation was performed with an air-cooled Argon ion laser (LASOS, RMC 7812Z, 488 nm) for GFP and a HeNE (LASOS, SAN 7450A, 543 nm) laser for DyLight. MES-1 cells were imaged using a conventional Nikon eclipse 80i light microscopy equipped with a 40× objective (NA 0.75).

Results
V93I and D172N mutations do not interfere with the PA-6 K$_{IR}2.1$ channel interaction
To determine whether V93I or D172N mutations in the K$_{IR}2.1$ channel may interfere with PA-6 current block,
mutations and PA-6 channel interaction were modelled. Figure 1b shows the docking result of PA-6 into the V93I/D172N double mutant homology model of KIR2.1. The results demonstrate that these mutations are unlikely to influence binding of PA-6 to the previously identified binding site in the cytoplasmic domain [20]. Both mutations are located in the transmembrane domain and thus >30 Å away from the binding site.

**PA-6 inhibits inward rectifier currents carried by V93I and D172N mutant KIR2.1 channels**

To determine the functional effects of PA-6 on the KIR2.1 gain-of-function mutant channels, expression constructs were transiently transfected into HEK293T cells. Currents were measured in the inside-out mode, to allow for direct access of PA-6 to the cytoplasmic channel pore, of the patch-clamp technique using a ramp protocol from −100 to +100 mV. PA-6 dose dependently inhibited inward rectifier inward (at −80 mV) and outward (at +50 mV) currents carried by WT, V93I and D172N KIR2.1 channels (Fig. 2a). A small voltage dependency of block was observed for WT and both mutant channels as reflected in small, but not significant, changes in IC₅₀ values obtained at −80 and +50 mV for each KIR2.1 type. Interestingly, in contrast to WT and V93I, D172N outward current was less sensitive for PA-6 block than inward current. Whereas WT and V93I displayed virtually identical dose dependent block (IC₅₀ of 52.9 and 58.0 nM at −80 mV and 35.5 and 43.6 nM at +50 mV for WT and V93I channels respectively), D172N channels were approximately two to three fold less sensitive (IC₅₀ 109.3 nM at −80 mV and 128.9 nM at +50 mV) (Fig. 2b).

Using the whole cell mode of the patch clamp technique, gain-of-function of D172N mutant became apparent as a change in the rectification index ((1−(outward current at −40 mV divided by inward current at −100 mV)) [30], not correct for liquid junction potential) from 0.83 ± 0.11 (n = 9, mean ± s.d.) for WT channels to 0.70 ± 0.06 (n = 10, P < 0.01) in D172N channels (Fig. 3a), in accordance with earlier findings [2]. No change in rectification index of V93I (0.87 ± 0.09, n = 10, n.s.) was found. In addition, whereas PA-6 sensitivity was decreased in the whole cell mode, compared to the inside-out mode no differences were observed between WT and mutant channels (Fig. 3b). In the presence of 1 μM of PA-6, a non-significant (n.s.) reduction of outward Iₖᵢ at −50 mV by 28 ± 36%, 18 ± 20% and 10 ± 6%, for WT, V93I and D172N channels respectively was observed. At 3 μM, PA-6 significantly inhibited Iₖᵢ at −50 mV by 94 ± 6%.

![Fig. 2](image-url) PA-6 dose dependently inhibits Iₖᵢ currents carried by homotypic wild-type (WT), V93I and D172N KIR2.1 channels measured in the inside out mode. a Steady state Kᵢᵢ current traces from WT, V93I and D172N KIR2.1 channel containing inside-out patches elicited by a voltage ramp protocol from −100 to 100 mV, under baseline conditions (C) and upon application of 10, 100 and 1000 nM of PA-6. Measurements were performed using symmetrical high potassium concentrations at both sides of the patch. b IC₅₀ curves of PA-6 for the inward (at −80 mV) and outward (at +50 mV) Kᵢᵢ currents of WT (n = 10), V93I (n = 9) and D172N (n = 11) channel containing patches. Error bars indicate s.e.m.
(P < 0.05), 77 ± 29% (P < 0.05) and 86 ± 7% (P < 0.01), for WT, V93I and D172N channels, respectively. For the inward current at −110 mV, 1 and 3 μM PA-6 inhibited I_{K1} by 30 ± 16% (n.s.) and 68 ± 15% (P < 0.01), 28 ± 14% (P < 0.05) and 64 ± 4% (P < 0.001), 30 ± 10% (n.s.) and 83 ± 1% (P < 0.01) for WT, V93I and D172N channels, respectively. We conclude that in the whole cell mode, PA-6 inhibits WT, V93I and D172N channels with similar efficacy resulting in IC_{50} values between 1 and 3 μM.

V93I and D172N mutations do not affect PA-6 mediated increase in Kir2.1 expression and intracellular accumulation

When applied at concentrations of 10 μM, PA-6 is able to enhance expression of WT Kir2.1 in cells stably expressing GFP-tagged Kir2.1 [20]. To assess the effects of PA-6 on expression of WT and mutant channels in transiently transfected HEK293T cells, cultures were treated with 0, 0.2, 1 and 5 μM PA-6 for 24 h after which expression levels were detected by Western blot analysis. PA-6 treatment increased Kir2.1 expression levels for all three variants (Fig. 4a). Strongest responses were obtained with 5 μM PA-6 added to the medium that reached significance for WT (P < 0.001) and V93I (P < 0.01) whereas a trend was observed for D172N (P = 0.09) (Fig. 4b). Lower concentrations of PA-6 did not result in significant increased expression.

To detect the subcellular location of the WT, V93I and D172N channels following PA-6 application, confocal immunofluorescent microscopy was performed in HEK293T cells. In non-treated control cells, both WT and mutant channels were mainly expressed at the plasma membrane, where they demonstrate colocalization with Cadherin (Fig. 5 left column), whereas PA-6 treatment (1 μM) for 24 h induced an intracellular increase of the channel proteins in small aggregates, irrespectively whether these are WT or mutant (Fig. 5, middle column). PA-6 treatment at 5 μM for 24 further increased the level of intracellular accumulation (Fig. 5, right column). To exclude any potential effect of cell-type specific response, immune detection was also performed in the mesoderm-like cell line MES-1 as shown previously [29] which yielded similar responses (Fig. 6). In control cells, WT and mutant channels were
localized mainly at the plasma membrane, whereas 5 μM of PA-6 (24 h) resulted in intracellular accumulation, in a fashion similar as for chloroquine although the latter treatment resulted in aggregates more equally in size [27, 31].

Discussion

Due to the high risk of sudden cardiac death, ICD implantation is indicated in congenital SQT patients. However, pharmacological treatment is warranted in young children not amenable for ICD implantation, in patients refusing an ICD and as a bridge to ICD therapy [10]. In congenital SQT patients, responsiveness of the QTc interval towards class IC and class III antiarrhythmics were unsatisfactory. SQT patients did not show anticipated responses to flecainide, d-sotalol or ibutilide [32, 33]. Only hydroquinidine was able to prolong QTc to borderline or normal duration [32, 33]. Short QT syndrome type 1 (SQT1) results from gain-of-function mutations in the Kv11.1 (hERG) channel encoded by the KCNH2 gene, and is the best studied SQT subtype with respect to pharmacological treatment. The N588K gain-of-function mutation appears a hotspot in SQT1. Interestingly, N558K channels were less sensitive for Class III antiarrhythmics like d-sotalol [34], and E-4031 (11-fold) [11]. Accordingly, d-sotalol was unable to prolong the QT interval in SQT1 N558K patients [34]. In contrast, disopyramide (1.5-fold) and hydroquinidine (3.5-fold) displayed smaller differences in IC50 values for WT and N558K Kv11.1 channels, respectively. Clinical studies indeed showed favourable responses to hydroquinidine in SQT1 [33, 35], whereas QTc prolongation in non-Kv11.1 SQT patients was smaller [33]. The SQT2 associated mutation V307L in the Kv7.1 channel was shown to be equally sensitive for mefloquine as its WT variant, on
which basis the authors suggested that this drug may be an effective treatment strategy in this patient population [14]. Interestingly, the same V307L mutation increased the IC\textsubscript{50} value for the K\textsubscript{v}7.1 inhibitor Chromanol293B by 7-fold [36], indicating again that a mutation specific pharmacological approach is favourable. The SQT3 associated D172N mutation in K\textsubscript{IR2.1} was equally sensitive for chloroquine (1.2-3.3 μM) as its WT counterpart (1.4-2.4 μM) measured in the whole cell mode [12, 13]. Here we demonstrate that upon acute superfusion, PA-6 is also able to inhibit D172N mutant K\textsubscript{IR2.1} channel with an IC\textsubscript{50} only two to three fold higher than that of WT channels, measured in the inside-out mode, and that potency of inhibition of the V93I channel was similar as for WT. In the whole cell mode, acute PA-6 superfusion inhibits WT, V93I and D172N with similar efficacy. Therefore, PA-6 could potentially be effective in addressing SQT3 and AF associated with each of these two mutations.

In contrast to PA-6, chloroquine suffers from lack of specificity as it significantly inhibits delayed rectifier (I\textsubscript{K1}), sodium (I\textsubscript{Na}) and l-type calcium (I\textsubscript{Ca,l}) currents [20, 37]. Upon long-term exposure however, both chloroquine and PA-6 are able to increase K\textsubscript{IR2.1} channel expression [20, 27, 31, 38]. However, due to its lower potency, the concentrations at which chloroquine affected trafficking (5 μM) are slightly closer to its IC\textsubscript{50} for acute blockade than seen for PA-6. Furthermore, the majority of PA-6 induced increase of K\textsubscript{IR2.1} channel expression as detected by Western blot upon chronic exposure results from intracellular accumulation instead of functional channels at the plasma membrane.

The acute channel inhibiting effect of a drug may or may be not be correlated with its chronic effect on ion channel expression. For example, pentamidine inhibits the K\textsubscript{IR2.1} channel acutely and decreases K\textsubscript{IR2.1} expression upon chronic treatment [38, 39], but its structural derivative PA-6 inhibits K\textsubscript{IR2.1} currents acutely while it increases channel expression chronically as shown here and previously [20]. In the former case, both drug effects are additive and maybe synergistic. In the case of PA-6, acute and chronic effects are opposite, but at 1 and 3 μM, the acute effects on current inhibition are stronger than the increases in channel expression levels upon chronic exposure. Only upon acute termination of PA-6 application, the effects on expression may temporarily prevail over the acute blocking effect (or absence thereof) resulting in enhanced I\textsubscript{K1}. 

**Fig. 5** PA-6 treatment for 24 h induces intracellular K\textsubscript{IR2.1} accumulation of WT, V93I and D172N channel proteins in HEK293T cells. Confocal microscopy optical slices (0.7-0.8 μm) displaying K\textsubscript{IR2.1} (green) localization in HEK293T cells transfected with WT, V93A and D172N channels under control conditions and following 24 h of PA-6 (1 and 5 μM). Cadherin (red) co-staining identifies the position of the plasma membrane. DAPI (blue) is used to visualize nuclei. Scale bars represent 10 μm.
Limitations
The effects of PA-6 on WT, V93I and D172N K\textsubscript{IR2.1} channels have only been tested in ectopic expression systems and therefore the effects of their blockade on cardiac action potential characteristics could not be evaluated.

Conclusions
In the K\textsubscript{IR2.1} ion channel, V93I and D172N gain-of-function mutations do not blunt the inhibitory capacity of PA-6. PA-6 application results in enhanced K\textsubscript{IR2.1} protein expression, mainly localized in intracellular aggregates. From our findings presented here, we conclude that PA-6 may be considered for further preclinical evaluation for treatment of congenital SQT3 and AF.

Abbreviations
AF: Atrial fibrillation; GFP: Green fluorescent protein; HEK: Human embryonal kidney; I\textsubscript{C\textsubscript{a}-L}: L-type Calcium current; I\textsubscript{CD}: Implantable cardioverter-defibrillator; I\textsubscript{K1}: Inward rectifier potassium current; I\textsubscript{KAC}: Acetylcholine activated inward rectifier potassium current; I\textsubscript{Na}: Delayed rectifier current; I\textsubscript{Na\textsubscript{v}}: Sodium current; PA-6: Pentamidine analogue-6; SQT\textsubscript{x}: Short QT syndrome type x; WT: Wild-type

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Availability of data and materials
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Authors' contributions
YJ, MGV, HT and MJCH performed electrophysiological recordings; KD, GvH generated V93I and D172N expression constructs; EMZP and ASW performed and analyzed molecular modelling; JZ performed and analyzed western blot experiments; FLR and MAGvdH performed and analyzed the subcellular localization experiments; MAGvdH and YJ designed the study. YJ, MAGvdH, GvH and ASW wrote the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate
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Competing interests

The authors declare that they have no competing interest.

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