Supporting material

Effect of Selective $I_{K,ACh}$ Inhibition by XAF-1407 in an Equine Model of Tachypacing-induced Persistent Atrial Fibrillation

Running title: $I_{K,ACh}$ Inhibition in an equine model of persistent AF

Fenner MF[1], Carstensen H[1], Nissen SD[1], Hesselkilde EZ[1], Lunddahl C[1], Jensen MA[1], Loft-Andersen AV[1], Sattler SM[2,3], Platonov PG[4], El-Haou S[5], Jackson C[5], Tang R[5], Kirby R[5], Ford JW[5], Schotten U[6], Milnes JT[5], Sørensen US[7], Jespersen T[8], Buhl R[1]

[1]Department for Veterinary Clinical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Taastrup, Denmark. [2]Department of Cardiology, The Heart Centre, Copenhagen University Hospital,Copenhagen, Denmark. [3]Department of Medicine I, University Hospital Munich, Campus Grosshadern, Ludwig-Maximilians University Munich (LMU), Munich, Germany. [4]Arrhythmia Clinic, Skåne University Hospital and Department of Cardiology, Clinical Sciences, Lund University, Lund, Sweden. [5]Xention Ltd., Cambridge, UK. [6] Department of Physiology, Cardiovascular Research Institute Maastricht, Maastricht, The Netherlands. [7]Acesion A/S, Copenhagen, Denmark. [8]Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark

Material and Methods:

In-vitro electrophysiology and pharmacology

Maintenance of ion channel expressing cell lines

Experiments were performed on a number of cell lines stably expressing a variety of ion channels. Cell line expressing $K_{ir}3.4$ was created in-house using standard techniques, of sub-cloning an appropriate DNA sequences into a mammalian expression vector together with a gene for specific antibiotic resistance (see Table S1). Expression constructs were transfected into HEK293 cells prior to selection and sub-cloning before finally being assayed for current expression and validated electrophysiologically. All other cell lines were sourced externally ($K_{ir}3.1/3.4$, $K_{ir}6.2$/SUR2A [Prof. Andrew Tinker, UCL], $Na_v1.5$ [UPenn]; hERG [Cytomyxs or Cytocentrics]; $K_v1.5$ [BSYS] and $K_v4.3$, $K_{ir}2.1$, $K_v7.1$/KCNE1 [bioFocusDPI]).

All cell lines were grown adhered to T-175 flasks and passaged using an enzymatic agent (Accutase, ICT, Inc.). All the cell lines were plated out onto small sterilised glass coverslips in 35 mm x 10 mm Polystyrene Petri dishes (Corning) containing an appropriate medium, supplemented with 10 % fetal bovine serum (Hyclone Perbio). The cells were incubated at 37 or 30 °C (5 % CO2) for 1-4 days prior to any electrophysiological study.

Cell Culture Conditions

Table S1

	Media	Serum	Selection Antibiotic	Other Supplements
K_v1.5	JRH Ex-Cell 302 (JRH Biosciences)	-	2 µg / ml Blasticidin (Invitrogen)	8 mM L-Glutamine (Gibco), 1% HT Supplement
K_v1.3	Nutrient mixture IMDM#21980	10 % Fatalclone II (HyClone Perbio)	400 µg / ml Geneticin (Gibco)	1% HT Supplement
K_v4.3	Nutrient mixture F12 (Gibco)	10 % Fatalclone II (HyClone Perbio)	500 µg / ml Geneticin (Gibco)	1% NEAA
Na_v1.5	Nutrient mixture F12 (Gibco)	10 % Fatalclone II (HyClone Perbio)	500 µg / ml Geneticin (Gibco)	-
K_{ir}2.1	MEM #31095	10 % Fatalclone II (HyClone Perbio)	300 µg / ml Hygromycine B (Invitrogen)	1% NEAA
K_{ir}3.x	MEM #31095	10% Invitrogene FBS #16000	182 µg / ml Zeocin (Invitrogen)	-
hERG	Nutrient mixture DMEM	10 % Fatalclone II (HyClone Perbio)	200 µg / ml Geneticin (Gibco)	1% L-Glutamine (Gibco), 1% Na Pyruvate
Ca_v1.2	Nutrient mixture DMFM F12 #31331	10 % Fatalclone II (HyClone Perbio)	500 µg / ml Geneticin (Gibco)	-

Cloned cardiac ion channel conventional electrophysiology

Experimental solutions (external (S2) & internal pipette (S3) solution composition)

Table S2

	K_v1.5 K_v4.3 (mM)	**K_{ir}6.2 (mM)**	**K_v7.1 (mM)**	**Na_v1.5 (mM)**	**hERG (mM)**	**K_{ir}2.1 K_{ir}3.x (mM)**	**0 mM K^+ (mM)**
NaCl	150	150	-	140	140	150	150
KCl	10	10	-	5	4	10	0
NMDG		-	140	-	-	-	-
K-gluconate	-	-	5	-	-	-	-
$CaCl_2$	3	3	2	1	2	3	3
$MgCl_2$	1	1	1	1.2	1	1	1
Glucose	-	-	5	11.1	10	-	-
Sucrose	-	-	50	-	-	-	-
HEPES	10	10	10	5	5	10	10
pH	7.4	7.4	7.4	7.4	7.4	7.4	7.4
pH adjusted	NaOH	NaOH	CH_3SO_3H	NaOH	NaOH	NaOH	NaOH

Table S3

	K_v1.5/Kv4.3 (mM)	K_v7.1 (mM)	K_{ir}2.1 (mM)	K_{ir}3.x (mM)	K_{ir}6.2 (mM)	Na_v1.5 (mM)	hERG (mM)
KCl	20	-	110	110	125	-	130
K-gluconate	-	90	-	-	-	-	-
KF	90	20	-	-	-	-	-
CsF	-	-	-	-	-	120	-
NaCl	10	-	20	20	10	15	-
Na-gluconate	-	10	-	-	-	-	-
$MgCl_2$	1	1	1	1	1.1	-	1
$CaCl_2$	-	-	-	-	0.5	-	-
Mg-ATP	-	-	5	5	3.15	-	5
Na_2-ATP	5	5	-	-	-	-	-
HEPES	10	10	10	10	7.5	10	10
Sucrose	10	20	-	-	-	-	-
EGTA	10	10	5	5	7.5	10	5
EDTA	-	10	-	-	-	-	-
GTP-γS	-	-	-	0.9 (Added fresh)	-	-	-
pH	7.2	7.2	7.2	7.2	7.2	7.25	7.2
pH adjusted	KOH	KOH	KOH	KOH	KOH	CsOH	KOH

Assessment of cardiac calcium channel activity

Experimental solutions

Table S4

	$Ca_v1.2$ resting solution, RPS (mM)	**$Ca_v1.2$ Dye-loading solution (mM)**	**$Ca_v1.2$ stimulus solution (mM)**
NaCl	140	140	90
KCl	5	5	55
$CaCl_2$	5	5	5
$MgCl_2$	1	1	1
Glucose	10	10	10
Fluo4 NW	-	10 mg in 12 ml RPS	-
Probenecid	2.5	2.5	2.5
FPL-64176	-		6.1^{-4}-
HEPES	15	15	15
pH	7.4	7.4	7.4
pH adjusted	NaOH	NaOH	NaOH

***In-vivo* animal model**

Animals

Table S5

ID	Sex	Age	Height (cm)	Body weight arrival (kg)	Body weight day 29 (kg) **	Reason for exclusion
horse #1	F	8	160	520	532	
horse #2	F	7	163	455	468	
horse #3	F	5	158	452	446	
horse #4	F	3,5	151	432	450	
horse #5	F	3	152	505		septicaemia
horse #6	F	6	158	432	455	
horse #7	F	5	162	516	581	
horse #8	F	7	160	515	525	
horse #9	F	8	155	430	451	
horse #10	F	5	164	494	530	
horse #11	F	15	158	530		> cut-off age *
horse #12	F	12	155	506	500	
horse #13	F	10	151	320	338	

**The age cut-off for this study was >12 years of age. The day after this horses' arrival, we realised that there was a mismatch between the declared age at purchase and the age that could be deduced from the horses' official identification papers (15 years of age). Therefore, this horse was excluded from the study right from the start.*

**Weight changes/gains were expected as inter-individual metabolisation differences become easily recognisable in periods of low exercise. Some horses are "easy keepers" and take on weight easily, others have a higher maintenance and stay at a certain weight level. In terms of horses #3 and #12 it must be assumed, that those actually maintained their weight and the respective 6 kg difference can be accounted for as daily fluctuation.

Results:

XAF-1407: Highly selective $I_{K,ACh}$ inhibition

Radioligand binding assessment of pharmacology

XAF-1407 was screened in a commercially available pan-screen against a diverse panel of receptors, GPCR and kinases (Eurofins CEREP France, Celle-Lévescault, France). When less than 50% specific binding was observed at 10µM, these targets as such were not further investigated.

Specific binding was <50% at 10µM for 78 of the 81 targets:

Receptors

Adenosine A_1 ,A_{2A}, A_3

Adrenergic α_1, α_2, β_1, β_2

Angiotensin-II AT_1 , AT_2

BB (non-selective)

Bradykinin B_2

CGRP

Canabinoid CB_1

Cholecystokinin CCK_1 (CCK_A), CCK_2 (CCK_B)

Dopamine D_1, D_{2S}, $D_{4.4}$, D_5

Endothelin ET_A , ET_B

GABA (non-selective)

GAL_1 , GAL_2

PDGF

Chemokines CCR1, CXCR2 (IL-8B)

Cytokines TNF-α

Histamine H_1 , H_2

Melanocortin MC_4

Melatonin MT_1

Muscarinic M_1, M_3 , M_4 ,M_5

Neurokinin NK_1 , NK_2 , NK_3

Neuropeptide-Y Y_1 ,Y_2

NT_{S1} (NT_1)

Opioid and opioid-like δ2 (DOP), κ (KOP) µ (MOP), NOP (ORL1)

PAC_1 (PACAP)

EP_2 , EP_4

IP (PGI_2)

5-HT_{1A} , 5-HT_{1B} , 5-HT_{2A} , 5-HT_{2B} , 5-HT_{2C} ,5-HT_3, 5-HT_{5a} ,5-HT_6, 5-HT_7

sigma (non-selective)

sst (non-selective)

GR

$VPAC_1$ (VIP_1)

Vasopressin V_{1a}

Nuclear Receptors

Non-steroid nuclear receptors

PPARγ

Ion & ligand-gated channels

GABA BZD (central), BZD (peripheral) Cl- channel (GABA-gated)

Potassium channel SK_{Ca} channel

Glutamate Channels PCP

Purinergic Channels P2X , P2Y

Transporters

Norepinephrine transporter

Dopamine transporter

5-HT transporter

However, specific binding curves revealed IC_{50} and K_i of 7.4 µM and 1.6 µM for the human recombinant dopamine D_3 receptor, and 8.9 µM and 6.2 µM for the human recombinant muscarinic M_2 receptor. High specific binding was furthermore observed for the sigma receptor (IC_{50} 0.1, K_d 0.1 µM). Function studies revealed no functional, neither agonist nor antagonist, effect on guinea-pig vas deferens contraction at concentrations up to 1 µM, though.

	IC_{50}	K_i	n_H
D_3 (nM)	7400	1600	0.7
M_2 (nM)	8900	6200	0.6
δ (nM)	100	100	

Cloned cardiac ion channel conventional electrophysiology

The effect of a range of concentrations of XAF-1407 on recombinant $K_{ir}3.x$ and $K_{ir}2.1$ ion channels (Fig S1), other cardiac inward rectifiers ($K_{ir}6.2$/SUR2A), voltage dependent sodium ($Na_v1.5$) (Fig S2A) and potassium channels ($K_v11.1$, $K_v1.5$, $K_v4.3$, $K_v7.1$) (Fig S2B and Fig S3) was tested with standard giga seal whole cell patch clamp techniques. The effect of XAF-1407 on Na^+ channels was investigated at 1 Hz and 5 Hz and at RMPs of -70 mV and -100 mV and due to a lack of appreciable effect, full concentration response relationships were not investigated for $Na_v1.5$, $K_v11.1$ as well as $K_v4.3$ (10 and 3 µM).

XAF-1407 potently inhibited inward $K_{ir}3.1/3.4$ current (Fig S1A) and $K_{ir}3.4/3.4$ current (Fig S1B) at 10 nM measured at -140 mV, with minimal effect on $K_{ir}2.1$ inward current at 30 µM (Fig S1C). Concentration dependent effects on the remaining functionally important cardiac ion channels were first recorded at XAF-1407 concentrations >2000 times higher than concentrations effectively inhibiting $K_{ir}3.1/3.4$ current (Fig S2 and S3).

Figure S1

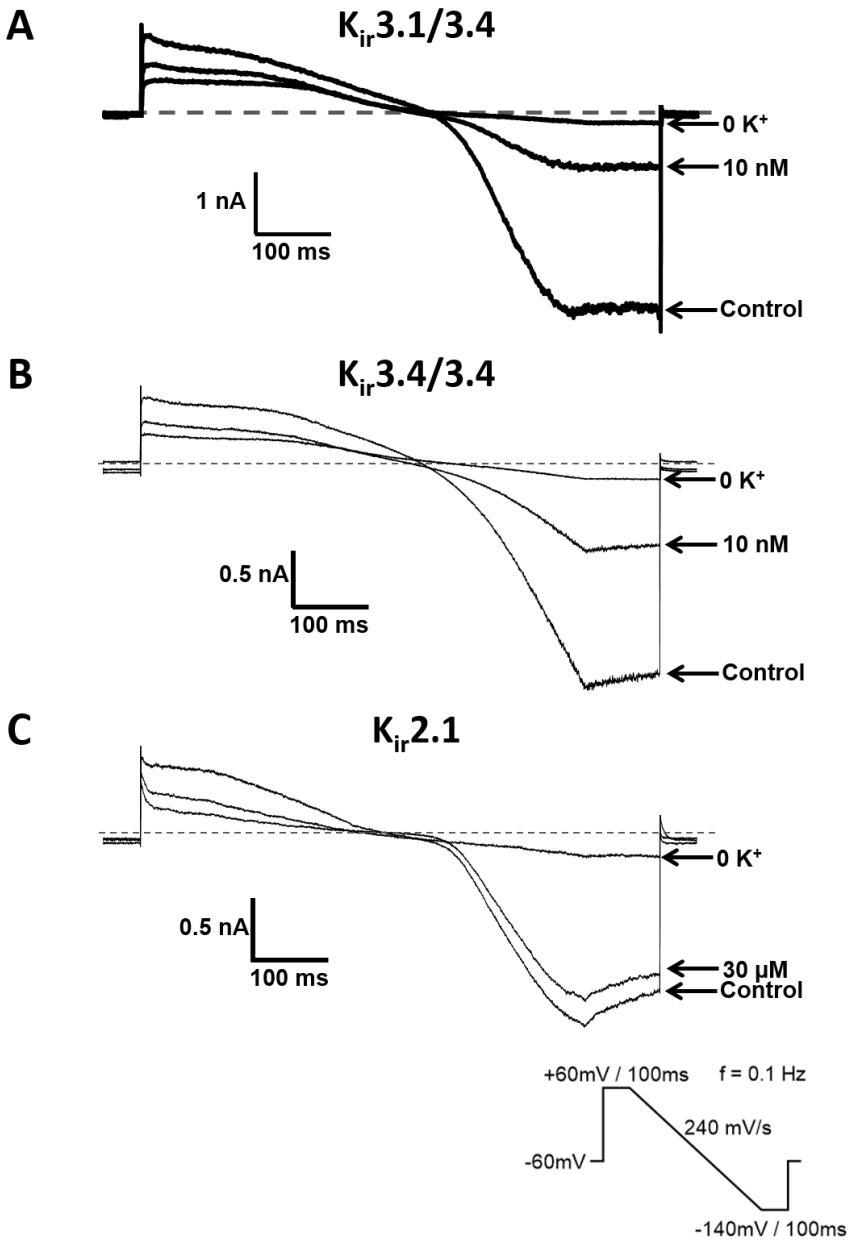

Figure S1: Effect of XAF-1407 on recombinant potassium inward rectifier currents. Original $K_{ir}3.1/3.4$ (**A**), $K_{ir}3.4/3.4$ (**B**) and $K_{ir}2.1$ (**C**) current traces are shown in the absence and presence of XAF-1407 and following removal of external K^+ to assess passive leak. Schematic of the voltage protocol used is shown at the bottom of the figure. A dashed line shows zero current.

Figure S2

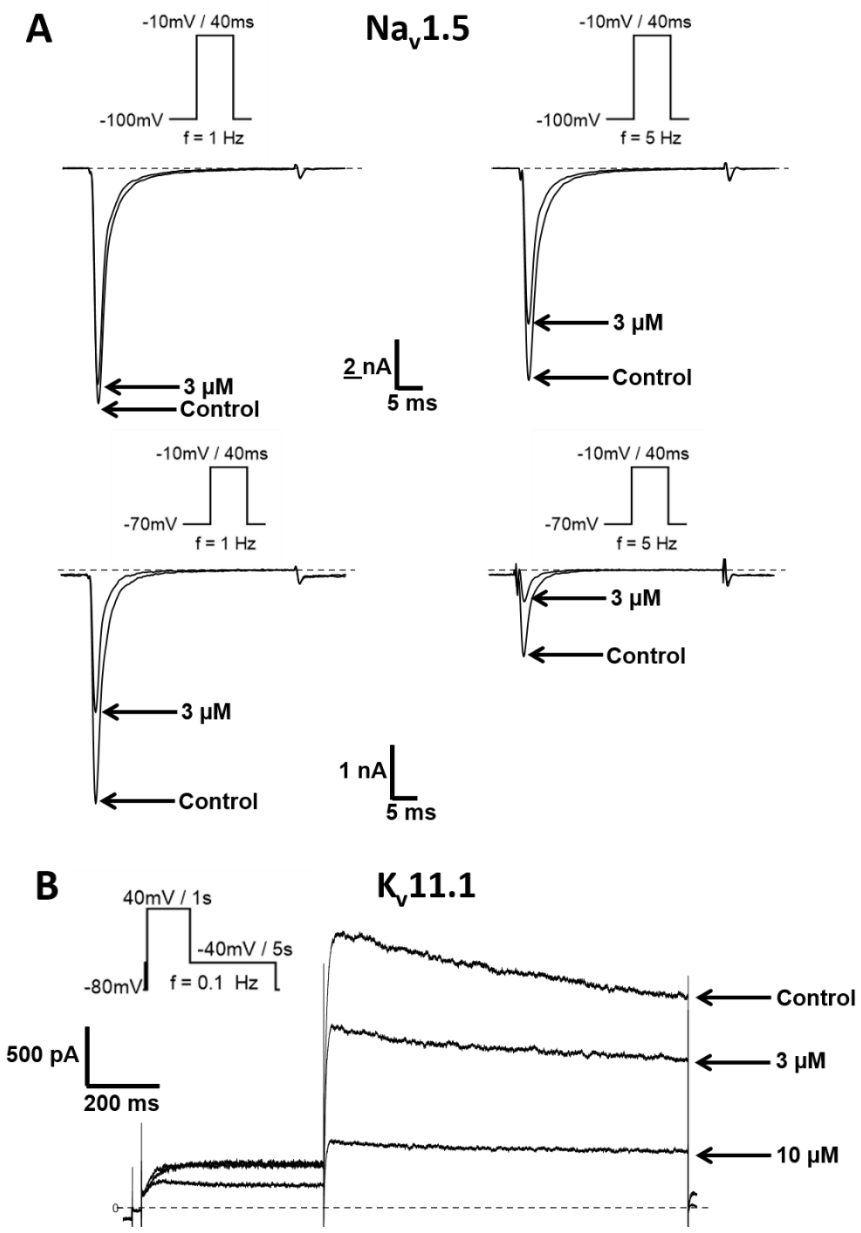

Figure S2: Effect of XAF-1407 on recombinant K_v11.1 (hERG) and Na_v1.5 current. Panel **A** shows the effect of 3 µM XAF-1407 on Na_v1.5 at different holding potentials (-70mV lower panel or 100 mV upper panel) and frequencies (1 Hz left panel and 5 Hz right panel).

Panel **B** shows original Kv11.1 current records in the absence and presence of 3 and 10 µM XAF-1407 record from the same cell. A schematic of the voltage protocol is shown inset at the top of each figure. A dashed line shows zero current.

Figure S3

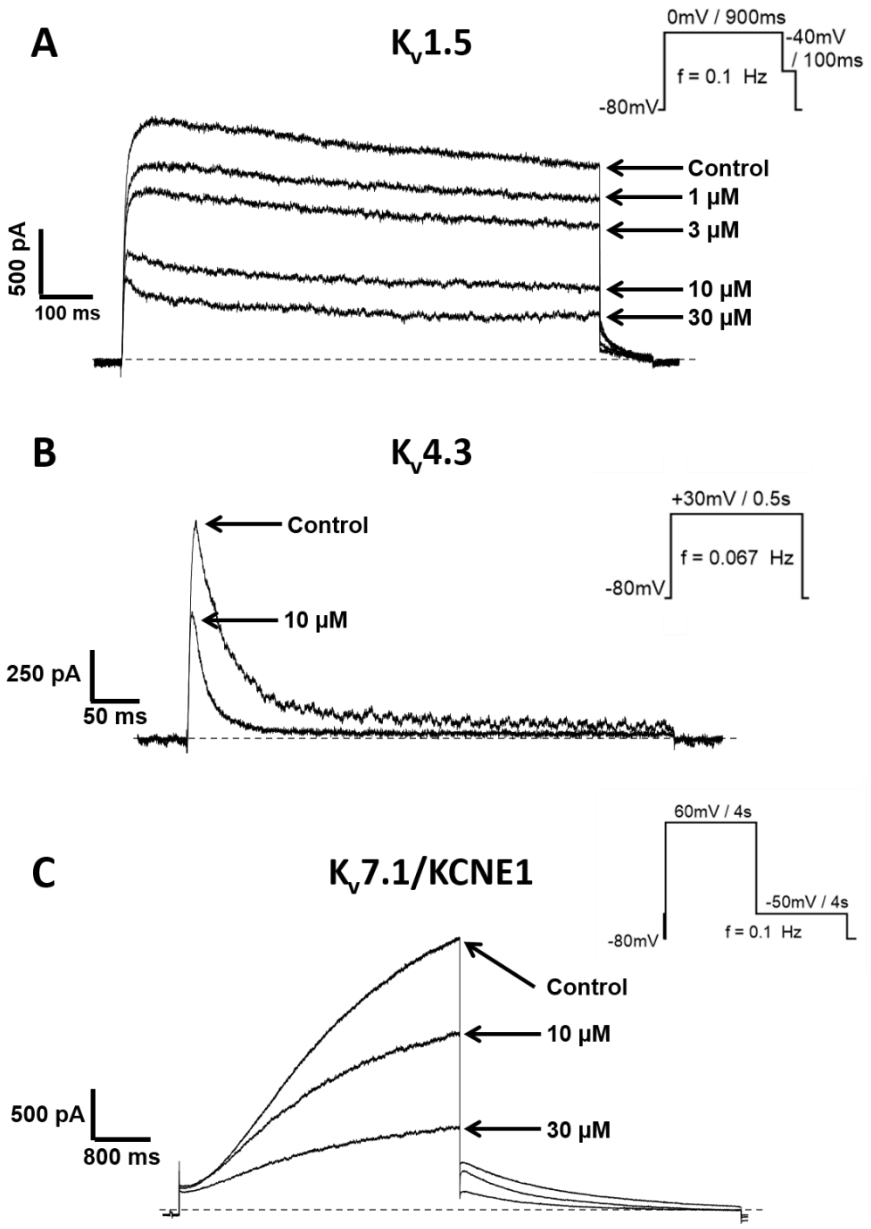

Figure S3: Effect of XAF-1407 on recombinant K_v ionic current. Panel **A** shows original K_v1.5 current records in the absence and presence of 1, 3, 10 and 30 µM XAF-1407 record from the same cell. Panel **B** shows original K_v4.3 current records in the absence and presence of 10 µM XAF-1407. Panel **C** shows original K_v7.1/KCEN1 current records in the absence and presence of 10 and 30 µM XAF-1407. A schematic of the voltage protocol is shown above each original trace. A dashed line shows zero current.