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RESEARCH PAPER

Effects of acute and chronic sunitinib treatment on cardiac function and calcium/calmodulin-dependent protein kinase II

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BACKGROUND AND PURPOSE

Calcium/calmodulin-dependent protein kinase IIδ (CaMKIIδ) is an important regulator of cardiac contractile function and dysfunction and may be an unwanted secondary target for anti-cancer drugs such as sunitinib and imatinib that have been reported to alter cardiac performance. This study aimed to determine whether anti-cancer kinase inhibitors may affect CaMKII activity and expression when administered *in vivo*.

EXPERIMENTAL APPROACH

Cardiovascular haemodynamics in response to acute and chronic sunitinib treatment, and chronic imatinib treatment, were assessed in guinea pigs and the effects compared with those of the known positive and negative inotropes, isoprenaline and verapamil. Parallel studies from the same animals assessed CaMKIIδ expression and CaMKII activity following drug treatments.

KEY RESULTS

Acute administration of sunitinib decreased left ventricular (LV) dP/dt_{max}. Acute administration of isoprenaline increased $LVdP/dt_{max}$ dose-dependently, while $LVdP/dt_{max}$ was decreased by verapamil. CaMKII activity was decreased by acute administration of sunitinib and was increased by acute administration of isoprenaline, and decreased by acute administration of verapamil. CaMKIIδ expression following all acute treatments remained unchanged. Chronic imatinib and sunitinib treatments did not alter fractional shortening; however, both CaMKIIδ expression and CaMKII activity were significantly increased. Chronic administration of isoprenaline and verapamil decreased LV fractional shortening with parallel increases in CaMKIIδ expression and CaMKII activity.

CONCLUSIONS AND IMPLICATIONS

Chronic sunitinib and imatinib treatment increased CaMKIIδ expression and CaMKII activity. As these compounds are associated with cardiac dysfunction, increased CaMKII expression could be an early indication of cellular cardiotoxicity marking potential progression of cardiac contractile dysfunction.

Abbreviations

AIP, autocamtide inhibitory peptide; CaMKII, calcium/calmodulin-dependent protein kinase II; FS, fractional shortening; LV, left ventricular; LVDD, left ventricular diastolic diameter; $LVdP/dt_{max}$, maximum rate of rise of left ventricular pressure; LVSD, left ventricular systolic diameter; ROS, reactive oxygen species; RyR, ryanodine receptor; SR, sarcoplasmic reticulum

Table of Links

TARGETS
Enzymes[a]
CaMKIIδ, calmodulin-dependent kinase II
Ion Channels[b]
RyR, ryanodine receptor

LIGANDS
Imatinib
Isoprenaline
Sunitinib
Verapamil

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b]Alexander *et al.*, 2013a,b).

Introduction

Cardiotoxicity is a common side effect of many 'successful' anti-cancer agents (Curigliano *et al.*, 2010). Although improvements in regression of cancer growth and metastasis have been observed with these agents, a range of cardiac toxicities can occur. These include ECG changes and left ventricular (LV) contractile dysfunction, leading in some cases to congestive heart failure.

One class of anti-cancer agents with proven cardiotoxic effects comprise the kinase inhibitors (Cheng and Force, 2010). Kinases, in particular tyrosine kinases, play an important role in tumour angiogenesis and cell proliferation. Inhibition of these enzymes slows tumour progression leading to shrinkage of the tumour mass. Unfortunately, and in spite of their potential for targeted effects, some members of this family of inhibitors exhibit marked cardiotoxicity. Imatinib was the first of these kinase inhibitors to gain FDA approval and cardiotoxicity was not predicted by preclinical studies. Following approval, however, it was reported that development of heart failure was associated with imatinib treatment in some patients (Kerkela *et al.*, 2006).

Unlike imatinib, sunitinib is a kinase inhibitor that is clearly associated with clinical cardiotoxicity. Preclinical evaluations have shown sunitinib to cause LV hypertrophy and hypertension (Blasi *et al.*, 2012; Maayah *et al.*, 2014). Clinically, prolongation of the QT interval has been observed (Bello *et al.*, 2009) and a retrospective study found that 15% of sunitinib-treated patients developed symptomatic grade 3/4 LV dysfunction (Telli *et al.*, 2008), while in another study 8% of patients developed heart failure following 33.6 weeks of median treatment duration (Chu *et al.*, 2007).

Sunitinib has multiple targets including VEGF receptors, platelet-derived growth factor receptors, FMS-like tyrosine kinase 3 and c-kit. A key issue with the limited selectivity of sunitinib is the likelihood of 'off-target toxicity' caused by the action of sunitinib on proteins other than those that are recognized targets (Cheng and Force, 2010). One example recently identified was AMP activated protein kinase. This kinase has an essential role in metabolic homeostasis in the heart and, as such, alterations in its activity following sunitinib treatment have been shown to compromise cardiac myocyte function (Kerkela *et al.*, 2009). There could potentially be a large number of 'off-target' effects of sunitinib (and indeed other members of the kinase inhibitor family) (Ghoreschi *et al.*, 2009). However, these additional targets and the contribution they make to the cardiotoxic properties of these low molecular weight inhibitors have yet to be fully assessed.

One kinase that plays a crucial role in cardiac function is calcium/calmodulin-dependent protein kinase II (CaMKII). CaMKII exists as four different isoforms, α, β, γ and δ, with the δ isoform being the predominant isoform expressed in the heart. As well as acting as a key modulator of cardiac calcium handling and contractility, CaMKIIδ has also been suggested to regulate the cardiac inflammatory response, transcriptional events and non-contractile cardiac cell proliferation (Currie *et al.*, 2011; Martin *et al.*, 2014). Importantly, CaMKIIδ is recognized not only as a critical modulator of normal cardiac function, but is also established as a fundamental molecular switch that, when excessively expressed and activated, can trigger pathophysiological events leading ultimately to heart failure (Anderson, 2007). Pathophysiological consequences of increased CaMKIIδ activity include a range of effects on cardiac ion channels influencing depolarization, cardiac excitability and repolarization of cardiac myocytes (Anderson, 2009). Dysfunctional sarcoplasmic reticulum (SR) Ca^{2+} handling is evident following CaMKIIδ-mediated hyperphosphorylation of the SR Ca^{2+} release channel, the ryanodine receptor (RyR), which leads to increased diastolic Ca^{2+} release and a propensity for cardiac arrhythmias (Wehrens *et al.*, 2004). As well as coordinating contractile dysfunction, CaMKIIδ activation can also increase the activity of pro-inflammatory mediators and the development of fibrosis. As such, it links a number of both acute and chronic pathological events in the progression of heart disease and is considered to be a prime candidate for therapeutic targeting (Anderson, 2007; Currie *et al.*, 2011).

The possibility that the cardiotoxic effects of kinase inhibitors may be mediated via CaMKII has not previously been investigated *in vivo*. This is surprising given the overwhelming recognition that elevated CaMKIIδ is central to cardiac pathophysiology. Previously, we have optimized conditions for studying the acute effects of drugs on indices of cardiac contractility and haemodynamics in an anaesthetized guinea pig model (Mooney *et al.*, 2012). In this study, we have also developed the guinea pig model to allow assessment of more prolonged dosing of drugs on cardiovascular function. Using these techniques, we have compared the effects of sunitinib and imatinib with recognized positive and

negative inotropic drugs. Applying an integrated approach, we have also assessed in parallel the effects of these drugs on CaMKIIδ expression and CaMKII activity in LV tissue taken from the same animals.

Methods

Animals and anaesthesia

All animal care and experimental procedures were performed in accordance with the UK Animals (Scientific Procedures) Act 1986, approved by institutional ethical review committees and conducted under the authority of project licences held at the University of Strathclyde or at AstraZeneca. A total of 50 male Dunkin Hartley guinea pigs (440–650 g) were purchased from Harlan (Bicester, UK). In acute experiments, animals were anaesthetized with fentanyl (50 $\mu g \cdot kg^{-1}$ s.c.) followed by sodium pentobarbital (50–60 $mg \cdot kg^{-1}$ i.p.). Throughout the haemodynamic assessments, anaesthesia was maintained by a continuous i.v. infusion of sodium pentobarbital (12 $mg \cdot mL^{-1}$) at 6 $mg \cdot kg^{-1} \cdot h^{-1}$ using an infusion pump. In chronic experiments, animals were anaesthetized using a combination of Hypnorm® (VetaPharma Ltd, Leeds, UK) and Hypnovel® (Roche, Welwyn Garden City, Herts, UK) [6 $mL \cdot kg^{-1}$ i.p. of a solution containing 1 part Hypnorm (fentanyl citrate 0.315 $mg \cdot mL^{-1}$ and fluanisone 10 $mg \cdot mL^{-1}$); 1 part Hypnovel (midazolam HCl 5 $mg \cdot mL^{-1}$) and two parts water made up freshly each day] for echocardiography assessments, and maintained for haemodynamic assessment with sodium pentobarbital as described above. A different anaesthetic regimen was used for the chronic experiments taking into account the requirement for recovery following initial echocardiography assessment. Although Hypnorm and Hypnovel can cause a decline in heart rate (Mooney *et al.*, 2012), this is not observed until after 15 min and at higher concentrations than that used here. Full details of animal housing conditions, diet, surgical preparation and all equipment have been published previously (Mooney *et al.*, 2012). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Haemodynamic assessment

Immediately after induction of an adequate level of anaesthesia, as determined by the absence of corneal and/or pedal withdrawal reflexes, the trachea was cannulated and animals were artificially ventilated with room air. Oxygen saturation and expired CO_2 were measured continuously using a pulse oximeter/capnograph (Medair Lifesense™, Colchester, CT, USA). If necessary, during the preparation and stabilization phases, the stroke volume of the pump was adjusted to keep the end-tidal CO_2 value between 35 and 45 mmHg and oxygen saturation above 95%. Blood gas values were kept within a set range: PO_2 >70 and <110 mmHg, PCO_2 >25 and <45 mmHg. ECG was recorded via subcutaneous limb leads (for monitoring only), arterial blood pressure (BP) via a fluid-filled cannula in the right carotid artery, and left ventricular pressure via a 2F or 3F Millar Mikro-tip® catheter pressure transducer (Millar Inc, Houston, TX, USA) or a 1.6F Scisense pressure catheter (SciSense Inc, London, ON, Canada) advanced through the left carotid artery so that its tip lay in the lumen of the LV. Drugs or vehicle were delivered via a jugular venous cannula. Body temperature was monitored throughout experiments via a rectal thermometer and maintained ~37°C using a heating lamp. All data were recorded continuously and processed using Ponemah P3 Plus software (St Paul, MN, USA).

Acute experimental protocol

Twenty guinea pigs weighing 460–650 g were used to assess acute drug effects. After completing the full surgical preparation a 20 min stabilization period was allowed. Guinea pigs received either sunitinib (0.3, 1.0 and 3 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) (1.8, 6 and 18 $mg \cdot kg^{-1}$), isoprenaline (0.1, 0.3 and 1.0 $nmol \cdot kg^{-1} \cdot min^{-1}$) (0.3, 0.9 and 3 $\mu g \cdot kg^{-1}$), verapamil (14, 42 and 140 $nmol \cdot kg^{-1} \cdot min^{-1}$) (95, 286 and 955 $\mu g \cdot kg^{-1}$) or vehicle (equal volumes of normal saline; 20, 60 and 200 $\mu L \cdot kg^{-1} \cdot min^{-1}$) as continuous i.v. infusions with each dose being infused for 15 min. Following infusion of the final dose animals were killed by removal of the heart.

Chronic experimental protocol

Thirty guinea pigs weighing 440–560 g at the start of procedures were used to assess chronic drug effects. On day 0, guinea pigs were anaesthetized and transthoracic echocardiography was performed using a 13 MHz linear array transducer and HDI® 3000 ultrasound system (ASL Ultrasound, MiuS, Gloucester, UK). M-mode short-axis images of the LV were obtained and LV diastolic and systolic diameters (LVDD and LVSD, respectively) were measured to determine % fractional shortening (FS). Immediately following this, an osmotic mini-pump (Alzet® Model 2ML1; Charles River, Harlow, UK) was implanted subcutaneously at the neck to deliver sunitinib (40 $\mu mol \cdot kg^{-1} \cdot day^{-1}$) (16 $mg \cdot kg^{-1} \cdot day^{-1}$), imatinib (170 $\mu mol \cdot kg^{-1} \cdot day^{-1}$) (84 $mg \cdot kg^{-1} \cdot day^{-1}$), isoprenaline (1.5 $\mu mol \cdot kg^{-1} \cdot day^{-1}$) (317 $\mu g \cdot kg^{-1} \cdot day^{-1}$), verapamil (20 $\mu mol \cdot kg^{-1} \cdot day^{-1}$) (9.1 $mg \cdot kg^{-1} \cdot day^{-1}$) or vehicle (0.1% acidified saline) over 6 days. All drugs were dissolved in 0.1% ascorbic acid in 0.9% w/v NaCl except imatinib which was dissolved in 0.9% w/v NaCl. On day 6, guinea pigs were re-anaesthetized and echocardiography was repeated. Haemodynamic assessment was then performed as described above. At the end of this, animals were killed by removal of the heart.

Tissue preparation

The heart was weighed following removal and a transverse section was taken through the right and left ventricles and fixed in 10% formalin for subsequent histological analysis. The remaining LV was cut into chunks, snap frozen in liquid nitrogen and stored at –80°C. As required, LV tissue was homogenized in buffer containing 20 mM Tris-HCl, pH 7.2, 1 mM DTT, phosphatase inhibitors (0.5 mM calyculin A, 4 mM Na orthovanadate) and protease inhibitors (cocktail set V, EDTA free; Calbiochem, Merck Millipore, Manchester, UK). The total protein content was determined using the Coomassie Plus protein assay (Pierce, Fisher Scientific, Loughborough, UK).

Immunoblotting

LV homogenates were subjected to SDS-PAGE using the NuPAGE system (Invitrogen, Fisher Scientific); 10% Bis-Tris gels for CaMKII and 3–8% Tris-Acetate gels for RyR. Antibod-

ies were as follows: custom-made polyclonal antibody (rabbit IgG) against the C terminus of CaMKIIδ (1:5000; Eurogentec, Southampton, UK), rabbit polyclonal anti-phosphoRyR (Ser-2815) IgG (1:2000; Badrilla), mouse monoclonal anti-GAPDH IgG (1:80 000; Abcam). Either goat anti-mouse or anti-rabbit-HRP conjugate (Sigma and GE Healthcare) was used as secondaries. Films were quantified using a GS800 calibrated densitometer and Quantity One software (Bio-Rad Laboratories Ltd.). GAPDH was used for intra-gel normalization. CaMKII:GAPDH and pSer2815-RyR:GAPDH ratios were calculated for each sample and these were then normalized to an untreated sample (aliquots from the same preparation) included in every gel to allow inter-gel normalization. The latter normalization was performed due to the number of samples, which had to be run across several different gels.

CaMKII activity assay

CaMKII activity was determined in triplicate in LV tissue homogenates by measuring the incorporation of γ-^{32}P into a CaMKII peptide substrate, autocamtide-2, as described previously (Anthony *et al.*, 2007). Controls included homogenates measured in the absence of Ca^{2+} (5 mM EGTA), absence of substrate and inclusion of autocamtide inhibitory peptide (AIP), a recognized CaMKII inhibitor. Individual activity values were converted to pmol of phosphate incorporated per min per μg protein and normalized to the calculated activity of an untreated sample included in every assay, in line with immunoblotting analyses.

Plasma drug and cardiac troponin I concentrations

In acute and chronic experiments, blood samples were collected in 1.3 mL lithium heparin tubes from the open chest at the end of the haemodynamic assessment and immediately after removal of the heart. Plasma was prepared by centrifugation (AccuSpin MicroR centrifuge; Fisher Scientific) at 2500× *g* for 10 min, aliquoted and stored at –80°C until analysis. Total plasma drug concentrations were determined by HPLC-MS/MS. On day 6 of the chronic dosing study, blood samples were also collected for determination of plasma cardiac troponin I using the ADVIA Centaur® CP Immunosystem cTnI-Ultra™ assay (Medical Siemens, Malvern, PA, USA).

Data analysis

Results are presented as mean ± SEM. In acute experiments, haemodynamic data were averaged over 5 s at 1, 2, 3, 4, 5, 10 and 15 min after the start of each infusion and drug effects were determined by Kruskal–Wallis tests. At the end of the chronic dosing experiments, haemodynamics were averaged over 15 min and drug effects were assessed by comparison to the vehicle group using a one-way ANOVA followed by Dunnett's test. Haemodynamic effects are presented as a % change from baseline to allow clear comparison across drug groups. For echocardiography experiments, pre- (day 0) and post- (day 6) treatment measurements were compared using Student's paired *t*-test. Kruskal–Wallis tests were used for comparisons of CaMKIIδ expression, CaMKII activity, pSer^{2815}-RyR expression and cardiac troponin I concentrations among groups because some of the data were not distributed normally.

Materials

Fentanyl, Hypnorm and Hypnovel were obtained from veterinary wholesalers through the Biological Procedures Unit at the University of Strathclyde. Sodium pentobarbital, isoprenaline HCl and verapamil were obtained from Sigma-Aldrich (Poole, Dorset, UK). Sunitinib and imatinib were purchased from Sequoia Research Products Ltd. (Pangbourne, UK). All drugs were dissolved in 0.9% w/v NaCl for acute administration or 0.1% acidified saline for chronic administration.

Results

Acute sunitinib treatment caused a decrease in LVdP/dt$_{max}$ accompanied by a fall in heart rate and BP

Sunitinib decreased LVdP/dt$_{max}$ in a dose-dependent manner with significant changes appearing at the beginning of infusion of the second dose (Figure 1). As expected, the negative inotrope verapamil also reduced LVdP/dt$_{max}$ whereas the positive inotrope isoprenaline caused rapid dose-dependent increases (Figure 1). The changes in heart rate induced by these drugs were similar to their effects on LVdP/dt$_{max}$; sunitinib and verapamil decreased heart rate and isoprenaline caused an increase (Figure 2A). However, although sunitinib and verapamil caused significant, dose-dependent reductions in systolic and diastolic arterial BPs, isoprenaline did not alter either parameter significantly (Figure 2B and C). Acute treatment with vehicle had no significant effects on any of the parameters.

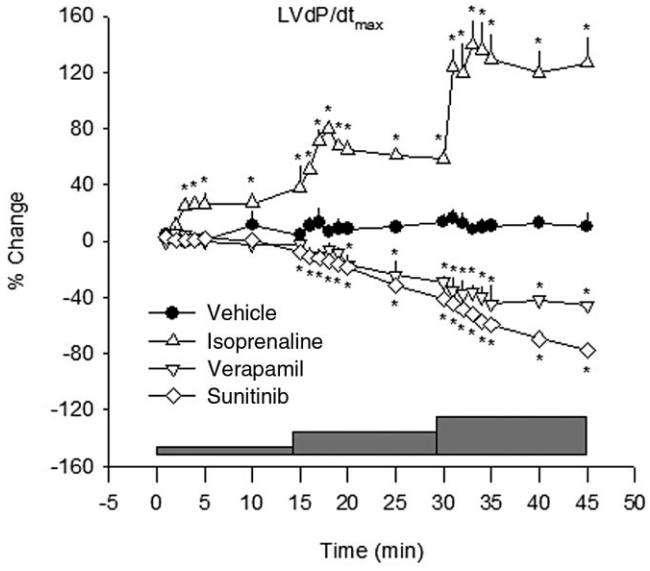

Figure 1

LV dP/dt$_{max}$ is reduced following acute sunitinib administration. LV dP/dt$_{max}$ responses (% change from baseline) following acute drug administration are shown for vehicle, isoprenaline, verapamil and sunitinib. The grey bars indicate increasing drug doses. Baseline values (mmHg·s^{-1}): isoprenaline 2610 ± 500, verapamil 3627 ± 355, sunitinib 1803 ± 163, vehicle 2948 ± 580. Mean ± SEM; $n = 4$. *$P <$ 0.05 compared with value within group at t = 0 min, Kruskal–Wallis test.

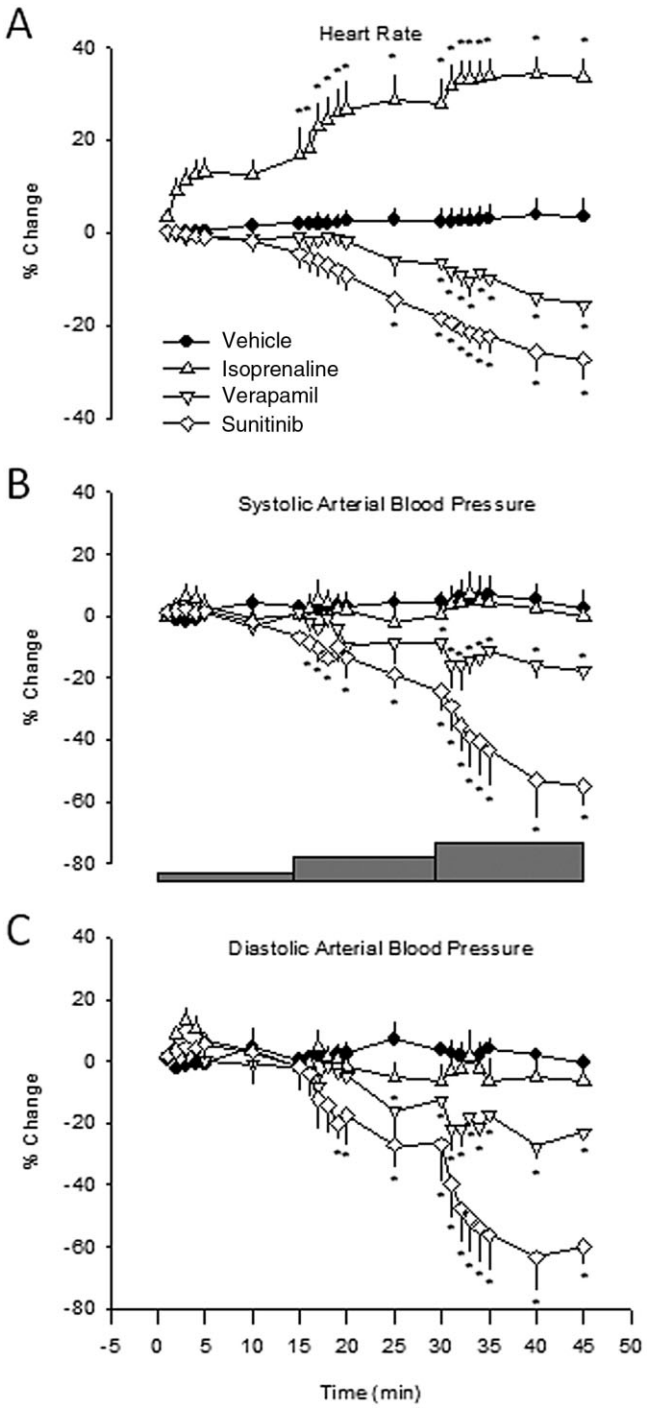

Figure 2

Heart rate and arterial BP are decreased following acute sunitinib administration. Heart rate (A), systolic BP (B) and diastolic BP (C) (% change from baseline) are shown following acute drug administration of vehicle, isoprenaline, verapamil and sunitinib respectively. The grey bars indicate increasing drug doses. Baseline values: heart rate (bpm), isoprenaline 266 ± 6, verapamil 284 ± 9, sunitinib 238 ± 7, vehicle 267 ± 16; systolic BP (mmHg), isoprenaline 62 ± 7, verapamil 59 ± 1, sunitinib 56 ± 2, vehicle 57 ± 3; diastolic BP (mmHg), isoprenaline 39 ± 5, verapamil 33 ± 2, sunitinib 39 ± 2, vehicle 33 ± 2. Mean ± SEM; $n = 4$. *$P < 0.05$, compared with value within group at t = 0 min, Kruskal–Wallis test.

CaMKII activity was significantly reduced following acute sunitinib treatment

Quantitative immunoblotting for CaMKIIδ revealed no differences in expression of the protein in LV homogenates across all acute treatment groups (Figure 3A). The same homogenates were then assessed for CaMKII activity. When compared with vehicle, sunitinib and verapamil treatment significantly decreased CaMKII activity and, as expected, acute isoprenaline administration increased CaMKII activity (Figure 3B).

To test the sensitivity of the CaMKII activity assay, LV homogenate was assessed alone, in the presence of the CaMKII inhibitor AIP (3 and 10 μM), in the absence of CaMKII peptide substrate and in the presence of 5 mM EGTA to remove extracellular Ca^{2+}. All of these treatments resulted in inhibition of CaMKII basal activity as expected, confirming the sensitivity of the assay (Figure 3C).

Chronic administration of either sunitinib or imatinib had no significant effects on $LVdP/dt_{max}$, heart rate, BP or FS

Echocardiography was used to assess LV diameter and FS before and after chronic drug administration. A short-axis view of the guinea pig heart is shown in Figure 4A along with a corresponding M-mode recording. LV diameters and FS remained unchanged following chronic administration with sunitinib and imatinib. LVDD was increased by 23% and LVSD by 89% in the isoprenaline group. LVSD was increased by 34% following verapamil administration. FS was decreased significantly in both the isoprenaline and verapamil groups by 28 and 24% respectively (Figure 4B).

Sunitinib and imatinib also had no effect on $LVdP/dt_{max}$ during the final haemodynamic assessment whereas this index of cardiac contractility was decreased significantly in the verapamil group (Table 1). None of the drugs caused significant changes in heart rate and the only drug that affected BP was verapamil which reduced both systolic and diastolic arterial BP significantly (Table 1).

There were no changes in heart : body weight ratio (Table 1) and no cardiac histopathological changes following any of the chronic drug treatments.

CaMKIIδ expression and CaMKII activity were both significantly increased by chronic sunitinib and imatinib administration

Expression of CaMKIIδ protein increased in response to chronic treatment in all drug groups compared with the vehicle group (Figure 5). Verapamil caused the greatest increase in expression (96%), followed by imatinib (62%), sunitinib (23%) and isoprenaline (22%). Parallel experiments were performed to measure changes in CaMKII activity following chronic drug treatment. The greatest increase was in the sunitinib group (110%), followed by isoprenaline (78%), verapamil (70%) and imatinib (50%) (Figure 6A).

CaMKII-dependent phosphorylation of RyR was increased following chronic administration of sunitinib and imatinib

In order to confirm the increase in activity measured using the CaMKII activity assay, quantitative immunoblotting of

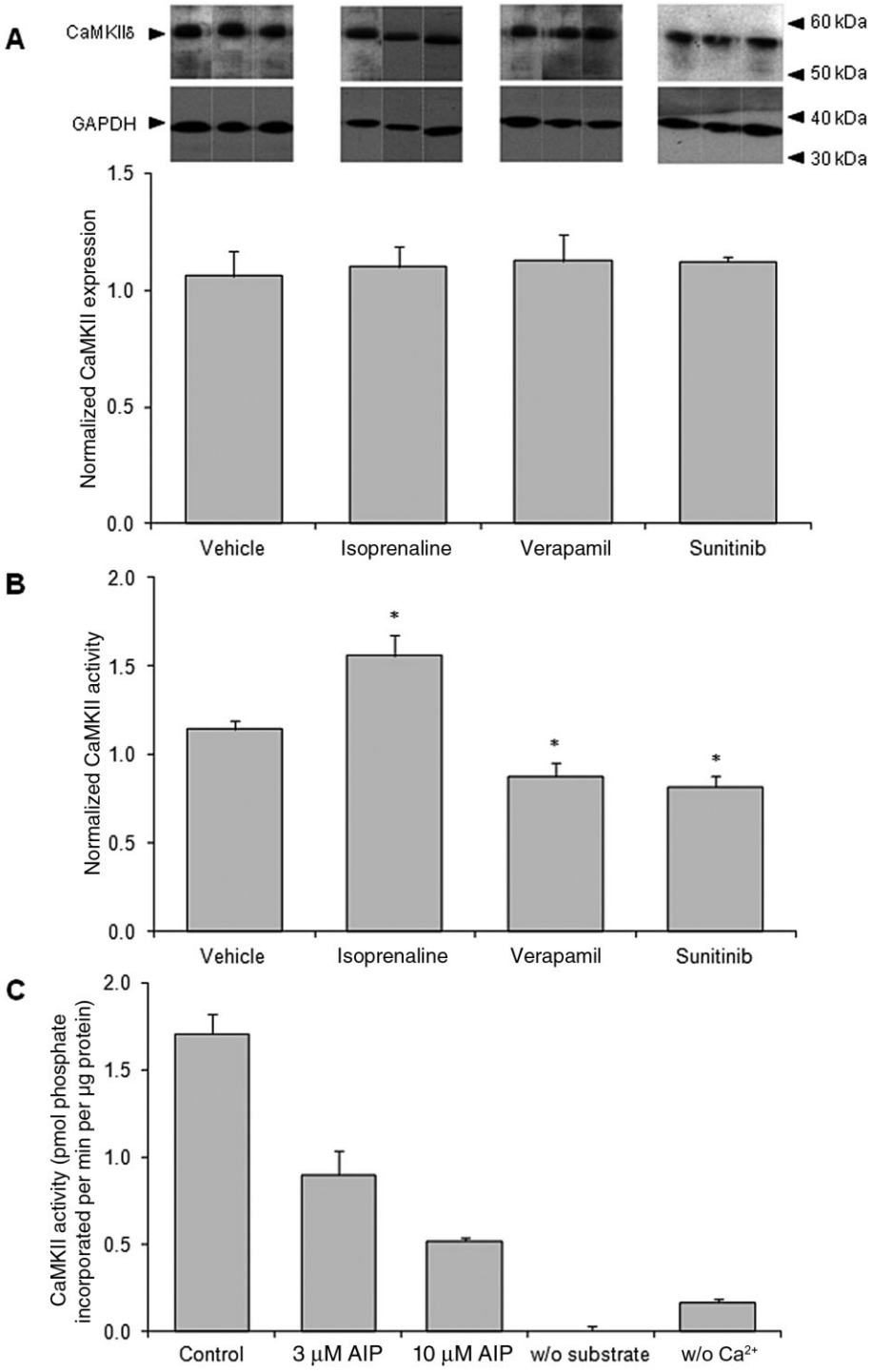

Figure 3

Protein expression of CaMKIIδ and CaMKII activity are not altered significantly following acute sunitinib administration. (A) Representative CaMKIIδ immunoblots are shown for each treatment from three separate samples (6 µg total protein) and pooled histogram data showing quantification of CaMKIIδ (calculated as CaMKIIδ:GAPDH and normalized to a control sample included in each blot) are shown below in the accompanying histogram normalized to GAPDH. (B) Histogram showing CaMKII activity in guinea pig LV homogenate (10 µg) following acute *in vivo* drug administration. For activity assays, data were normalized to the same control untreated sample used across assays for consistency. Histogram data are mean ± SEM; vehicle $n = 6$, isoprenaline $n = 5$, verapamil $n = 5$, sunitinib $n = 4$. *$P < 0.05$ compared with vehicle, Kruskal–Wallis test. (C) Histogram showing CaMKII activity determined in triplicate in guinea pig LV homogenate (10 µg) either receiving no treatment (control), pretreated with 3 and 10 µM autocamtide-2 related inhibitory peptide (AIP), without (w/o) autocamtide substrate or without Ca^{2+}.

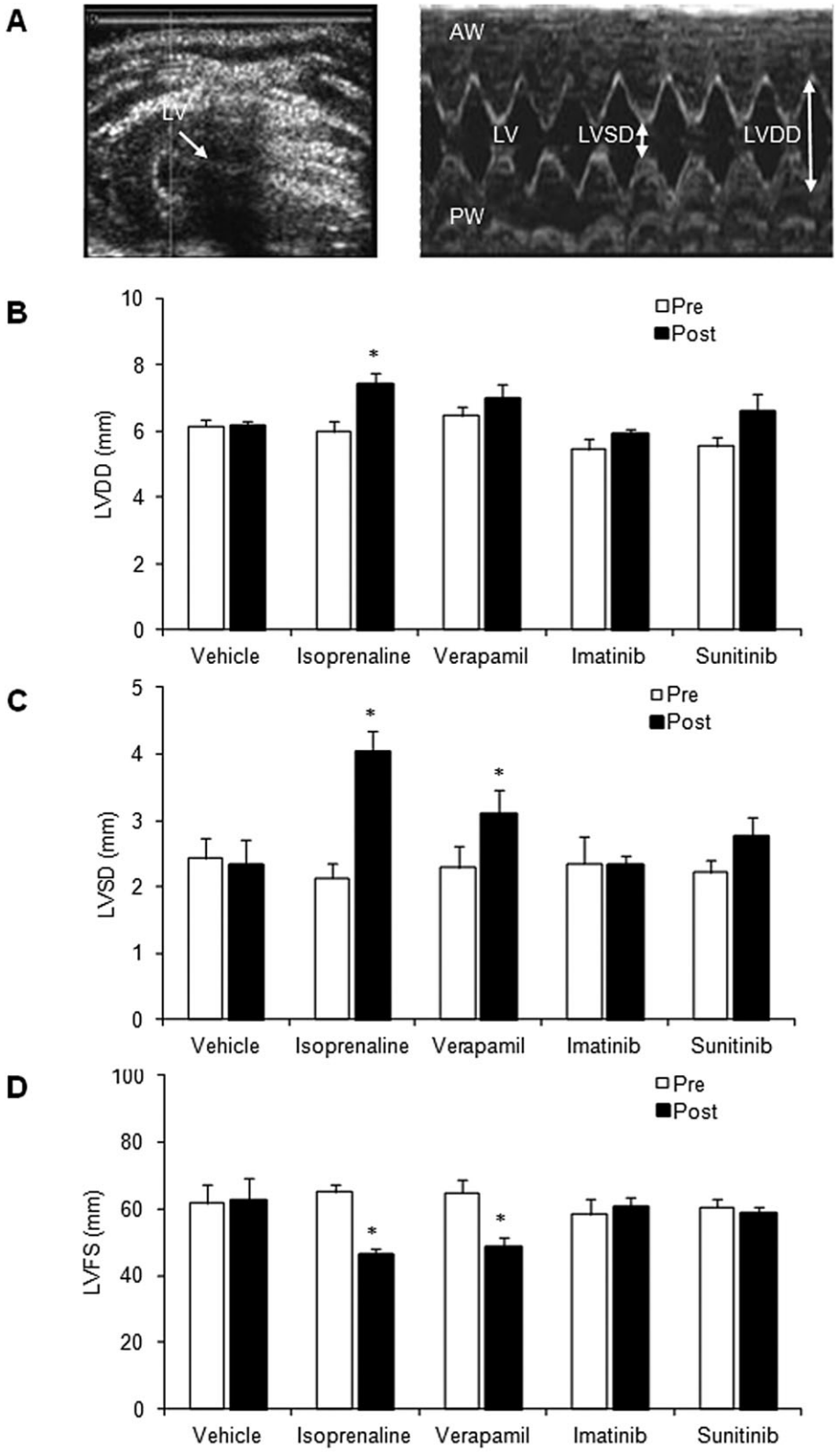

Figure 4

Chronic administration of sunitinib and imatinib does not significantly alter LV contractility. (A) Echocardiography was used to calculate % FS. The left hand panel shows a two-dimensional short-axis view of the left ventricle (LV); the right hand panel shows an M-mode recording indicating the lumen of the left ventricle (LV), anterior wall (AW), posterior wall (PW), LVSD and LVDD. (B) LVDD, (C) LVSD and (D) LV fractional shortening (%FS) are shown for pre- and post-chronic drug administration. Mean ± SEM; $n = 6$. *$P < 0.05$ compared with pre-drug value, paired t-test.

Table 1

Haemodynamic measurements following chronic drug administration

	$LVdP/dt_{max}$ ($mmHg \cdot s^{-1}$)	LVEDP (mmHg)	Heart rate (bpm)	SBP (mmHg)	DBP (mmHg)	H : BW ratio (×1000)
Vehicle	4809 ± 484	−3 ± 2	288 ± 20	57 ± 3	36 ± 2	3.31 ± 0.12
Isoprenaline	4779 ± 169	4 ± 3*	300 ± 5	58 ± 2	37 ± 2	2.91 ± 0.04
Verapamil	3176 ± 436*	−6 ± 1	264 ± 14	42 ± 3*	24 ± 3*	3.25 ± 0.11
Imatinib	4938 ± 574	−6 ± 1	318 ± 17	55 ± 3	28 ± 3	3.20 ± 0.11
Sunitinib	4548 ± 156	−2 ± 1	283 ± 4	53 ± 3	29 ± 1	3.25 ± 0.25

DBP, diastolic BP; LVEDP, left ventricular end-diastolic pressure; SBP, systolic BP; H : BW ratio, heart : body weight ratio. Mean ± SEM; $n = 6$. *$P < 0.05$ compared with vehicle group, one-way ANOVA with Dunnett's test.

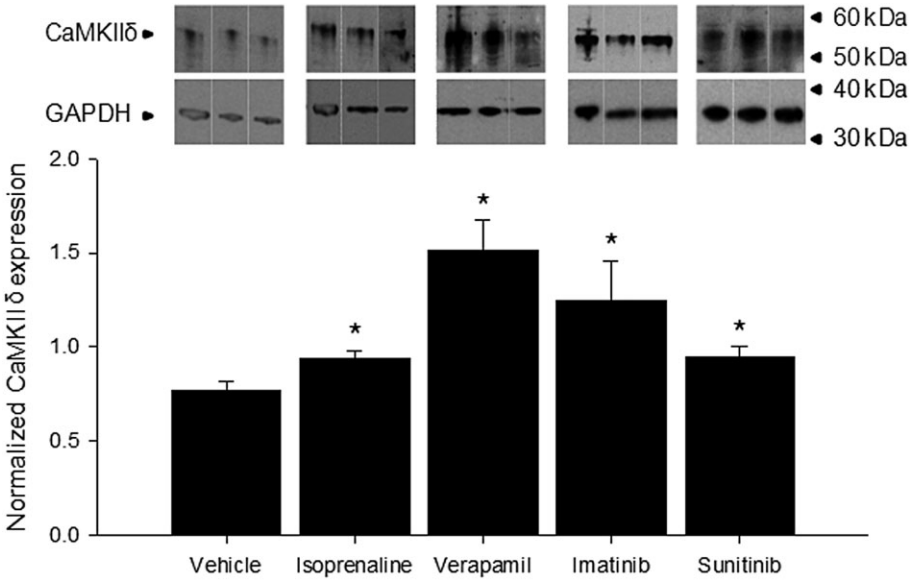

Figure 5

Chronic administration of either sunitinib or imatinib causes a significant increase in CaMKIIδ expression. Representative signals for CaMKIIδ and GAPDH from three individual blots are shown for each treatment (6 μg total protein), as indicated. Corresponding mean data for each treatment (calculated as CaMKIIδ:GAPDH and normalized to a control sample included in each blot) are shown below in the accompanying histogram. Mean ± SEM; $n = 6$. *$P < 0.05$, compared with the vehicle group; Kruskal–Wallis test.

the CaMKII-selective phosphorylation site on RyR, Ser^{2815} ($pSer^{2815}$-RyR), was used as a second assessment of CaMKII activity. All chronic treatments resulted in an increase in $pSer^{2815}$-RyR expression when compared with vehicle treatment (Figure 6B). Again, the largest increase was with sunitinib treatment (123%), but this time it was followed by imatinib (61%) then isoprenaline and verapamil which were similarly effective causing increases in $pSer^{2815}$-RyR expression of 16 and 12% respectively.

Plasma drug and cardiac troponin I concentrations

In the acute phase studies, total plasma drug concentrations achieved at the end of the dosing period were: sunitinib 25.4 ± 8.3 μM and verapamil 7.8 ± 2.9 μM. In the chronic dosing groups, total plasma drug concentrations, respectively, were: sunitinib 0.01 ± 0.002 μM (clinically relevant concentration is 0.06 μM); imatinib 1.36 ± 0.38 μM (clinically relevant concentration is 2 μM); verapamil 0.16 ± 0.02 μM. Plasma drug concentrations for isoprenaline could not be determined as a suitable method could not be established.

Plasma cardiac troponin I concentrations for each chronic treatment group were: sunitinib 3.56 ± 1.13 $ng \cdot mL^{-1}$; imatinib 2.24 ± 0.54 $ng \cdot mL^{-1}$; verapamil 9.16 ± 4.76* $ng \cdot mL^{-1}$; isoprenaline 1.80 ± 0.37 $ng \cdot mL^{-1}$; vehicle 1.42 ± 0.47 $ng \cdot mL^{-1}$ (* indicates $P < 0.05$ compared with vehicle group, Kruskal–Wallis test).

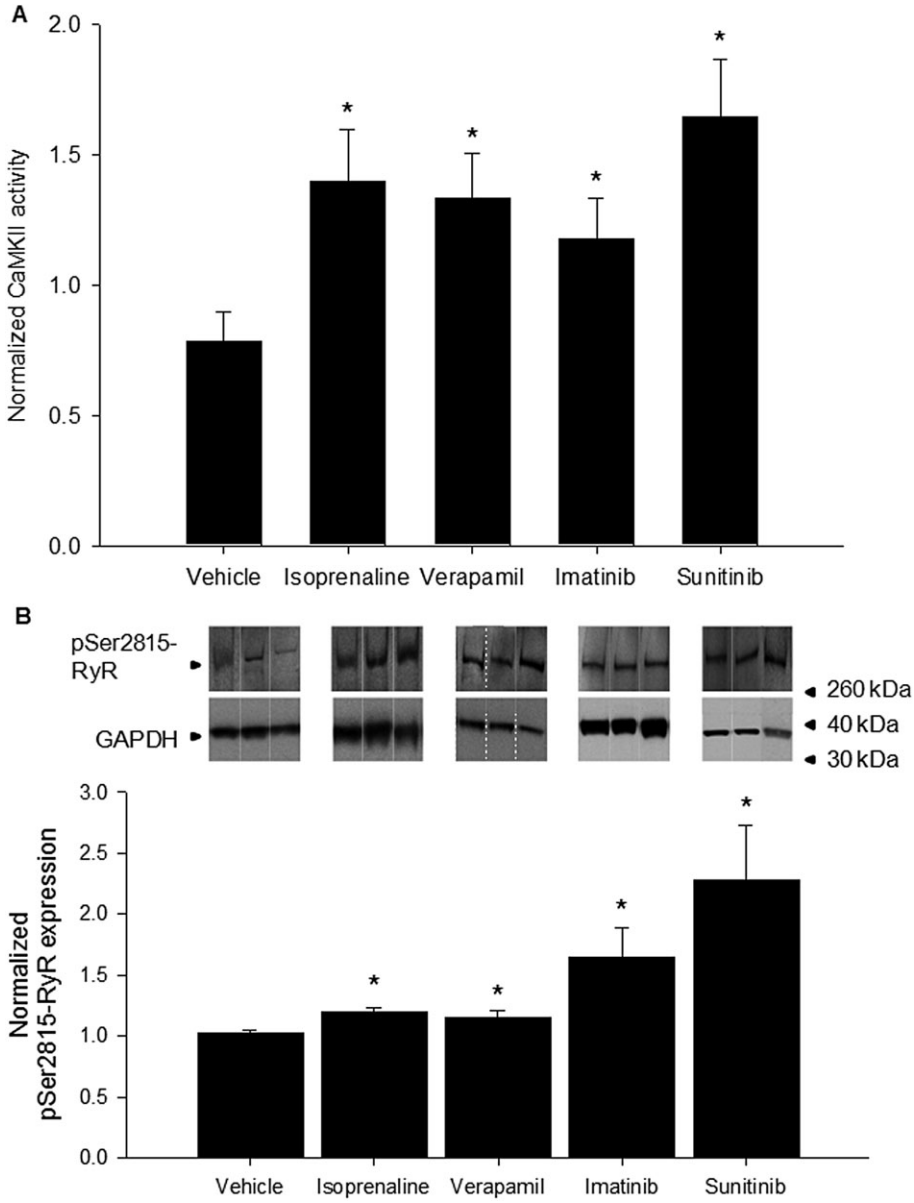

Figure 6

CaMKII activity is significantly increased following chronic sunitinib and imatinib treatment. (A) Histogram showing mean CaMKII activity following chronic *in vivo* drug administration. (B) Immunoblots from three individual experiments showing pSer^{2815}-RyR expression in representative LV homogenates (10 μg total protein) following chronic *in vivo* drug administration. Corresponding mean data for each treatment (expressed as pSer^{2815}RyR:GAPDH and normalized to a control sample included in each blot) are shown in the accompanying histogram. Mean ± SEM, n = 6. *P < 0.05 compared with vehicle group; Kruskal–Wallis test.

Discussion

The central finding of this study is that chronic treatment with anti-cancer kinase inhibitors, sunitinib and imatinib, increased cardiac CaMKIIδ expression and CaMKII activity in guinea pigs in the absence of any overt cardiac dysfunction. As both agents have been associated with cardiotoxicity in the clinic, these changes could reflect early adaptations by the heart to these anti-cancer kinase inhibitors which could precede the onset of contractile dysfunction. We have specifically examined the anti-cancer agents sunitinib and imatinib; however, the potential role of CaMKII in modulating cardiotoxic effects of other anti-cancer therapies should not be overlooked.

Acute drug effects

Acute treatment with sunitinib (3 × 15 min infusions) caused significant haemodynamic effects in anaesthetized guinea pigs. With cumulative infusions, there was a significant decrease in LVdP/dt$_{max}$ as well as significant decreases in both systolic and diastolic BPs. Decreases in BP of such a magnitude would be expected to result in a reflex tachycardia based

on the known baroreflex sensitivity of this model (Mooney *et al.*, 2012); however, sunitinib caused a dose-dependent decrease in heart rate. In the acute studies, a large dose range was explored from an initial dose, expected to yield clinically relevant plasma concentrations, to higher doses that yielded concentrations well in excess of effective concentrations. As sunitinib is a relatively non-selective kinase inhibitor, the cardiovascular effects, observed at supratherapeutic exposures, are likely to be due to actions at other secondary molecular targets, such as tyrosine kinases or GPCRs (Sutent FDA Pharm Review). Previous experiments with sunitinib in rats have shown that it causes a dose-dependent hypertension possibly due to its inhibition of VEGF signalling; however, this effect is only apparent after repeated dosing (Blasi *et al.*, 2012; Isobe *et al.*, 2014).

Overall, these results suggest that acute administration of sunitinib, at high doses, causes a negative chronotropy, and may also reduce inotropy. A similar haemodynamic profile was observed with the calcium channel antagonist verapamil in agreement with previous studies in this model (Marks *et al.*, 2012). In contrast, isoprenaline increased $LVdP/dt_{max}$ and heart rate, in line with its positive inotropic and chronotropic actions, and had no effect on systolic or diastolic arterial BP.

It is well known that $LVdP/dt_{max}$ can be influenced by changes in cardiac loading and heart rate (Wallace *et al.*, 1963; Markert *et al.*, 2012). Previous studies in guinea pigs have shown $LVdP/dt_{max}$ to be positively influenced when mean arterial BP is >80 ± 8 mmHg and negatively influenced when mean arterial BP is <39 ± 3 mmHg (Mooney, 2012). In addition, $LVdP/dt_{max}$ increased with increases in heart rate to peak values of 220–249 bpm, after which $LVdP/dt_{max}$ decreased with further increases in heart rate (Mooney, 2012). In the present study, during the final infusions of both sunitinib and verapamil, mean arterial BP and heart rate decreased below 39 mmHg and 220 bpm respectively. In isoprenaline experiments, mean arterial BP was not significantly altered and heart rate was maintained above 249 bpm. Therefore, it must be borne in mind that the changes in $LVdP/dt_{max}$ induced with sunitinib and verapamil administration may also be a consequence of the concurrent changes in heart rate and/or arterial BP.

Interestingly, when CaMKII was assessed in acute drug-treated hearts, although there was no change in CaMKIIδ protein expression with any of the drug treatments (which is unsurprising over such a short timescale), changes in activity were observed. Specifically, sunitinib caused a significant decrease in CaMKII activity as did verapamil. Acute administration of isoprenaline caused CaMKII activity to increase. This is in agreement with previous studies in rats and mice (Yoo *et al.*, 2009; Grimm and Heller Brown, 2010) but is the first time this relationship has been demonstrated in guinea pigs *in vivo*. Previous work has suggested that a significant element of β-adrenoceptor signalling is mediated via CaMKII and that this contributes to the pathophysiology observed in heart failure (Grimm and Heller Brown, 2010). It may also be the case that drugs already used in the treatment of various cardiomyopathies (such as β-blockers) can target CaMKII albeit non-specifically (Grimm and Heller Brown, 2010; Currie *et al.*, 2011). Given the pivotal role that CaMKII plays in cardiac function and dysfunction and the potential it has as a target for mediating therapeutic effects, it seems likely that it could also be an unwanted secondary target for drugs exhibiting adverse cardiac side effects. This is of interest when examining acute sunitinib treatment where there is evidence for significantly altered CaMKII activity.

Chronic drug effects

Chronic drug administration via mini-pump delivery over 6 days used the same standard inotropes and included imatinib as well as sunitinib. Ideally, it would have been beneficial to include chronic administration of a CaMKII-selective modulator. We had originally planned to include AIP; however, the costs involved in administering this peptide over 6 days in the guinea pig were prohibitive. Chronic selective inhibition of CaMKII has proved feasible in mice where studies have used the CaMKII inhibitor KN-93 or genetic models where the autoinhibitory domain of the kinase is targeted (Zhang *et al.*, 2005; Kaurstad *et al.*, 2012). These studies provide support to the feasibility of chronic and selective targeting of CaMKII.

In the present study, chronic administration of sunitinib and imatinib had no obvious adverse effects on cardiac function and haemodynamics. Larger group sizes may have increased the chance of finding rare cardiotoxic effects, but the guinea pigs used here were healthy with no co-morbidities which may predispose to such effects. Another likely explanation for the absence of effects on cardiac function is related to the duration of treatment which is far short of the weeks/months of treatment given to patients. Other *in vivo* animal studies investigating cardiotoxicity have been performed over longer durations with periods of up to 6 weeks for imatinib (Kerkela *et al.*, 2006; Wolf *et al.*, 2010) and up to 5 weeks for sunitinib (Kerkela *et al.*, 2009); however, it is of interest that in the later study, sunitinib had no effects on cardiac function in the mouse and caused only an apparent loss of cardiomyocytes. In the current study, the effects of imatinib and sunitinib, in particular on CaMKII expression and activity, were highly significant over 6 days of treatment. Despite CaMKII being highlighted in reports reviewing drug-induced cardiotoxicity (Stummann *et al.*, 2009; Zhou, 2010), no previous studies have investigated the effects of imatinib or sunitinib on cardiac contractility and CaMKII expression and/or activity. However, interestingly, a recent study examining the effects of imatinib on rat neonatal cardiomyocytes has reported significant activation of CaMKII activity in response to the anti-cancer drug (Barr *et al.*, 2014). Clinically relevant doses of 2 and 5 μM imatinib were used and, in response to both concentrations, there was significant elevation of Thr^{17} phosphorylation of phospholamban and Ser^{2814} phosphorylation of the RyR – both selective substrates for CaMKII. This resulted in enhanced SR function as well as activation of NFAT signalling. This imatinib-dependent activation of Ca^{2+} handling resulted in pathological hypertrophy and necrotic cell death. These results are particularly pertinent to the current study where along with measurement of CaMKII activity via selective incorporation of phosphate into a specific peptide substrate, we also show evidence for increased $pSer^{2815}$-RyR following chronic imatinib and sunitinib treatment.

In the current study, echocardiography showed significant decreases in FS following chronic isoprenaline treat-

ment. However, there was no increase in heart : body weight ratio and no histopathological effects suggesting that chronic β-adrenoceptor activation had not yet induced heart failure. The decrease in FS was associated with LV dilatation indicated by the significant increase in both LV end-diastolic and end-systolic diameters and also the increase in left ventricular end-diastolic pressure. Decreased contractile performance and increased CaMKII expression and activity are indicative of compromised cardiac function which can ultimately lead to heart failure (Anderson, 2007). Isoprenaline has been administered chronically to mice (Kudej *et al.*, 1997; Friddle *et al.*, 2000) and rats (Kitagawa *et al.*, 1997; Takeshita *et al.*, 2008), but less commonly in guinea pigs (Maisel *et al.*, 1987). In all of these studies, chronic isoprenaline administration caused cardiac hypertrophy. CaMKII expression and activity are known to be increased following chronic β-adrenoceptor stimulation and in hypertrophy and heart failure (Zhang *et al.*, 2003). Taken together, this evidence supports the effects of chronic isoprenaline treatment observed in the current study.

A decrease in FS and $LVdP/dt_{max}$ was observed following administration of verapamil. It was anticipated that chronic verapamil treatment would decrease contractile performance, given the negative inotropic effects of this drug. This has been shown previously in rabbits chronically treated with verapamil (2 $mg \cdot kg^{-1} \cdot day^{-1}$, 28 days) where a significantly lower force of contraction and a depressed contractile response to adrenaline in papillary muscles isolated from verapamil-treated animals were observed (Bosnjak *et al.*, 1991). By blocking Ca^{2+} entry into cardiomyocytes, theoretically, verapamil should result in a decrease in CaMKII activity and this was apparent following acute verapamil treatment. However, chronic verapamil administration caused CaMKIIδ expression and CaMKII activity to increase. Although this was initially unexpected, similar effects of chronic verapamil treatment have been reported elsewhere in the rat (Zhou, 2010). Chronic verapamil treatment (15 $mg \cdot kg^{-1} \cdot day^{-1}$) caused several biochemical and functional changes to Ca^{2+} handling proteins, resulting in altered cardiac function. Of particular relevance to the findings here were the observations of increased CaMKII expression and hyperphosphorylation of RyR. Interestingly, these changes occurred in the absence of cardiac hypertrophy or fibrosis following chronic verapamil treatment (Zhou, 2010). Although plasma cardiac troponin I was significantly increased in the current study, perhaps indicating cardiac damage, histological analysis revealed no morphological abnormalities. Thus, biochemical changes may adversely affect cardiac function, without evidence of structural deficits.

Potential mechanisms involved in the effects on CaMKII

It seems unlikely that imatinib and sunitinib are directly targeting CaMKII as these drugs were designed as kinase *inhibitors* rather than kinase *activators*. It is possible that these drugs affect other signalling molecules up- or downstream of CaMKII which subsequently increase CaMKIIδ expression and CaMKII activity. A potential link lies in the fact that CaMKII can be activated *in vitro* and *in vivo* by reactive oxygen species (ROS) acting via oxidation of $Met^{281/282}$ in the autoregulatory domain (Erickson *et al.*, 2008). Mitochondria are an important source of ROS within most mammalian cells and this source underlies oxidative damage in many disease states (Murphy, 2009). Mitochondrial perturbation and toxicity have been identified as a 'common theme' in drug-induced cardiotoxicity (Force and Kolaja, 2011), and imatinib and sunitinib have been linked to mitochondrial injury and abnormalities in mice, rat and humans (Kerkela *et al.*, 2006; Wolf *et al.*, 2010). In addition, sunitinib increased the generation of ROS *in vitro* (Mellor *et al.*, 2011; Zhuang *et al.*, 2011). More recently, CaMKII activity has been identified as having a central role in the mitochondrial Ca^{2+} entry that precedes myocardial dysfunction and cell death (Joiner *et al.*, 2012). Taken together, these findings suggest CaMKII can be activated by oxidation resulting from ROS, in conjunction with crosstalk and potential feedback to the mitochondria.

It is interesting to note that *in vitro* studies in rat neonatal cardiac myocytes suggested that CaMKII inhibition could at least partly reverse the cardiotoxic effects of imatinib (Barr *et al.*, 2014). Myocytes treated with AIP or infected with a virus containing a dominant negative form of CaMKII showed less imatinib-induced hypertrophic remodelling. In addition, imatinib-induced nuclear translocation of NFAT was blocked by the calcineurin inhibitor FK506, the CaMKII inhibitor AIP and by the L-type Ca^{2+} channel blocker nifedipine. The authors suggest that imatinib increases the pool of cellular Ca^{2+} available for activation of CaMKII and calcineurin-NFAT nuclear translocation which ultimately results in pathological hypertrophy.

In the present study, we aimed to achieve clinically relevant concentrations of the test drugs in the chronic dosing protocol. In general, this was achieved with a mean total plasma concentration of 1.36 and 0.01 μM achieved in the chronic phase for imatinib and sunitinib, respectively, compared with clinically effective concentrations of 2 μM (980 $ng \cdot mL^{-1}$) and 0.06 μM (25 $ng \cdot mL^{-1}$) respectively (Kitagawa *et al.*, 2013). It is worth noting that the concentration of imatinib achieved in our chronic dosing is compatible with the concentration (2 μM) used *in vitro* (Barr *et al.*, 2014) where significant CaMKII activation was observed. The concentration of verapamil achieved was also in the clinical range [0.16 μM vs. 0.23 μM (105 $ng \cdot mL^{-1}$) (Freedman *et al.*, 1981)].

In conclusion, findings presented here show for the first time that sunitinib and imatinib treatments affect CaMKII in the heart with chronic treatments leading to significantly increased CaMKII expression and activity. Given the strong link between chronic CaMKII elevation and cardiovascular dysfunction, this may be a mechanism by which sunitinib and imatinib exert at least part of their cardiotoxic side effects. Further investigation is required to verify the mechanism of action of these kinase inhibitors upon CaMKII.

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Conflict of interest

None.

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