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

L Mooney¹, M Skinner², S. J. Coker¹ and S. Currie^{1*}

¹Strathclyde Institute of Pharmacy & Biomedical Sciences, Hamnett Building, University of Strathclyde, Glasgow G4 0RE, U.K., ²Safety Assessment UK, AstraZeneca R&D, Alderley Park, Macclesfield, SK10 4TG, U.K.

*To whom all correspondence should be addressed

E-mail: susan.currie@strath.ac.uk

Tel: +44 141 548 2405

Fax: +44 141 552 2562

E-mail addresses: m.l.mooney@gmail.com (Laura Mooney); Matthew.Skinner@astrazeneca.com (Matthew Skinner); susan.currie@strath.ac.uk (Susan Currie).

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Background and Purpose

Calcium/calmodulin-dependent protein kinase II δ (CaMKII δ) 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

A guinea pig model was used to assess cardiovascular haemodynamics in response to acute and chronic sunitinib treatment, and chronic imatinib treatment. The effects were compared with known positive and negative inotropes isoprenaline and verapamil. Parallel studies from the same animals assessed CaMKII δ expression and CaMKII activity following drug treatments. Acute administration of sunitinib decreased left ventricular (LV) dP/dt_{max} . Acute administration of isoprenaline increased LV dP/dt_{max} in a dose-dependent manner while LV dP/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. CaMKII δ expression following all acute treatments remained unchanged. Chronic imatinib and sunitinib treatments did not alter fractional shortening; however, both CaMKII δ expression and CaMKII activity were significantly increased. Chronic administration of isoprenaline and verapamil decreased left ventricular fractional shortening with parallel increases in CaMKII δ expression and CaMKII activity.

Conclusions and Implications

Using an integrated approach, this study indicates increased CaMKII δ expression and CaMKII activity occur following chronic sunitinib and imatinib treatment. Given the association of these compounds 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; DBP, diastolic blood pressure; FS, fractional shortening; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LV, left ventricular; LV dP/dt_{max} , maximum rate of rise of left ventricular pressure; LVDD, left ventricular diastolic diameter; LVEDP, left ventricular end diastolic pressure; LVP, left ventricular pressure; LVSD, left ventricular systolic diameter; ROS, reactive oxygen species; RyR, ryanodine receptor; SBP, systolic blood pressure; SR, sarcoplasmic reticulum.

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 are 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 (Maayah et al., 2014; Blasi et al., 2012). 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) whilst in another study 8% of patients developed heart failure following 33.6 weeks median treatment duration (Chu et al., 2007).

Sunitinib has multiple targets including vascular endothelial growth factor 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 recognised 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 cause compromised 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 small molecule 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, CaMKII δ 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, CaMKII δ is recognised 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 et al., 2007). Pathophysiological consequences of increased CaMKII δ activity include a range of effects on cardiac ion channels influencing depolarisation, cardiac excitability and repolarisation of cardiac myocytes (Anderson, 2007). Dysfunctional sarcoplasmic reticulum (SR) Ca²⁺ handling is evident following CaMKII δ -mediated hyperphosphorylation of the SR Ca²⁺ release channel, the ryanodine receptor (RyR), which leads to increased diastolic Ca²⁺ release and a propensity for cardiac arrhythmias (Wehrens et al., 2004). As well as coordinating contractile dysfunction, CaMKII δ 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 has been referred to as a prime candidate for therapeutic targeting (Currie et al., 2011; Anderson, 2007).

The possibility that 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 CaMKII δ is central to cardiac pathophysiology. Previously, we have optimised conditions for studying the acute effects of drugs on indices of cardiac contractility and haemodynamics in an anaesthetised 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 recognised positive and negative inotropes. Applying an integrated approach we have also assessed in parallel, the effects of these drugs on CaMKII δ expression and CaMKII activity in LV tissue taken from the same animals.

Methods

Animals and anaesthesia

All animal experiments 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 fifty male

Dunkin Hartley guinea pigs (440 – 650 g) were purchased from Harlan (Bicester, UK). In acute experiments animals were anaesthetised with fentanyl ($50 \mu\text{g kg}^{-1}$ s.c.) followed by sodium pentobarbital ($50\text{-}60 \text{ mg kg}^{-1}$ i.p.). Throughout haemodynamic assessments anaesthesia was maintained by a continuous i.v. infusion of sodium pentobarbital (12 mg ml^{-1}) at $6 \text{ mg kg}^{-1} \text{ hr}^{-1}$ using an infusion pump. In chronic experiments animals were anaesthetised using a combination of Hypnorm® and Hypnovel® (6 ml kg^{-1} i.p. of a solution containing 1 part Hypnorm (fentanyl citrate 0.315 mg ml^{-1} and fluanisone 10 mg ml^{-1}); 1 part Hypnovel (midazolam HCl 5 mg ml^{-1}) and 2 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 minutes 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).

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™).** If necessary, during the preparation and stabilisation phases, the stroke volume of the pump was adjusted to keep the end-tidal CO_2 value between 35 and 45mmHg and oxygen saturation above 95%. **Blood gas values were kept within a set range: $\text{PO}_2 >70$ and $<110\text{mmHg}$, $\text{PCO}_2 >25$ and $<45\text{mmHg}$.** 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 (LVP) via a 2F or 3F Millar Mikro-tip® catheter pressure transducer (Millar Inc, 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 $\sim 37^\circ\text{C}$ using a heating lamp. All data were recorded continuously and processed using Ponemah P3 Plus software.

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 stabilisation period was allowed. Guinea pigs received either sunitinib ($0.3, 1.0$ and $3 \mu\text{mol kg}^{-1} \text{ min}^{-1}$)($1.8, 6$ and 18 mg kg^{-1}), isoprenaline ($0.1, 0.3, 1.0 \text{ nmol kg}^{-1}$

$^{-1}$ min $^{-1}$) (0.3, 0.9 and 3 μ g kg $^{-1}$), verapamil (14, 42 and 140 nmol kg $^{-1}$ min $^{-1}$)(95, 286, 955 μ g kg $^{-1}$) or vehicle (equal volumes of normal saline; 20, 60, 200 μ l kg $^{-1}$ min $^{-1}$) as continuous i.v. infusions with each dose being infused for 15 min. Following infusion of the final dose animals were euthanised 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 anaesthetised and transthoracic echocardiography was performed using a 13-MHz linear array transducer and HDI® 3000 ultrasound system (ASL Ultrasound). 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 minipump (Alzet® Model 2ML1; Charles River) was implanted subcutaneously at the neck to deliver sunitinib (40 μ mol kg $^{-1}$ day $^{-1}$)(16mg kg $^{-1}$ day $^{-1}$), imatinib (170 μ mol kg $^{-1}$ day $^{-1}$)(84mg kg $^{-1}$ day $^{-1}$), isoprenaline (1.5 μ mol kg $^{-1}$ day $^{-1}$)(317 μ g kg $^{-1}$ day $^{-1}$), verapamil (20 μ mol kg $^{-1}$ day $^{-1}$)(9.1 mg kg $^{-1}$ 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-anaesthetised and echocardiography was repeated. Haemodynamic assessment was then performed as described above. At the end of this animals were euthanised by removal of the heart.

Tissue preparation

The heart was weighed following removal and a transverse section taken through the right and left ventricle and fixed in 10% formalin for subsequently histological analysis. The remaining LV was cut into chunks, snap frozen in liquid nitrogen and stored at -80°C. As required, LV tissue was homogenised in buffer containing 20 mM Tris-HCl, pH7.2, 1 mM DTT, phosphatase inhibitors (0.5 mM calyculin A, 4 mM Na orthovanadate) and protease inhibitors (cocktail set V, EDTA free; Calbiochem). The total protein content was determined using the Coomassie Plus protein assay (Pierce).

Immunoblotting

LV homogenates were subjected to SDS-PAGE using the NuPAGE system (Invitrogen); 10% Bis-Tris gels for CaMKII and 3-8% Tris-Acetate gels for RyR. Antibodies were as follows: Custom made polyclonal antibody (rabbit IgG) against the C terminus of CaMKII δ (1:5000, Eurogentec), 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-horseradish peroxidase

conjugate (Sigma and GE Healthcare) were 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 normalisation. CaMKII:GAPDH and pSer2815-RyR:GAPDH ratios were calculated for each sample and these were then normalised to an untreated sample (aliquots from the same preparation) included in every gel to allow inter-gel normalisation. The latter normalisation 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, autocalmitide-2 as described previously (Anthony et al., 2007). Controls included homogenates measured in the absence of Ca^{2+} (5mM EGTA), absence of substrate, and inclusion of autocalmitide inhibitory peptide (AIP), a recognised CaMKII inhibitor. Individual activity values were converted to pmol of phosphate incorporated $\text{min}^{-1} \mu\text{g protein}^{-1}$ and normalised 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 3000 rpm 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).

Drugs

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. Sunitinib and imatinib were purchased from Sequoia Research Products Ltd. All drugs were dissolved in 0.9% w/v NaCl for acute administration or 0.1% acidified saline for chronic administration.

Data Analysis

Results are presented as mean \pm 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 CaMKII δ expression, CaMKII activity, pSer2815-RyR expression and cardiac troponin I concentrations among groups because some of the data were not distributed normally.

Results

Acute sunitinib treatment caused a decrease in LVdP/dt_{max} that was accompanied by a fall in heart rate and blood pressure

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 BP, isoprenaline did not alter either parameter significantly (Figure 2B and C). Acute treatment with vehicle had no significant effects on any of the parameters.

CaMKII activity was significantly reduced following acute sunitinib treatment

Quantitative immunoblotting for CaMKII δ 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 μ M and 10 μ M), in the absence of CaMKII peptide substrate and in the presence of 5 mM EGTA to remove extracellular Ca²⁺. 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, blood pressure or fractional shortening

Echocardiography was used to assess left ventricular diameter and fractional shortening (FS) before and after chronic drug administration. A short-axis view of the guinea pig heart is shown in Fig. 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 which 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.**

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

Expression of CaMKII δ protein increased in response to chronic treatment in all drug groups compared to 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 the CaMKII-selective phosphorylation site on RyR, serine 2815 (pSer2815-

RyR), was used as a second assessment of CaMKII activity. All chronic treatments resulted in an increase in pSer2815-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 pSer2815-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 $25.4 \pm 8.3 \mu\text{M}$ and verapamil $7.8 \pm 2.9 \mu\text{M}$. In the chronic dosing groups, total plasma drug concentrations, respectively, were: sunitinib $0.01 \pm 0.002 \mu\text{M}$ (clinically relevant concentration is $0.06\mu\text{M}$); imatinib $1.36 \pm 0.38 \mu\text{M}$ (clinically relevant concentration is $2\mu\text{M}$); verapamil $0.16 \pm 0.02 \mu\text{M}$. 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 $3.56 \pm 1.13 \text{ ng ml}^{-1}$; imatinib $2.24 \pm 0.54 \text{ ng ml}^{-1}$; verapamil $9.16 \pm 4.76^* \text{ ng ml}^{-1}$; isoprenaline $1.80 \pm 0.37 \text{ ng ml}^{-1}$; vehicle $1.42 \pm 0.47 \text{ ng ml}^{-1}$ (* indicates $P < 0.05$ compared to 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 CaMKII δ expression and CaMKII activity in guinea pigs in the absence of any overt cardiac dysfunction. Since 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 x 15 min infusions) caused significant haemodynamic effects in anaesthetised guinea pigs. With cumulative infusions there was a significant decrease in $\text{LVdP/dt}_{\text{max}}$ as well as significant decreases in both systolic and diastolic BP. Decreases in blood pressure 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 efficacious concentrations. Since sunitinib is a relatively non-selective kinase inhibitor, the cardiovascular effects, observed at suprathreshold exposures, are likely due to actions at other secondary molecular targets (eg 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 (Isobe et al., 2014, Blasi et al., 2012).

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 $>80 \pm 8$ mmHg and negatively influenced when mean arterial BP is $<39 \pm 3$ mmHg (Mooney, 2012). In addition, LVdP/dt_{max} increased with increases in heart rate to peak values of 220-249 beats min⁻¹, 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 220bpm, respectively. In isoprenaline experiments mean arterial BP was not significantly altered and heart rate was maintained above 249 beats min⁻¹. 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 from acute drug-treated hearts, although there was no change in CaMKII δ protein expression with any of the drug treatments (which is unsurprising over such a short time scale), 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 & 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 -adrenergic signalling is mediated via CaMKII and that this contributes to the pathophysiology observed in heart failure (Grimm & 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 (Currie et al., 2011; Grimm & Brown 2010). 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 administered the CaMKII inhibitor KN-93 or used 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 capability 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 (Zhou et al., 2010; Stumman et al., 2009), 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 μ M and 5 μ M imatinib were used and, in response to both concentrations, there was significant elevation of Thr17 phosphorylation of phospholamban and Ser

2814 phosphorylation of the ryanodine receptor – both selective substrates for CaMKII. This resulted in enhanced sarcoplasmic reticulum function as well as activation of NFAT (nuclear factor of activated T cells) signalling. This imatinib-dependent activation of Ca²⁺-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 pSer2815-RyR following chronic imatinib and sunitinib treatment.

In the current study echocardiography assessment showed significant decreases in FS following chronic isoprenaline treatment; 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 left ventricular (LV) dilatation indicated by the significant increase in both LV end-diastolic and end-systolic diameter and also the increase in LVEDP. Decreased contractile performance and increased CaMKII expression and activity are indicative of compromised cardiac function which can ultimately lead to heart failure (Anderson et al., 2007). Isoprenaline has been administered chronically in several studies in mice (Kudej et al., 1997; Friddle et al., 2000) and rat (Kitagawa et al., 1997, Takeshita et al., 2008), but less commonly in guinea pig (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 fractional shortening 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 kg⁻¹ day⁻¹, 28 days) where a significantly lower force of contraction and a depressed contractile response to epinephrine in papillary muscles isolated from verapamil- treated animals was observed (Bosnjak et al., 1991). By blocking Ca²⁺ 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 CaMKII δ 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 kg⁻¹ day⁻¹) caused several biochemical and functional changes to Ca²⁺ 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 result in adverse effects on cardiac function without evidence of structural deficits.

Potential mechanisms involved in 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 CaMKII δ 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 Met281/282 in the autoregulatory domain (Erickson et al., 2008). Mitochondria are an important source of ROS within most mammalian cells, and this 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 et al., 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 Ca²⁺ 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 cross-talk and potential feedback to the mitochondria.

It is interesting to note that in vitro studies in rat neonatal cardiac myocytes suggest CaMKII inhibition can at least partly reverse the cardiotoxic effects of imatinib (Barr et al., 2014). Myocytes treated with AIP or infected with a virus containing a dn-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 Ca²⁺ channel blocker Nifedipine. The authors suggest that imatinib increases the pool of cellular Ca²⁺ 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 μ M and 0.01 μ M achieved in the chronic phase for imatinib and sunitinib, respectively,

compared to clinically efficacious concentrations of 2 μM (980 ng/mL) and 0.06 μM (25 ng/mL), respectively (Kitagawa et al., 2013). It is worth noting that the concentration of imatinib achieved in our chronic dosing is in line 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.mL) (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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Table 1. Haemodynamic measurements following chronic drug administration.

	LVdP/dt_{max} (mmHg s ⁻¹)	LVEDP (mmHg)	Heart rate (beats min ⁻¹)	SBP (mmHg)	DBP (mmHg)	H:BW Ratio (x1000)
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 blood pressure; LVdP/dt_{max}, left ventricular dP/dt_{max}; LVEDP, left ventricular end diastolic pressure; SBP, systolic blood pressure; H:BW ratio, heart:body weight ratio (multiplied by 1000). Mean ± S.E.M; n=6. *p<0.05 compared to vehicle group, one way ANOVA plus Dunnett's test.

Figure Legends

Figure 1. Left ventricular (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 \pm SEM; $n=4$. * $p<0.05$ compared to value within group at time 0 min, Kruskal-Wallis test.

Figure 2. Heart rate and arterial blood pressure are decreased following acute sunitinib administration. Heart rate (A), systolic blood pressure (BP) (B), and diastolic (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 (beats min^{-1}), isoprenaline 266 ± 6 , verapamil 284 ± 9 , sunitinib 238 ± 7 , vehicle 267 ± 16 ; Systolic blood pressure (mmHg), isoprenaline 62 ± 7 , verapamil 59 ± 1 , sunitinib 56 ± 2 , vehicle 57 ± 3 . Diastolic blood pressure (mmHg), isoprenaline 39 ± 5 , verapamil 33 ± 2 , sunitinib 39 ± 2 , vehicle 33 ± 2 . Mean \pm SEM; $n=4$. * $p<0.05$ compared to value within group at time 0 min, Kruskal-Wallis test.

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

Figure 4. Chronic administration of sunitinib and imatinib do not significantly alter left ventricular contractility. (A) Echocardiography was used to calculate % fractional shortening (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), LV systolic diameter (LVSD) and LV diastolic diameter (LVDD). (B) LV diastolic diameter (LVDD), (C) LV systolic diameter (LVSD) and (D) Left ventricular (LV) fractional shortening (%FS) are shown for pre and post chronic drug administration. Mean \pm SEM; n = 6. *p<0.05 compared to pre-drug value, paired t-test.

Figure 5. Chronic administration of either sunitinib or imatinib causes a significant increase in CaMKII δ expression. Representative signals for CaMKII δ and GAPDH from three individual blots are shown for each treatment (6 μ g total protein) as indicated. Corresponding mean data for each treatment (calculated as CaMKII δ :GAPDH and normalised to a control sample included in each blot) is shown below in the accompanying histogram. Mean \pm SEM; n = 6. *p<0.05 compared to value 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 pSer2815-RyR expression in representative LV homogenates (10 μ g total protein) following chronic in vivo drug administration. Corresponding mean data for each treatment (expressed as pSer2815RyR:GAPDH and normalised to a control sample included in each blot) is shown in the accompanying histogram. Mean \pm SEM, n = 6. *p<0.05 compared to vehicle group, Kruskal-Wallis test.

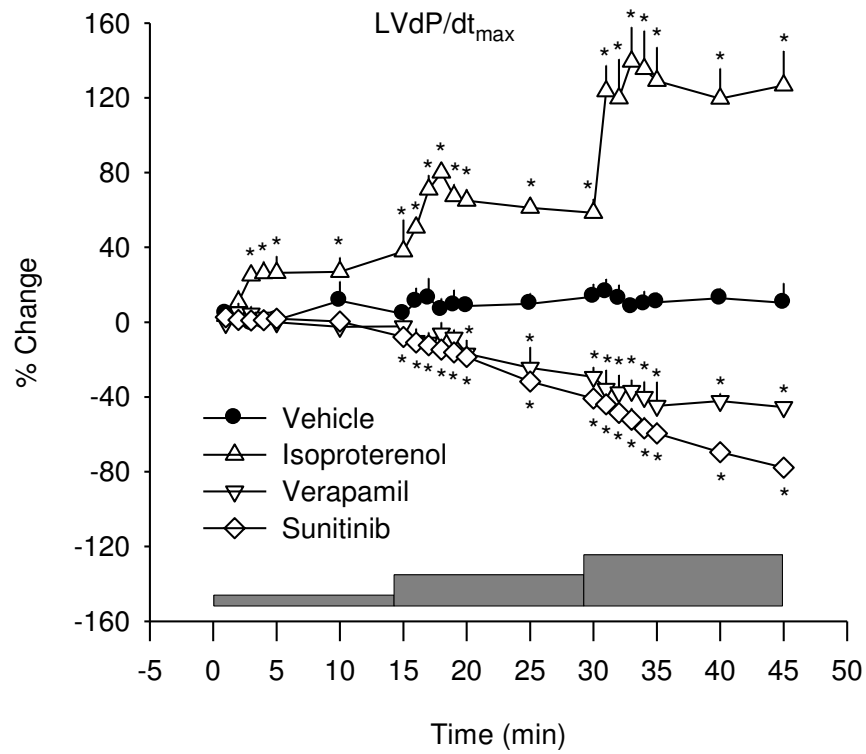


Figure 1

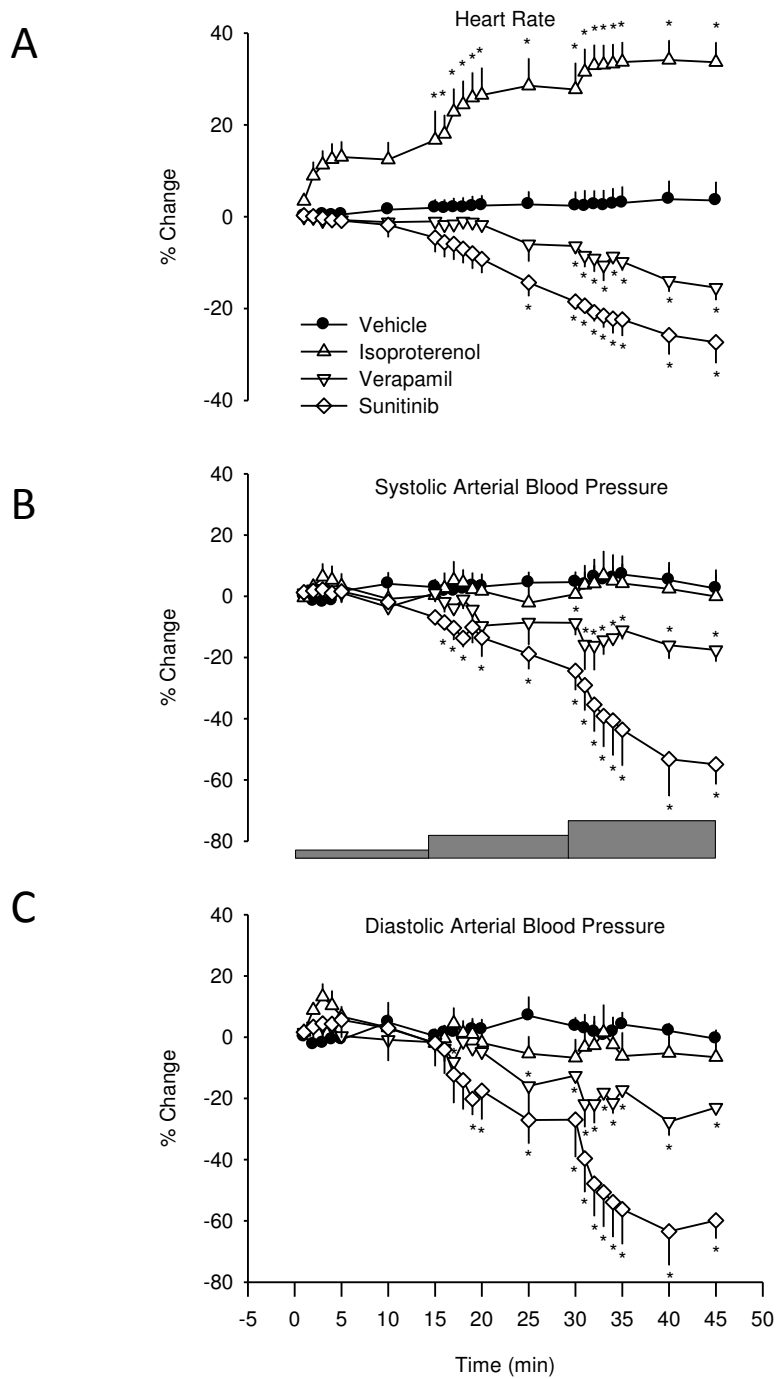


Figure 2

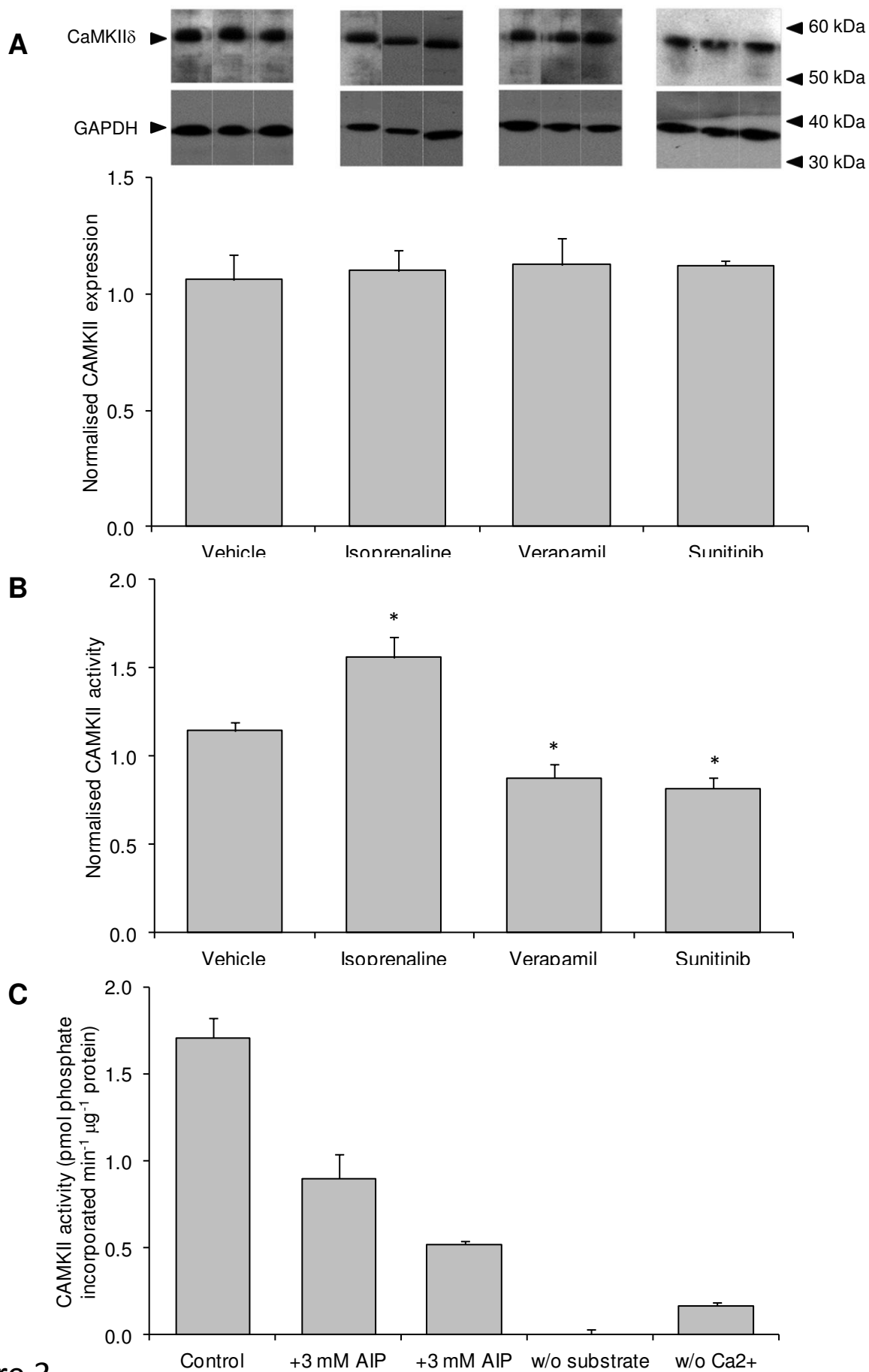


Figure 3

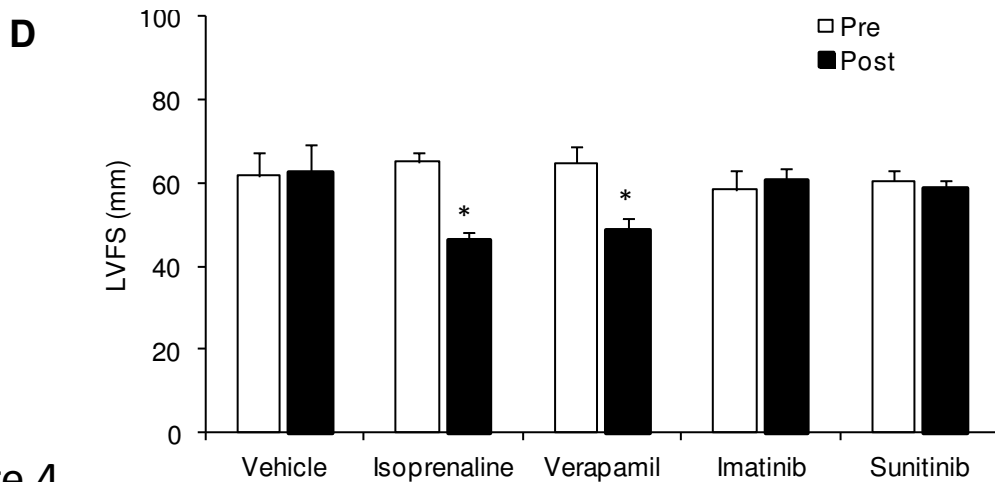
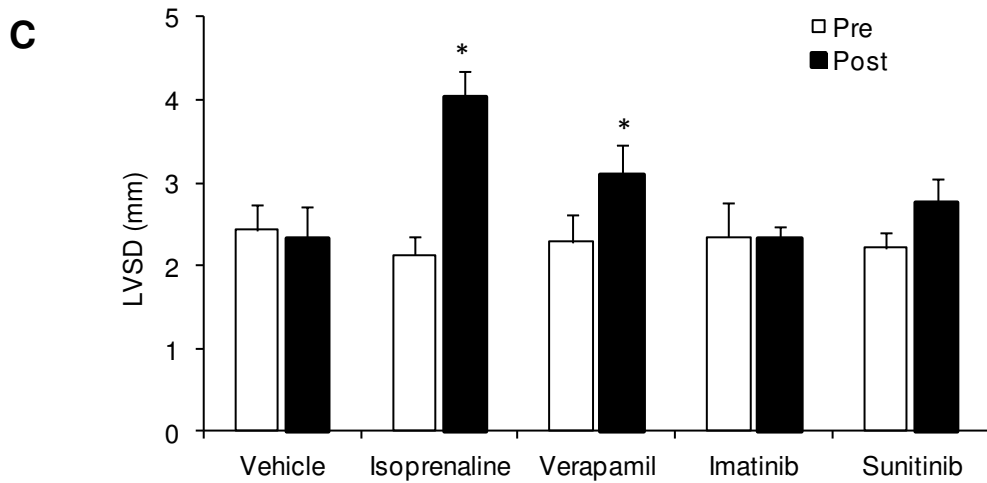
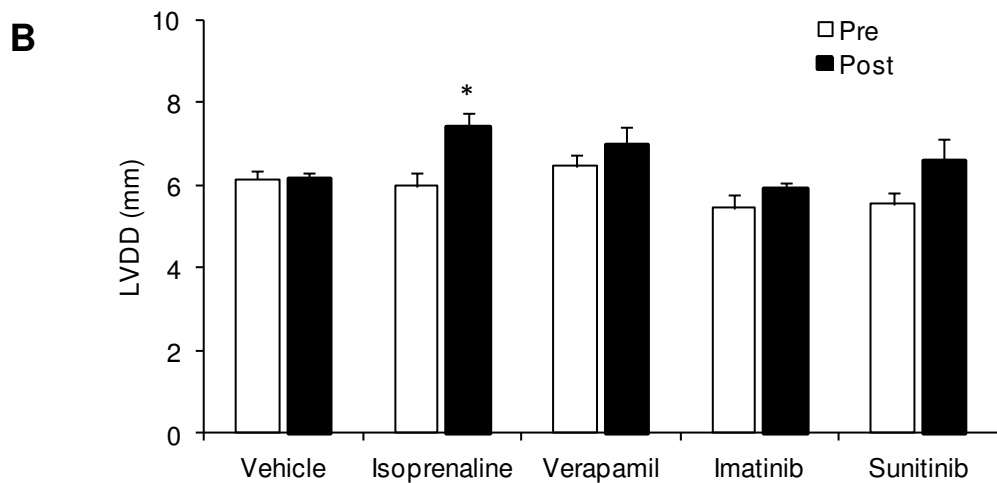
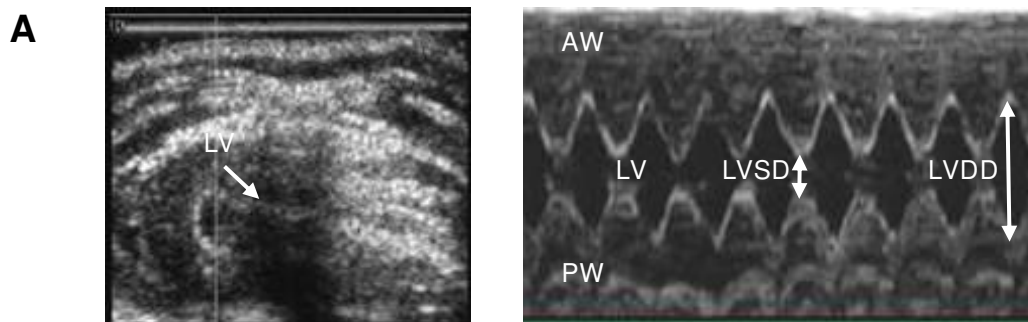


Figure 4

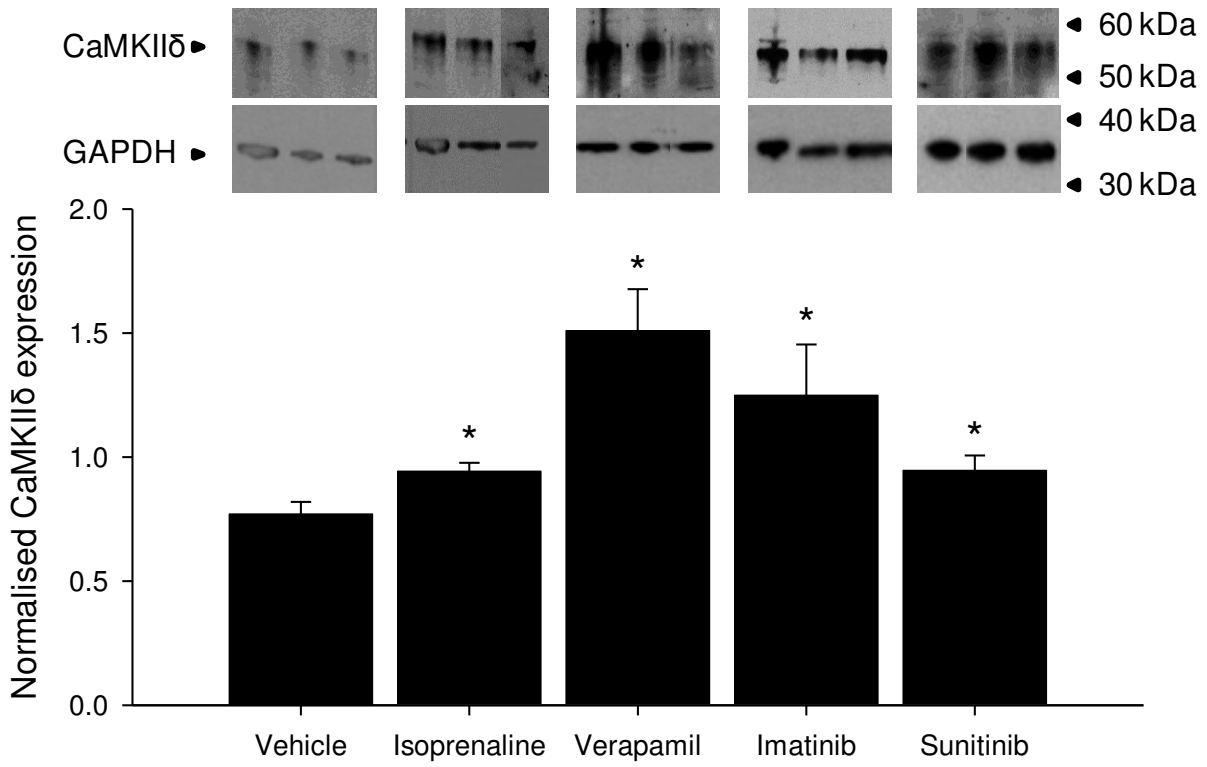


Figure 5

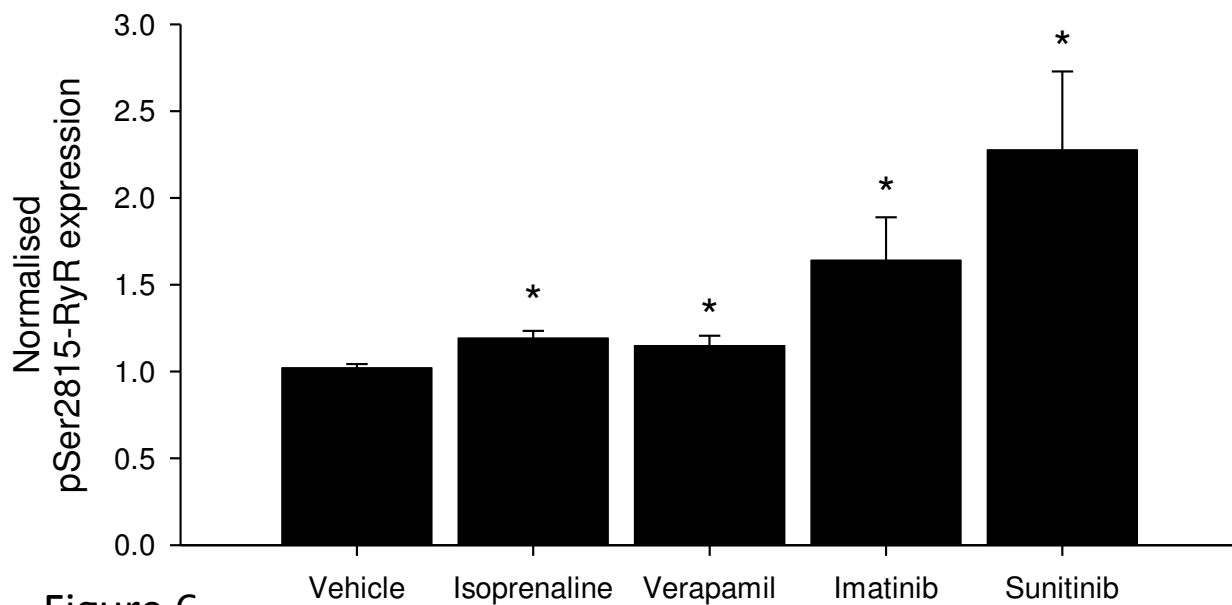
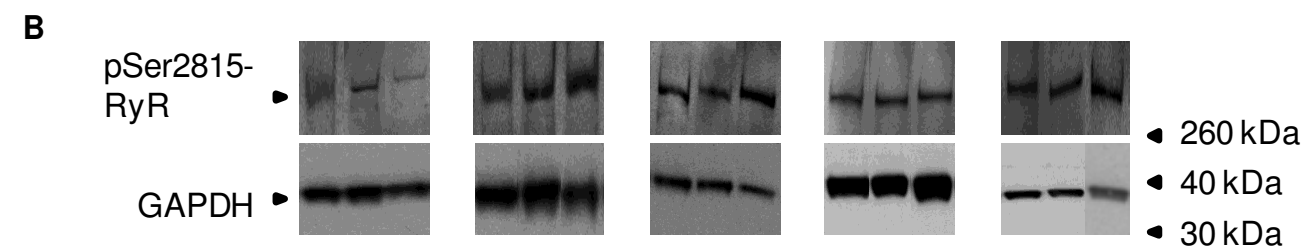
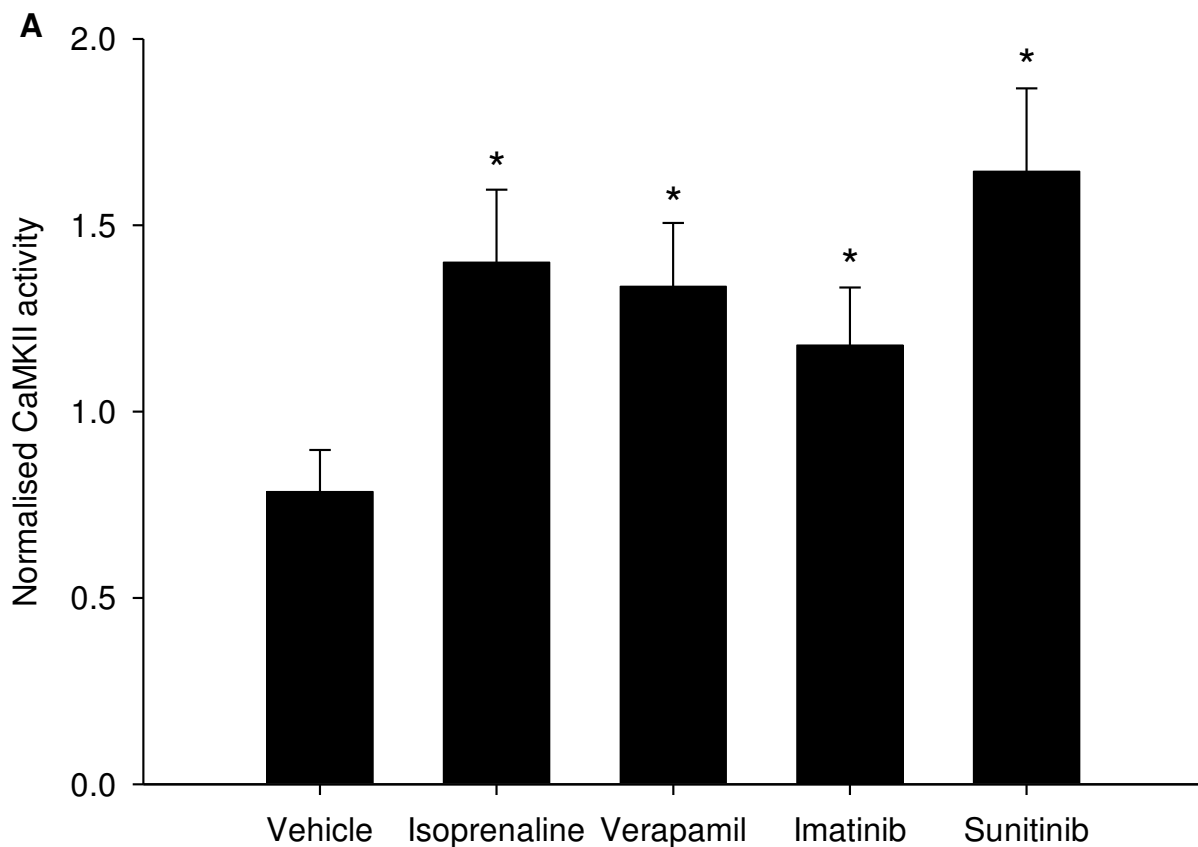


Figure 6