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Title:

Metformin protects against sunitinib-induced cardiotoxicity: Investigating the role of AMPK

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Abstract

Sunitinib is associated with cardiotoxicity through inhibition of AMP-protein kinase (AMPK) signalling. In contrast, the common anti-diabetic agent metformin has demonstrated cardioprotection via indirect AMPK activation. Here we investigate the effects of metformin during sunitinib-induced cytotoxicity. Left ventricular developed pressure (LVDP), coronary flow (CF), heart rate (HR) and infarct size was measured in Langendorff perfused rat hearts treated with 1 µM sunitinib ± 50 µM metformin ± 1 µM human equilibrative nucleoside transporter inhibitor S-(4-Nitrobenzyl)-6-thionosine (NBTI). Western blot analysis was carried out for p-AMPKα levels. Primary isolated cardiac myocytes from the left ventricular tissue were used to measure live cell population levels. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess adjunctive treatment of and metformin in human hepatoma G2 (HepG2) and promyelocytic leukaemia (HL-60) cells treated with 0.1-100 µM sunitinib ± 50 µM metformin. In the perfused hearts co-administration of metformin attenuated the sunitinib-induced changes to LVDP, infarct size and cardiac myocyte population. Western blot analysis revealed a significant decrease in p-AMPKα during sunitinib treatment, which was attenuated following co-administration with metformin. All metformin-induced effects were attenuated NBTI was co-administered. The MTT assay demonstrated an increase in the EC_{50}-value during co-administration of metformin with sunitinib compared to sunitinib mono-therapy in HepG2 and HL-60 cell lines, demonstrating the impact and complexity of metformin co-administration and the possible role of AMPK signalling**.** This study highlights the novel cardioprotective properties of metformin and AMPK activation during sunitinib-induced cardiotoxicity when administered together in the Langendorff heart model.

Keywords: Sunitinib, Metformin, AMP-protein kinase, Cardiotoxicity, Cardioprotection, Cardiac myocytes.

1 Introduction

The multi-TKI sunitinib is used for the treatment of various cancers, including renal cell carcinoma, and as a second-line treatment for advanced gastrointestinal stromal tumour following imatinib resistance (1). However, the off-target effect of sunitinib can lead to serious cardiac dysfunction, such as hypertension, asymptomatic QT prolongation, reduction in left ventricular ejection fraction, acute coronary syndrome, myocardial infarction, and symptomatic congestive heart failure (2-5). The occurrence of congestive heart failure, myocardial injury, or cardiac death has been shown to be as high as 11 % in metastatic gastrointestinal stromal tumour patients treated with sunitinib (2).

Sunitinib-associated cardiac dysfunction is attributed to alterations in mitochondrial structure, including the induction of apoptosis and mitochondrial permeability transition pore (mPTP) opening, all of which contribute towards cardiac myocyte death and the induction of ischaemia (2, 6). Energy metabolism perturbations during pathological states such as ischaemia and cardiac hypertrophy activate the AMPK pathway inhibiting ATP-consuming anabolic pathways to conserve ATP and increase ADP levels (7-9), however, during sunitinib treatment the off-target AMPK signalling pathway is inhibited (4).

In the clinic metformin is used for the treatment of type 2 diabetes (10). Intracellularly metformin is known to inhibit the mitochondrial respiratory chain complex 1 in hepatocytes, which leads to a reduced cellular energy charge and an increase in the AMP/ATP ratio (11). This results in the activation of the AMPK pathway to facilitate glucose uptake in skeletal muscles, hepatocyte glucose production attenuation, and an increased level of free fatty acids oxidation (12, 13). Non-clinical and clinical studies have linked metformin with cardioprotective properties (14-16). The United Kingdom Prospective Diabetes study group showed that type 2 diabetes patients co-treated with metformin in addition to sulphonylurea had a 39% lower risk of developing myocardial infarction compared with conventional sulphonylurea mono-treatment (16). Multiple murine studies have shown that metformin administration, either before the ischaemic insult or at the moment of reperfusion, dramatically reduces the myocardial injury by activating the AMPK pathway, endothelial nitric oxide

synthase, and elevation of adenosine receptor stimulation (17-23). Moreover, the pre-conditioning effects of metformin involving AMPK activation further demonstrated that AMPK acts to regulate sarcolemmal K_{ATP} channels, indirectly of pathways associated with mitochondrial membrane potential (24, 25).

It is important to investigate if metformin offers cardioprotection during sunitinib-induced cardiotoxicity, and the role of AMPK during the metformin-induced cardioprotection in the whole heart, in cardiac myocytes, and at an intracellular level. Further to this, it is also imperative to investigate if metformin jeopardises the anti-proliferative properties of sunitinib in relevant cancer cell lines.

2 Materials and Methods

2.1 Compliance with ethical standards

Experiments were approved by Coventry University's ethics review board (project licence number P43367) according to Coventry University Policy and Standards (Animal Research: Reporting of *In Vivo* Experiments guidelines), which follows the United Kingdom Home office Guide on the Operation of the Animals (Scientific Procedures) Act 1986. Male Sprague–Dawley (SD) rats were used throughout this study due to general purpose model and based on previous worked carried out at Coventry University (26-28), and the housing and husbandry of the animals were followed as described previously by Andrag and Curtis (29). Studies of the animals were reported in compliance with the Animal Research: Reporting of *In Vivo* Experiments guidelines (30, 31). A total of 36 animals were used for the Langendorff and triphenyl tetrazolium chloride (TTC) experimental model, 49 for the western blot analysis and 7 for the cardiac myocyte experimental model

2.2 Heart isolation prior to Langendorff and cardiac myocyte isolation

For the Langendorff perfusion (n=6) and cardiac myocyte isolation (n=7) experiments male 2-3 month old SD rats (Charles River laboratories, UK), weighing between 345-375 g, were selected at

random for each experimental group (i.e. vehicle, sunitinib, metformin, NBTI, sunitinib + metformin, and sunitinib + metformin + NBTI) (Sigma Aldrich, UK).

The rats were sacrificed by cerebral dislocation and their hearts were rapidly isolated and placed into ice cold 4^{o}C Krebs-Henseleit (KH) bicarbonate buffer (NaCl 118.5 mM, $NaHCO_3$ 25.0 mM, KCL 4.8 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $CaCl_2$ 1.7 mM, glucose 11 mM, continuously gassed with 95% O_2 and 5% CO_2 and maintained at pH 7.4±0.2 and 37±1^{o}C). Hearts were immediately mounted onto the Langendorff perfusion system and cannulated via the aorta with a 1.4 mm inner diameter cannula, whilst retrogradely being perfused with KH buffer. The left atrium was trimmed away, and the latex isovolumic balloon was carefully introduced into the left ventricle and inflated up to 5–10 mmHg end diastolic pressure. *Ex-vivo* perfused rat hearts were equilibrated and stabilised for 20 min prior to starting the experiment. Functional recordings were measured using the physiological pressure transducer connected to a Bridge Amp and a PowerLab™ system (ADInstruments, UK).

2.3 Langendorff perfusion protocol

Sunitinib was administered at a concentration of 1 µM in accordance with steady state blood concentrations of sunitinib corresponding to 50 ng·ml^{-1} in patients (1, 32). During previous work carried out by our team a concentration of 1 µM sunitinib have shown to induce cardiotoxicity in Langendorff perfused rat heart (26-28). Metformin was administered at a concentration of 50 µM in agreement to previous studies looking at the cardioprotective properties of metformin (17, 21). NBTI was administered at a concentration of 1 µM as previously studies using this concentration of NBTI had shown to inhibit the facilitated diffusion of adenosine through the human equilibrative nucleoside transporter, thus preventing the stimulation of the adenosine receptor to provide cardioprotection through the activation of AMPK (33). All drugs were dissolved in dimethyl-sulfoxide (DMSO) and sterile filtered using 5 µm pore size filters ensuring that the final DMSO concentration was <0.1 % in the perfusate KH bicarbonate buffer, as previously studies have shown that there are no changes in

contractility in cardiac *ex-vivo* experiments at this volume of DMSO in the KH bicarbonate buffer (26-28).

Hearts were perfused with at a constant pressure generated by the height of the perfusion column. Hearts were stabilised with KH bicarbonate buffer for 20 min and excluded if pre-determined exclusion criteria were met (i.e. CF: < 10 ml/min and > 28 ml/min; HR: < 70 beats/min and > 400 beats/min; LVDP: < 70 mmHg and > 130 mmHg) (34). LVDP, CF, and HR were measured at regular intervals of 5 min for the first 20 min during stabilisation, intervals of 5 min for 35 min during initial drug perfusion, and afterward at 15 min intervals for the remainder of 120 min drug perfusion (i.e. a 20 min stabilisation followed by a total of 155 min drug perfusion). During this study 1 µM Sunitinib ± 50 µM Metformin ± 1 µM adenosine transporter inhibitor NBTI was administered through the KH bicarbonate buffer perfusate following 20 min of stabilisation for each heart during the 155 min drug perfusion. Control Langendorff hearts were perfused with vehicle. Following the Langendorff perfusion experiment the left ventricular tissue was dissected free and the tissue was divided into two portions for either TTC staining or Western blotting analysis.

2.4 TTC staining

Following Langendorff perfusion the hearts were dismounted, weighed, snap-frozen in liquid nitrogen, and stored at -20^{o}C. Frozen hearts were sliced into 2 mm thick transverse sections before being incubated in TTC solution (1% concentration in phosphate buffer NaH_2PO_4 100 mM and Na_2HPO_4 100 mM), at 37±1^{o}C for 10–12 min and fixed in 10% formalin for a minimum of 4 h. The heart slices were monitored for discolouration and identity of the treatment group was kept anonymous to reduce error and bias. Risk zone and infarct area were traced onto acetate sheets. Computerised software ImageJ 1.52 (National Institutes of Health, USA) was used to analyse the percentage of infarct tissue. Infarct size was normalised to the total area of each heart slice.

2.5 Western blotting

Following Langendorff perfusion the hearts were dismounted, and the left atrium coronary organ tissue was dissected, snap-frozen in liquid nitrogen, and stored in -80^{o}C. The tissue was homogenised in Protein Lysis Buffer (tris-base, EDTA, SDS, NaCl, Sodium Pyruvate, NaF, β-glycophosphate, protease inhibitor cocktail tablet and phosphostop tablet). A total of 60 µg of protein concentration was determined using Pierce™ BCA Protein Assay Kit (Thermo Scientific, UK). Samples were mixed 1:1 with Laemmli buffer (tris-HCl, pH 6.8, SDS, glycerol, β-mercaptoethanol, and bromophenol blue ultra) and heated to 100^{o}C. Samples were run using Mini-PROTEAN® TGX stain-free Gel™ (BioRad, UK), and were membrane transferred via electrophoresis with the Trans-Blot® Turbo™ Transfer (BioRad, UK). Immunoblots were incubated with 5 % bovine serum albumin powder in x1 concentration tris-buffered saline solution supplemented with tween for a 10 % tween concentration. Immunoblots were analysed using primary antibodies phosphorylated AMPKα (1:1000 dilution, p-AMPKα, Thr^{172}, #2531, RRID: AB_330330, Cell Signalling Technologies, UK), and GAPDH (1:1000 dilution, D16H11, #5174, RRID:AB_10622025, Cell Signalling Technologies, UK) for overnight incubation in 4^{o}C followed by incubation with secondary antibody anti-rabbit IgG (1:1000 dilution, #7074, RRID: AB_2099233, Cell Signalling Technologies, UK). Chemiluminescence detection kit SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific, UK) was used to visualise membranes. Images were taken using ChemiDoc™ System (BioRad, UK). Density was analysed with the software Quantity One® (BioRad, UK). Analysis of p-AMPKα protein receptor bands was normalised to GAPDH and quantified using ImageJ 1.52 (National Institutes of Health, USA). Bands were analysed for the following treatment groups; vehicle (n=7), metformin (n=7), sunitinib (n=6), metformin + sunitinib (n=7), NBTI (n=7) and metformin + sunitinib + NBTI (n=7).

2.6 Cardiac myocyte isolation and trypan blue staining

Rat left ventricular cardiac myocytes were isolated by conventional enzymatic dissociation. Following cerebral dislocation the rat hearts were immediately isolated and mounted onto a modified Langendorff apparatus. Hearts were perfused with *modified* KH bicarbonate buffer (116 mM NaCl,

5.4 mM KCl, 0.4 mM $MgSO_4 \cdot 7H2O$, 10 mM Glucose, 20 mM Taurine, 5 mM Pyruvate, 2.4 mM $NaHCO_3$ and 12 mM KH_2PO_4, continuously gassed with 95% O2 and 5% CO2 and maintained at pH 7.4±0.2 and 37±1ºC) for stabilisation followed by perfusion with collagenase buffer (pH 7.4±0.2, 1 $mg \cdot mL^{-1}$ Type II collagenase powder, 1 M $CaCl_2$) in *modified* KH buffer. The effluent was collected and reused following perfusion, the rat hearts were dismounted, and the atrium was cut away following completion of cardiac myocyte isolation. Left ventricular tissue was manually dissociated in collagenase buffer. Following centrifugation and removal of supernatant the pellet was redistributed in restoration buffer prepared in *modified* KH bicarbonate buffer at pH 7.4±0.2, containing 1% Bovine Serum Albumin, 1% pen-strep, creatine, and 100 mM $CaCl_2$. The calcium concentration was gradually brought back to 1.25 mM and the myocyte viability was assessed under light microscopy. Cardiac myocytes were seeded and incubated for 4 h in 37±1ºC with vehicle, 1 µM sunitinib ± 50 µM metformin ± 1 µM NBTI prior to undergoing trypan blue (0.4% $w \cdot v^{-1}$ in 1x PBS filtered, 1:1 dilution, 2x concentration) staining for assessment of live cell population counts.

2.7 Cancer cell lines experimental protocol

HepG2 (n=6) and HL-60 (n=6) cells were investigated for assessing cell metabolic activity with nicotinamide adenine dinucleotide phosphate dependent cellular oxidoreductase enzymes to reduce tetrazolium dye MTT to the insoluble formazin product. Experiments were performed using HepG2 (RRID: CVCL_0027) and HL-60 (CVCL_0002) cell lines acquired from The American Type Culture Centre and brought up from at Coventry University in accordance with Coventry University ethical approval procedures. Sunitinib is used to treat leukaemia by inhibiting the overactive BCR-ABL tyrosine kinase detected during acute myeloid leukaemia (35), therefore the HL-60 cell line was deemed as a suitable model to investigate the cytotoxic properties of sunitinib. Moreover HepG2 cell line was deemed suitable for a comparison study to investigate the potential anti-proliferative properties with metformin in the treatment of liver carcinoma but also in alignment with studies demonstrating sunitinib for treatment of hepatocellular carcinoma (36). HepG2 and HL-60 cell lines were incubated with 0.1-100 µM Sunitinib ± 50 µM Metformin for 24 h (37 ºC, 5% CO_2) prior to the

media being replaced with MTT solution (concentration 5 $mg \cdot mL^{-1}$ in sterile x1 concentration PBS) for 6 h. Media containing MTT solution was aspirated and replaced with DMSO prior to plates being read on a microtiter plate reader at 595 nm (reference 690 nm). Metformin mono-treatment in concentrations of 6 µM – 1 mM in HepG2 and 30 µM – 1 mM in HL-60 cells was also investigated.

2.8 Data and statistical analysis

Data are presented as means ± SEM. Statistical comparisons of a single variable was analysed by one way ANOVA with LSD post hoc test between vehicle ± sunitinib ± metformin ± NBTI using GraphPad Prism. Values of $p < 0.05$ were considered statistically significant. In the cancer cell lines the EC_{50}-values of the sunitinib ± metformin dose-response curves were determined using GraphPad Prism.

2.9 Drugs and materials

Sunitinib malate (PZ0012-25 mg), Metformin hydrochloride (PHR1084-500 mg), and S-(4-Nitrobenzyl)-6-thionosine (NBTI) (N2255-100 mg) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (M2128-250 mg) were purchased from Sigma Aldrich (Merck), UK. Phospho-AMPKα (Thr172) (#2531, RRID:AB_330330), Total AMPKα (Cat:#2532, RRID:AB_330331), GAPDH (Cat:#5174, RRID:AB_10622025) and anti-rabbit IgG HRP-linked (Cat:#7074, RRID:AB_2099233) were purchased from Cell Signalling Technologies, UK. The western blots kits Mini-PROTEAN® TGX stain-free GelTM and Trans-Blot® TurboTM Transfer were purchased from BioRad, UK, while PiercetTM BCA Protein Assay Kit and SuperSignalTM West Femto Maximum Sensitivity Substrate were from Thermo Fisher Scientific, UK.

3 Results

3.1 Metformin co-administration attenuates sunitinib-induced changes in LVDP through AMPK in the perfused heart

Measurements of LVDP, CF, and HR were carried out to investigate the role of sunitinib and metformin adjuvant therapy in Langendorff perfused rat hearts. Sunitinib administration significantly decreased LVDP compared to vehicle perfused hearts at the selected time-points: 145 min (vehicle = 79.6±3.3% vs. sunitinib = 63.6±5.2%), 160 min (vehicle = 78.6±3.7% vs. sunitinib = 57.4±5.6%), and 175 min (vehicle = 77.2±3.3% vs. sunitinib = 58.3±5.9%). Co-administration of sunitinib and metformin perfusion in hearts significantly attenuated the sunitinib-induced decrease in LVDP at the same selected time-points mentioned above: 145 min (sunitinib = 63.6±5.2% vs. sunitinib + metformin = 80.1±5.6%), 160 min (sunitinib = 57.4±5.6% vs. sunitinib + metformin = 75.1±3.4%), and 175 min (sunitinib = 58.3±5.9% vs. sunitinib + metformin = 73.9±3.5%). To investigate the role of intracellular adenosine needed for AMPK signalling during metformin-induced cardioprotection, NBTI was added to the perfusate containing sunitinib and metformin throughout the Langendorff experiments. The co-administration of sunitinib and metformin with NBTI during perfusion significantly attenuated the metformin-induced restoration of LVDP again at the specific time-points highlighted above: 145 min (sunitinib + metformin = 80.1±5.6% vs. sunitinib + metformin + NBTI = 65.4±3%), 160 min (sunitinib + metformin = 75.1±3.4% vs. sunitinib + metformin + NBTI = 64.01±1.63%), and 175 min (sunitinib + metformin = 73.9±3.5% vs. sunitinib + metformin + NBTI = 63.73±2.28%) (Figure 1A). Sunitinib administration did not significantly decrease CF when compared to vehicle perfused hearts, however, the co-administration of metformin and sunitinib significantly reduced CF compared to vehicle perfused hearts at the selected time points: 145 min (vehicle = 80.8±4% vs. sunitinib + metformin = 70.1±4%), 160 min (vehicle = 79.9±4% vs. sunitinib + metformin = 65.3±3.3%), and 175 min (vehicle = 77.8±2.7% vs. sunitinib + metformin = 58.9±3.5%). The addition of NBTI with sunitinib and metformin significantly decreased the CF compared to sunitinib and metformin co-administered hearts at the selected time-points: 100 min (sunitinib + metformin = 80.3±3.3% vs. sunitinib + metformin + NBTI = 59.3±6.8%), 115 min (sunitinib + metformin = 76.2±4.6% vs. sunitinib + metformin + NBTI = 56.5±6.4%), 130 min (sunitinib + metformin = 74.7±4.4% vs. sunitinib + metformin + NBTI = 54.8±5.5%), 145 min (sunitinib + metformin = 70.1±4.0% vs. sunitinib + metformin + NBTI = 52.2±6.6%), 160 min (sunitinib +

metformin = 65.3±3.3% vs. sunitinib + metformin + NBTI = 47.7±6.4%), and 175 min (sunitinib + metformin = 58.9±3.5% vs. sunitinib + metformin + NBTI = 39.5±5.2%) (Figure 1B). The administration of sunitinib and metformin did not significantly alter the HR when compared to vehicle perfused hearts (Figure 1C).

3.2 Metformin protects the perfused heart from sunitinib-induced infarction through AMPK

To determine the potential cardioprotective properties of metformin following co-administration with Sunitinib, we assessed the infarct percentage of rat heart tissue following the Langendorff perfusion using TTC staining. Sunitinib perfusion of hearts resulted in a significant increase in infarct size percentage when compared to vehicle perfused control hearts (vehicle = 11.4±0.5% vs. sunitinib = 31.3±2.1%). The sunitinib-induced increase in infarct percentage was significantly attenuated following metformin co-administration with sunitinib (metformin + sunitinib = 20.2±1.8%). Perfusion of NBTI together with sunitinib and metformin attenuated the metformin-induced decrease in infarct size, thus highlighting the involvement of AMPK and adenosine signalling in metformin-induced cardioprotection (sunitinib + metformin + NBTI = 29.8±1.6%) (Figure 2).

3.3 Sunitinib and metformin perfusion alters the level of p-AMPKα in the cardiac tissue

To determine the involvement of AMPK signalling during metformin co-administration with sunitinib the level of p-AMPKα at Thr^{172} was determined by Western blot analysis with SDS-PAGE using the left ventricular tissue obtained from the hearts following Langendorff perfusion. The p-AMPKα band intensities were normalised to vehicle and standardised against GAPDH band intensity levels. Here we demonstrate that p-AMPKα levels were decreased significantly following sunitinib perfusion of hearts when compared to vehicle perfused hearts (vehicle = 100% vs. sunitinib = 56 ± 10%). Metformin co-administration with sunitinib was able to significantly attenuate the decrease in p-AMPKα levels compared to sunitinib mono-treatment hearts (metformin + sunitinib = 118±17%). The metformin-induced increase in p-AMPKα levels was significantly attenuated by co-administration

with NBTI along with sunitinib and metformin, which almost brought the p-AMPKα levels back to sunitinib mono-treatment levels (sunitinib + metformin + NBTI = 63±13%) (Figure 3).

3.4 Activation of AMPK prevents sunitinib-induced reduction in live cell population of cardiac myocytes during co-administration with metformin

Trypan blue staining of live cell population of isolated cardiac myocytes was used to demonstrate metformin's cardioprotective properties during incubation with sunitinib. Incubation with sunitinib was shown to significantly decrease the live cell population of isolated cardiac myocytes compared to vehicle (vehicle = 39.8±3.6% vs. sunitinib = 11.6±1.8%). Co-incubation of isolated cardiac myocytes with sunitinib and metformin significantly attenuated the decrease in live cell population induced by sunitinib mono-treatment (metformin + sunitinib = 41.3±3.2%). To determine the role of AMPK during metformin-induced cardioprotection of isolated cardiac myocytes NBTI was incubated with sunitinib and metformin. This demonstrated a significant attenuation of metformin's cardioprotective effect during the sunitinib-induced cell death of isolated cardiac myocytes, highlighting the crucial involvement of the AMPK signalling pathway during metformin-induced cardioprotection (16.0±3.0%) (Figure 4).

3.5 Anti-neoplastic properties of sunitinib and metformin

To determine if metformin jeopardised the anti-neoplastic properties of sunitinib HepG2 and HL-60 cancer cell lines were treated with 50 µM metformin in combination with 0.1-100 µM sunitinib using the MTT assay. Sunitinib demonstrated a dose-dependent decrease in cell viability in HepG2 (EC_{50} = 18.1 µM) and HL-60 cells (EC_{50} = 12.0 µM) when standardised to vehicle treated cancer cells (Figure 5A-B). Compared to the vehicle group metformin mono-treatment did not alter cell viability of HepG2 cells (6 µM – 1 mM metformin) or HL-60 cells (30 µM – 1 mM metformin) (data not shown). Co-administration of 50 µM metformin with sunitinib demonstrated a right-shift of the sunitinib dose-response curve, thus an increase in the EC_{50} values for HepG2 (EC_{50} = 43.6 µM) and HL-60 cells (EC_{50} = 27.6 µM) was observed when compared to sunitinib mono-treatment (Figure 5A-B).

4 Discussion

Our study demonstrates the novel adjunctive use of metformin together with sunitinib and the role of AMPK signalling during metformin-induced cardioprotection and sunitinib-induced cytotoxicity. In comparison to other studies that have used metformin and failed to demonstrate cardioprotection as pre-treatment (4, 37, 38), we highlight the significance and importance of co-administering metformin and activating AMPK signalling together with existing chemotherapeutic agents such as sunitinib in the Langendorff perfused animal heart model. Unlike studies by Cohen et al. 2011, we used a clinically relevant dose of sunitinib (1µM) in our animal heart study in order to closely monitor effects similar to patients receiving sunitinib (37). Metformin was administered for the same period of time as sunitinib, as well as adjunctively, in our *ex vivo* and *in vitro* models to closely monitor pleiotropic effects of metformin unlike studies by Cohen et al. 2011 and Hasinoff et al. 2008 who exposed cardiac cells for a shorter duration of time compared to sunitinib, but also removed metformin prior to exposing cardiac cells to sunitinib, which has the ability to inactivate AMPK once introduced (37, 38). For this reason, it did not make sense to remove the potential source of AMPK activation before administering toxicity. Our study highlights the potential for metformin to overcome sunitinib-mediated effects of AMPK signalling as indicated by the study conducted by Kerkela et al. 2009 (4).

In this study, Langendorff perfusion of sunitinib significantly increased the infarct size and decreased LVDP in male SD rat hearts. In isolated cardiac myocytes sunitinib administration decreased live cell population. Western blotting analysis of the left ventricular tissue of sunitinib perfused hearts demonstrated a decrease in p-AMPKα signalling. Our findings on sunitinib-induced infarct increase and LVDP are supported by previous studies, where a similar decrease in whole rat heart sunitinib perfusion settings was observed (26-28). The observed changes in infarct size, LVDP, and cardiac myocytes cell viability following sunitinib administration in this study can be attributed to sunitinib initiating a pro-apoptotic response in cardiac myocytes (2, 6).

The inhibition of p-AMPKα by sunitinib treatment shown in this study is supported by previous studies involving *in vitro* and *in vivo* rodent experiments (3, 39). AMPK activation results in anti-apoptotic response to (a) promote mitochondrial biogenesis through PGC1-α and NRF1/2 gene expression stimulation to preserve mitochondrial homeostasis, (b) support the mitochondrial fission of oxidatively-damaged mitochondria through fission modulator dynamin-related protein 1 activation, and (c) preventing the opening of the mPTP (40). The decrease in p-AMPKα in our study can potentially be attributed to the sunitinib associated opening of the mPTP and mitochondrial dysfunction. AMPK is vital for cell survival during mitochondrial impairment, thus the inhibition of the AMPK pathway by sunitinib impairs energy homeostasis in cardiac myocytes and accelerate hypertrophy through release of eukaryotic elongation factor-2 and acetyl-coenzyme A carboxylase (3, 38). In the heart sunitinib-induced inhibition of ribosomal S6 kinase activity can trigger the release of pro-apoptotic factor BCL2-antagonist of cell death, leading to BCL2 associated X protein activation and cytochrome c release and activation of the intrinsic apoptotic pathway, resulting in ATP depletion, left ventricular dysfunction, and cardiac myocyte loss (3, 38). The cascade of these events contributes to mitochondrial impairment and dysfunction due to the sunitinib-induced cardiotoxicity (6). Therefore, another potential explanation for the reduction in LVDP and the reduction in p-AMPKα signalling in the left cardiac tissue following sunitinib perfusion could be due to the sunitinib-induced energy deprivation and ATP depletion, which could lead to a reduction in ATP availability for high energy-demanding cardiac myocytes (4). Further to this, p-AMPKα is vital in preventing cardiac myocyte death, as AMPK activation leads to activation of the catabolic pathway, suppressing non-essential ATP-consuming processes, thus re-distributing energy for cardiac myocyte and myocardium function (7, 41), and as a result of this sunitinib treatment reduces the cardiac myocyte cell viability and increases the infarct size in the whole heart.

Co-administration of metformin was able to abolish the cardiotoxic effects of sunitinib in the whole heart and in cardiac myocytes. Further to this, we demonstrated that the metformin associated

cardioprotective properties and the reactivation of p-AMPKα were attenuated by co-administration of NBTI. In line with the results demonstrated in this study, clinical studies have revealed that metformin therapy improves the survival outcome in diabetic patients suffering from metastatic renal cell carcinoma when treated with sunitinib when compared to metformin non-users (42, 43). Reperfusion studies using rat hearts subjected to regional ischemia and reperfusion showed a reduction in the myocardial infarct size through increased AMPK activation when 50 μM metformin was administered during the first 15 min of reperfusion, with a 45% reduction in infarct size observed in SD rats (21), and a 34 % reduction in infarct size detected in Wistar rats, (22). Metformin's cardioprotective properties have been previously be attributed to attenuating the reperfusion-induced mPTP opening (17). There are no studies involving co-administration of metformin to prevent sunitinib-induced mPTP opening, however as previously noted, evidence exists of metformin-induced AMPK activation, which would lead to attenuation of the mPTP opening (40). Indeed studies have shown that the metformin-induced activation of AMPK signalling is required for the prevention of calcium-overload-mediated mPTP opening, mitochondrial oxidative phosphorylation, and the cascading pro-inflammatory response due to hypoxia, which are all the traits associated with cardiotoxicity (17, 44). In agreement with these studies metformin co-administration with sunitinib attenuated the sunitinib-mediated decrease in p-AMPKα levels in our study. Activation of AMPK signalling results in activation of endothelial nitric oxide synthase and nitric oxide bioavailability, thus preventing oxidative stress and apoptosis development (45-47). Metformin mediated increase in the AMP:ATP ratio levels facilitate the extracellular diffusion of adenosine via the equilibrative nucleoside transporter, which activates downstream cardioprotective pathways (48). The metformin-induced AMPK activation can be attenuated by inhibiting the equilibrative nucleoside transporter involved in the facilitated diffusion of adenosine across the cardiac myocyte cell membrane (33, 48). NBTI is demonstrated to attenuate intracellular adenosine signalling that results from metformin-induced increase in intracellular formation of adenosine by dephosphorylation of AMP (21, 35), the change in the ATP:AMP ratio and increase in cytosolic AMP signalling indirectly activates AMPK signalling (49-52). Previous studies demonstrated that free

adenosine in the cytosol provides a pivotal role in the formation of the endogenous purine nucleoside adenosine and acts to limit infarct size and mPTP formation (53, 54).

To investigate if metformin interfered with the anti-neoplastic properties of sunitinib, HepG2 and HL-60 cancer cell lines were incubated with sunitinib in the absence and presence of metformin. Sunitinib inhibited angiogenesis and induced a pro-apoptotic response in both HepG2 cells (EC_{50} = 18.1 µM) and HL-60 cells (EC_{50} = 12.0 µM). Addition of 50 µM metformin with increasing sunitinib doses resulted in a right-shift of the sunitinib dose-response curve, and thus an increase in EC_{50} values (HepG2 EC_{50} = 43.6 µM and HL-60 EC_{50} = 27.6 µM). These results from our study suggest that the activation of AMPK signalling may potentially reduce the sunitinib-induced ATP depletion in HepG2 and Hl-60 cancer cells, and potentially protect cancer cells against oxidative stress and DNA damage (55). Even in the presence of metformin-induced activation of AMPK here we demonstrate that sunitinib still carries out anti-proliferative properties, indicating that sunitinib may be dependent on other intracellular pathways and mechanisms than an inactive AMPK pathway for its anti-cancer properties. Moreover, studies have shown that metformin has favourable effects on overall survival rates with sunitinib (43, 56), studies have shown that another multi-TKI sorafenib acts to activate AMPK (57) emphasise that activation of AMPK may not be the only cause of the metformin-induced right-shift and increase in EC_{50} values of the sunitinib dose-response curve observed in our study. The cause may lie with metformin's downstream targets, as metformin has been shown to suppress cisplatin-induced apoptotic cell death via an AMPK-independent manner, possibly by upregulation of the Akt survival pathway (58). In addition to metformin's cardioprotective properties, recent studies suggest that metformin has cytostatic effects in cancer cells rather than inducing apoptosis directly. Metformin was shown to reduce colony formation of colorectal cancer cells without arresting cell growth in the absence of apoptosis (59), and inhibition of AMPK did not prevent the metformin-induced anti-proliferative properties, but rather induced cell senescence by inhibiting the cell cycle at the G_0/G_1 phase in a cytostatic manner (60). With this knowledge, the cytostatic effects of metformin to reduce cancer cell growth could compromise the cytotoxic effects of chemotherapeutic agents,

which would otherwise be more sensitive to more rapidly dividing cells. This highlights the complexity of adjunctive treatment with metformin. In order to further evaluate the role of metformin during sunitinib-induced cardiotoxicity a series of further *in vivo* experiments will be needed to be conducted in order to unravel the specific underlying pathways and mechanisms involved.

5 Conclusion

Sunitinib is associated with severe cardiotoxic adverse effects, thus identification of adjunctive therapy that will alleviate sunitinib-induced cardiotoxicity is vital for increasing the outcome in cancer patients treated with sunitinib. This study highlights metformin's potential cardioprotective property during sunitinib-induced cardiotoxicity. The sunitinib-induced increase in infarct size, decreases in LVDP, and decrease in isolated cardiac myocyte cell viability were attenuated by metformin co-administration, which was abolished by NBTI, thus emphasising on the involvement and importance of AMPK during metformin-induced cardioprotection. Importantly, metformin co-administration with sunitinib in relevant cancer cell lines did increase the EC_{50} values. Our study highlights the complexity of adjunctive treatment and the fine balancing act of cardioprotection and treating carcinoma. Understanding the key intracellular pathways and mechanisms in both cardiac and cancer cells will unravel the potential of metformin as an adjunct therapy option in cancer patients treated with sunitinib.

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- Figure Legends -

Figure 1. Haemodynamic changes in rat hearts Langendorff perfused with 1μM sunitinib ± 50μM metformin ± 1μM NBTI; mean ± SEM; (A) LVDP, (B) CF, and (C) HR; n = 6 for all groups; * = $p<0.05$ for vehicle versus sunitinib; # = $p<0.05$ for sunitinib versus sunitinib + metformin; $ = $p<0.05$ for sunitinib + metformin versus sunitinib + metformin + NBTI (Sun = sunitinib; Met = metformin).

Figure 2. TTC staining for infarct percentage size of heart tissue following Langendorff perfusion with 1μM sunitinib ± 50μM metformin ± 1μM NBTI; mean ± SEM; n = 6 for all groups; *** = $p<0.001$ vehicle versus sunitinib; # = $p<0.05$ sunitinib versus sunitinib + metformin; $ = $p<0.05$ sunitinib + metformin versus sunitinib + metformin + NBTI (Sun = sunitinib; Met = metformin).

Figure 3. Western blotting analysis for p-AMPKα and GAPDH protein expression of left ventricular tissue following Langendorff perfusion with 1μM sunitinib ± 50μM metformin ± 1μM NBTI; mean ± SEM; n = 7 for vehicle; n = 7 for sunitinib; n = 6 for metformin; n = 7 for sunitinib + metformin; n = 7 for NBTI; n = 7 for sunitinib + metformin + NBTI; p-AMPKα bands standardised to GAPDH. *** = $p<0.001$ vehicle versus sunitinib; # = $p<0.05$ sunitinib versus sunitinib + metformin; $ = $p<0.05$ sunitinib + metformin versus sunitinib + metformin + NBTI (Sun = sunitinib; Met = metformin).

Figure 4. Trypan blue staining for live cell population of isolated cardiac myocytes following 4 h incubation with 1μM sunitinib ± 50μM metformin ± 1μM NBTI; mean ± SEM; n = 7 for all groups; *** = $p<0.001$ vehicle versus sunitinib; # = $p<0.05$ sunitinib versus sunitinib + metformin; $ = $p<0.05$ sunitinib + metformin versus sunitinib + metformin + NBTI (Sun = sunitinib; Met = metformin).

Figure 5. MTT cell viability assay for 0.1-100μM sunitinib ± 50μM metformin in (A) HepG2 cells and (B) HL-60 cells; mean ± SEM; n = 6 for all groups; # = $p<0.05$ sunitinib versus sunitinib + metformin (Sun = sunitinib; Met = metformin).

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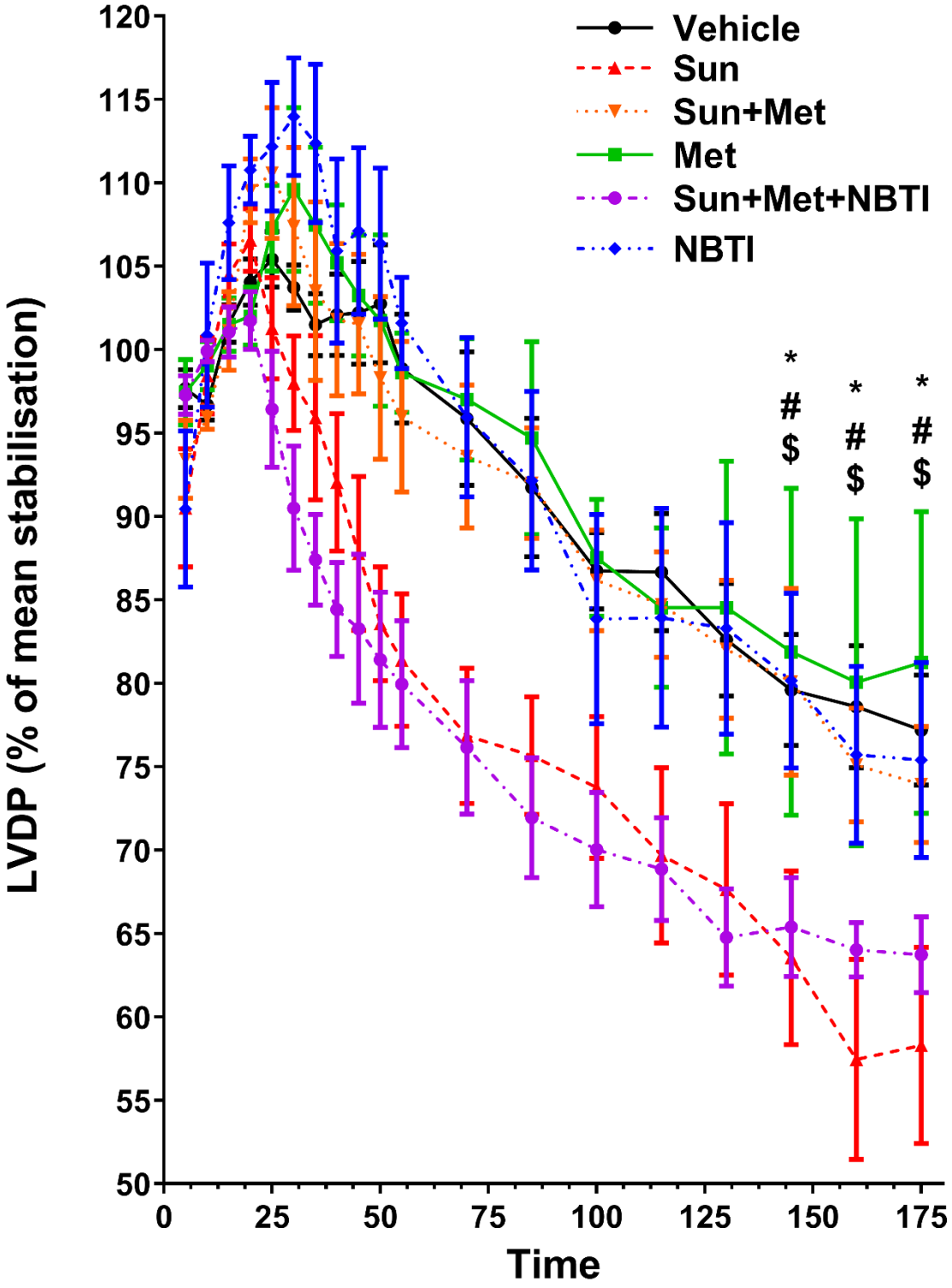
648 Figure1A



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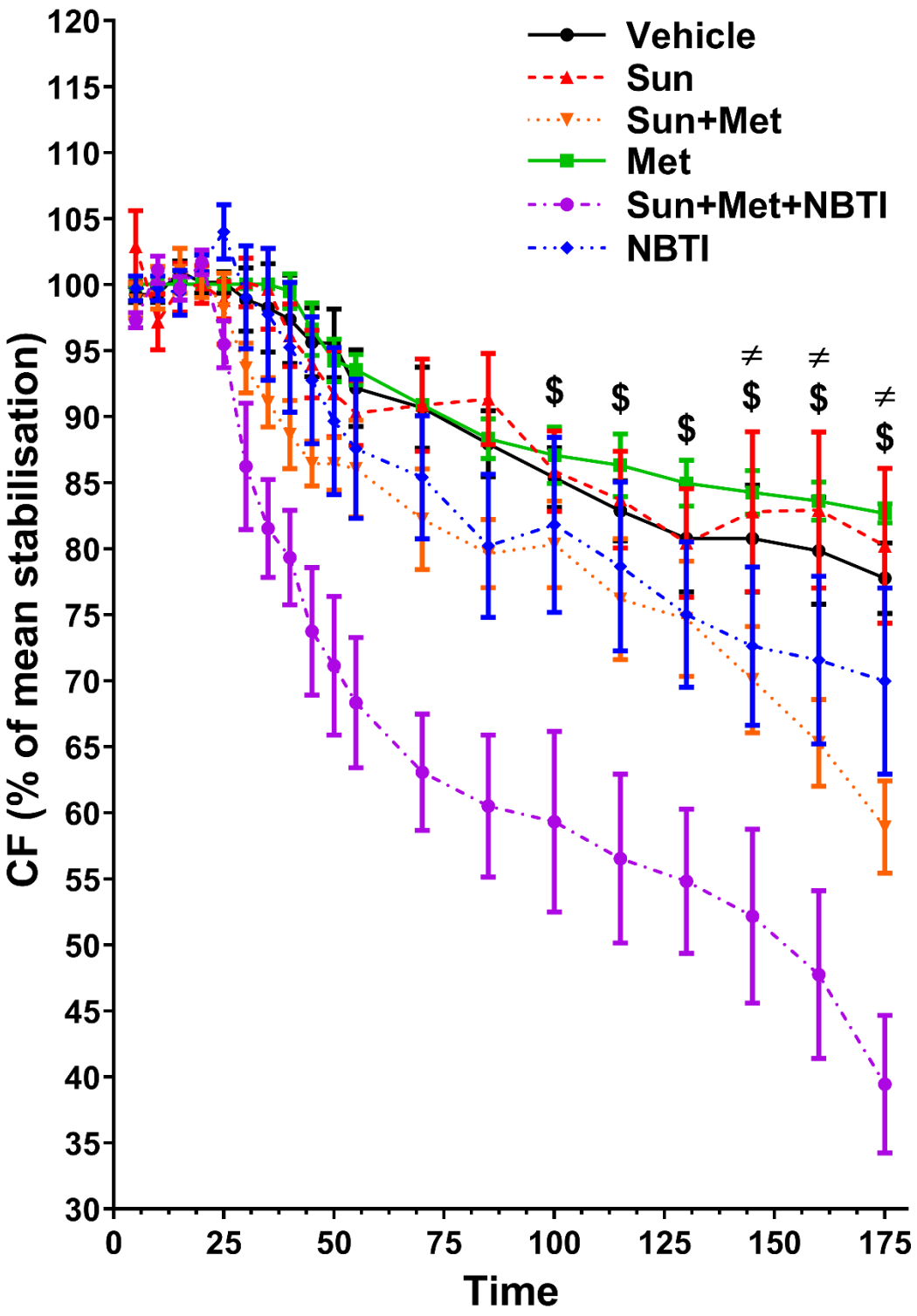
651 Figure 1B



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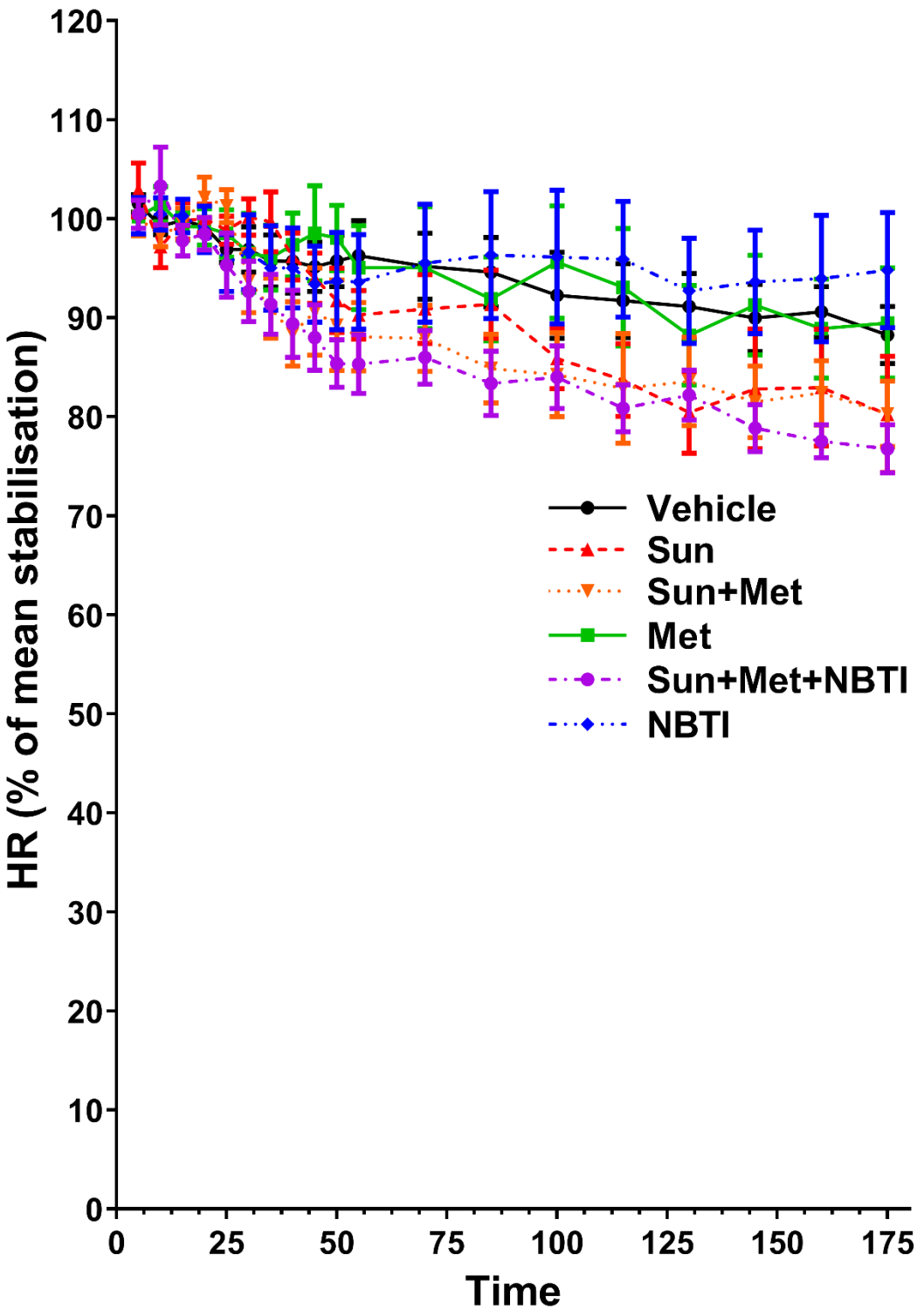
655 Figure 1C



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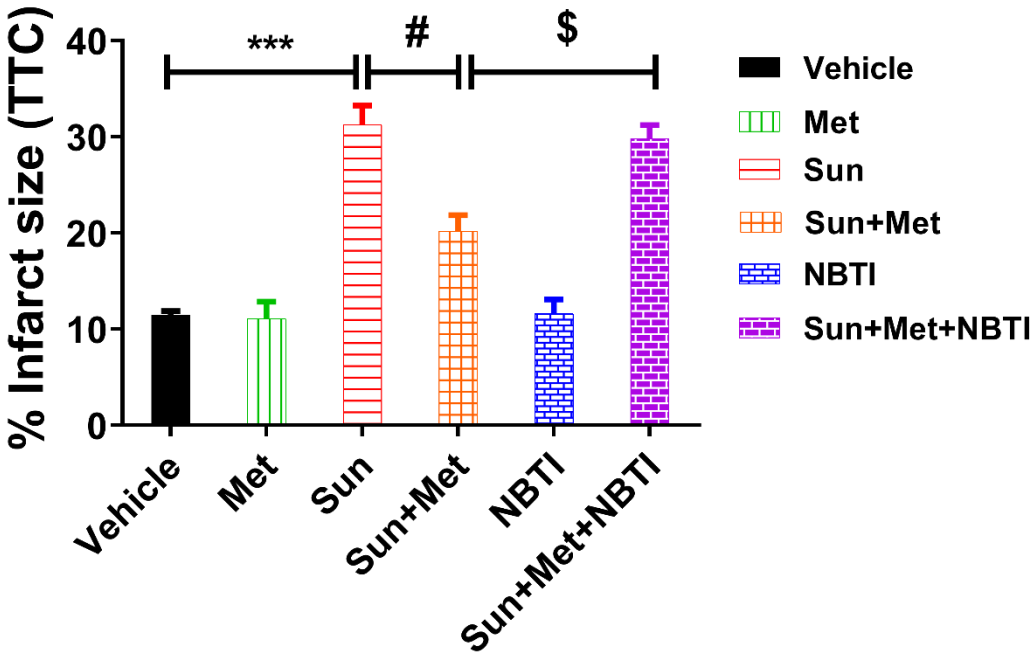
658 Figure 2



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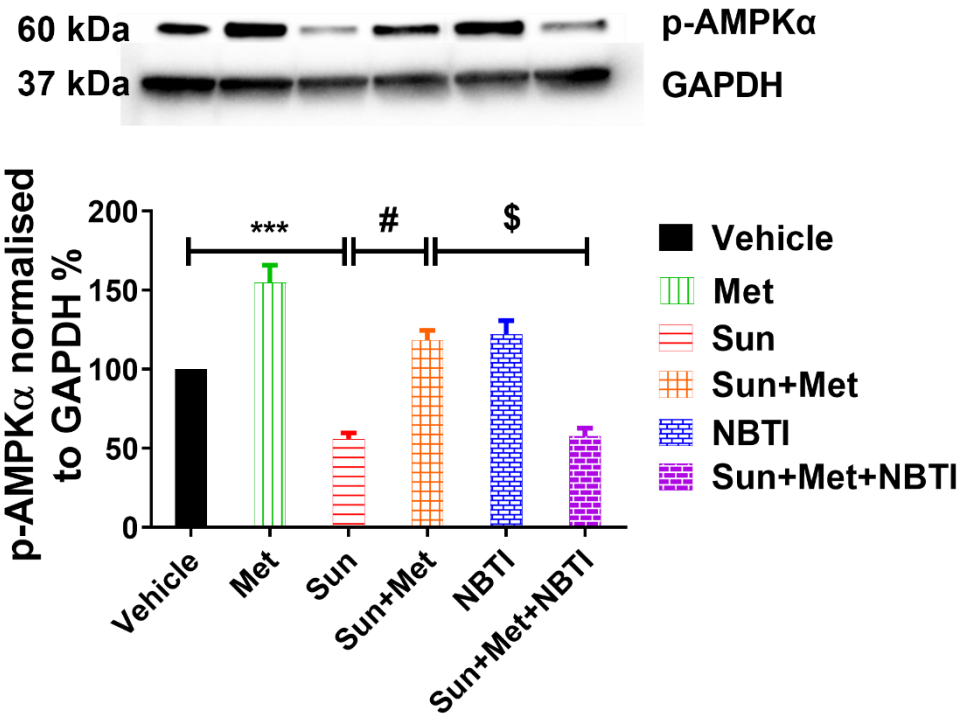
661 Figure 3



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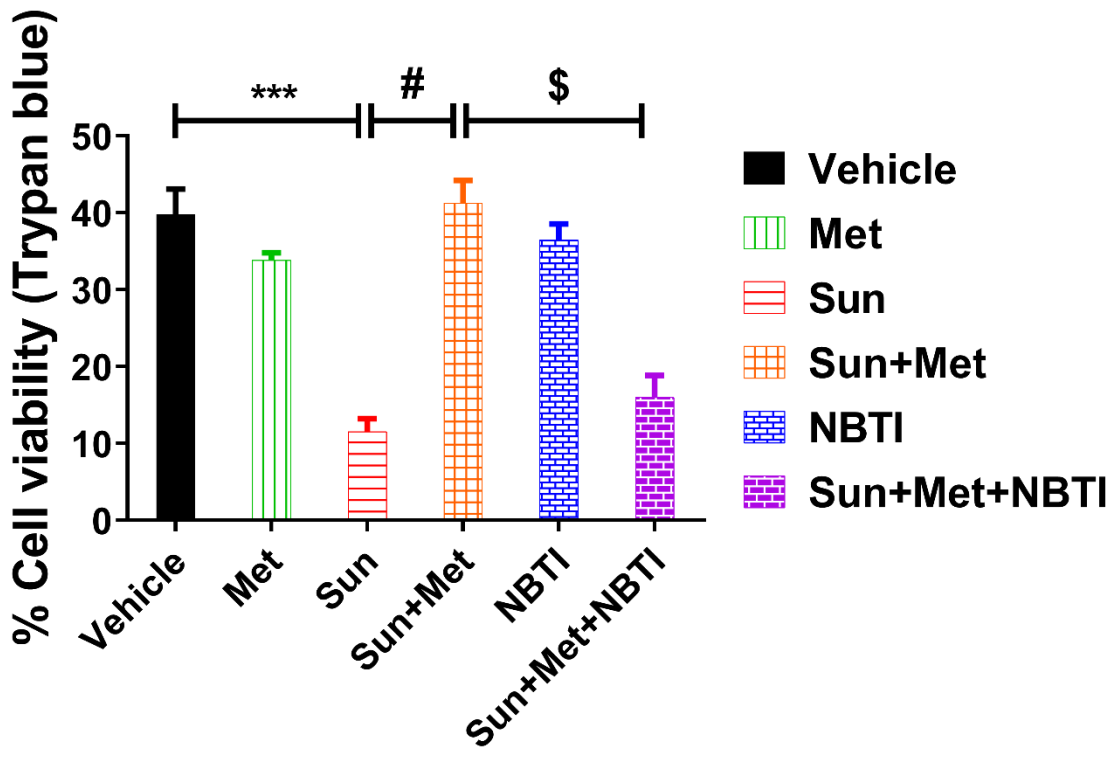
664 Figure 4



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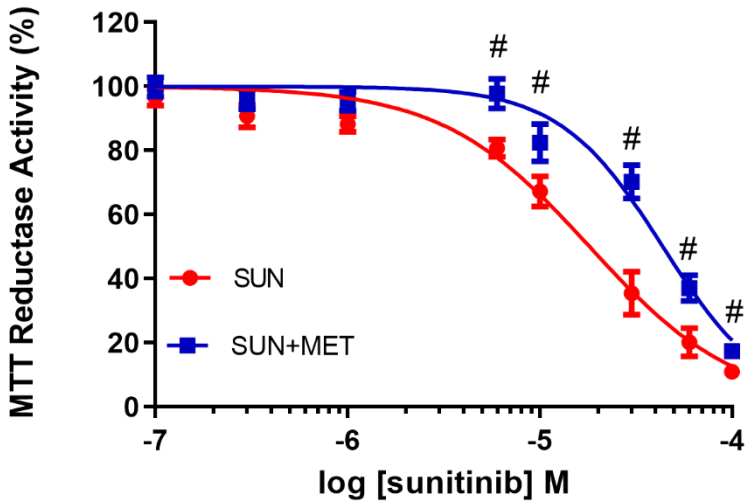
667 Figure 5A



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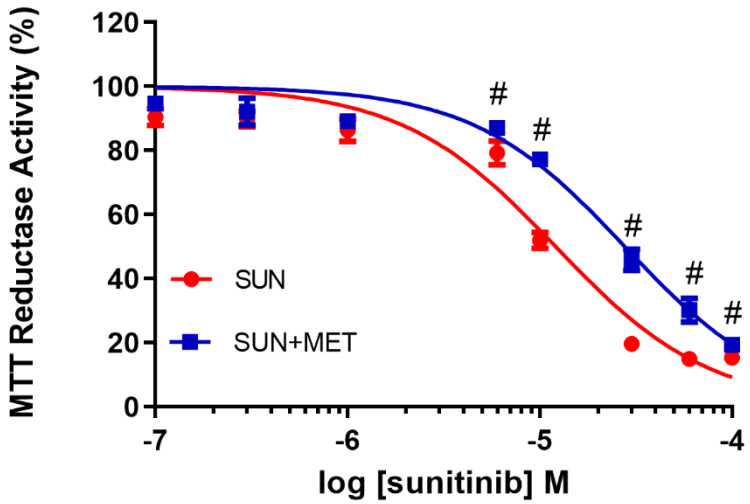
670 Figure 5B



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