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Mechanisms of myocyte cytotoxicity induced by the multiple receptor tyrosine kinase inhibitor sunitinib

Brian B. Hasinoff, Daywin Patel and Kimberley A. O'Hara

Faculty of Pharmacy, Apotex Centre, University of Manitoba, Winnipeg, Manitoba R3E 0T5, Canada

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b) Address correspondence to:
Dr. Brian Hasinoff
Faculty of Pharmacy
Apotex Centre
University of Manitoba
750 McDermot Avenue
Winnipeg, Manitoba R3E 0T5, Canada
Tel: (204) 474-8325
FAX: (204) 474-7617
E-Mail: B_Hasinoff@UManitoba.ca

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d) ABBREVIATIONS:

ACC, acetyl-Coenzyme A carboxylase; ACCα and ACCβ, alpha and beta isoforms of ACC; AMPK, AMP-activated protein kinase; DF-*x*, Dulbecco's Modified Eagle Medium/Ham's F-12 Medium, 1:1 where *x* is % (v/v) serum; K_d, kinase binding dissociation constant; LDH, lactate dehydrogenase; pACCα and pACCβ, phosphorylated alpha and beta isoforms of ACC; PBS, Dulbecco's phosphate buffered saline (pH 7.4); RSK, ribosomal S6 kinase.

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ABSTRACT

The anticancer tyrosine kinase inhibitor sunitinib has recently been shown to be cardiotoxic. Using a neonatal rat myocyte model we investigated various mechanisms that might be responsible for its cardiotoxicity. Sunitinib potently inhibited the enzyme activity of both AMP-activated protein kinase (AMPK) and the ribosomal S6 kinase RSK1 at therapeutically relevant concentrations. Heart tissue with its high energy needs might be particularly sensitive to inhibition of AMPK because of its role as an energy sensor regulating ATP levels. As measured by lactate dehydrogenase release sunitinib treatment of myocytes caused dose-dependent damage at therapeutic levels. Sunitinib treatment also caused a dose-dependent reduction in myocyte protein levels of the phosphorylated alpha and beta isoforms of the AMPK phosphorylation target acetyl-Coenzyme A carboxylase (ACC). However, myocytes were not protected from sunitinib treatment by pretreating them with the AMPK activating antidiabetic drug metformin. Sunitinib treatment of myocytes also did not affect cellular ATP levels. Together these last two results do not suggest a major role for inhibition of AMPK in sunitinib-induced myocyte damage. Dexrazoxane, which is a clinically approved doxorubicin cardioprotective agent, also did not protect myocytes from damage, which suggests that sunitinib did not induce oxidative damage. In conclusion, even though sunitinib potently inhibits AMPK and RSK1, given the extreme lack of kinase selectivity that sunitinib exhibits it is likely that inhibition of other kinases or combinations of kinases are responsible for the cardiotoxic effects of sunitinib.

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Sunitinib (Fig. 1, Sutent®) is a new multitargeted oral tyrosine kinase inhibitor that has both antiangiogenic and antitumor activity. It is approved in the US for treatment of gastrointestinal stromal tumors after disease progression on, or intolerance to, imatinib, and for the treatment of advanced renal cell carcinoma (Faivre et al., 2007). However, two recent clinical studies in patients treated for gastrointestinal stromal tumors or renal cell carcinoma have shown evidence of sunitinib-associated cardiotoxicity as evidenced by heart failure, left ventricular systolic dysfunction, and hypertension (Chu et al., 2007; Telli et al., 2008). Sunitinib joins the other tyrosine kinase-targeted anticancer drugs trastuzumab (Herceptin®), and possibly imatinib (Gleevec®), dasatinib (Sprycel®), sorafenib (Nexavar®) and bevacizumab (Avastin®) in having cardiotoxic effects (Force et al., 2007; Mego et al., 2007; Menna et al., 2008). Sunitinib inhibits many kinases including vascular endothelial growth factor receptors (VEGFR), platelet-derived-growth factor receptors (PDGFR), stem-cell-factor receptor (KIT), colony-stimulating factor 1 receptor (CSF1R), FMS-related tyrosine kinase-3 (FLT3) and rearranged-during-transfection (RET) receptor tyrosine kinases (Force et al., 2007). While sunitinib's anticancer efficacy is likely due to its being a multitargeted kinase inhibitor, its lack of specificity also carries with it a concomitant greater risk of collateral toxicity. A recent study (Karaman et al., 2008) using a competition binding assay investigated the binding of 38 kinase inhibitors to 317 kinases representing more than 50% of the predicted human kinome. Of the seven small molecule anticancer kinase inhibitors (sunitinib, imatinib, dasatinib, sorafenib, lapatinib, gefitinib and erlotinib) that are currently approved in the US, sunitinib is the most promiscuous, binding to 57% and 18% of the kinases tested at 3 and 0.1 μM, respectively. In fact, of the 38 kinase inhibitors tested only the well characterized promiscuous inhibitor staurosporine was less selective

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(Karaman et al., 2008).

A recent review (Force et al., 2007) of the possible cardiotoxic mechanisms of tyrosine kinase-targeting drugs hypothesized that inhibition of AMPK through inhibition of pro-survival signals in the heart (Dyck and Lopaschuk, 2006; Terai et al., 2005) and/or inhibition of the ribosomal S6 kinases (RSK), which signals survival through inhibitory phosphorylation of the pro-apoptotic factor Bad may, in part, be responsible for the cardiotoxicity of sunitinib. Thus, in order to investigate these two possible targets studies were carried out to determine if sunitinib inhibited AMPK and RSK at clinically relevant concentrations, damaged cardiac myocytes, induced apoptosis in myocytes, and finally whether sunitinib-induced myocyte damage could be prevented by the clinically approved doxorubicin cardioprotective agent dexrazoxane (ICRF-187, Zinecard®) (Cvetkovic and Scott, 2005; Hasinoff and Herman, 2007). We previously showed in an in vitro myocyte model that dexrazoxane strongly protects myocytes from doxorubicin-induced damage (Hasinoff and Herman, 2007; Hasinoff et al., 2003).

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Materials and Methods

Materials. Sunitinib malate (ChemPacific, Baltimore, MD) stock solutions were prepared in sterile water. Trypsin, collagenase, and deoxyribonuclease were from Worthington (Freehold, NJ). Dexrazoxane hydrochloride was a gift from Adria Laboratories (Columbus, OH). Unless specified, other reagents were obtained from Sigma (Oakville, Canada). DF-15 medium (with 7.5% (v/v) FBS and 7.5% (v/v) horse serum), fetal bovine and horse serum, penicillin, streptomycin, and fungizone were obtained from Invitrogen (Burlington, Canada). The errors shown are SE. Where significance is indicated ($p < 0.05$), an unpaired *t*-test was used (SigmaPlot, San Rafael, CA). Non-linear least squares curve fitting was done with SigmaPlot.

Myocyte isolation and culture and epifluorescence microscopy. Ventricular myocytes were isolated from 2 – 3 d old Sprague-Dawley rats as described (Hasinoff et al., 2003; Hasinoff et al., 2007; Schroeder et al., 2008). Briefly, minced ventricles were serially digested with collagenase and trypsin in PBS/1 % (wt/v) glucose at 37˚C in the presence of deoxyribonuclease and preplated in large petri dishes to deplete fibroblasts. The preparation, which was typically greater than 90% viable by trypan blue exclusion, yielded an almost confluent layer of uniformly beating cardiac myocytes by day 2. For the LDH release experiments the myocyte-rich supernatant was plated on day 0 in 24-well plastic culture dishes (5 x 10^5 myocytes/well, 750 µl/well) in DF-15. On day 2 and 3 the medium was replaced with 750 µl of fresh DF-10 containing 10% (v/v) FBS. In order to lower the background LDH levels, on day 4, 24 h before the drug treatments, the medium was changed to DF-2 and again on day 5 just before the addition of drugs. The animal protocol was approved by the

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University of Manitoba Animal Care Committee. The anti-α-actinin/Hoechst 33258 staining and imaging by epifluorescence microscopy of fixed myocytes to score for both myofibrillar disruption and for apoptotic nuclei was carried out as we previously described (Hasinoff et al., 2007).

Drug treatments and LDH determination. Myocytes were either treated with sunitinib for the times indicated, or they were pretreated with either dexrazoxane or metformin as indicated. Starting on day 6 after plating, samples (80 μl) of the myocyte supernatant were collected every 24 h for 3 d after treatment. The samples were frozen at -80˚C and analyzed within 1 week. After the last supernatant sample was taken the myocytes were lysed with 250 μl of 1% (v/v) Triton X-100/2 mM EDTA/1 mM dithiothreitol/0.1 M phosphate buffer (pH 7.8) for 20 min at room temperature. The total cellular LDH activity, from which the percentage of LDH release was calculated, was determined from the activity of the lysate plus the activity of the three 80 μl samples previously taken. The LDH activity was determined in quadruplicate in a spectrophotometric kinetic assay in 96-well plate in a Molecular Devices (Menlo Park, CA) plate reader as previously described (Hasinoff et al., 2003; Hasinoff et al., 2007; Schroeder et al., 2008).

Protein isolation and western blotting analysis. Neonatal rat myocytes (2.5×10^6) were isolated and plated as described above 5 d prior to treatment. On the day of treatment fresh medium was added and the cells were treated with sunitinib or water for 2 h. Cells were rinsed twice in PBS, and lysed in buffer (0.125 mM Tris-HCl (pH 6.8)/4% SDS, supplemented with 1 mM sodium orthovanadate and protease and phosphatase inhibitors). The amount of protein in the lysate was

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determined using the QuantiPro BCA Assay Kit (Sigma) using bovine serum albumin as a reference standard. Sample buffer (5% glycerol/0.003% bromophenol blue (w/v)/1% (v/v) 2-mercaptoethanol) was added and lysates were boiled for 5 min. Proteins were separated for analysis by SDS/polyacrylamide gel electrophoresis on either 5% (w/v) gels for ACCα, ACCβ, pACCα and pACCβ or 12% (w/v) gels for Bax. Proteins were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA) after which Ponceau staining was used to verify transfer efficiency. Membranes were blocked in 5% (w/v) milk protein dissolved in Tris-buffered saline containing 0.05% (v/v) Tween 20 (pH 8.0). Membranes were rinsed three times for 5 min in this buffer and then incubated overnight with primary antibodies at 4˚C. Antibodies recognizing ACC and ACC phosphorylated at Ser79 (Cell Signaling Technology, Pickering, Canada), N-terminal reactive Bax (N-20, Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (AC-15, Sigma) were used. After rinsing in Tris-buffered saline containing 0.05% (v/v) Tween 20 (pH 8.0), the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Pierce Biotechnology, Rockford, IL) for 1 h at room temperature. Reactive bands were detected using enhanced chemiluminescence on an Alpha Innotech (San Leandro, CA) Fluorochem 8900 imaging system equipped with a charge-coupled-device camera.

Kinase assays. The AMPK and RSK kinase inhibition assays were carried out in duplicate by SignalChem (Richmond, Canada) using a radiolabeled ATP filter binding assay. The AMPK kinase assay mixture contained recombinant full-length human AMPK (combination of A1/B1/G2 subunits), peptide (40 μM) with a sequence (HMRSAMSGLHLVKRR) based on mouse ACCα

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(amino acids 73-85), and 50 μM ATP. The RSK kinase assay mixture contained recombinant full-length human RSK1, S6K sub peptide (40 μM) with a sequence (KRRRLASLR) based on human 40S ribosomal protein S6 (amino acids 230-238), and 50 μM ATP. Staurosporine (1 μM) was used as a positive control.

Oxidation of intracellular 2',7'-dichlorofluorescin, caspase-3/7 and cellular ATP assays. The oxidation of intracellular dichlorofluorescin assay was carried out as we previously described (Hasinoff et al., 2007). 2',7'-dichlorofluorescin diacetate was loaded into attached myocytes 3 d after plating in 96-well plates (125,000 myocytes/well, 200 μl medium/well) by incubating myocytes with 50 μM 2',7'-dichlorofluorescin diacetate, the cell permeant ester of 2',7'-dichlorofluorescin, for 20 min at 37˚C in Hanks' balanced salt solution, supplemented with added Ca^{2+} (1.2 mM), Mg^{2+} (0.4 mM) and $NaHCO_3$ (4 mM), followed by two changes of the supplemented Hanks' balanced salt solution at room temperature to remove extracellular ester. The kinetic fluorescence measurements were made on a BMG (Durham, NC) Fluostar Galaxy fluorescence plate reader (excitation wavelength of 485 nm, emission wavelength of 520 nm, 37˚C) equipped with excitation and emission probes directed to the bottom of the plate. After initial baseline fluorescence intensity data was collected for 10 min, the drugs were added to the attached myocytes and gently mixed with a pipette and the increase in fluorescence was recorded for 60 min. The change in the rate of fluorescence increase was computed from data directly before, and 55 min directly after addition of the drug. Hydrogen peroxide, which rapidly enters myocytes and oxidizes 2',7'-dichlorofluorescin, was used as a positive control.

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The caspase-3/7 assay that measures caspase-3 and caspase-7 activities was carried out on myocyte lysate as we previously described (Hasinoff et al., 2007) on a BMG Fluostar Galaxy plate reader in luminescence mode according to the manufacturer's directions (Caspase-Glo® 3/7, Promega, Madison, WI). The assay utilizes a proluminescent caspase-3/7 DEVD-aminoluciferin substrate to produce a luminescent signal proportional to caspase-3/7 activity.

The relative ATP content of the myocytes was measured on a BMG Fluostar Galaxy plate reader in luminescence mode at 30˚C according to the manufacturer's directions (CellTiter-Glo Luminescent Cell Viability Assay®, Promega). The assay (total volume 50 µl) was carried out in DF-15 (10,000 myocytes/well, 100 µl medium/well) in one-half well 96-well white plates. The luminescence signal that is proportional to the cellular ATP content was measured for 1 h after the addition of the assay reagent and the average maximum luminescence observed was recorded.

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Results

Effects of sunitinib on LDH release in myocytes. LDH release is a widely used measure of drug-induced damage to myocytes (Adderley and Fitzgerald, 1999; Hasinoff et al., 2007; Schroeder et al., 2008). Using the LDH release assay we examined the ability of sunitinib to damage myocytes 5 d after isolation (Fig. 1A) when the myocytes would be essentially non-proliferating (Li et al., 1996). As shown in Fig. 1B, at all concentrations between 0.1 and 10 μM, sunitinib significantly increased LDH release (p values of 0.002 for 0.1 μM, and < 0.001 for all other concentrations, respectively) up to 2.6-fold at 72 h compared to untreated control myocytes. For comparison we showed in a previous study (Hasinoff et al., 2003) that a 3 h treatment with 1.5 μM doxorubicin resulted in about the same percentage LDH release (~ 9%) as a 72 h treatment with 1 μM sunitinib (Fig. 1). The submicromolar concentrations of sunitinib used in this study at which significant LDH release was observed are within both peak and trough plasma concentrations observed when a therapeutic dose of sunitinib is given (Deeks and Keating, 2006; Faivre et al., 2006). The mean maximum plasma concentration (C_{max}) of an oral once daily dose of 50 mg/day of sunitinib is reported to be 0.18 μM (Deeks and Keating, 2006). With repeated daily dosing trough plasma levels of sunitinib of 0.11 μM are achieved (Faivre et al., 2006).

Attempts to score sunitinib-treated fixed myocytes by epifluorescence microscopy using muscle specific anti-α-actinin staining along with a Hoechst 33258 counter stain for both myofibrillar disruption and for apoptotic nuclei using methodology we previously described (Hasinoff et al., 2007) were not successful. These attempts were confounded by sunitinib causing myocyte detachment from the coverslips even with a 3 h sunitinib treatment. As measured by the number of

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myocytes per microscopic field both 5 and 10 μM sunitinib treatment caused a significant loss ($p < 0.001$) in the number of myocytes per field (30 and 35%, respectively). The myocytes that remained attached showed no measurable myofibrillar disruption ($p > 0.05$) compared to controls.

Inhibition of AMPK and RSK by sunitinib. The inhibition of AMPK was measured because AMPK is a key regulator of energy metabolism in the heart (Dyck and Lopaschuk, 2006; Force et al., 2007; Terai et al., 2005). As shown in Fig. 2 sunitinib inhibited AMPK with an IC_{50} value of 0.32 μM, which is within a range that is clinically achieved in plasma (Deeks and Keating, 2006; Faivre et al., 2006). For comparison a recent redetermination (Karaman et al., 2008) of the binding dissociation constant (K_d) for the alpha1 and alpha2 isoforms of AMPK yielded values of 0.019 and 0.089 μM, respectively. It was also hypothesized (Force et al., 2007) that inhibition of RSK, which signals survival through inhibitory phosphorylation of the pro-apoptotic factor Bad, may also, in part, be responsible for the cardiotoxicity of sunitinib. As shown in Fig. 2 sunitinib inhibited RSK1 with an IC_{50} value of 0.36 μM. This compares to the K_d value for the RSK1 isoform of 0.14 μM (Karaman et al., 2008). Other RSK isoforms have K_d values in the range of 0.017 to 8.4 μM (Karaman et al., 2008).

Sunitinib inhibits phosphorylation of ACCα and ACCβ in myocytes. To confirm our finding that sunitinib inhibited AMPK (Fig. 2), we next determined whether sunitinib functionally inhibited AMPK in neonatal rat myocytes by measuring the inhibition of phosphorylation of ACC, which is one of its phosphorylation targets. The activity of ACC, the rate-limiting enzyme in fatty acid

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biosynthesis, is primarily regulated by AMPK (Davies et al., 1990; Hardie, 2003; Hardie, 2004). AMPK phosphorylates ACC on Ser79, which appears to mediate AMPK-induced inactivation of ACC (Davies et al., 1990). Therefore, decreasing AMPK activity results in less phosphorylation and thus enhanced activity of ACC and usage of ATP. There are two isoforms of ACC, 265 kDa ACCα and 276 kDa ACCβ, of which ACCβ is the predominant form in mitochondrial-rich cardiac tissue (Kim et al., 1998). As measured by reductions in pACCα and pACCβ protein levels (Fig. 3A and 3B), treatment of myocytes for 2 h with sunitinib decreased the phosphorylation of the two ACC isoforms in a dose dependent manner. Treatment with 5 or 10 μM sunitinib significantly (p of 0.0002 and < 0.0001, respectively) reduced phosphorylation of ACCα by nearly 50 and 60%, respectively. Reduction in protein levels of pACCβ was significantly (p = 0.002) achieved only with a 10 μM sunitinib treatment.

Sunitinib induces apoptosis in myocytes. It has been shown that treatment of myocytes with 1 μM sunitinib for 30 h resulted in activation of caspase-9, cytochrome *c* release and an increase in terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick end labeling (TUNEL) (Chu et al., 2007) which is suggestive of induction of apoptosis. Sunitinib was examined to see if it was also able to induce caspase-3/7 activity as an indication of apoptosis in non-proliferating myocytes. Caspase-3 and -7 are effector caspases that cleave cellular substrates and initiate apoptotic death. We previously showed that doxorubicin treatment induced a significant increase in caspase-3/7 activity in myocytes (Hasinoff et al., 2007). As shown in Fig. 4A myocytes treated for 4 h with sunitinib at concentrations as low as 0.5 μM showed significant increases in

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caspase-3/7 activity ($p < 0.05$ at 0.5 μM and $p < 0.001$ at all other higher concentrations) which indicates that it has strong apoptosis-inducing effects on myocytes.

Activation of Bax, a pro-apoptotic protein Bcl-2 family member, leads to its translocation from the cytosol to the mitochondrial membrane where it causes permeabilization of the mitochondria, resulting in cytochrome *c* release and downstream caspase activation (Li et al., 1997). Because sunitinib treatment increased both LDH release (Fig. 1A) and caspase-3/7 activity (Fig. 4A), we next examined whether protein levels of mitochondrial membrane associated N-terminal accessible Bax (Capano and Crompton, 2006) were also affected. As shown in Fig. 3C and 3D treatment of myocytes with sunitinib for 2 h at concentrations up to 10 μM did not significantly change Bax protein levels.

Effect of sunitinib on ATP levels and oxidation of DCF in myocytes. AMPK is a sensor of cellular energy status and its activation switches on catabolic pathways that generate ATP while switching off ATP-consuming processes (Hardie, 2004). Conversely, inhibition of AMPK might be expected to decrease ATP levels. Thus, in this functional test experiments were done in order to determine if cellular ATP levels were affected by sunitinib treatment. As shown in Fig. 4B treatment of myocytes with concentrations of sunitinib from 0.5 to 10 μM for up to 4 h resulted in no significant change in cellular ATP levels.

The oxidation of 2',7'-dichlorofluorescin to fluorescent 2',7'-dichlorofluorescein, is commonly used to quantitatively measure oxidative stress in cells such as that induced by drugs or hypoxia (Wang et al., 2008). Thus, in order to investigate whether sunitinib directly or indirectly produced

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oxidative stress in myocytes, the oxidation of 2',7'-dichlorofluorescin loaded into myocytes was followed in a fluorescence plate reader. As the results in Fig. 4C show, that while treatment with 20 and 50 μM H_2O_2 significantly (p of < 0.001) increased the rate of 2',7'-dichlorofluorescin oxidation, sunitinib treatment at concentrations from 10 to 40 μM did not significantly affect the rate of 2',7'-dichlorofluorescin oxidation. Thus, it can be concluded, that at least over the short times that these experiments were carried out, that the toxic effects of sunitinib on myocytes were not due to oxidative stress.

We previously showed that the clinically approved doxorubicin cardioprotective agent dexrazoxane (ICRF-187, Zinecard™, Totect™, Savene™), which is a pro-drug iron chelator, reduced doxorubicin-induced LDH release in a neonatal rat cardiac myocyte model (Hasinoff et al., 2003). Dexrazoxane is clinically used to reduce doxorubicin-induced cardiotoxicity (Cvetkovic and Scott, 2005; Hasinoff and Herman, 2007) and has just been approved in the US and Europe for the prevention of anthracycline-induced extravasation injury (Hasinoff, 2008). Drug-induced damage to myocytes can involve a series of events culminating in membrane damage and the release of iron from the cell, which could then involve a secondary indirect iron-induced oxidative stress on other healthy myocytes. Thus experiments were carried out to determine if dexrazoxane might also be cardioprotective against sunitinib-induced damage to myocytes. In contrast to the protection against doxorubicin-induced LDH release we previously observed (Hasinoff et al., 2003), pretreatment of myocytes with 100 μM dexrazoxane did not significantly change the cumulative amount of 5 or 10 μM sunitinib-induced LDH release at 72 h (Fig. 5A). There is considerable evidence that the cardiotoxicity of doxorubicin may be due to iron-dependent oxygen free radical formation (Hasinoff

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and Herman, 2007) on the relatively unprotected cardiac muscle. Thus, the fact that dexrazoxane did not protect against sunitinib-induced damage is in accord with the lack of any effect of sunitinib on the oxidation of 2',7'-dichlorofluorescin (Fig. 4C).

Effect of metformin and phenformin on sunitinib-induced LDH release in myocytes. The antidiabetic biguanide drug metformin is able to activate AMPK in myocytes to induce ACC phosphorylation (Bertrand et al., 2006). Thus if sunitinib is cytotoxic through its inhibition of AMPK, pretreatment of myocytes with metformin might be expected to protect them from sunitinib-induced damage by increasing pACCα and pACCβ levels. In this third functional test, experiments were carried out in which myocytes were pretreated with either 0.1 or 1 mM metformin for 4 h. This was followed by a 5 μM sunitinib treatment for 3 h, after which both drugs were washed off. As shown in Fig. 5B metformin did not significantly protect against sunitinib-induced LDH release. Metformin treatment alone compared to control had no significant ($p = 0.5$) effect on LDH release at 72 h. Similar experiments with the more potent (Bertrand et al., 2006) metformin analog phenformin also had no significant effect (data not shown). Thus, even though a 5 μM sunitinib treatment was able to reduce pACCα levels (Fig. 3B), the fact that pretreatment of myocytes with metformin was not protective suggests that sunitinib-induced damage was not AMPK mediated. It is also worthy of note that a 3 h treatment of myocytes with 5 μM sunitinib (Fig. 5B) caused almost as much LDH release at 72 h as continuous treatment of myocytes for 72 h (Fig. 5B). If the two 20-min washes were effective in reducing intracellular concentrations of sunitinib, this suggests that initiation of sunitinib-induced damage occurred quickly and was not reversed by removal of the drug.

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Discussion

The results of this study have shown that either a continuous (Fig. 1) or a 3 h treatment (Fig. 5B) of myocytes with sunitinib causes a dose-dependent LDH release at concentrations that are similar to peak plasma or even trough concentrations observed when a therapeutic once-daily dose of sunitinib is given (Deeks and Keating, 2006; Faivre et al., 2006). Based on the observation that sunitinib bound strongly to both AMPK and RSK kinases (Fabian et al., 2005; Karaman et al., 2008), it had been hypothesized (Force et al., 2007) that inhibition of AMPK and/or inhibition of RSK might be responsible for sunitinib-induced cardiotoxicity. However, because a competition binding assay was used, it had not been previously shown (Fabian et al., 2005; Karaman et al., 2008) whether sunitinib is able to inhibit the enzyme activity of AMPK and RSK. Sunitinib did, in fact, potently inhibit the enzyme activity of both AMPK and RSK1 (Fig. 2) with IC_{50} values of about 0.32 and 0.36 μM, respectively. Because these IC_{50} values are in the therapeutic plasma concentration range for sunitinib (Deeks and Keating, 2006; Faivre et al., 2006), they both could potentially contribute to the cardiotoxicity (Chu et al., 2007; Telli et al., 2008) of sunitinib. It should be also noted that because the kinase inhibition assays were measured with 50 μM ATP, which is much less than the millimolar intracellular ATP concentrations, the sunitinib IC_{50} values would increase due to competition with ATP.

AMPK is a sensor of cellular energy status and its activation controls ATP levels (Hardie, 2004). Because cardiac tissue, unlike other tissues, has only a small reserve of ATP, impaired ATP production could be a factor in sunitinib-induced cardiotoxicity. In order to see if sunitinib could functionally affect one of the phosphorylation targets of AMPK, its effect on the phosphorylation

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of ACC in myocytes was studied. While sunitinib did decrease pACCα levels (Fig. 3B) in a dose-dependent manner, significance was only achieved at a sunitinib concentration of 5 μM. Because this concentration is about 25-fold higher than therapeutic sunitinib plasma levels (Deeks and Keating, 2006; Faivre et al., 2006), it is unclear whether therapeutic levels could significantly inhibit AMPK to contribute to the cardiotoxicity of sunitinib. However, it must be noted that these experiments were carried out with a 2 h treatment of the myocytes with sunitinib, while patients are dosed daily with sunitinib for extended periods of time. Thus continual inhibition of AMPK activity by sunitinib due to long term dosing could have more subtle effects in inducing cardiotoxicity. Also the cellular levels of sunitinib that are achieved with daily dosing are unknown and could be higher than plasma levels. Thus we cannot rule out that more subtle cardiotoxic effects can occur due to long term partial inhibition of AMPK when patients are treated with sunitinib for extended periods of time. It should also be noted that sunitinib causes hypertension (Deeks and Keating, 2006), which may modify cardiac energy metabolism and make the heart more sensitive to inhibition of AMPK. The relative decrease in the levels of mitochondrial-associated pACCβ were not significantly different from those observed for pACCα (Fig. 3B), and thus sunitinib does not preferentially affect pACCβ in mitochondrial-rich myocytes.

A second functional test of whether inhibition of AMPK by sunitinib may be causing cardiotoxicity was carried out by measuring its effect on intracellular levels of ATP in myocytes (Fig. 4B). However, the lack of any significant effects, at least with this short 4 h treatment, suggests that sunitinib did not induce damage in myocytes by reducing ATP levels.

As measured by caspase-3/7 activity, sunitinib treatment did induce a rapid apoptotic response

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in myocytes (Fig. 4A) indicating that this pathway may be significant in producing cardiotoxicity. However, Bax levels were not significantly changed by sunitinib treatment (Fig. 4B), indicating there is no role for this pathway in the induction of apoptosis. It would also appear, that as measured by oxidation of intracellular oxidation of DCF, that sunitinib did not cause oxidative damage to myocytes. This conclusion was also supported by experiments in which myocytes were pretreated with the clinically approved doxorubicin cardioprotective agent dexrazoxane which showed that dexrazoxane provided no significant protective effect (Fig. 5A) against sunitinib-induced damage. This result suggests that dexrazoxane would not be clinically useful as a sunitinib protective agent.

In a final functional test, pretreatment of myocytes with either the antidiabetic drug metformin or phenformin did not protect myocytes from sunitinib. Metformin and phenformin activate AMPK (Bertrand et al., 2006), and its activation could in principle partially compensate for the AMPK inhibitory effects of sunitinib. The failure of metformin and phenformin to protect myocytes also suggests that sunitinib-induced damage to myocytes is not AMPK-mediated.

In this study we investigated the inhibition of only two potential kinase targets (AMPK and RSK1) that might be involved in the cardiotoxicity of sunitinib. In a recent extensive profiling of the binding of 38 kinase inhibitors to 317 kinases representing more than 50% of the predicted human kinome, sunitinib as measured by its K_d, was the second most promiscuous inhibitor (next only to staurosporine) (Karaman et al., 2008). At a sunitinib concentration of 0.1 μM, which is approximately equal to therapeutic plasma levels (Deeks and Keating, 2006; Faivre et al., 2006), sunitinib strongly bound to 18% of the kinases tested (Karaman et al., 2008). If this degree of binding, and hence inhibition, is maintained over the whole human kinome of more than 500

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proteins, this result suggests that approximately 90 kinases are potently inhibited at therapeutic plasma levels of sunitinib. Inhibition of which kinase, or even which combination of kinases, is responsible for the cardiotoxicity may be very difficult to ever definitively identify. While this study did show that sunitinib inhibited AMPK within its therapeutic range, other functional tests of downstream effects of this inhibition were, for the most part, not supportive of a major role for sunitinib inhibition of AMPK. Balanced against this it should be noted that all of these functional tests were carried out over a period of a few hours only.

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Footnotes

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b) Address correspondence to:

Dr. Brian Hasinoff, Faculty of Pharmacy, Apotex Centre, University of Manitoba, 750 McDermot Avenue, Winnipeg, Manitoba R3E 0T5, Canada. E-Mail: B_Hasinoff@UManitoba.ca

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Legends for Figures

Fig. 1. As measured by LDH release sunitinib damaged cardiac myocytes. A, Plot of cumulative percentage LDH release from myocytes that were untreated (○), or continuously treated with 0.1 μM (△), 0.5 μM (▽), 1 μM (□), 5 μM (◇), or 10 μM (●) sunitinib. B, Plot of percentage LDH release at 72 h after treatment with sunitinib. Treatment of myocytes with either 0.1, 0.5, 1, 5, or 10 μM sunitinib significantly increased LDH release at 72 h compared to untreated myocytes. Significance relative to the untreated controls: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. The leftmost zero data point on the abscissa was measured in the absence of sunitinib. While the results shown were from single experiments measured in 4 (8 for the control) replicate wells, they were typical of 3 separate myocyte isolations. Where error bars are not seen, they are smaller than the size of the symbol. The structure of sunitinib is shown in the inset.

Fig. 2. Sunitinib inhibits AMPK and RSK1 kinase activity. Sunitinib inhibited AMPK and RSK1 kinase activities with IC_{50} values of 0.32 ± 0.02 and 0.36 ± 0.03 μM, respectively. The smooth (AMPK) and broken (RSK1) lines are the best fit calculated values from 4-parameter non-linear least squares fits to the activity-concentration data. The leftmost zero data points on the abscissa are the activities measured in the absence of sunitinib.

Fig. 3. Treating myocytes with sunitinib reduces pACC levels but has no effect on Bax. A, Western blots for pACCα, pACCβ, ACCα and ACCβ showing a sunitinib concentration dependent decrease

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for each of the pACC isoforms. Myocytes were treated with sunitinib for 2 h, lysed, and subjected to SDS-polyacrylamide gel electrophoresis and western blotting. The blots were first probed with antibody to pACC, stripped, and reprobed with antibody to ACC. The blots are representative of five independent experiments. B, Quantitation of western blots by densitometry showing a sunitinib concentration dependent decrease in pACCα and pACCβ protein levels. The results of five independent experiments are reported as the mean ± SE. The pACCα or pACCβ protein levels were measured relative to ACCα or ACCβ levels, respectively, and normalized to the controls for each isoform. Significance relative to the untreated controls: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. C, Western blots for Bax showing lack of an effect of sunitinib treatment. Myocytes were treated with sunitinib for 2 h, lysed, and subjected to SDS-polyacrylamide gel electrophoresis and western blotting. The blots were probed with antibodies to Bax and β-actin (as a loading control). D, Quantitation of western blots by densitometry showing a lack of sunitinib concentration dependence in Bax protein levels. The results of three independent experiments are reported as the mean ± SE. Significance relative to untreated controls was not achieved with treatment at any sunitinib concentration.

Fig. 4. Effect of sunitinib on induction of caspase-3/7 apoptosis, ATP levels, and DCF oxidation in myocytes. A, Sunitinib treatment induces caspase-3/7 activity in myocytes after 2 and 4 h of treatment. Caspase-3/7 induced rate of increase in luminescence was measured over the first 8 min in a luminescence assay using a proluminescent caspase-3/7 DEVD-aminoluciferin substrate. The results are from an average of 5 wells (10 for the control). Significance relative to the untreated

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controls: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. B, Sunitinib treatment has no effect on cellular ATP levels in myocytes. ATP levels were measured directly after addition of sunitinib (0.1 h) or after 1, 2 or 4 h of treatment. The results are from an average of 4 wells (16 for the control). Significance was not achieved relative to the untreated controls for any treatments. C, Sunitinib treatment has no affect on cellular DCF oxidation in myocytes. The change in the rate of fluorescence increase due to intracellular oxidation of DCF was measured after the treatment with either H_2O_2 or sunitinib at the concentrations indicated. The results are from an average of 4 wells (8 for the control). Relative to untreated controls significance (***$p < 0.001$) was only achieved for both H_2O_2 treatments.

Fig. 5. Neither the doxorubicin cardioprotective agent dexrazoxane, nor the AMPK activator metformin protected myocytes for sunitinib-induced LDH release. A, Plot of cumulative percentage LDH release from myocytes that were untreated (○); or treated with 5 μM sunitinib (△) alone; or pretreated with 100 μM dexrazoxane and then treated with 5 μM sunitinib (▲); or treated with 10 μM sunitinib (▽) alone; or pretreated with 100 μM dexrazoxane and then treated with 10 μM sunitinib (▼); or continuously treated with 100 μM dexrazoxane (□) alone. The dexrazoxane pretreatments were for 3 h prior to treatment with sunitinib. Dexrazoxane pretreatment did not significantly protect myocytes from any sunitinib-induced LDH release at 72 h. While the results shown are from a single myocyte isolation, they are typical of results of five experiments from two separate isolations. B, Plot of cumulative percentage LDH release from myocytes that were untreated (○); or treated with 5 μM sunitinib (△) for 3 h; or pretreated with 100 μM metformin for 4 h and then treated with 5 μM sunitinib for 3 h (▲); or treated with 100 μM metformin (□) alone for 7 h; or

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pretreated with 1 mM metformin for 4 h and then treated with 5 μM sunitinib for 3 h (▼); or treated with 1 mM metformin (∇) alone for 7 h. At the end of the drug incubations both drugs were washed off by replacing the medium twice for 20 min. Metformin pretreatment did not significantly protect myocytes from any sunitinib-induced LDH release at 72 h. While the results shown are from a single myocyte isolation they are typical of results from two other isolations. Where error bars are not seen, they are smaller than the size of the symbol.

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Fig. 1

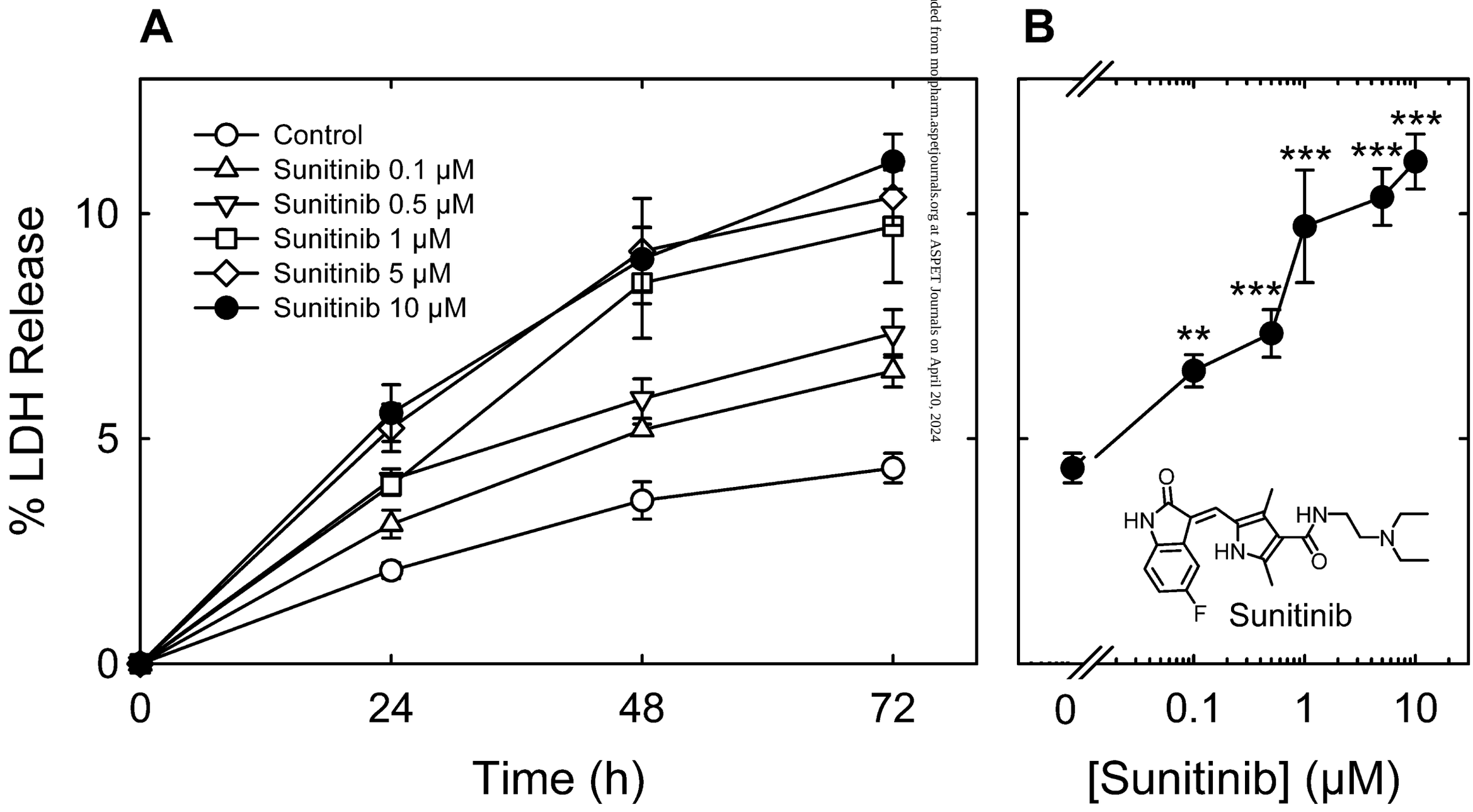

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Fig. 2

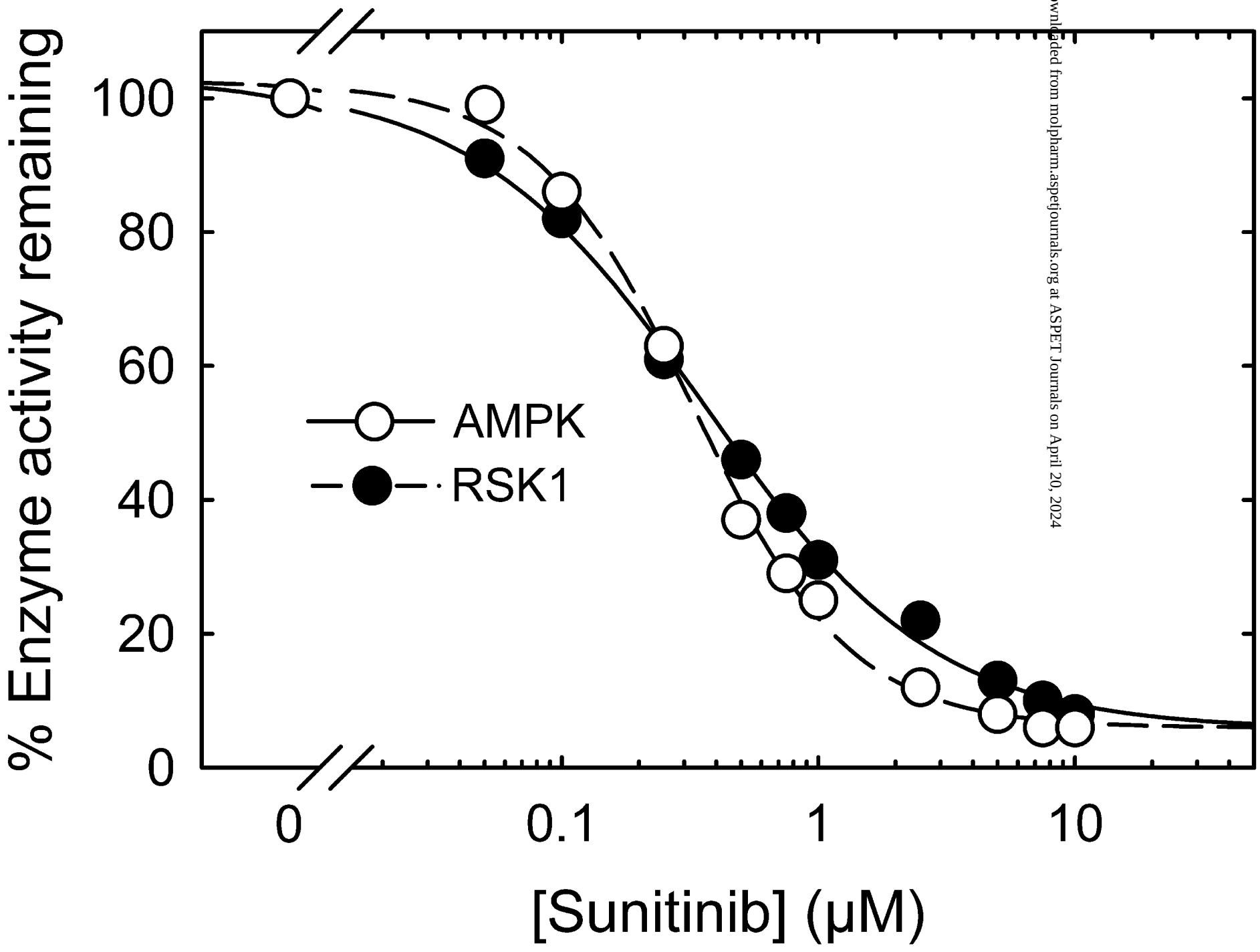

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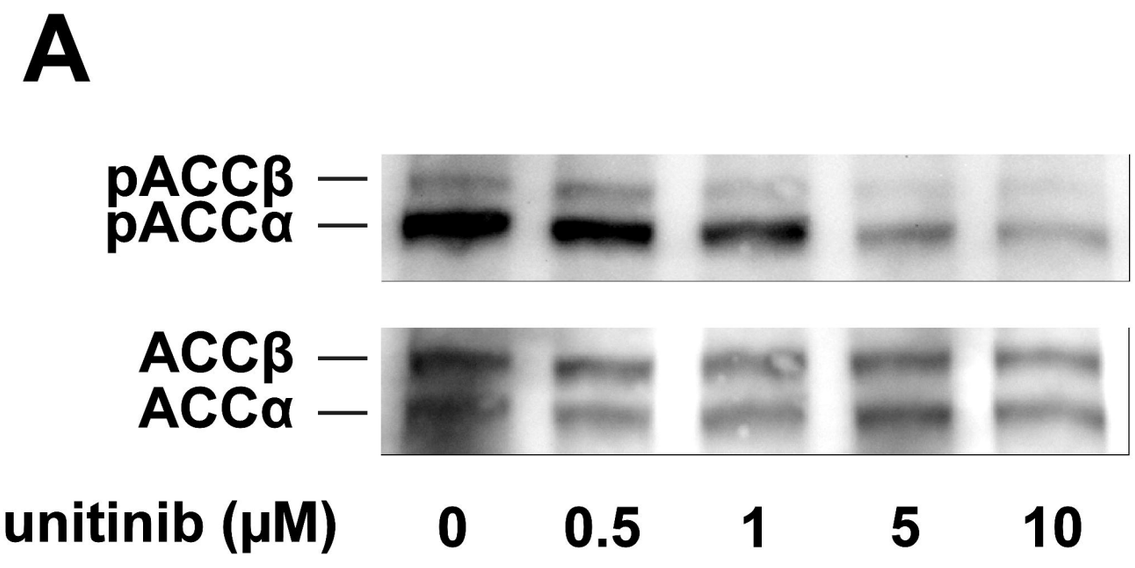
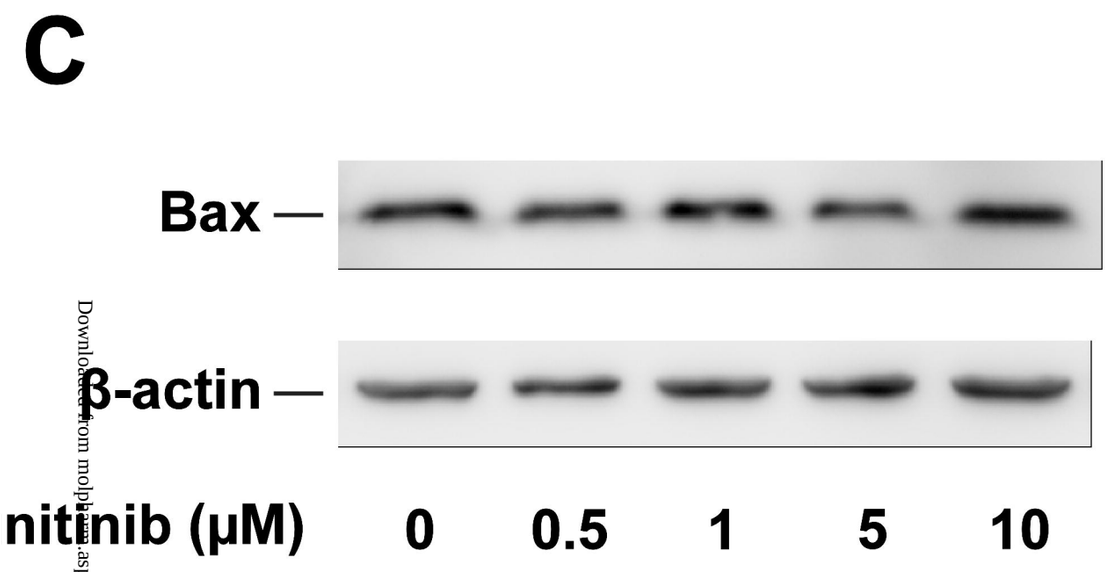
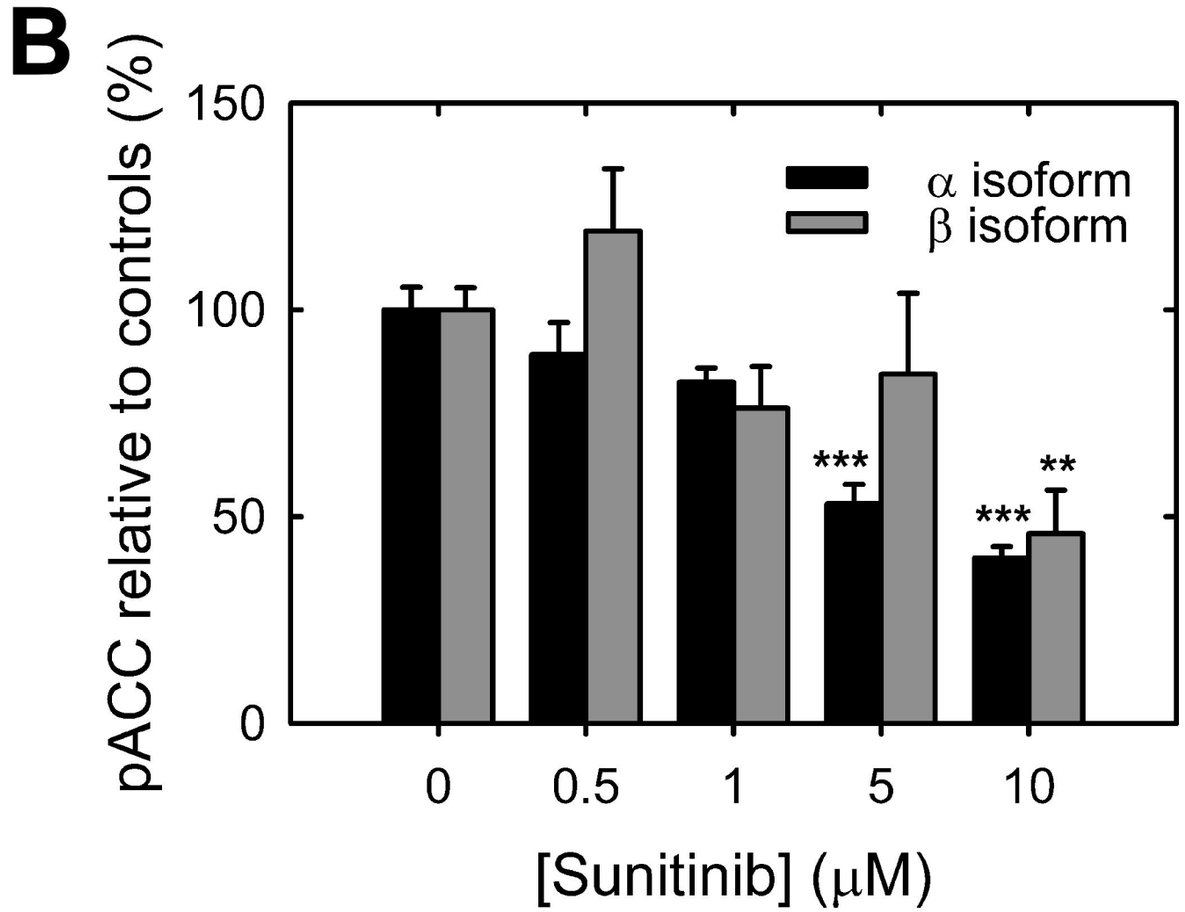
Fig. 3







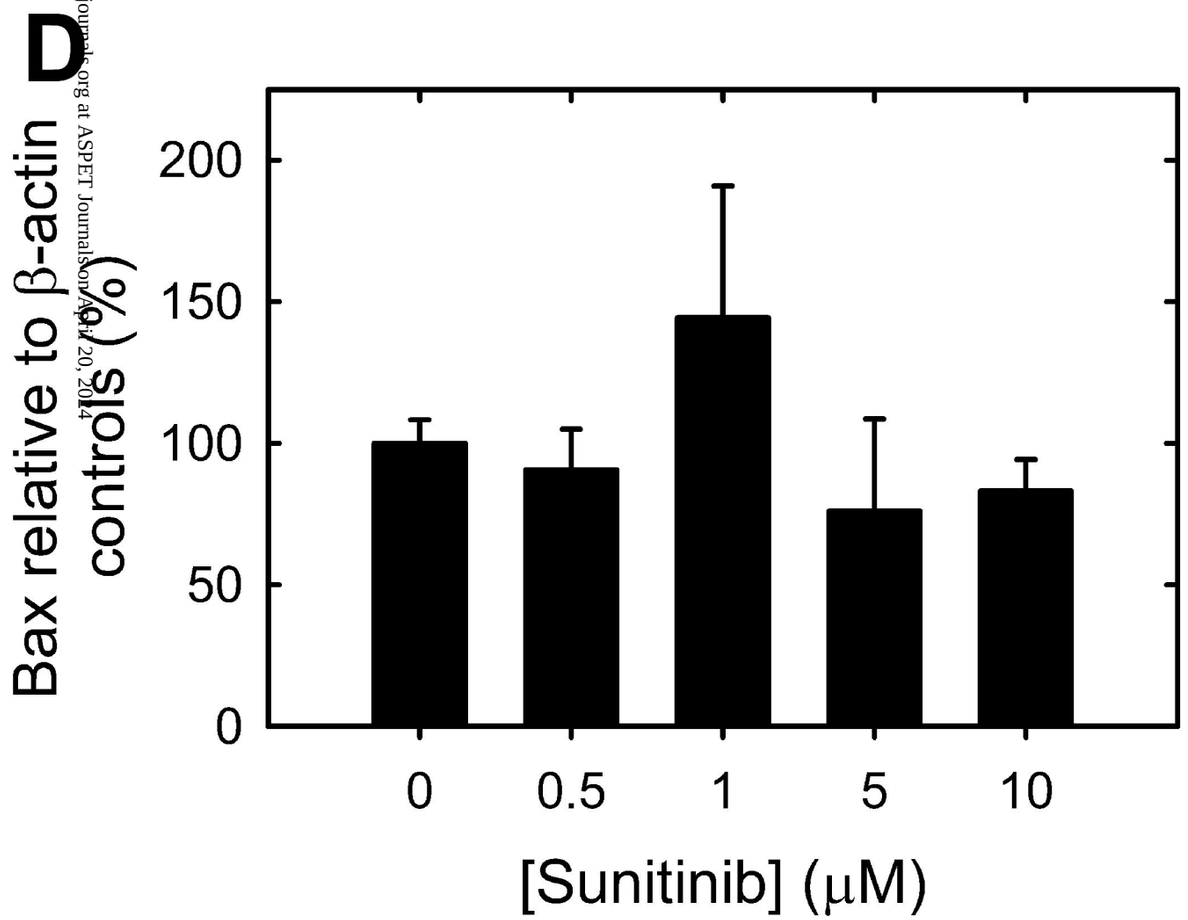


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Fig. 4

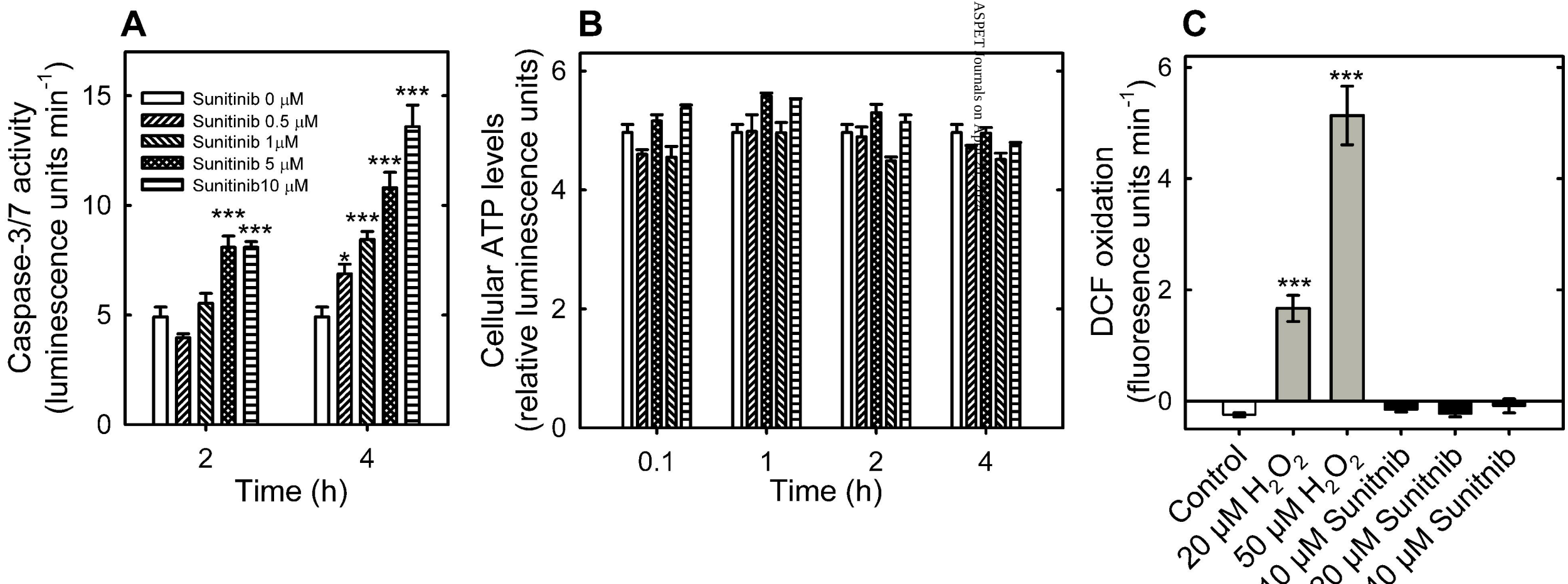

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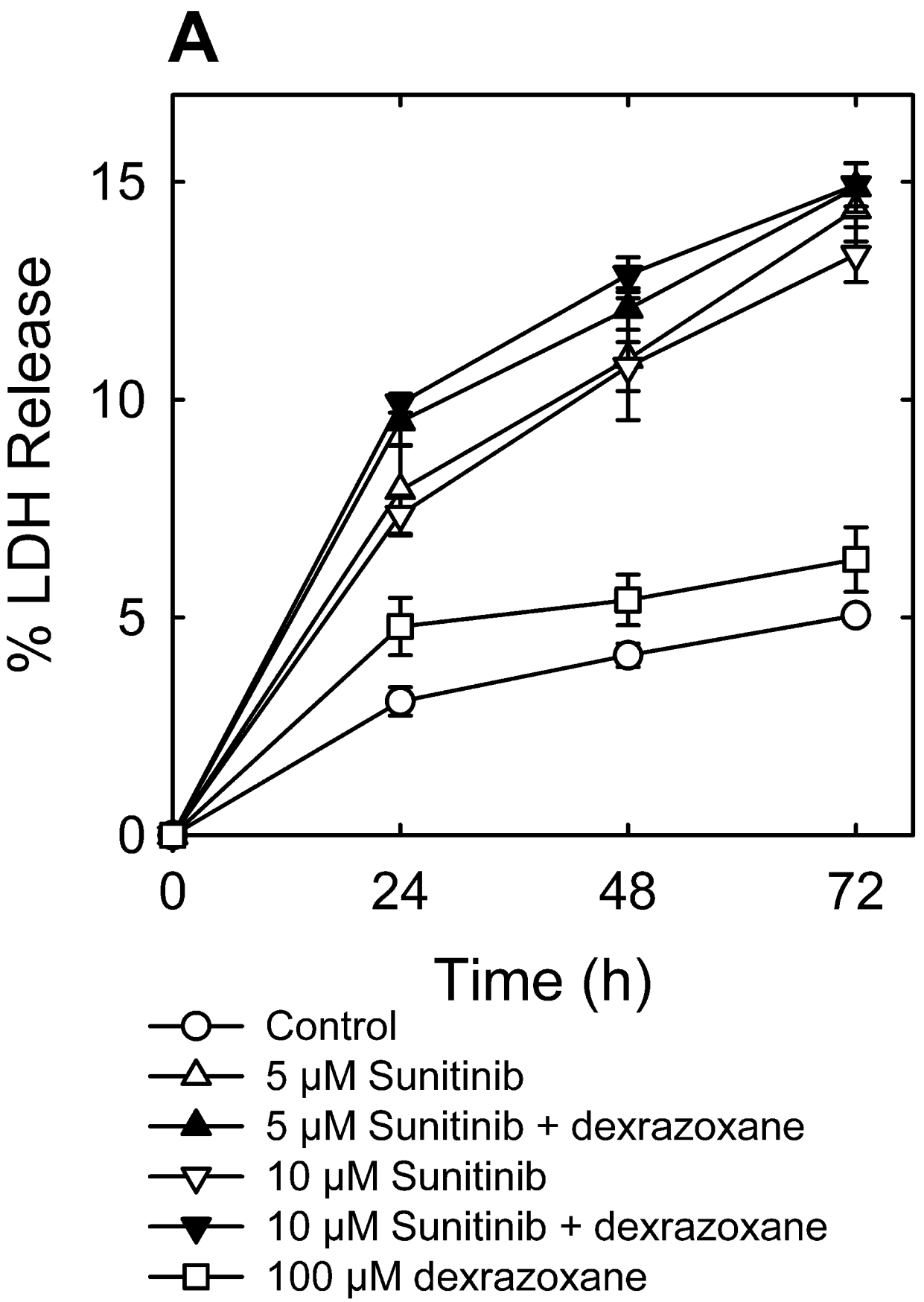
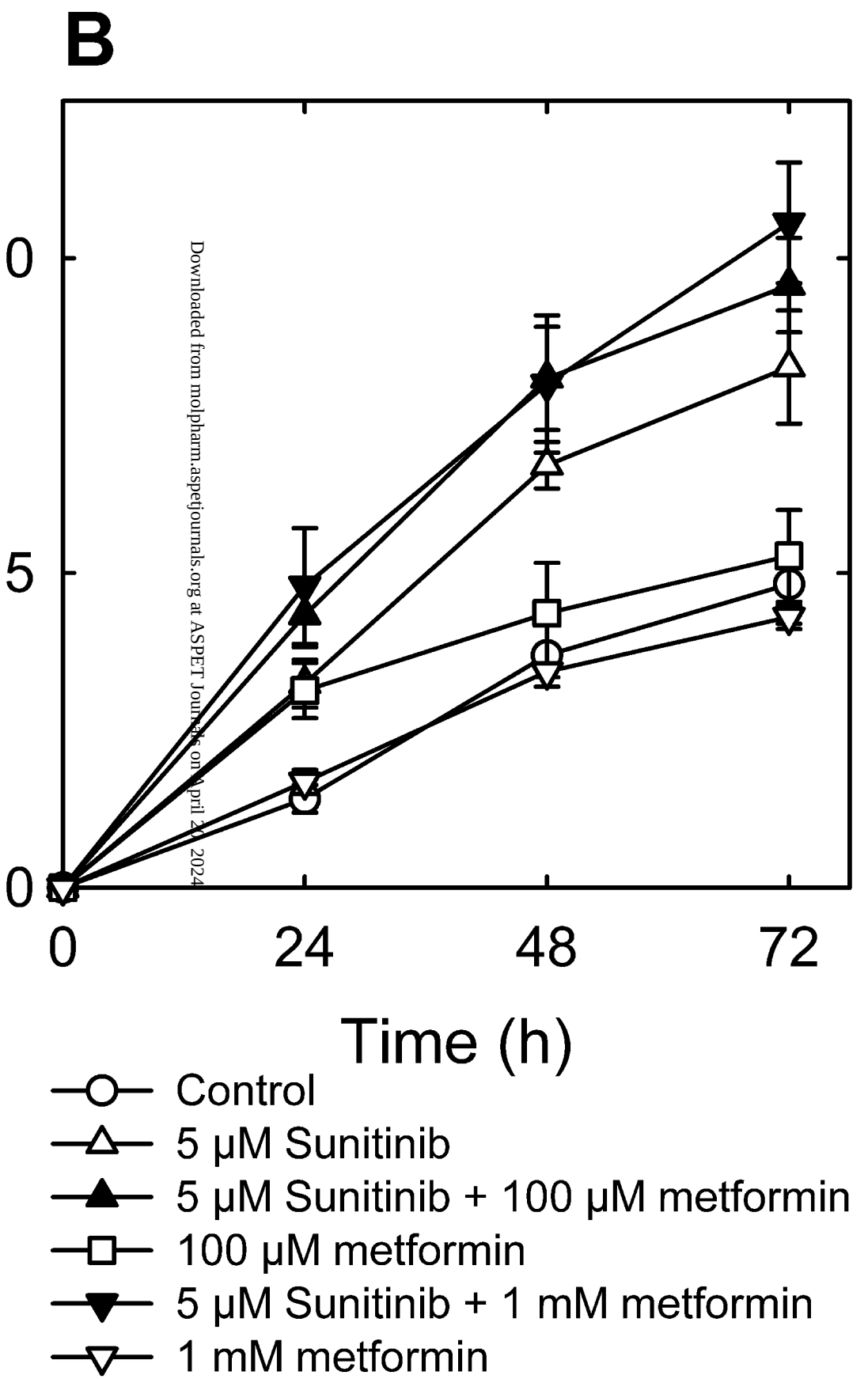
Fig. 5





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