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Keywords: Rat Langendorff heart model; tyrosine kinase inhibitor Sunitinib; PKCα pathway; HL60 cell line; microRNAs; A3 adenosine receptor agonist IB-MECA.

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Abstract: Sunitinib is an anti-cancer tyrosine kinase inhibitor associated with severe cardiotoxic adverse effects. Using rat Langendorff heart model and human acute myeloid leukemia 60 (HL60) cell line we detected the involvement of protein kinase C (PKC) α during Sunitinib-induced cardiotoxicity and the effect of Sunitinib on cancer progression. The cardioprotective and anti-cancer properties of the A3 adenosine receptor agonist 2-chloro-N6-(3-iodobenzyl)-adenosine-5′-N-methyluronamide (IB-MECA) were investigated.
The cardiac effect of Sunitinib (1μM) and IB-MECA (1nM) treatment was measured through haemodynamic and infarct size assessment. The cytotoxic effect of Sunitinib (0.1 - 10 μM) and IB-MECA (10 nM - 10 μM) on HL60 cells was assessed using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay technique. Myocardial injury associated microRNAs (miR-1, miR-27a, miR-133a and miR-133b) and cancer associated microRNAs (miR-15a, miR-16-1 and miR-155) were profiled by qRT-PCR in the cardiac tissue and HL60 cells, while phosphorylated PKCα levels were measured by Western Blot analysis.
Sunitinib treatment increased infarct size and decreased left ventricular developed pressure and heart rate. Co-treatment of IB-MECA reversed the myocardial injury produced by Sunitinib administration. IB-MECA did not jeopardize the anti-cancer effect of Sunitinib in HL60 cells. The expression signature of the specific microRNAs in cardiac tissue and HL60 cells showed an altered expression profile when treated with Sunitinib and IB-MECA. pPKCα levels were increased by Sunitinib treatment in cardiac tissue and HL60 cells and co-administration of IB-MECA attenuated this increase in the cardiac tissue.
This study reveals that A3 adenosine receptor activation by IB-MECA attenuates Sunitinib-induced cardiotoxicity through the involvement of PKCα.

Cover Letter

Faculty Research Centre in Applied Biological and Exercise Sciences, Faculty of Health and Life Sciences, Coventry University, Coventry, CV1 5RW,United Kingdom

08/08/2017

Dear Sir/Madam,

Please find the resubmission our research paper with the title: "**Attenuation of Sunitinib-induced cardiotoxicity through the A3 adenosine receptor activation**".

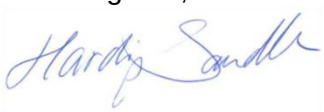
The total text count excluding References is 6444 words (Word count per section: Abstract: 249; Introduction: 500; Material and methods: 2214; Results: 2025; Discussion: 1359). We have cited 59 papers in the Reference list. All figures have been designed using the GraphPad Prism programme (version 5.03) and are attached individually as 300 dpi TIFF formatted. GraphPad Prism was furthermore used for statistical analysis of data.

The manuscript was submitted to European Journal of Pharmacology on the 5th of May 2017 and we received with the feedback with major corrections from European Journal of Pharmacology the 23rd of June 2017. We amended the manuscript according to the feedback. On the 19th of July and the 4th of August we received some final minor corrections and we have hence updated the manuscript accordingly. We trust you will find the manuscript enclosed to your satisfaction.

All the authors have read the manuscript and approve the publication with European Journal of Pharmacology, and we accept the publishing term and conditions of European Journal of Pharmacology.

Please do not hesitate to contact us if you need any additional information regarding this manuscript.

Kind Regards,



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20170808

Attenuation of Sunitinib-induced cardiotoxicity through the A3 adenosine receptor activation

EJP Editorial Office comments are in grey fonts
Corresponding author's response is in black Italic fonts

04-08-2017

Re: EJP-46152R2 Title: Attenuation of Sunitinib-induced cardiotoxicity through the A3 adenosine receptor activation Dear Dr. Sandhu, Your submission entitled "Attenuation of Sunitinib-induced cardiotoxicity through the A3 adenosine receptor activation" has been received by European Journal of Pharmacology .

However, before we can send your manuscript to the publisher we ask you to make the following changes to your submission:

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- To indicate statistical significance in Figures, please use symbols such as *, **, ***, instead of letters a, b, c. Lower case letters are used for tables only.

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Yours sincerely, EJP Editorial Office

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Response:
The manuscript and figure legends has been amended according to these instructions.

Title

Attenuation of Sunitinib-induced cardiotoxicity through the A3 adenosine receptor activation

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Abstract

Sunitinib is an anti-cancer tyrosine kinase inhibitor associated with severe cardiotoxic adverse effects. Using rat Langendorff heart model and human acute myeloid leukemia 60 (HL60) cell line we detected the involvement of protein kinase C (PKC) α during Sunitinib-induced cardiotoxicity and the effect of Sunitinib on cancer progression. The cardioprotective and anti-cancer properties of the A_3 adenosine receptor agonist 2-chloro-N6-(3-iodobenzyl)-adenosine-5′-N-methyluronamide (IB-MECA) were investigated.

The cardiac effect of Sunitinib (1μM) and IB-MECA (1nM) treatment was measured through haemodynamic and infarct size assessment. The cytotoxic effect of Sunitinib (0.1 – 10 μM) and IB-MECA (10 nM – 10 μM) on HL60 cells was assessed using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay technique. Myocardial injury associated microRNAs (miR-1, miR-27a, miR-133a and miR-133b) and cancer associated microRNAs (miR-15a, miR-16-1 and miR-155) were profiled by qRT-PCR in the cardiac tissue and HL60 cells, while phosphorylated PKCα levels were measured by Western Blot analysis.

Sunitinib treatment increased infarct size and decreased left ventricular developed pressure and heart rate. Co-treatment of IB-MECA reversed the myocardial injury produced by Sunitinib administration. IB-MECA did not jeopardize the anti-cancer effect of Sunitinib in HL60 cells. The expression signature of the specific microRNAs in cardiac tissue and HL60 cells showed an altered expression profile when treated with Sunitinib and IB-MECA. pPKCα levels were increased by Sunitinib treatment in cardiac tissue and HL60 cells and co-administration of IB-MECA attenuated this increase in the cardiac tissue.

This study reveals that A_3 adenosine receptor activation by IB-MECA attenuates Sunitinib-induced cardiotoxicity through the involvement of PKCα.

Keywords:

Rat Langendorff heart model; tyrosine kinase inhibitor Sunitinib; PKCα pathway; HL60 cell line; microRNAs; A_3 adenosine receptor agonist IB-MECA.

1 Introduction

Sunitinib belongs to the tyrosine kinase inhibitor family and is used to treat various cancer forms, such as renal cell carcinoma, gastrointestinal stromal tumours and colorectal cancers (Le Tourneau et al., 2007). Sunitinib inhibits cancer specific cellular signalling by targeting the adenosine 5'-triphosphate (ATP) binding site of multiple receptor tyrosine kinases involved in tumour angiogenesis and tumour cell proliferation: these include receptors for platelet-derived growth factor and vascular endothelial growth factor. Attenuation of receptors for platelet-derived growth factor and vascular endothelial growth factor signalling by Sunitinib is a powerful tumour treatment as both tumour vascularisation is reduced and cancer cell apoptosis is initiated (Mendel et al., 2003).

Sunitinib has displayed a lack of kinase selectivity resulting in the cardiotoxic adverse effects of Sunitinib (Hasinoff and Patel 2010; Hasinoff, et al., 2008; Krause and Van Etten 2005). Cardiotoxic adverse effects of tyrosine kinase inhibitor therapy range from asymptomatic QT prolongation, reduction in left ventricular ejection fraction, acute coronary syndromes, myocardial infarction and symptomatic congestive heart failure (Chu et al., 2007; Kerkelä et al., 2006; Force et al., 2007; Khakoo et al., 2008). Sunitinib inactivates the adenosine monophosphate-activated protein kinase, which is crucial for cell survival after hypoxia, causing cardiomyocyte death and hypertrophy (Force et al., 2007). Adenosine monophosphate-activated protein kinase has the potential to inhibit the activation of PKC (Ceolotto et al., 2007). PKC has been shown to have an important role on cardiac contractility (Braz et al., 2004). Braz *et al.* 2004 demonstrated that over expression of PKCα causes hypo-contractility which is associated with cardiomyopathy (Braz et al., 2004).

The A_3 adenosine receptor agonist IB-MECA has been shown to have powerful cardioprotective effects against cardiac damage caused by hypoxia, ischaemia/reperfusion injury and anti-cancer treatment with Doxorubicin (Carr et al., 1997; Tracey et al., 1997; Maddock et al., 2002a; Shneyvays et al., 2002; Maddock et al., 2003; Emanuelov et al.,

2010). IB-MECA has been shown to reduce the level of ischaemia and infarct size in the heart by reducing abnormal Ca^{2+} levels and the accumulation of free radicals. It has been suggested that the cardioprotection generated by IB-MECA is mediated through the PKC pathway (Auchampach et al., 1997), as inhibition of the PKC pathway has been shown to produce cardioprotective results by reducing apoptosis (Thuc et al., 2012; Rakkar and Bayraktutan, 2016).

The expression of miRNAs miR-155 and the miR-15a-miR-16-1 cluster have been associated with cancer development (Calin et al., 2002; Faraoni et al., 2009), while miR-1, miR-27, miR-133a and miR-133b have been linked to myocardial injury (Sandhu and Maddock, 2014; Ai et al., 2010; D'Alessandra et al., 2010; Wang et al., 2010; Yang et al., 2007).

We investigated the ability of the A_3 adenosine receptor agonist, IB-MECA to reduce the level of Sunitinib induced cardiotoxicity in Langendorff heart experiments. The intracellular signalling molecule PKCα levels were assessed to determine PKCα's involvement in cardioprotection elicited by IB-MECA. The cytotoxic effect of Sunitinib ± IB-MECA was investigated in HL60 cells. Furthermore, microRNAs associated cardiotoxicity and cancer development were also profiled.

2 Materials and methods

2.1 Cell line and reagents

The HL60 cell line were obtained from European Collection of Cell Culture (England). RPMI 1640 medium and MTT was purchased from Sigma Aldrich (USA) and the medium supplements L-Glutamine, HEPES and antibiotics mix (100 U/ml penicillin and 100 µg/ml of streptomycin) were from Invitrogen (UK), while foetal bovine serum (FBS) was from Biosera (UK). Formaldehyde was bought from Fisher Scientific (USA). Protease inhibitor cocktail was purchased from Roche (UK). Sunitinib malate and 2,3,5-triphenyltetrazolium chloride

(TTC) was purchased from Sigma Aldrich (USA) and IB-MECA was purchased from Tocris Bioscience (UK). Antibodies anti-PKCα (phospho T497) pPKCα and total PKC were from Abcam (UK), while glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (14C10) rabbit mAb and anti-rabbit linked IgG antibody conjugated to horseradish peroxidase were from Cell Signalling (UK). RNAlater, Ambion mirVana miRNA Isolation Kit, Applied Biosystems MicroRNA Reverse Transcription Kit, TaqMan Universal PCR Master Mix II (uracil N-Glycosylase not included), Applied Biosystems primers assays (U6, hsa-miR155, hsa-miR-15a, hsa-miR-16-1, rno-miR-1, hsa-miR-27a, hsa-miR-133a and hsa-miR-133b) were purchased from Life Technologies (USA).

2.2 Animals and Ethics

Adult male Sprague-Dawley rats (300-350 g in body weight); were purchased from Charles River UK Ltd (UK) and housed suitably, received humane care and had free access to standard diet according to "The Guidance on the Operation of the Animals (Scientific Procedures) Act of 1986". Animals were selected at random for all treatment groups and the collected tissue was blinded for infarct size assessment. The experiments were performed after approval of the protocol by the Coventry University Ethics Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments.

2.3 Langendorff perfused model using rat hearts

Rats were sacrificed by cervical dislocation (Schedule 1 Home Office procedure) and the hearts were rapidly excised and placed into ice-cold Krebs Henseleit buffer (118.5 mM NaCl, 25 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.7 mM $CaCl_2$, and 12 mM glucose, pH=7.4). The hearts were mounted onto the Langendorff system and retrogradely perfused with Krebs Henseleit buffer. The pH of the Krebs Henseleit buffer was maintained at 7.4 by gassing continuously with 95 % O_2 and 5 % CO_2 and maintained at 37 ± 0.5 °C using a water-jacketed organ chamber. The left atrium was removed and a latex iso-volumic

balloon was carefully introduced into the left ventricle and inflated up to 5-10 mmHg. Functional recordings (left ventricular developed pressure and heart rate) were taken via a physiological pressure transducer and data recorded using Powerlab, AD Instruments Ltd. (UK). Coronary flow was measured by collecting and measuring the volume of perfusate for 1 min, thereafter it was disposed of. Haemodynamic measurements were taken immediately after the 20 min stabilisation period (time point 0 min) and then at a 5 min interval up to 35 min followed by measurements taken at 15 min interval until the drug exposure. Haemodynamic effects are presented as a percentage of the mean stabilisation period for each parameter to allow clear comparison across drug groups.

Each Langendorff study was conducted for 145 min: a 20 min stabilisation period and 125 min of drug or control perfusion in normoxic conditions. Hearts were included in the study with heart rate between 225-325 beats per min, left ventricular developed pressure between 80-150 mmHg and a coronary flow between 3.5-12.0 ml/g (weight of the rat heart) during the stabilisation period. Sunitinib malate (1 µM) was administered throughout the perfusion period in the presence or absence of IB-MECA (1 nM). The concentration of 1 µM Sunitinib was chosen in line with the clinically relevant study by Goodman et al. 2007, where patients suffering from imatinib refractory or intolerant gastrointestinal stromal tumour, and patients with metastatic renal cell carcinoma where treated with Sunitinib. The steady state blood concentrations of Sunitinib was reported to be in the range of 0.1 – 1.0 µM (Henderson et al., 2013; Goodman et al., 2007). The dose of 1 nM IB-MECA was chosen in line with previous *in vitro* studies (Maddock et al., 2002b).

Langendorff perfused hearts treated with dimethyl sulfoxide (DMSO) as vehicle were recorded as control group. The hearts were then weighed and either stored at -20 °C for TTC staining or the left ventricular tissue was dissected free and immersed in RNAlater for qRT-PCR or snap frozen by liquid nitrogen for Western blot analysis.

2.4 Infarct size analysis

Frozen whole hearts were sliced into 4-6 transverse sections approximately 2 mm thick and incubated in 0.1 % TTC solution in phosphate buffer (2 ml of 100 mM $NaH_2PO_4.2H_2O$ and 8 ml of 100 mM NaH_2PO_4, pH = 7.4) at 37 °C for 15 min and fixed in 10 % formaldehyde (Fisher Scientific, UK) for 4 h. The risk zone and infarct areas were traced onto acetate sheets. The tissue at risk stained red and infarct tissue appeared pale. The acetate sheet was scanned and ImageTool from UTHSCSA (USA) software was used to measure the area of infarct and the area of risk. The infarct to risk size was calculated (Infarct size (%) = (Area of infarct / Total area of heart slice) X 100) for each individual slice, and an average was taken of all of the slices from each heart to give the percentage infarct size of the whole heart. The mean of infarct to risk ratio for each treatment group and the mean ± S.E.M. was plotted as a bar chart. The infarct size determination was randomised and blinded.

2.5 Human acute myeloid leukaemia HL60 cell studies

2.5.1 HL60 cell culture

The HL60 cell line were maintained in RPMI 1640 medium supplemented with L-Glutamine (2 mM) and 10 % heat-inactivated FBS and antibiotics mix at 37 °C in a humidified incubator under 5 % CO_2/95 % air. Cells were counted with nucleoCounter (Chemometec, Denmark) and split in a 1:5 ratio every 2-3 days. Cells were incubated with (i) vehicle (Control) or increasing concentrations of (ii) Sunitinib (0.1 – 10 μM), (iii) Sunitinib (0.1 – 10 μM) + IB-MECA (1 nM) or (iv) IB-MECA (10 nM – 10 μM) for 24 h. The dose range for Sunitinib (0.1 – 10 μM) and IB-MECA (10 nM – 10 μM) was based from cell viability studies, where these drugs were showing to have an apoptotic effect on HL60 cells. Sunitinib has shown to induced apoptosis in HL60 cells in the dose range of 1-9 μM through G_1 cell cycle arrest (Teng et al., 2013), while the study by Kohno *et al.* 1996 showed that IB-MECA concentrations ≥ 10 μM caused apoptosis in HL-60 cells (Kohno et al., 1996). Sunitinib and IB-MECA were dissolved in DMSO and the DMSO concentration was < 0.05 % (v/v) during the *in vitro* studies.

2.5.2 Cell viability assessed by MTT assay

HL60 cells were incubated in 100 µl of RPMI media in 96-well plates with the above indicated concentration of the drugs for 24 h at an initial cell density of 10^5 cells/ml. After each period of incubation 30 µl of MTT solution (5 mg MTT/ml H_2O) was added and the HL60 cells were incubated for a further 24 h, thus to make sure that the HL60 cells in suspension had adequate time to interact with the diluted MTT solution. A volume of 100 µl of DMSO was added to each culture and mixed by pipetting to release reduced MTT crystals from the cells. Relative cell viability was obtained by scanning with an ELISA reader (Anthos Labtech AR 2001 Multiplate Reader, *A*nthos Labtec Instruments, Austria) with a 480 nm filter. Results were expressed as a percentage of viable cells relative to untreated cells/control. Experiments were performed in triplicates and repeated ≥ 4 times. Cells treated with drugs were normalised against untreated cells, and pEC_{50} values were calculated using the Matlab prism program.

2.6 Real time PCR analysis of miRNA

2.6.1 miRNA extraction of HL60 cells and Langendorff perfused heart samples

The HL60 cells were cultured in 6-well plates - each well containing 10^6 cells - for 24 h with (i) Control, (ii) Sunitinib (7 µM), (iii) Sunitinib (7 µM)+ IB-MECA (1nM) or (iv) IB-MECA (1nM). The 7 µM Sunitinib dose was based from the IC_{50} value from the 0.1 – 10 µM Sunitinib dose response curve on HL60 cell viability assay. Langendorff perfused hearts were treated for 125 min with (i) Control, (ii) Sunitinib (1 µM), (iii) Sunitinib (1 µM) + IB-MECA 1nM or (iv) IB-MECA (1 nM). After treatment miRNA from cells/tissue was extracted with the mirVana miRNA Isolation Kit (Ambion, USA) according to the manufacturer´s instructions. The miRNA quantity and quality was detected by NanoDrop-1000 (NanoDrop Products, USA) measuring the absorbance at 260nm and 280 nm to ensure high RNA quality.

2.6.2 Real time PCR of HL60 cells and Langendorff perfused samples

A total of 500 ng miRNA was reverse transcribed into cDNA using primers specific for housekeeping reference RNA U6 snRNA and target microRNAs: hsa-miR-155, hsa-miR-15a, hsa-miR-16-1, hsa-miR-1, rno-miR-1, hsa-miR-27a, hsa-miR-133a or hsa-miR-133b (all human hsa-miR assays are compatible with rat samples as well) using the MicroRNA Reverse Transcription Kit according to the manufacturer's instructions. The reverse transcription PCR reaction was performed with the following setup: 1) 16 °C for 30 min, 2) 42°C for 30 min, 3) 85 °C for 5 min and 4) ∞ at 4°C. The qRT-PCR was performed using the TaqMan Universal PCR Master Mix II (no UNG) protocol on the 7500 HT Real Time PCR sequence detection system from Applied Biosystems (USA). A 20 µl reaction mixture containing 100 ng cDNA, specific primer assays mentioned and the TaqMan Universal PCR Master Mix II (no UNG) was used in the qRT-PCR reaction in triplicates. A non-template control was included in all experiments. The real time PCR reaction was performed using the program: 1) 2 min at 50°C, 2) 10 min at 95°C, 3) 15 s at 95°C, 4) 1 min at 60°C. Steps 3) and 4) were repeated 39 times. The microRNAs data analysis was calculated using the ΔΔCT method using the formula $X_0/R_0=2^{CTR-CTX}$ (Sandhu et al., 2010), where X_0 is the original amount of target microRNAs (hsa-miR-155, hsa-miR-15a, hsa-miR-16-1, hsa-miR-1, rno-miR-1, hsa-miR-27a, hsa-miR-133a or hsa-miR-133b), R_0 is the original amount of U6 snRNA, CTR is the CT value for U6 snRNA, and CTX is the CT value for the target microRNAs (hsa-miR-155, hsa-miR-15a, hsa-miR-16-1, hsa-miR-1, rno-miR-1, hsa-miR-27a, hsa-miR-133a or hsa-miR-133b). Each individual primer set were calculated and bar charts were plotted with mean ± S.E.M. The mean of the control group was set as 1 for the miRNA study.

2.7 Western blot assay

2.7.1 Protein preparation of HL60 cells

$5x10^6$ cells were incubated for 24 h with (i) Control, (ii) Sunitinib (7 µM), (iii) Sunitinib (7 µM) + IB-MECA (1 nM) or (iv) IB-MECA (1 nM). A concentration 7 µM Sunitinib was chosen to reflect the pEC_{50} value determined during the MTT assay. After treatment cells were

harvested and washed with ice-cold phosphate buffered saline. The cell pellet was dissolved in ice-cold Protein Lysis Buffer (25 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10 % v/v Glycerol, 1 % (v/v) Triton X-100) and cells were lysed by homogenising with a syringe needle. After centrifugation at 14,000 rpm for 30 min at 4°C the supernatant was collected and the amount of protein extracted from cells was detected using NanoDrop-1000 (NanoDrop Products, USA) measuring the absorbance at A260.

2.7.2 Protein preparation of Langendorff perfused heart samples

A total 45-55 mg of the frozen left ventricular tissue was lysed in lysis buffer (NaCl 0.1 M, Tris base 10 µM, EDTA 1 mM, sodium pyrophosphate 2 mM, NaF 2 mM, β-glycaophosphate 2 mM, 4-(2-Aminoethyl, pH = 7.6) benzenesulfonyl fluoride hydrochloride (0.1 mg/ml, 1/1.5 of protease cocktail tablet) using a IKA Overtechnical T25homogeniser (UK) at 11,000 RPM. The supernatants were measured for protein content using NanoDrop from Nanoid Technology (USA).

2.7.3 Western blot analysis of HL60 cells and Langendorff perfused heart samples

A total of 60 µg of protein was loaded to Any kDa Mini-Protean TGX Gel from BioRad (UK) and separated at 200 V for 60 min. After separation, the proteins were transferred to the Bond-P polyvinylidene difluoride membrane from BioRad (UK) by using the Trans-Blot Turbo transfer system from BioRad (UK) and probed for the phosphorylated form pPKCα and total form. The membranes were stripped by boiling and the PVDF membrane was used for total PKC. The relative changes in the pPKCα protein levels were measured and corrected for differences in protein loading as established by probing for total PKC. Phosphorylated antibody levels were normalised to total antibody levels in order to correlate for unequal loading of protein and differential blot transfer and to identify the level of active vs inactive protein levels. Results were expressed as a percentage of the density of phosphorylated protein relative to the density of total protein using Image Lab 4.1 from BioRad (UK). The phosphorylated antibody levels determination was randomised and blinded.

2.8 Statistical analysis

Results are presented as mean ± S.E.M. Significance of all data sets was measured by one-way ANOVA analysis with the Tukey post hoc test using the Matlab prism program. The following groups were compared during ANOVA analysis: Control versus Sunitinib, control versus Sunitinib + IB-MECA, control versus IB-MECA (statistical significance symbol: *) or Sunitinib versus Sunitinib + IB-MECA (statistical significance symbol: $^{\$}$). P-values <0.05 were considered statistically significant.

3 Results

3.1 Sunitinib treatment injures the heart dramatically and results in decreased cardiac haemodynamic parameters heart rate and left ventricular developed pressure

To characterise the levels of Sunitinib induced cardiotoxicity with and without the co-administration of cardioprotective IB-MECA the Langendorff perfused heart model was used. Hearts were perfused with (i) Control, (ii) Sunitinib (1 µM), (iii) Sunitinib (1 µM) ± IB-MECA (1 nM), or (iv) IB-MECA (1 nM) and haemodynamic data was collected: left ventricular developed pressure, heart rate and coronary flow measurements. The hearts were stabilised for a period of 20 min, followed by 125 min of drug perfusion.

Haemodynamic assessment detected at significant decrease in left ventricular developed pressure in Sunitinib treated hearts compared to control at time points at time points: 15, 20, 30, 35, 50, 65, 80, 95, 110, and 125 mins, while left ventricular developed pressure in the Sunitinib + IB-MECA group was significantly altered compared to Sunitinib at time point 20 min (Fig. A.1 and Table 1).

Table 1: Left ventricular developed pressure (LVDP) values in Control, 1 µM Sunitinib, 1 µM Sunitinib = 1 nM IB-MECA, and IB-MECA treated hearts throughout the 125 min Langendorff perfusion. Statistics: Groups compared during One-Way ANOVA analysis: Control versus Sunitinib, Sunitinib + IB-MECA, or IB-MECA ([a]), or Sunitinib versus Sunitinib + IB-MECA ([b]). [a] or [b] = P<0.05 (n=6-9).

LVDP	**Control (n=6)**		**Sunitinib (n=9)**		**Sunitinib + IB-MECA (n=9)**		**IB-MECA (n=9)**	
Time / min	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
0	101.40	0.92	98.48	1.11	102.38	1.21	102.48	1.72
5	111.19	4.16	120.61	4.24	108.98	2.62	108.72	1.77
10	109.04	4.98	114.91	8.74	111.50	3.13	111.30	3.20
15	109.62	5.69	99.01 [a]	4.96	105.66	2.97	107.36	3.30
20	106.26	5.79	94.77 [a]	3.80	106.76 [b]	3.30	104.04	3.84
25	102.62	4.33	96.53	4.06	98.37	5.41	101.35	4.93
30	106.29	6.06	88.01 [a]	3.70	98.96	5.44	94.50	4.64
35	101.69	5.88	90.44 [a]	3.21	96.76	6.44	97.01	3.68
50	99.40	8.51	78.25 [a]	4.52	92.43	6.92	87.31	3.59
65	92.04	6.98	76.32 [a]	3.55	87.35	6.35	84.99	4.38
80	88.70	3.72	76.26 [a]	3.72	82.23	4.08	82.45	3.93
95	90.98	4.52	76.68 [a]	4.89	82.38	5.04	82.97	7.16
110	84.26	3.96	69.61 [a]	2.37	80.30	3.53	79.83	7.54
125	86.08	2.60	65.63	2.37	78.36	4.84	76.16	8.40

There was also a significant decline in heart rate in the Sunitinib treatment group compared to Control throughout the 125 min drug perfusion at time points: 15, 20, 30, 35, 50, 65, 80, and 125 mins. (Fig. A.2 and Table 2).

Table 2: Heart rate (HR) values in Control, 1 µM Sunitinib, 1 µM Sunitinib = 1 nM IB-MECA, and IB-MECA treated hearts throughout the 125 min Langendorff perfusion. Statistics: Groups compared during One-Way ANOVA analysis: Control versus Sunitinib, Sunitinib + IB-MECA, or IB-MECA ([a]), or Sunitinib versus Sunitinib + IB-MECA. [a] = P<0.05 (n=8-9).

HR	Control (n=8)		Sunitinib (n=9)		Sunitinib + IB-MECA (n=9)		IB-MECA (n=9)	
Time / min	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
0	100.14	0.71	98.22	0.94	99.95	0.97	99.53	1.29
5	96.74	2.79	100.15	4.50	98.63	2.27	98.51	1.98
10	98.36	3.80	94.57	3.07	94.62	2.86	99.81	2.36
15	97.76	4.95	89.78 [a]	2.33	95.66	1.13	99.87	3.26
20	98.22	4.87	88.97 [a]	2.19	92.32	3.60	100.35	3.96
25	96.92	5.73	91.41	3.13	92.08	1.80	100.06	4.54
30	99.35	5.74	85.51 [a]	3.47	89.07	2.38	99.01	5.52
35	101.60	5.59	88.83 [a]	3.98	88.72	2.09	101.00	5.07
50	99.65	6.16	84.82 [a]	4.05	89.39	3.28	104.13	4.36
65	96.78	6.27	85.58 [a]	4.26	88.09	3.42	105.96	4.24
80	94.95	6.61	85.33 [a]	4.07	86.13	3.05	102.80	4.77
95	93.00	3.55	86.34	3.62	87.19	4.14	97.90	6.92
110	95.96	5.28	85.55	5.53	86.67	4.00	93.76	8.48
125	95.97	5.51	82.53 [a]	5.11	86.37	5.55	96.97	8.60

Coronary flow was not affected by Sunitinib, IB-MECA or Sunitinib + IB-MECA treatment compared to Control (Fig. A.3 and Table 3).

Table 3: Coronary flow (CF) values in Control, 1 µM Sunitinib, 1 µM Sunitinib = 1 nM IB-MECA, and IB-MECA treated hearts throughout the 125 min Langendorff perfusion. Statistics: Groups compared during One-Way ANOVA analysis: Control versus Sunitinib, Sunitinib + IB-MECA, or IB-MECA, or Sunitinib versus Sunitinib + IB-MECA (n=8-9).

CF	**Control (n=8)**		**Sunitinib (n=9)**		**Sunitinib + IB-MECA (n=9)**		**IB-MECA (n=9)**	
Time / min	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
0	103.69	2.86	96.13	1.22	99.71	0.64	102.09	0.86
5	97.35	2.20	104.43	2.08	100.08	2.75	99.38	2.39
10	97.03	2.13	103.73	4.14	98.01	4.25	99.66	2.59
15	96.99	4.78	98.15	5.25	100.92	3.86	98.71	2.32
20	98.00	5.04	94.34	3.23	98.78	5.32	98.42	3.14
25	97.02	6.44	92.72	3.71	94.73	5.10	96.50	4.15
30	97.46	6.46	83.56	5.00	96.97	5.29	93.72	3.35
35	96.79	6.41	85.63	4.29	94.91	5.54	93.43	4.49
50	93.78	7.12	84.30	5.52	89.99	6.34	87.69	3.58
65	92.54	10.56	80.40	4.24	85.33	6.61	88.71	4.48
80	88.87	9.42	79.01	3.53	87.08	6.33	86.37	5.47
95	86.70	6.67	78.76	4.75	87.94	8.36	80.77	5.77
110	80.04	6.95	75.01	5.59	83.95	8.30	77.43	6.70
125	79.89	8.23	72.54	5.24	77.92	7.40	71.20	4.93

Administration of Sunitinib (1 µM) for 125 min resulted in a significant increase in infarct size compared with non-treated hearts (Control: 8.47 ± 0.67 %; Sunitinib: 43.02 ± 3.15 %, n=6, P<0.001) (Fig. A.4). This demonstrated that Sunitinib treatment resulted in a drastic increase

in cardiac injury and there is also an effect on the cardiac function of the heart as observed by a reduction in heart rate and left ventricular developed pressure.

3.2 Sunitinib-mediated cardiotoxicity is reduced by A_3 adenosine receptor agonist IB-MECA

The effect of the A_3 adenosine receptor agonist on cardiac function and infarction was investigated. IB-MECA significantly counteracted Suntinib's effect on left ventricular developed pressure and coronary flow, although it could not protect against the bradycardiac effect of Sunitinib. In addition, IB-MECA treatment alone did not induce any significant change in all studied parameters as compared to the control group (Fig. A.1, A.2 and A.3).

Co-administration of Sunitinib with IB-MECA significantly decreased infarct size compared to Sunitinib treated hearts (Sunitinib: 43.02 ± 3.15 %; Sunitinib + IB-MECA: 20.46 ± 3.13 %, n=6, P<0.001). Administration of IB-MECA alone did not significantly affect infarct size compared to control (Control: 8.47 ± 0.67 %; IB-MECA: 13.80 ± 3.40 %, n=6, P<0.05) (Fig. A.4). This demonstrates that IB-MECA is effective in attenuating Sunitinib induced cardiac injury.

3.3 IB-MECA does not jeopardize the anti-cancer property of Sunitinib

To investigate the anti-cancer properties of Sunitinib in combination with the cardioprotective agent IB-MECA, HL60 cells were incubated with (i) Control or increasing concentrations of (ii) Sunitinib (0.1 – 10 µM), (iii) Sunitinib (0.1 – 10 µM) + 1 nM IB-MECA or (iv) IB-MECA (10 nM – 10 µM). Cell viability was measured using the MTT assay method after 24 h of treatment. Addition of Sunitinib to the HL60 cells decreased cell viability in a dose dependent manner (Fig. B.1). The cell viability was decreased significantly from 102.0 ± 1.4 % in control to 43.2 ± 6.3 % (n=5-7, P<0.001) when 10 µM Sunitinib was added to the cell culture. Co-administration of 1 nM IB-MECA did not significantly alter the Sunitinib treatment dose curve (pEC_{50} of Sunitinib = 8.4 ± 1.3 and pEC_{50} of Sunitinib + 1 nM IB-MECA = 7.0 ± 1.6, n=5-6).

Administration of IB-MECA by itself to HL60 cells did not have any impact on cell viability in the dose 10 nM – 1 µM, however, addition of a high dose of 10 µM IB-MECA did decrease the cell viability significantly. Cell viability decreased significantly from 102.0 ± 1.4 % in control to 72.1 ± 17.5 % (n=7, P<0.001) when treated with 10 µM IB-MECA (Fig. B.2).

3.4 microRNAs associated with myocardial injury: miR-1, miR-27a, miR-133a and miR-133b in heart tissue

The expression of microRNAs associated with cardiac injury were analysed in left ventricular heart tissue, collected after Langendorff perfusion with Sunitinib in the absence or presence of IB-MECA. There was a tendency for a decrease in miR-1 (Fig. C.1) and miR-27a (Fig. C.2), while miR-133a (Fig. C.3) and miR-133b (Fig. C.4) tended to increase in the Sunitinib treatment group when compared to control (Table 4).

Table 4: Summary of miRNA expression of cardiotoxicity-linked miRNAs (miR-1, miR-27a, miR-133a and miR-133b) in cardiac tissue and cancer-linked miRNAs (miR-15a, miR-16-1 and miR-155) in HL60 cells (↑=increase in expression, *=statistical significance when compare to Control, $^{\$}$=statistical significance when compare to Sunitinib, *=P<0.05, *** or $^{\$\$\$}$=P<0.001, n=5-6).

	Cardiac tissue				**HL60 cells**		
	Cardiotoxicity-linked miRNAs				**Cancer-linked miRNAs**		
	miR-1	**miR-27a**	**miR-133a**	**miR-133b**	**miR-15a**	**miR-16-1**	**miR-155**
Control versus Sunitinib	-	-	-	-	-	-	-
Control versus Sunitinib+IB-MECA	-	-	↑ ***	↑ ***	-	↑ *	-
Control versus IB-MECA	-	-	-	-	-	↑ ***	↑ ***
Sunitinib versus Sunitinib+IB-MECA	-	↑ $^{\$\$\$}$	-	-	-	-	-

Co-administration of IB-MECA in the presence of Sunitinib increased miR-27a 16.5 folds (n=5-6, P<0.001) (Fig. C.2) when compared to hearts treated with Sunitinib alone. The remaining three microRNAs also followed the trend with miR-1 (Fig. C.1), miR-133a (Fig. C.3) and miR-133b (Fig. C.4) showing a tendency to increase when IB-MECA was co-administrated with Sunitinib treatment group when compared to Sunitinib treatment group.

Co-administration of IB-MECA in the presence of Sunitinib significantly increased miR-133a 5.07 folds (n=5-6, p<0.0001) (Fig C.3) and miR-133b 23.38 folds (n=5-6, p<0.0001) (Fig C.4) when compared to control hearts. The same pattern for an increase in miR-1 (Fig. C.1) and

miR-27a (Fig. C.2) was observed in the Sunitinib + IB-MECA group when compared to control group, however this increase was not significant.

The miRNA levels were not altered in hearts treated with IB-MECA alone compared to control hearts in any of the microRNAs investigated.

3.5 microRNAs associated with apoptosis and cancer development: miR-155, miR-15a and miR-16-1

Treatment of HL60 cells with Sunitinib in the absence and presence of IB-MECA altered the expression of microRNAs associated with apoptosis and cancer development. The qRT-PCR analysis of miR-15a showed a slight increase in miR-15a expression in HL60 cells treated with Sunitinib (1.4 folds) and Sunitinib + IB-MECA (1.5 folds) when compared to control, while IB-MECA treatment showed a 0.4 fold decrease, however, these changes were not significant (Fig. C.5). The expression of miR-15a's cluster partner, miR-16-1 was slightly increased by 3.6 folds in Sunitinib treated HL60 cells when compared to control, and co-administration of IB-MECA significantly increased the miR-16-1 expression further 7.7 folds ($n=6$, $p<0.05$) when compare to Sunitinib treated HL60 cells. Treatment with IB-MECA showed a significant 46.8 folds increase ($n=6$, $P<0.001$) in miR-16-1 expression compared to control treated HL60 cells (Fig. C.6). The expression of miR-155 was increased with 2.3 folds in Sunitinib treated HL60 cells and co-administration of IB-MECA did not alter this increase. Treating the HL60 cells with IB-MECA showed a significant 82.2 folds increase ($n=6$, $P<0.001$) in miR-155 expression compared to control (Fig. C.7) (Table 4).

3.6 IB-MECA protects against Sunitinib-induced cardiotoxicity in Langendorff perfused hearts through PKCα signaling pathway

PKCα has been previously shown to have a major contribution to heart function (Braz et al., 2004; Lange et al., 2016). We investigated whether PKCα signaling contributed to Sunitinib induced cardiotoxicity. PKCα phosphorylation was significantly increased 1.68 fold in the

Sunitinib (1μM) treatment group compared to control, and IB-MECA co-administration significantly attenuated the increase in PKCα by 0.9 fold (Fig. D.1). The pPKCα levels were normalised to total PKCα. The changes in PKCα phosphorylation correlate with the level of infarct size in each of the treatment groups (Fig. A.4).

3.7 HL60 cell viability and PKCα signaling

PKCα has been shown to have a crucial role in cancer progression (Antal et al., 2015). The level of pPKCα expression was determined after 24 h treatment of control, Sunitinib (7 μM) ± IB-MECA (1 nM) and IB-MECA (1 nM). The pPKCα levels in HL60 cells were significantly increased by administration of Sunitinib (7 μM) alone 1.7 fold and by IB-MECA (1 nM) alone 2.67 fold compared to control. The co-treatment of Sunitinib with IB-MECA also demonstrated a significant increase in pPKC levels compared to control, however, the combination of Sunitinib and IB-MECA did not reduce the level of PKC phosphorylation compared to Sunitinib (Fig. D.2).

4 Discussion

Sunitinib has very effective antineoplastic activity; however in the clinic it has been shown to cause cardiomyopathy, in some patients, that can potentially lead to congestive heart failure or even sudden death (Khakoo et al., 2008; Uraizee et al., 2011). Sunitinib is known to cause adverse cardiovascular events (Telli et al., 2008) through both on-target inhibition of vascular endothelial growth factor, receptors for platelet-derived growth factor, c-KIT and fms-like tyrosine kinase-3, and also off-target inhibition of various other kinases essential in the maintenance of cardiac function (Ghoreschi et al., 2009; Force and Kolaja, 2011; de Jesus-Gonzalez et al., 2012).

We determined the level of heart tissue apoptosis and necrosis caused by Sunitinib and the co-treatment of Sunitinib with IB-MECA. Infarct size was significantly increased in hearts treated with 1 μM Sunitinib compared to control hearts (Fig. A.4) and Sunitinib treatment

significantly decreased both left ventricular developed pressure and heart rate compared to control (Fig. A.1 and A.2). This is in accordance with other studies investigating the level of cardiotoxicity induced by Sunitinib and supports existing evidence that Sunitinib treatment could result in left ventricular dysfunction and even heart failure in patients (Henderson et al., 2013; Mooney et al., 2015; Chu et al., 2007; Di Lorenzo et al., 2009).

It is well known that A_3 adenosine receptor stimulation produced cardioprotective results (Carr et al., 1997; McIntosh and Lasley, 2012). In rat hearts, IB-MECA has shown to be beneficial for ischaemia (Hochhauser et al., 2007) and IB-MECA has been shown to have powerful cardioprotective effects against cardiac damage caused by hypoxia, ischaemia/reperfusion injury and anti-cancer treatment with Doxorubicin (Shneyvays et al., 2002). We show the co-treatment of the A_3 adenosine receptor selective agonist IB-MECA attenuated the Sunitinib-induced cardiac injury, as the infarct size was significantly decreased by the co-administration of IB-MECA (Fig. A.4). Interestingly, at time point 20 min, IB-MECA was shown to attenuate Sunitinib induced left ventricular developed pressure decline (Fig. A.1). For the remainder of the experiment, haemodynamic parameters left ventricular developed pressure and coronary flow were not significantly altered during the co-treatment of Sunitinib and IB-MECA compared to control. Haemodynamic parameters were not affected by IB-MECA treatment alone and infarct size was not changed by IB-MECA compared to control (Fig. A.1-4), therefore, IB-MECA did not produce cardiotoxicity, when administered alone.

The key cardioprotective functions mediated by IB-MECA to reduced ischaemia and infarct size are through (i) mitochondrial K_{ATP} activation: this increase the O_2 consumption and ATP production in mitochondria (Lipshultz et al., 2013; Sandhu and Maddock, 2014), (ii) protect against myofibril damage, (iii) reduced ATP catabolism rate, (iv) reduced intracellular Ca^{2+} levels , (v) activation of PKC-δ, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ), inducible nitric oxide synthase, mitogen activated protein kinase/extracellular-

signal-regulated kinase and Akt/ phosphoinositide 3-kinase (Hussain et al., 2014) and (vi) activation of mitochondrial permeability transition pore.

Cardiac injury associated microRNAs miR-1, miR-27a, miR-133a and miR-133b have been linked to cardiotoxicity (Sandhu and Maddock, 2014). Both miR-1 and miR-133 regulate heart development (Chen et al., 2008) and are dysregulated in patients with cardiac hypertrophy and heart failure (Care et al., 2007). The miR-133a has a partial complimentary target site in the 3'untranslated region region of ether-a-go-go gene and a reduction in ether-a-go-go levels can cause the delayed myocyte repolarisation attributed to a long QT interval (Xiao et al., 2007). And miR-27a is important in the regulation of contractility within the heart (Nishi et al., 2011). On our study we see an increase in miR-27a expression in Sunitinib + IB-MECA treated hearts when compared to Sunitinib (Fig. C.2), and both miR-133a and miR-133b are increased in Sunitinib + IB-MECA treated hearts when compared to Sunitinib (Fig. C.3 and C.4). The expression of miR-1 was not altered when the hearts were treated with Sunitinib or IB-MECA (Fig. C.1). The increase in miR-27a, miR-133a and miR-133b in the co-treatment group indicates that the combination of Sunitinib and IB-MECA induces stress at a cellular miRNA level, however, in the perfused heart tissue IB-MECA does attenuate Sunitinib induced cardiotoxicity. Further investigations unravelling miR-27a, miR-133a and miR-133b expression levels during Sunitinib and IB-MECA treatment are required in order to clarify this.

The tumour suppressor miR-15a/16 cluster is involved in proliferation and growth regulation of multiple myeloma cells by blocking protein kinase B serine/threonine-protein-kinase, ribosomal-protein-S6, mitogen activated protein kinase and NF-κβ activator MAP3KIP3 (Roccaro et al., 2009). The role of miR-15a and miR-16-1 in HL60 cells during IB-MECA treatment seems complex as the miR-15a unaltered by Sunitinib and IB-MECA treatment (Fig. C.5), however, Sunitinib + IB-MECA and IB-MECA treatment significantly increases miR-16-1 expression when compared to control (Fig. C.6).

The oncogene miR-155 was the first miRNA transcript shown to possess tumour-promoting activity (Eis et al., 2005) and miR-155 have been linked to diverse tumour types including B-cell lymphoma (Eis et al., 2005), and breast (Iorio et al., 2005), lung (Yanaihara et al., 2006) and pancreatic adenocarcinoma (Lee et al., 2007). A significant increase in miR-155 was detected in HL60 cells treated with IB-MECA alone (Fig. C.7). This is the same pattern identified for pPKCα in the HL60 cell western blot (Fig. D.2). A study by Kluiver et al. 2006, is in agreement with our findings. Their study showed the expression of mature miR-155 from primary miR-155 (pri-miR-155) in both Hodgekin's lymphoma cell lines and normal lymphoid tonsillar B cells was strongly linked to activation by PKC (Kluiver et al., 2006). pri-miR-155 have shown oncogenic abilities in lymphoma and leukaemia by associating with the oncogene c myc (Tam et al., 2002), and Sunitinib has shown to increase phosphorylated levels of PKC α/β, this interaction between miR-155 and PKC. This suggests that an increase in miR-155 and an increase in pPKCα could indicate anti-cancer properties of Sunitinib and IB-MECA.

PKCα is fundamental in stress signalling and it has been shown to have a pro-apoptotic effect in the heart (Steinberg, 2004). Elevated levels of the protein PKCα in cardiomyocytes is an important contributor to cardiomyopathy which can lead to heart failure (Lange et al., 2016). We identify an increase in pPKCα levels in hearts treated with Sunitinib (1 μM) compared to control (Fig. D.1). This shows a clear involvement of PKCα in Sunitinib-induced cardiotoxicity, as co-administration with IB-MECA (1 nM) attenuated this increase in phosphorylated PKCα. Previous studies have linked tyrosine kinase inhibitor Imatinib therapy of hearts with increases PKC phosphorylation (Steinberg, 2004). This pPKCα expression pattern was similar in HL60 cells, where there is a significant increase in PKCα phosphorylation by Sunitinib (7μM) (Fig. D.2). Our findings are in agreement with previous findings published in Teng et al. 2013 where treatment of HL60 cells with 0.1-1 μM Sunitinib resulted in increased phosphorylation of PKCα in a Sunitinib dose dependent manner (Teng

et al., 2013). Addition of 1 nM IB-MECA in HL60 cells does increase pPKCα expression significantly (Fig. D.2). Interestingly, a study by Nayeem and Mustafa showed that PKCα phosphorylation was elevated by addition of 10 μM adenosine A1 receptor agonist (2s)-N 6-[2-endo-norbornyl]adenosine in porcine coronary smooth muscle cells (Nayeem and Mustafa, 2002). In mice aortic smooth muscle adenosine A1 receptor activation inhibits large conductance Ca^{2+}/voltage-sensitive K^+ channel activity in a PKCα dependent manner (Kunduri et al., 2013). However, it remains to be investigated whether the adenosine A3 receptor activation is directly correlated to elevated PKCα phosphorylation in cardiomyocytes.

Many cardioprotective strategies fail to demonstrate beneficial effects in clinical or *in vivo* settings as they interfere or reduce with the anti-cancer effects and thereby reduce the clinical utility (Granger, 2006). Our findings show that co-treatment of HL60 cells with the A_3 adenosine receptor agonist IB-MECA can ameliorate the cardiotoxic effects of Sunitinib without affecting its anti-cancer properties. However, A_3 adenosine receptor activation has shown to play a key role in adenosine-induced inhibition of various tumour cell proliferation (Fishman et al., 2000).

This study reveals for the first time that A_3 adenosine receptor activation improves myocardial survival by attenuating Sunitinib-induced myocardial injury without interfering with the anti-tumour efficacy of Sunitinib. Investigating the specific A_3 adenosine receptor associated signalling pathways and microRNAs involved in Sunitinib-induced cardiotoxicity, as well as further investigating PKC signaling, could be important in the development of adjunctive cardioprotective chemotherapy treatment.

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Figure legends for

Attenuation of Sunitinib-induced cardiotoxicity through the A3 adenosine receptor activation

Figure A: Representation of haemodynamic data collected during Langendorff experiments over time relative to the stabilisation (S) period and infarct size with the following groups (n=6 for all): (i)Control (black), (ii) Sunitinib (1µM) (red), (iii) Sunitinib (1µM) + IB-MECA (1nM) (blue) and (iv) IB-MECA (1nM) (green). (1) Change in LVDP (mmHg), (2) Heart Rate (HR) and (3) Coronary flow (CF) (ml). (4) Representation of infarct size. Data expressed as mean ± S.E.M. Statistics: Groups compared during One-Way ANOVA analysis: Control versus Sunitinib, Sunitinib + IB-MECA, or IB-MECA (*), Sunitinib versus Sunitinib + IB-MECA ($^{\$}$). * or $^{\$}$ = P<0.05 and *** or $^{\$\$\$}$ = P<0.001.

Figure B: Cell viability in % of HL60 cells (10^5 cells/ml) incubated for 24 h with control (Control) (n=7) or with increasing concentrations of (1) Sunitinib (0.1 – 10 µM) (red) (n=5) or Sunitinib (0.1 – 10 µM) + IB-MECA (1nM) (blue) (n=6) or (2) IB-MECA (10 nM – 10 µM) (green) (n=7). Statistics: Groups compared during One-Way ANOVA analysis: Control versus Sunitinib or Sunitinib + IB-MECA (*), and Control versus IB-MECA ($^{\$}$). *** or $^{\$\$\$}$ = P<0.001.

Figure C: qRT-PCR analysis of Langendorff hearts (n=5-6) treated with (i) Control (black), (ii) Sunitinib (1 µM) (red), (iii) Sunitinib (1 µM) + IB-MECA (1nM) (blue) or (iv) IB-MECA (1nM) (green), showing miRNA expression of (1) miR-1, (2) miR-27a, (3) miR-133a, (4) miR-133b, and qRT-PCR analysis of HL60 cells (n=6) treated with (i) Control, (ii) Sunitinib (7 µM) (iii) Sunitinib (7 µM) with IB-MECA (1 nM) and (iv) IB-MECA (1 nM), showing miRNA expression of (5) miR-15a, (6) miR-16-1 and (7) miR-155. Data expressed as mean ± S.E.M. Statistics: Groups compared during One-Way ANOVA analysis: Control versus Sunitinib, Sunitinib + IB-

MECA, or IB-MECA (*), Sunitinib versus Sunitinib + IB-MECA ($^{\$}$). * = P<0.05 and *** or $^{\$\$\$}$ = P<0.001.

Figure D: Western blot analysis showing fold change in pPKCα (1) Langendorff perfused heart tissue samples of groups (n=6) (i) Control, (ii) Sunitinib (1 µM) (red), (iii) Sunitinib (1 µM) + IB-MECA (1 nM) (blue) and (iv) IB-MECA (1 nM) (green) normalised to Total PKC. (2) pPKCα western blots of HL60 cell sample groups (n=5) (i) Control, (ii) Sunitinib (7 µM) (red), (iii) Sunitinib (7 µM) + IB-MECA (1 nM) (blue) and (iv) IB-MECA (1 nM) (green) normalised to Total PKC. Statistics: Groups compared during One-Way ANOVA analysis: Control versus Sunitinib, Sunitinib + IB-MECA, or IB-MECA (*), Sunitinib versus Sunitinib + IB-MECA ($^{\$}$). * = P<0.05, $^{\$\$}$ = P<0.01 and *** = P<0.001.

Figure A1

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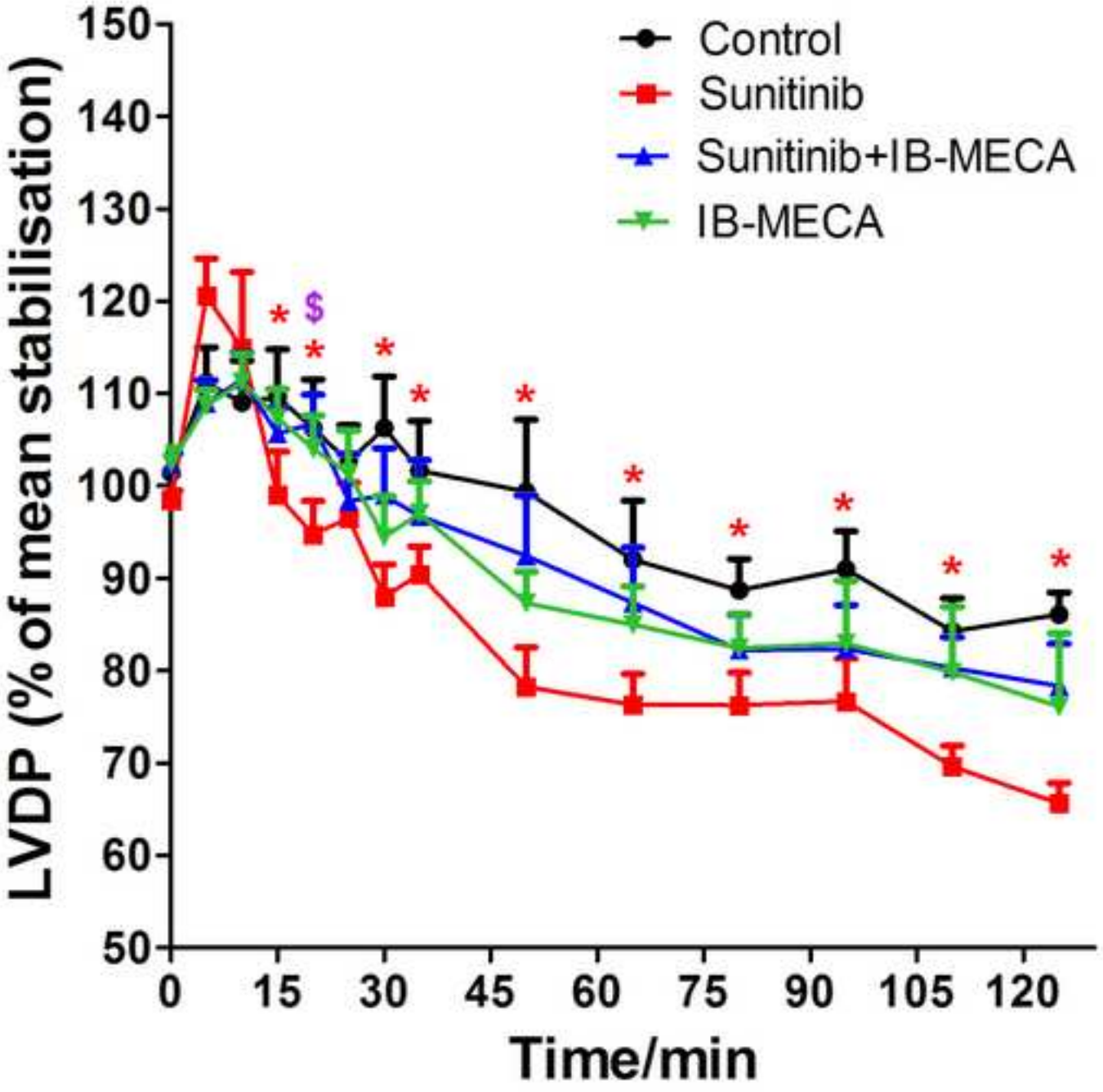

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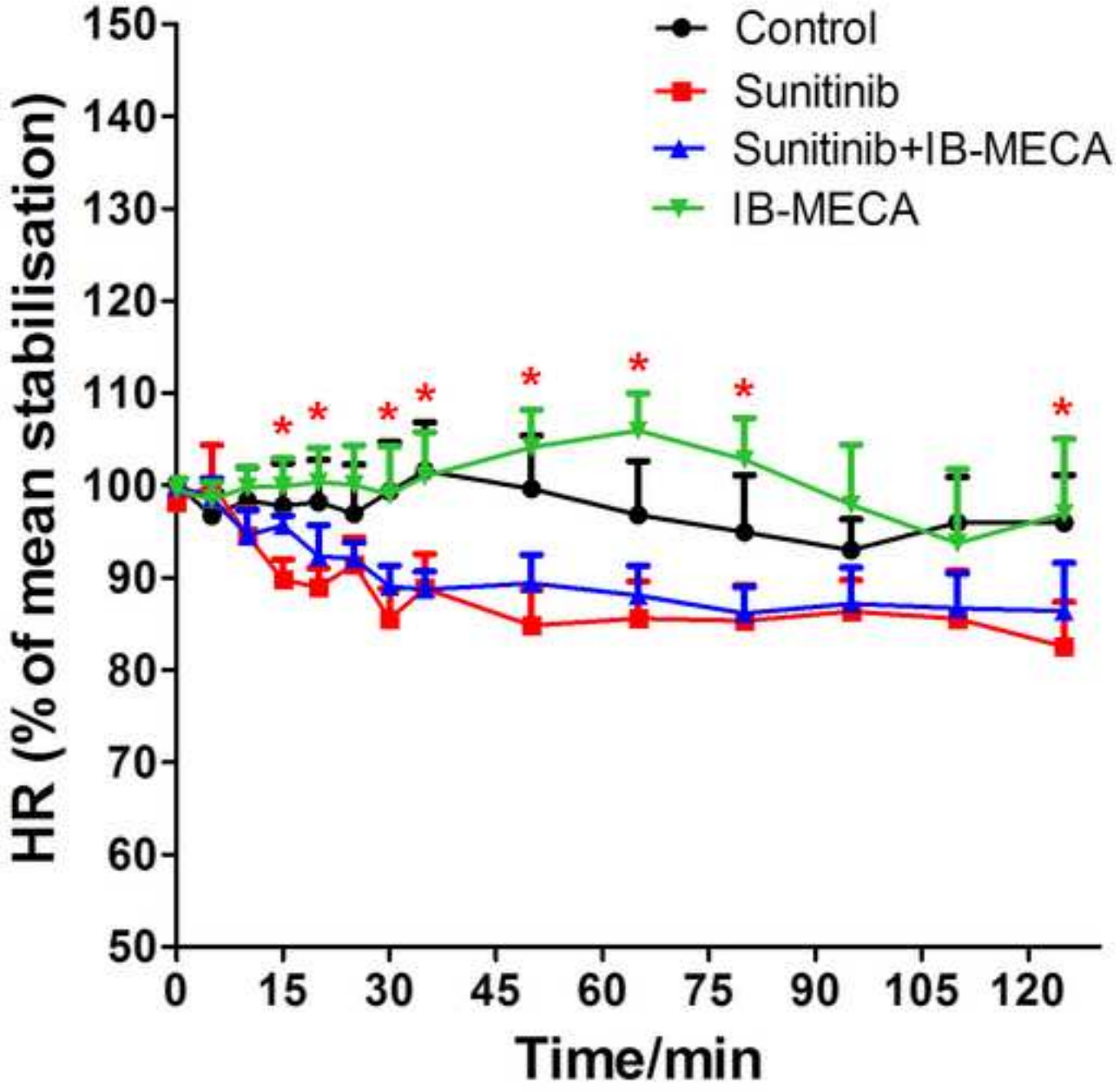

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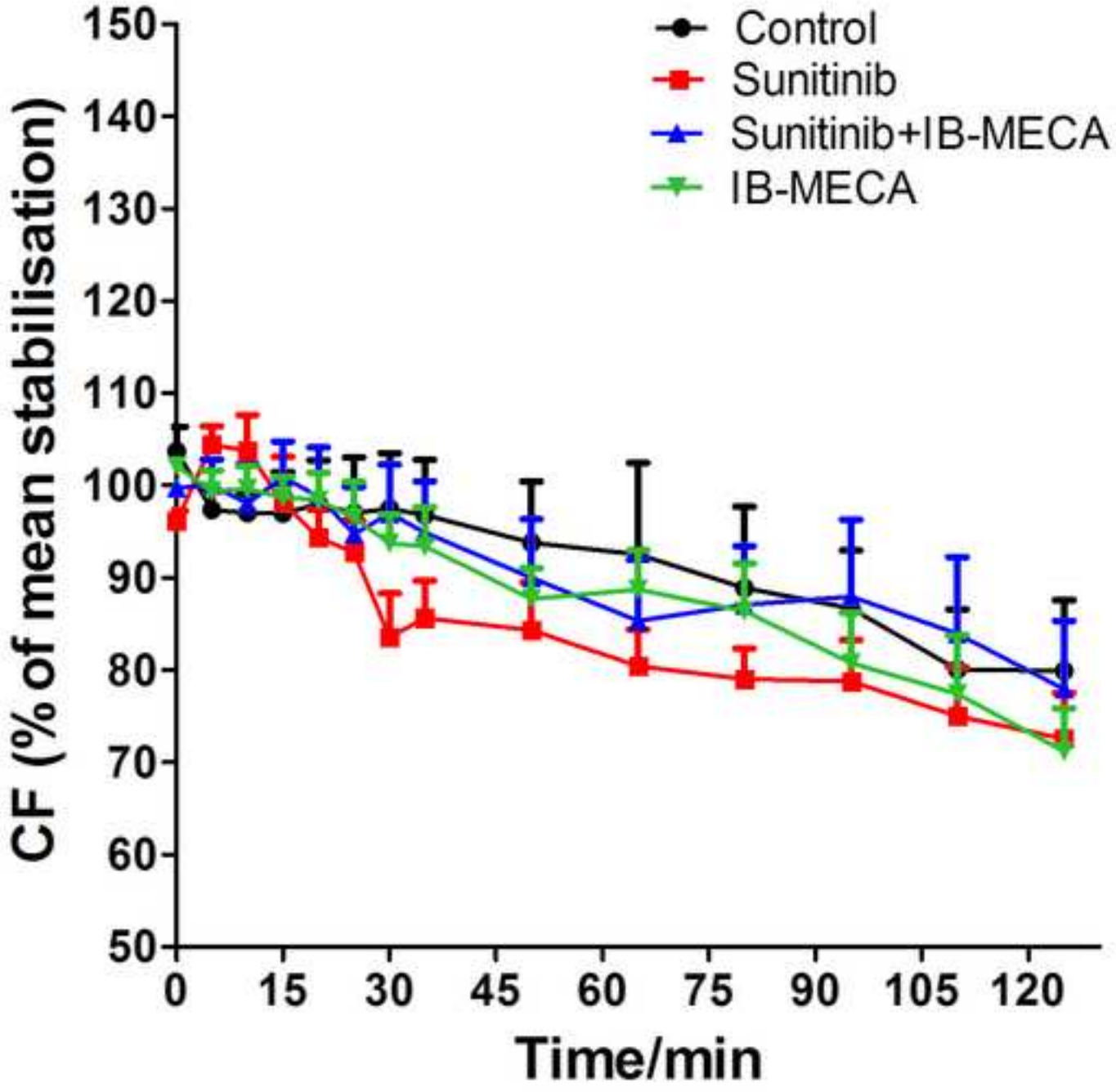

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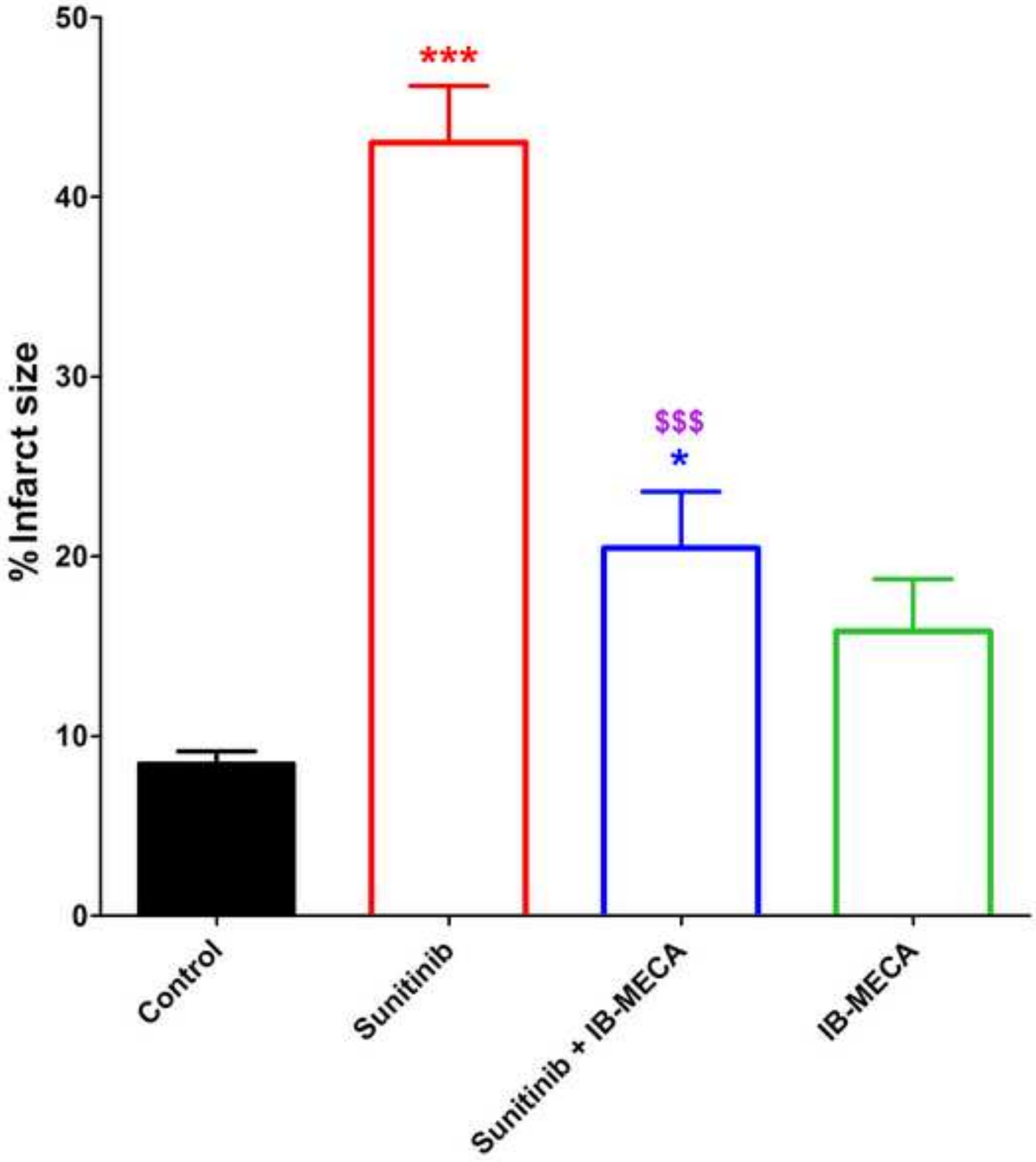

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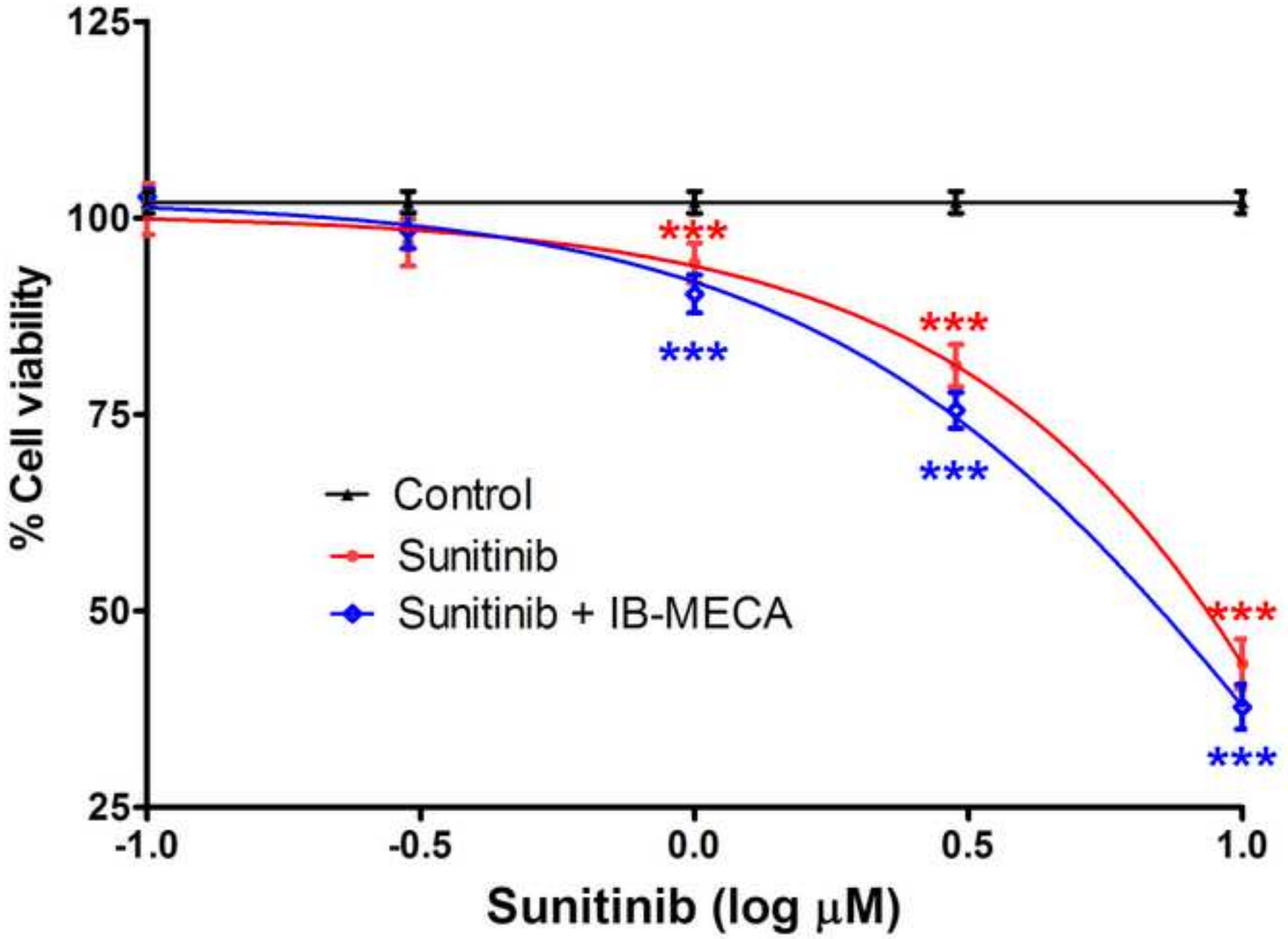

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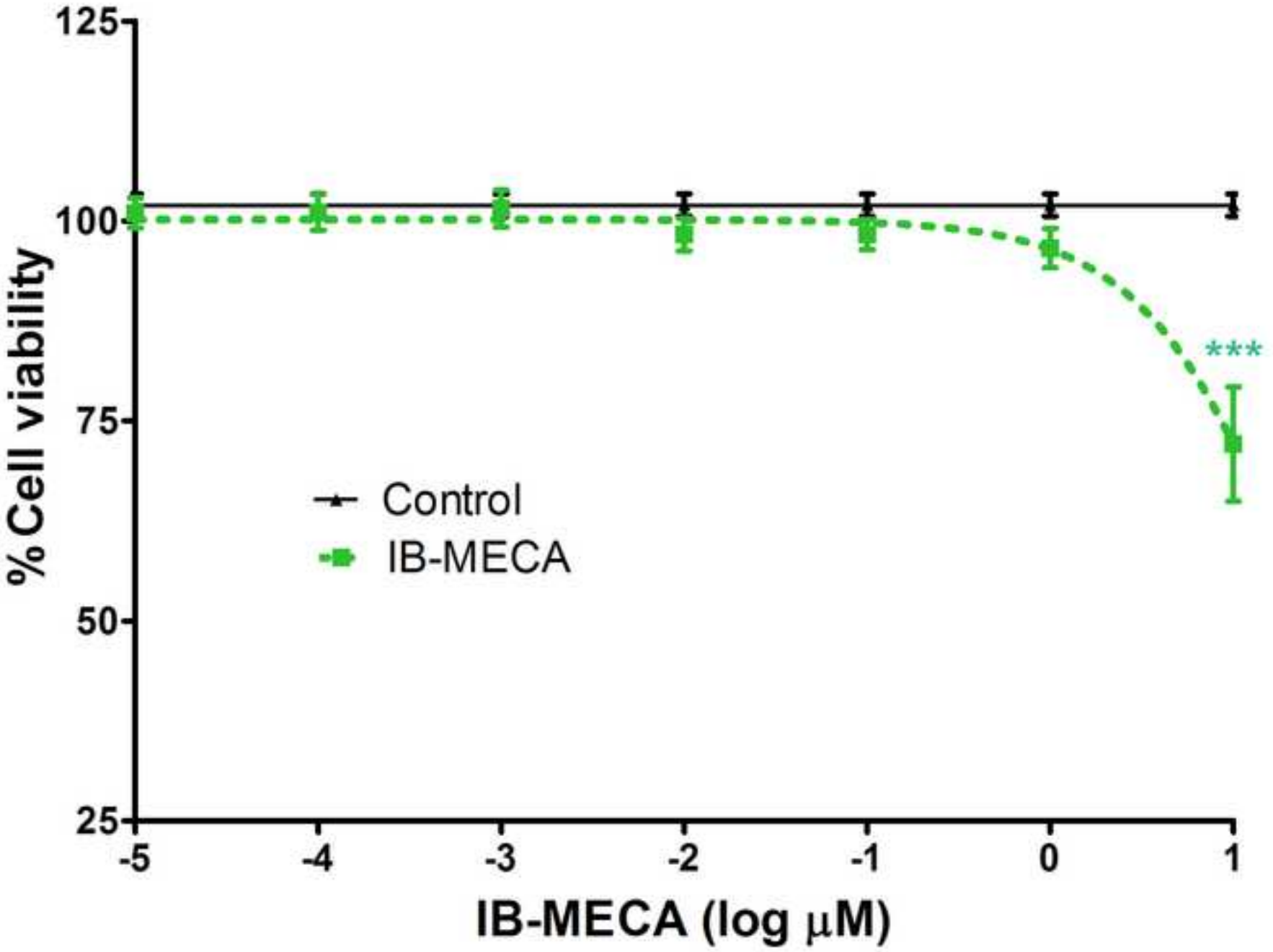

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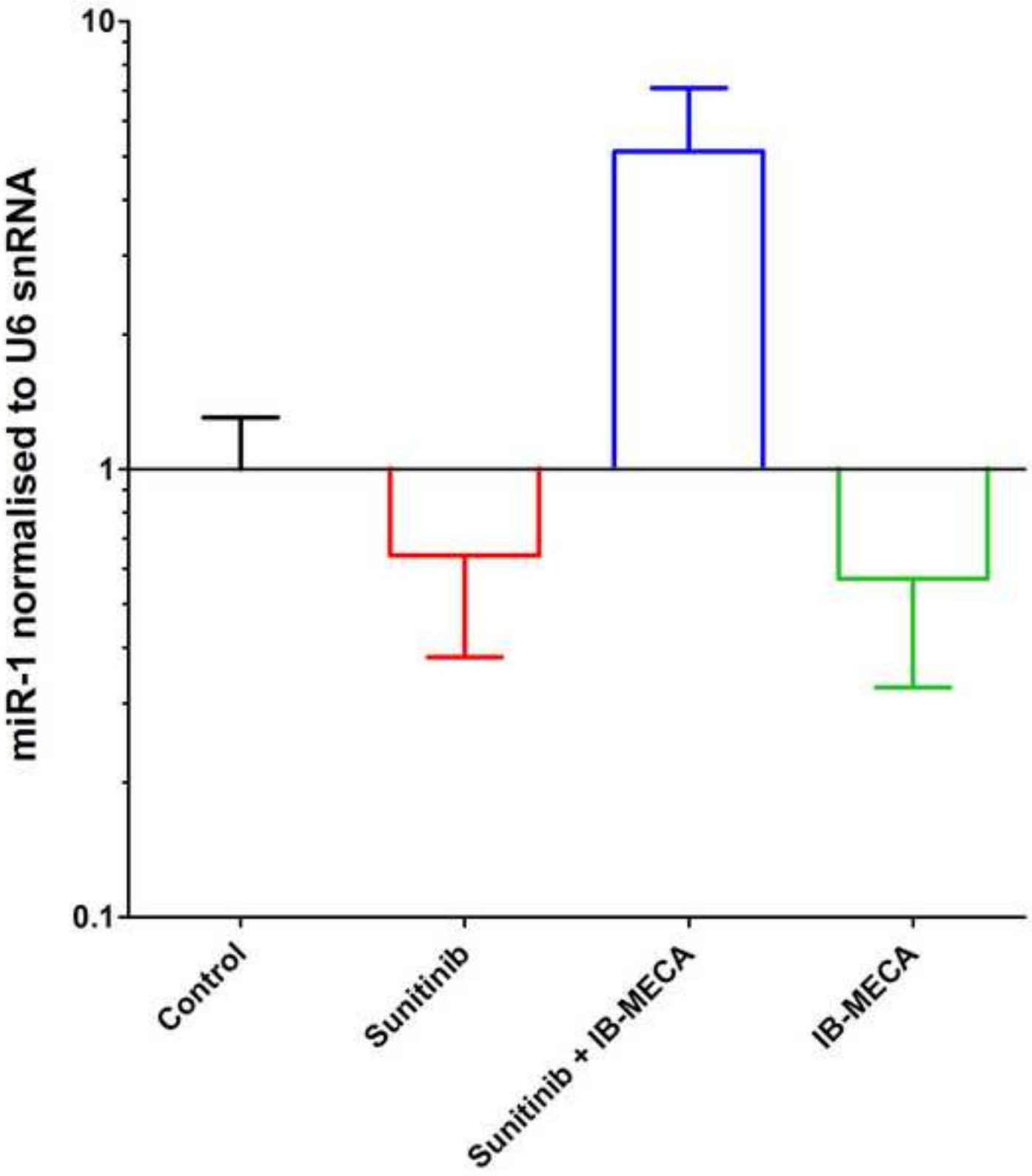

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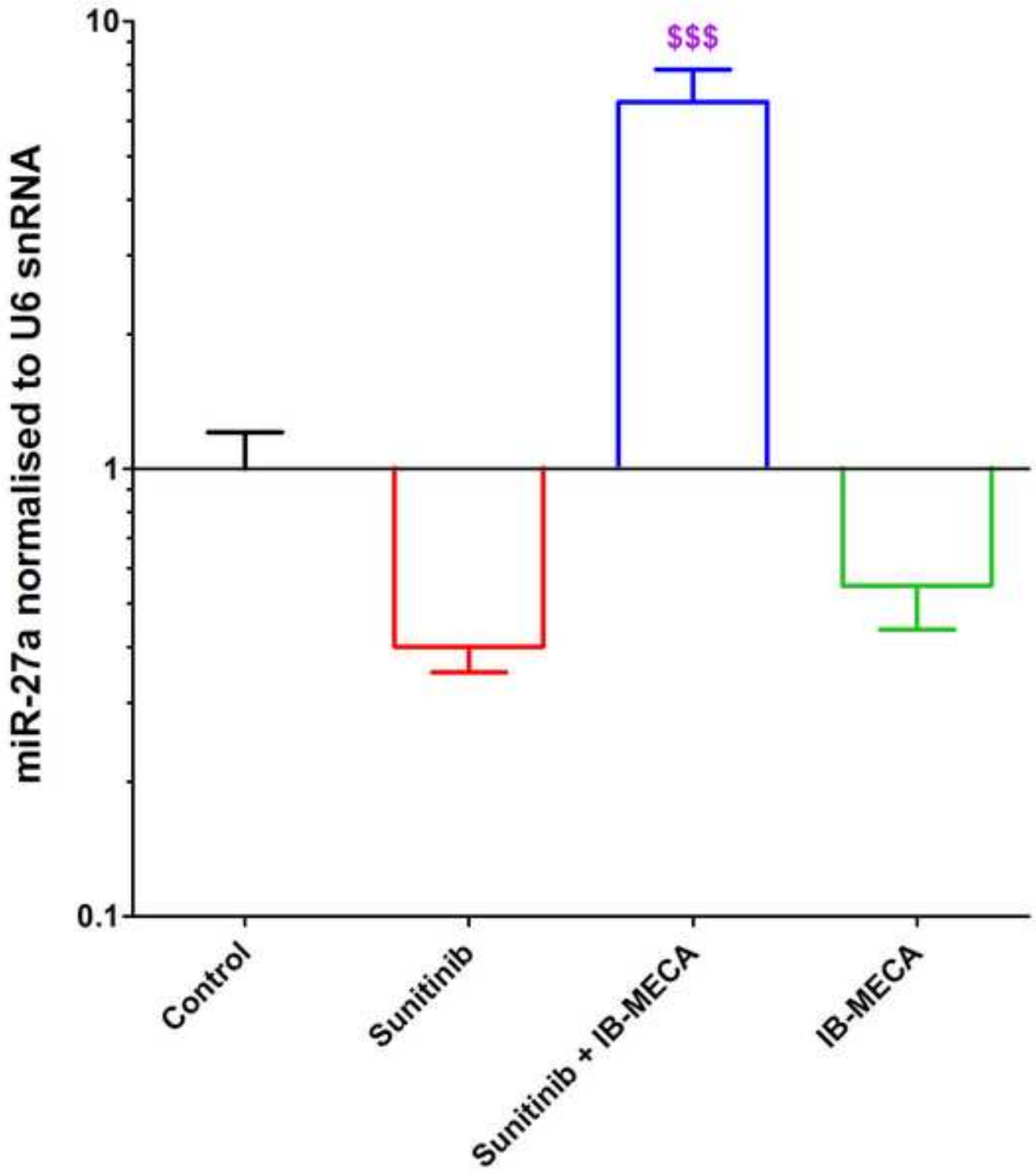

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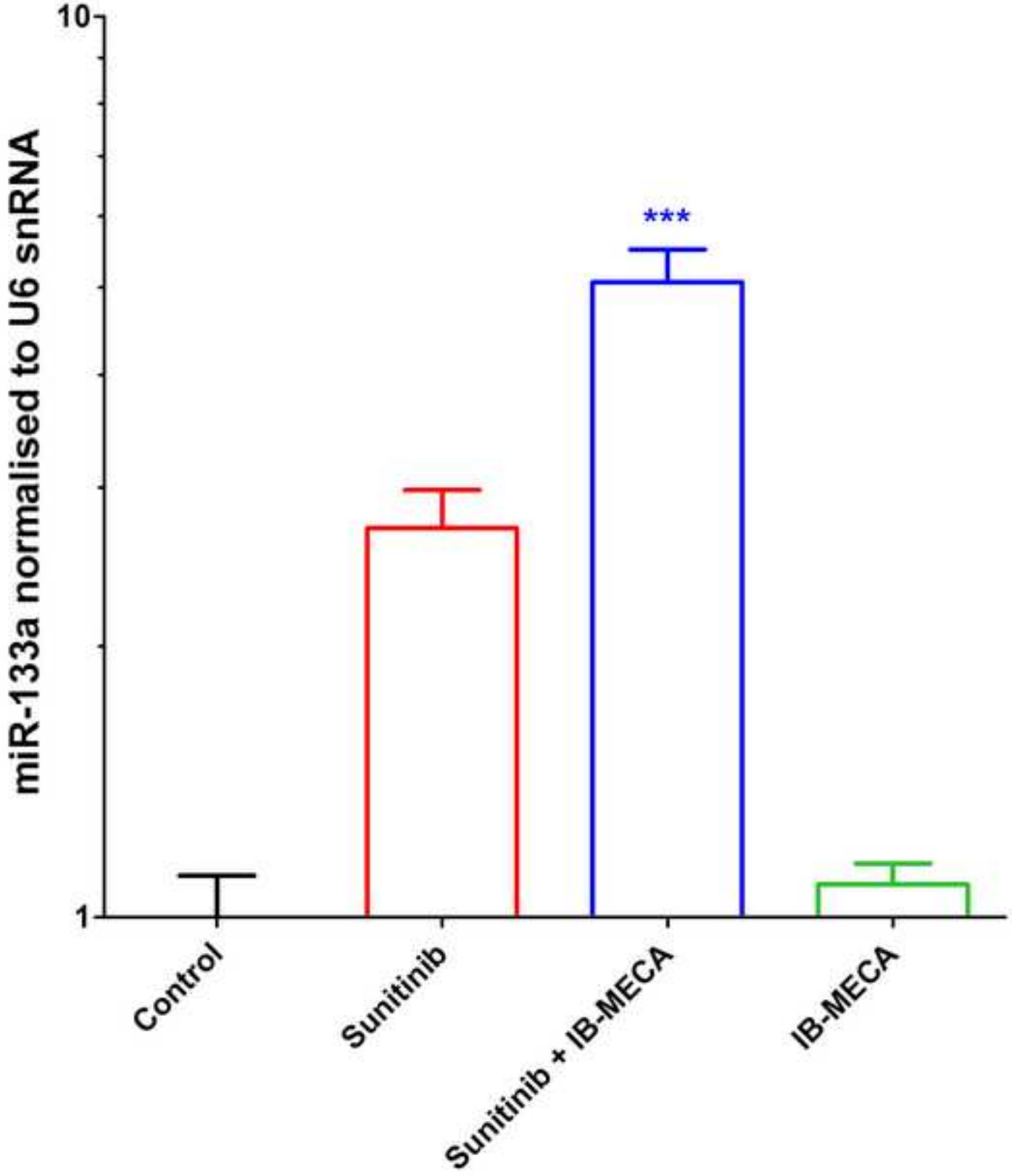

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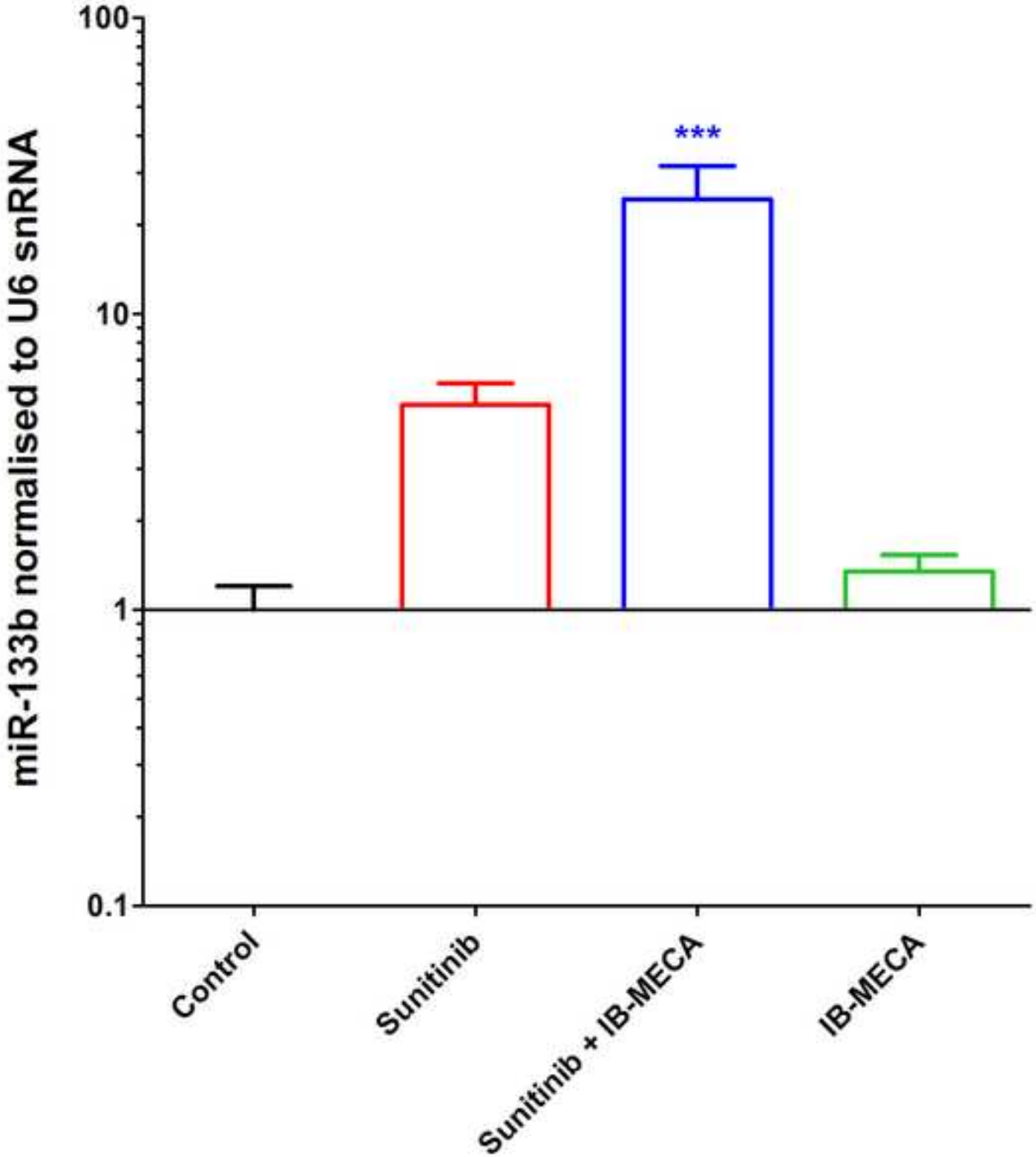

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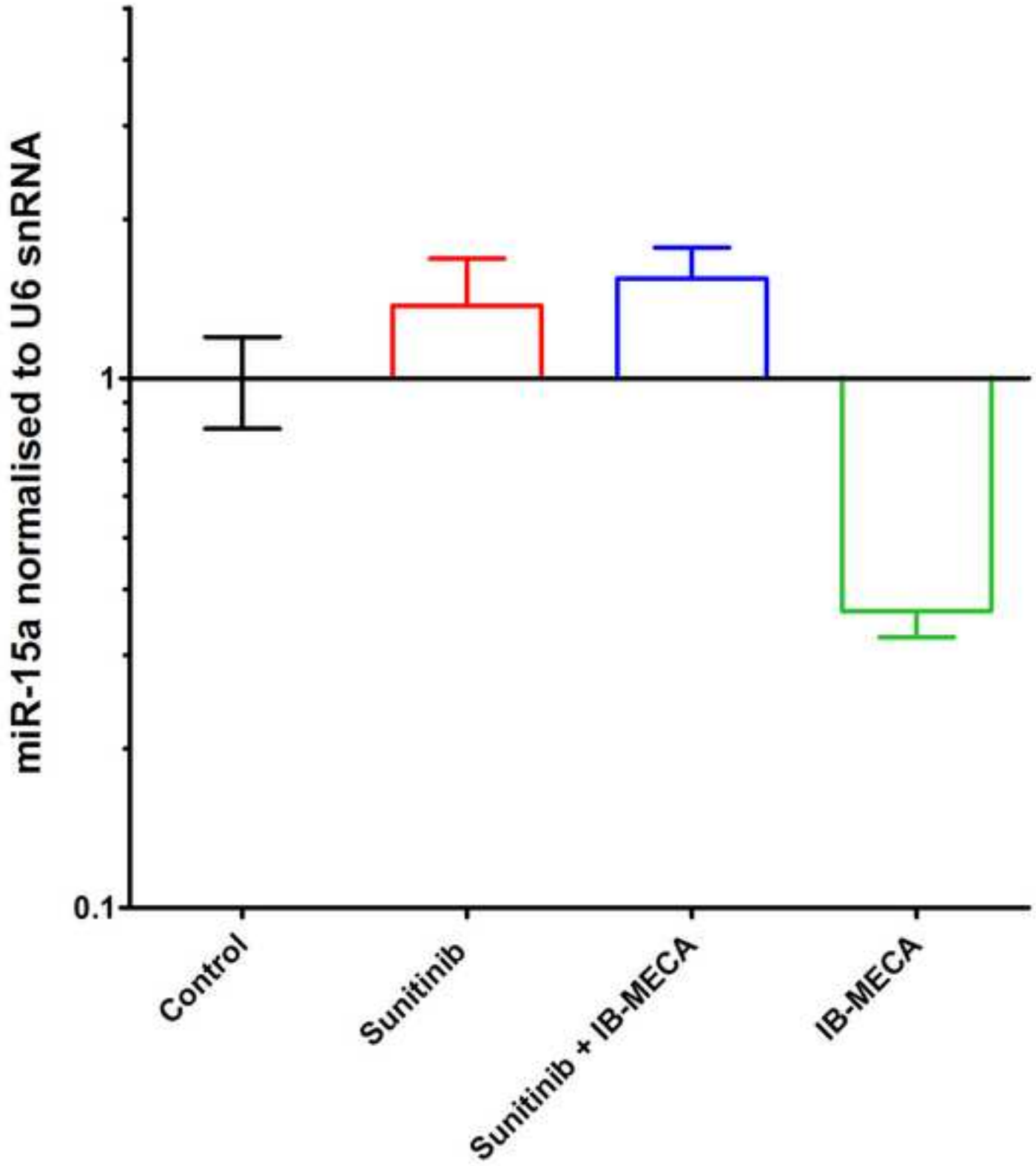

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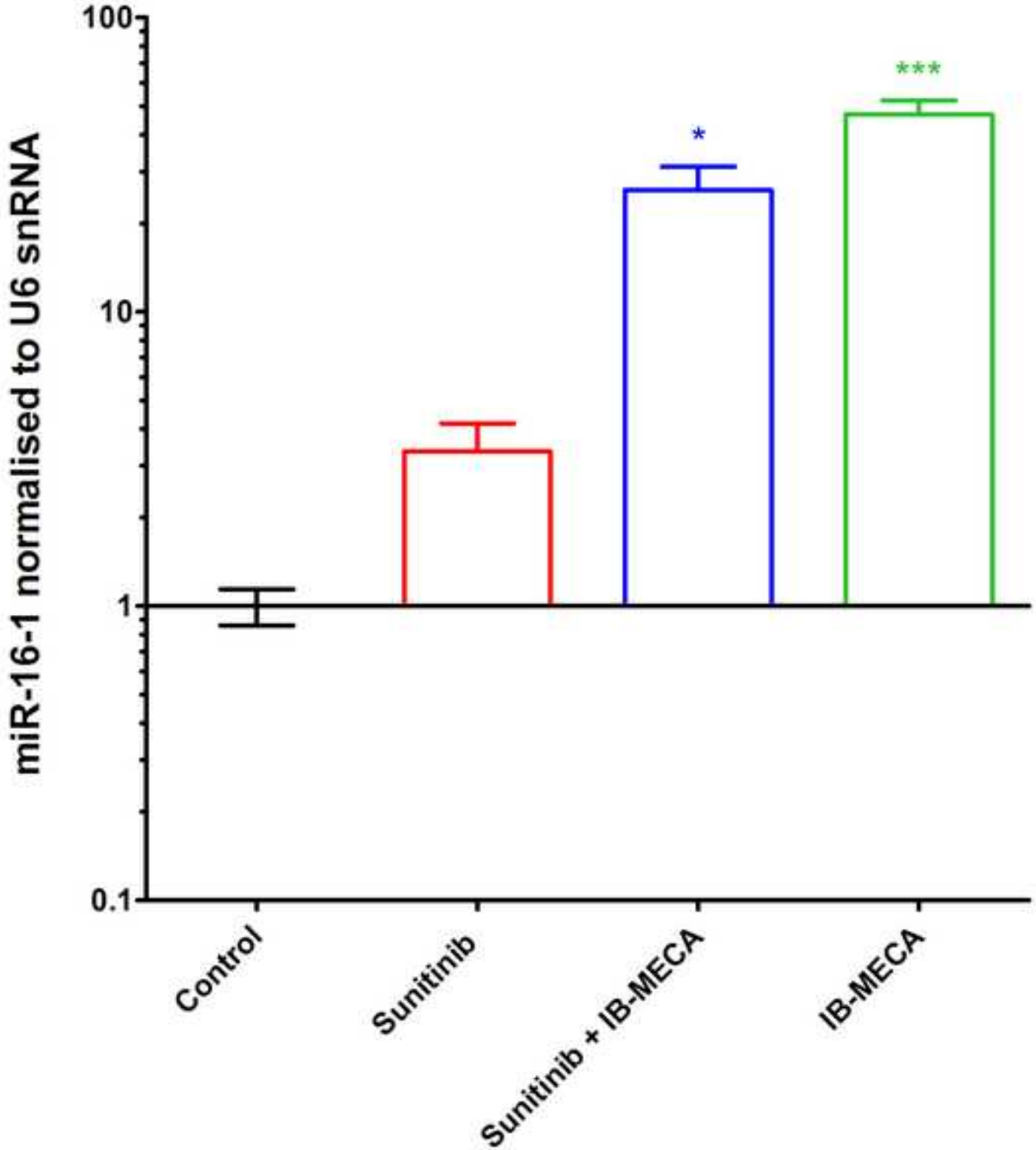

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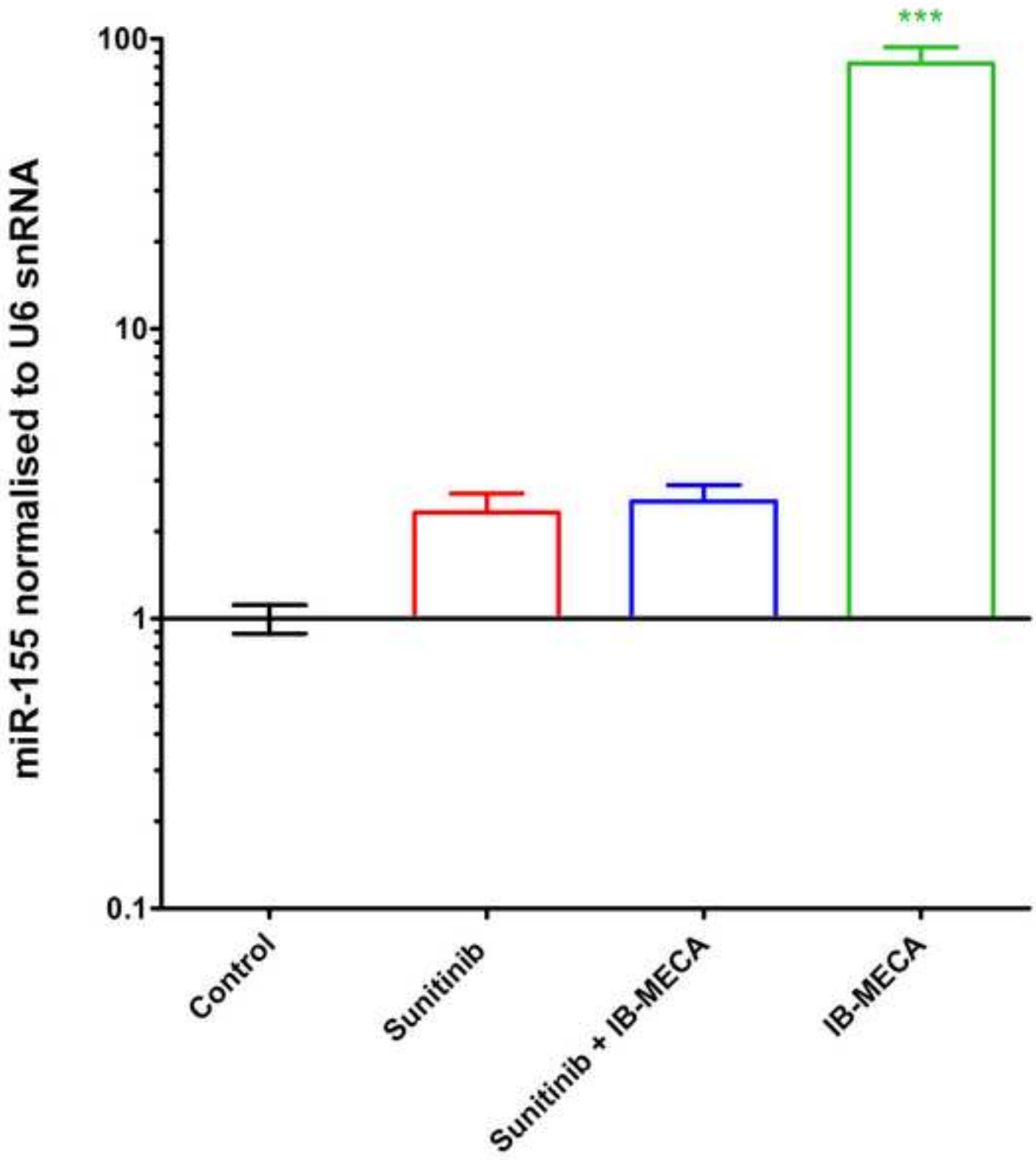

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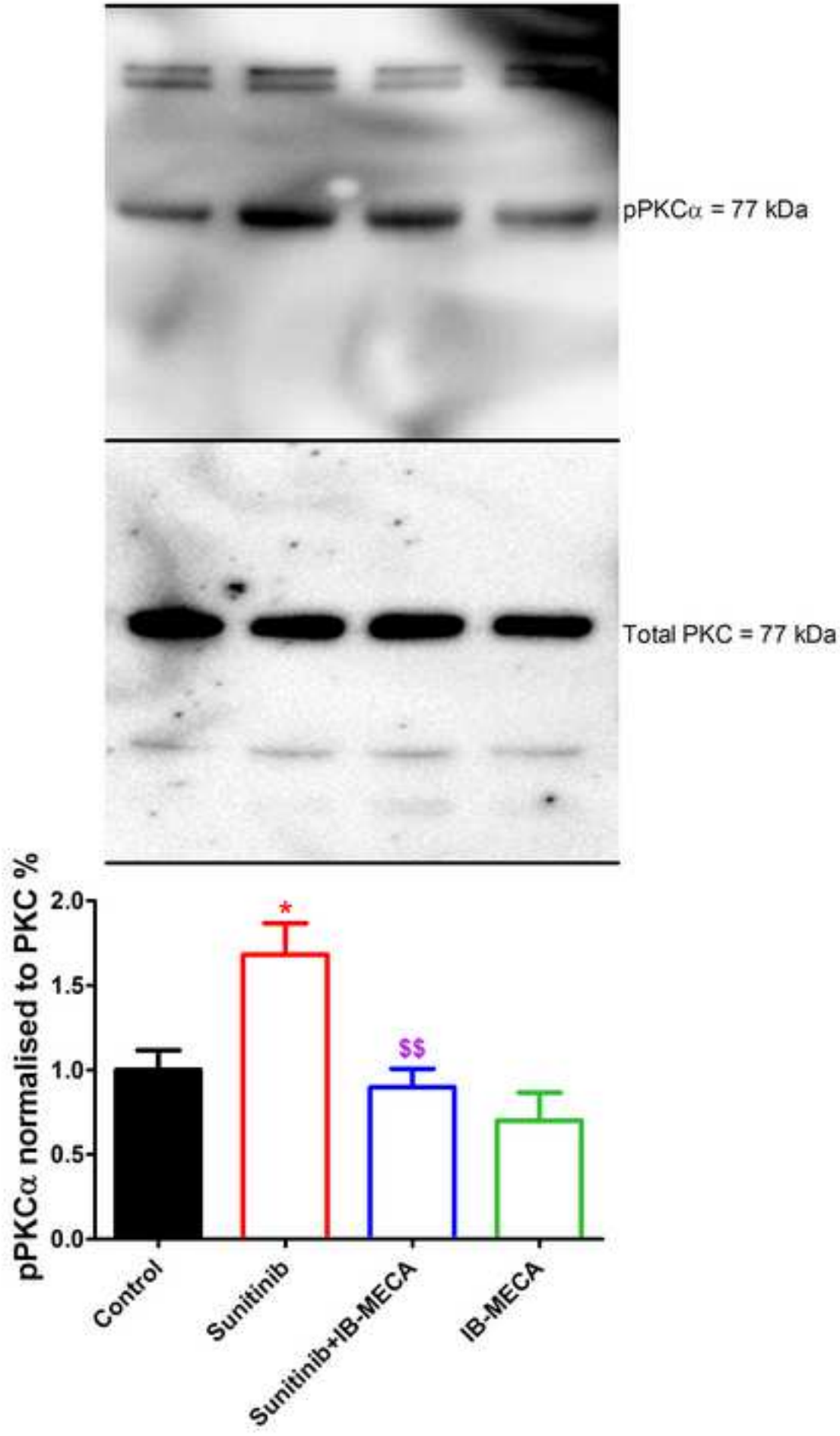

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