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TITLE: Ibuprofen inhibits migration and proliferation of human coronary artery smooth muscle cells by inducing a differentiated phenotype: Role of peroxisome proliferator-activated receptor γ

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Keywords: CAD, coronary artery disease; DES, drug eluting stent; HCASMCs, human coronary artery smooth muscle cells; HCAECs, human coronary artery endothelial cells; NSAIDs, non-steroidal antiinflammatory drugs; PCI, percutaneous coronary intervention; PPAR, peroxisome proliferator activated receptor.

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Running title: Ibuprofen modulates human VSMC phenotype

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Abstract

Objectives: The search for agents that are capable of preventing restenosis and reduce the risk of late thrombosis is of utmost importance. In this study we aim to evaluate the in vitro effects of ibuprofen on proliferation and migration of human coronary artery smooth muscle cells (HCASMCs) and on human coronary artery endothelial cells (HCAECs) migration.

Methods: Cell proliferation was evaluated by direct cell counting using trypan blue exclusion. Cell migration was assessed by wound healing “scratch” assay and by time lapse videomicroscopy. Protein expression was assessed by immunoblotting, and morphological changes were studied by immunocytochemistry. The involvement of the PPAR γ pathway was studied with the selective agonist troglitazone, and the use of selective antagonists of PPAR γ such as PGF2 α and GW9662.

Results: We demonstrate that ibuprofen inhibits proliferation and migration of HCASMCs and induces a switch in HCASMCs towards a differentiated and contractile phenotype, and that these effects are mediated through the PPAR γ pathway. Importantly we also show that the effects of ibuprofen are cell type specific as it does not affect migration and proliferation of endothelial cells.

Conclusions: Taken together, our results suggest that ibuprofen could be an effective drug for the development of novel drug eluting stents, which could lead reduced rates of restenosis and potentially other complications of DES stent implantation.

Introduction

Cardiovascular disease (CVD) remains the leading cause of morbidity and mortality worldwide, accounting for the death of one in three people each year.^[1, 2] Of the 17.3 million deaths per year due to CVD, coronary artery disease (CAD) was responsible for over 40% of cases.^[2] Current treatment modalities for CAD include percutaneous coronary intervention (PCI) with balloon angioplasty and stent implantation. However, despite advances in stent technology (e.g. drug eluting stents (DES)) that reduced substantially the rates of restenosis to less than 10%,^[3, 4] the rates of late in-stent thrombosis, a devastating complication in terms of morbidity and mortality, are higher with DES compared to bare metal stents.^[5] Recent reports suggest that the risk of late-stent thrombosis appears to be steadily present over time,^[6, 7] and, indeed, there have been reports of these events occurring 4.5 to 5 years after DES implantation.^[8, 9] Thus, late in-stent thrombosis remains a major problem in clinical practice.^[10, 11] DES contain antiproliferative agents (e.g. rapamycin or paclitaxel), which target vascular smooth muscle cells (VSMCs) and inhibit their migration and proliferation to the site of injury following stent deployment, thus ultimately preventing neointimal formation and restenosis. However, DES also interfere with re-endothelialization of the stent struts, which currently is thought to play a central role in the pathogenesis of very late (>12 months) in-stent thrombosis observed with DES.^[12, 13] With this in mind, the search for compounds capable of selectively inhibiting migration and proliferation of VSMCs, but sparing endothelial cell (EC) migration and proliferation, for use in DES is of utmost importance.

During restenosis following stent deployment, VSMCs undergo significant changes in morphology and function exhibiting a “synthetic” or “dedifferentiated” phenotype, whereby these cells synthesize components of the extracellular matrix and exhibit characteristics similar to immature cells found in blood vessels during development.^[14, 15] In the intact vessel wall, however, VSMCs exhibit a “differentiated” or contractile phenotype characterized by a spindle shape and increased expression of contractile proteins, which correlates with increased contractile ability.^[15]

Peroxisome proliferator-activated receptor γ (PPAR γ) is a member of the nuclear hormone-receptor superfamily.^[16] PPAR γ expression has been shown to increase in the intima in response to injury.^[17] It has been proposed that increased activity of PPAR γ in blood vessels is important for limiting neointimal formation by enhancing the differentiation of VSMCs and thereby reducing their migratory ability.^[18] Previous studies from our laboratory, and others, have demonstrated that ibuprofen, as well as certain other non-steroidal anti-inflammatory drugs (NSAIDs), reduces VSMC proliferation in a dose- and cell cycle-dependent manner.^{[19,}
^{20]} In addition, several lines of evidence indicate that NSAIDs, including ibuprofen, have the ability to activate PPAR γ receptors in several cell types and tissues.^[21-23] Here, we confirm that ibuprofen inhibits proliferation and report for the first time that ibuprofen inhibits migration of human coronary artery smooth muscle cells (HCASMCs), and that this correlates with the induction of a differentiated phenotype in these cells. We also report that at least some of the effects of ibuprofen on HCASMCs are mediated through PPAR γ . More importantly, we also show for the first time that the effect of ibuprofen is cell type specific as it does not affect migration and proliferation of endothelial cells. Taken together, our results suggest that ibuprofen could be a potential molecule for incorporation into DES which may prevent restenosis without interfering with the process of re-endothelialization, thus having the potential of preventing other complications stent implantation.

Materials and Methods

Antibodies and reagents

Antibodies raised against SM α -actin (ab5694), SM22 α (ab14106), GAPDH (ab8245), β -actin (ab6276) and Lamin A (ab8980) were from Abcam (UK); antibody raised against PPAR γ (sc-7273) was from Santa Cruz Biotechnology (USA), antibody raised against ATF-2 (9226) was from Cell Signalling Technology (USA). Alexa Fluor 488 goat anti-mouse (A-11001), Alexa Fluor 594 phalloidin (A12381) and prolong[®] Gold anti-fade reagent with DAPI (P36935) were from Life Technologies (UK). Most chemicals, including ibuprofen and troglitazone were obtained from Sigma (UK); Mitomycin C was from Calbiochem (UK); GW9662 was obtained from Biomol (UK); PGF2 α was from Merck Chemicals (UK). Tissue culture plates and dishes were from Thermo Fisher Nunc.

Cell culture

Primary human coronary artery smooth muscle cells (HCASMCs) and primary human coronary artery endothelial cells (HCAECs) were purchased from PromoCell (GmbH, Heidelberg, Germany), and cultured according to manufacturer's instructions using PromoCell's smooth muscle cell growth medium 2 (C-22062) or endothelial cell growth medium MV2 (C-22022), both of which contains 5% serum without antibiotics or antifungal agents. Passaging was according to the manufacturer's instructions using DetachKit, also from PromoCell. Primary human pulmonary microvascular endothelial cells (HPMECs) were purchased from PromoCell (GmbH, Heidelberg, Germany). Growth media for HPMECs was prepared to give a final concentration of 20% (v/v) serum, and a concentration of 50 μ g/mL endothelial cell growth supplement in M199 with GlutaMAX[™] medium.

For all experiments, cells were used between passages 3-5 and were cultured replacing with the same fresh growth medium described above. For proliferation assays, HCASMCs (1×10^4 /ml) and HPMECs (1×10^4 /ml) were seeded in 6-well plates. For wound healing

assays, HCASMCs (4×10^4 /ml) and HCAECs (5×10^4 /ml) were seeded overnight in 12-well plates to form a confluent monolayer. For time-lapse video microscopy experiments, both HCASMCs and HCAECs were seeded overnight at (1×10^4 /ml) in 12-well plates. For immunoblotting, HCASMCs (2×10^5) and HCAECs (3×10^5) were plated in 60 mm tissue culture dishes. For immunostaining experiments, glass cover slides were placed in 12-well plates and coated with sterile 1% (v/v) gelatin in PBS, then washed in PBS and HCASMCs seeded at a density of 1×10^4 /ml.

Proliferation analysis

Following an initial 24 h incubation, media was replaced without agonists (vehicle controls) or treated with different doses of ibuprofen. After a further 72 h, direct cell counting was performed by trypan blue exclusion.^[20]

Wound healing assay

Migration was investigated using a wound healing “scratch” assay as described before,^[24, 25] with minor modifications. Briefly, 24 h after plating both HCASMCs and HCAECs at the desired cell density, medium was replaced in the absence (control) or presence of different concentrations of ibuprofen or troglitazone, and in some instances with the selective PPAR γ inhibitors, GW9662 or PGF2 α .^[26-28] Mitomycin C, was added one hour before scratching at a concentration of 0.5 μ g/ml. This concentration of mitomycin C completely blocks proliferation of HCASMCs within 1 h of administration (data not shown). A scratch in the cell monolayer was then generated with a sterile 200 μ l micropipette tip and images of the gap were captured immediately (time 0 h) and repeated at 24 h (HCASMCs) or 10 h (HCAECs) using a phase contrast microscope with an attached ProgRes SpeedXT core 5 digital camera (Jenoptik Systems GmbH) with ProgRes CapturePro 2.8.8. The extent of closure of the gap

(HCAECs) or migrated cell number (HCASMCs) was analysed using ImageJ (NIH, Bethesda).

Time lapse videomicroscopy

Time lapse video microscopy was used for *in vitro* real time analysis of HCASMCs and HCAECs migration, as described before,^[29] with minor modifications. Cells were re-fed with fresh medium in the absence (control) or presence of agonists such as ibuprofen or troglitazone, plus in some instances antagonists, such as GW9662. Following treatments for 30 min, cells were tracked for 48 h at 15 min intervals. Over 90 cells were tracked in each of three separate wells per condition, with images captured by a Nikon digital camera DXM 1200 Japan (NIS-elements AR software). All microscopy stages were performed in a controlled environment (37°C and 5% CO₂). The distance that a particular cell had migrated within 48 h was analysed using ImageJ software (NIH, Bethesda).

Immunoblotting

Whole cell extracts were prepared as described previously with minor modifications.^[20] Briefly, HCASMCs or HCAECs were washed gently in ice cold PBS and scraped into 150 µl protein lysis buffer [50 mM Tris HCl pH 7.4, 250 mM NaCl, 5 mM EDTA pH 8.0, with freshly added Igepal (0.1 % v/v)], supplemented with protease inhibitors [0.3 mM AEBSF, 10 µg/ml Leupeptin and 2 µg/ml Aprotinin]. Samples were incubated on ice for 10 min and then centrifuged (10,000 ×g, 5 min, 4°C). Supernatants were transferred to fresh micro tubes and stored at -80°C until use.

Nuclear extracts were prepared as described previously, with minor modifications.^[30, 31] Briefly, HCASMCs or HCAECs were washed gently in ice cold PBS and scraped into 150 µl ice cold nuclear buffer A [10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.3 mM

Na₃VO₄], supplemented with protease inhibitors (0.3 mM AEBSF, 10 µg/ml Leupeptin and 2 µg/ml Aprotinin). Samples were incubated on ice for 5 min, then centrifuged (10,000 × g, 5 min, 4°C) and the pellets washed in 100 µl nuclear buffer A containing Igepal (0.1 % v/v). Samples were incubated for 5 min on ice, then centrifuged (10,000 × g, 5 min, 4°C) and the pellets re-suspended in 50 µl nuclear buffer C [20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.3 mM Na₃VO₄, 0.2 mM EDTA pH 8.0, 25% (v/v) glycerol] supplemented with protease inhibitors as above. Suspensions were incubated on ice for 1h with gentle vortexing every 15 min, then centrifuged (10,000 × g, 5 min, 4°C). Supernatants were transferred to fresh micro tubes and stored at -80°C until use. Protein concentrations were determined by the Bradford method.^[32]

Samples were boiled with 0.33 vol of SDS-polyacrylamide gel electrophoresis sample buffer [10% SDS (w/v), 13% glycerol v/v), 300 mM Tris HCl, pH 6.8, 130 mM DTT, 0.2% bromophenol blue (w/v)]. Proteins (20 µg) were separated by SDS-polyacrylamide gel electrophoresis using 10% (w/v) resolving gels and 6% (w/v) stacking gels, and transferred to nitrocellulose as described previously.^[31] Nonspecific binding sites were blocked with 5% (w/v) non-fat milk powder in TBS-T [20 mM Tris HCl pH 7.5, 137 mM NaCl, 0.1% (v/v) Tween 20]. Membranes were incubated (overnight, 4 °C) with primary antibodies diluted (1:1000 for PPAR γ to 1:20000) in TBS-T containing 5% non-fat milk powder for most antibodies, except PPAR γ and ATF2 which were incubated in 5% (w/v) bovine serum albumin. Membranes were washed in TBS-T, incubated (60 min, room temperature) with horseradish peroxidase- (HRP-) conjugated secondary antibodies from Dako (1/5000 to 1:25000 dilutions) in TBS-T containing 1% (w/v) non-fat milk powder, and were then washed in TBS-T. Bands were detected using enhanced chemiluminescence (ECL) Prime (GE Healthcare) and an ImageQuant LAS4000 mini digital imager (GE Healthcare). ImageQuant 8.1 (GE Healthcare) software was used for densitometric analysis.

Immunocytochemistry

HCASMCs were exposed to 500 μ M ibuprofen for 72 h, then washed with PBS and fixed in 4% (v/v) formaldehyde (10 min at room temperature). Following permeabilization with 0.1% Triton X-100 in PBS (10 min at room temperature) and incubation with 1% (w/v) BSA in PBS containing 0.1% Triton X-100 (20 min at room temperature) to block non-specific antibody binding, immunocytochemistry was performed as described previously with minor modifications.^[31] Briefly, cover slides were incubated with mouse monoclonal primary antibodies against β -actin (1:1000 dilution for 60 min at 37°C), followed by Alexa Fluor® 488 anti-mouse secondary antibodies (1/200 dilution for 60 min, 37°C), and then incubated with Alexa Fluor® 594 phalloidin (1:200 dilution for 20 min at 37°C). Washes (\times 3) were performed with PBS between incubations. After the final wash, cover slides were mounted with prolong® Gold anti-fade reagent with DAPI (Invitrogen) and visualized using a Zeiss AX10 Axioskop fluorescence microscope. Images were captured using a Zeiss AxioCam MRm digital camera with Software AxioVision Rel 4.8.2 (Carl Zeiss MicroImaging GmbH). Colour images were converted to greyscale using Adobe Photoshop.

Statistical Analysis

Statistical analysis was performed with Graphpad Prism 5.0. The data are presented as mean \pm SEM, and significance of results was determined by one-way ANOVA with Dunnet's or Tukey's post test, and, where appropriate, a paired two-tailed student *t* test. The results were considered significant when $p < 0.05$.

Results

Proliferation and migration of HCASMCs is inhibited by ibuprofen in a dose dependent manner

Proliferation and migration of VSMCs are key elements in the pathophysiology of restenosis post-stent implantation.^[33] Previous studies from our laboratory demonstrated that A10 rat VSMC proliferation was inhibited by several NSAIDs in a dose-dependent manner.^[20] Here, we first evaluated whether proliferation of human primary HCASMCs is similarly affected by ibuprofen. Treatment with different concentrations of ibuprofen did, indeed, cause an inhibitory effect on the proliferation of HCASMCs in a dose-dependent manner. Thus, whilst 100 μM ibuprofen had no significant effect, concentrations above 250 μM progressively inhibited proliferation of HCASMCs, with an IC_{50} of $680 \pm 120 \mu\text{M}$ (Figure 1A).

We then performed *in vitro* studies to evaluate the effects of ibuprofen on the migration of HCASMCs. Using a wound healing “scratch” assay (a well validated method for evaluating migration of cells *in vitro*),^[25] we first observed that HCASMCs migrate individually rather than as a population of cells. Subsequent observations indicated that ibuprofen inhibits the number of cells that migrate to the wound in a dose-dependent manner. Thus, doses up to 2000 μM ibuprofen were able to inhibit the migrated cell number with an IC_{50} of $410 \pm 60 \mu\text{M}$ (Figures 1B and 1C), without significant cytotoxicity for the cells (as determined by trypan blue exclusion assay; data not shown).

We then used time-lapse videomicroscopy to evaluate, in real time, the effects of ibuprofen on migration of HCASMCs. Ibuprofen (500 μM), caused a significant reduction in the average mean displacement by HCASMCs over a period of 48 h (Figure 2), further confirming the ability of ibuprofen to inhibit migration of HCASMCs cells *in vitro*.

Ibuprofen induces a phenotypic switch in HCASMCs

Previous studies have shown that, during culture in the presence of serum, VSMCs become de-differentiated, adopting an amoeboid shape and exhibiting increased synthesis of components of the extracellular matrix, thus the term “synthetic” that is commonly used to describe this phenotype.^[34] Associated with this change in phenotype, de-differentiated VSMCs also show a reduced expression of contractile proteins, including SM α -actin, SM22 α and F-actin.^[33, 34] In contrast, serum starvation or treatment with rapamycin, induces differentiation of VSMCs with a characteristic spindle shape and increased expression of contractile proteins, giving rise to the commonly used term, “contractile” phenotype ^[33-35] Importantly, during vascular damage (e.g. stent deployment), VSMCs migrate and proliferate to the site of injury, and this correlates with a change from a contractile towards a synthetic phenotype.^[15]

Given the effects of ibuprofen on the proliferation and migration of HCASMCs, we tested the possibility that these effects correlate with a change in the de-differentiation state of these cells. Therefore, we investigated the morphology of HCASMCs using immunocytochemistry. Treatment with 500 μ M ibuprofen for 72 h induced a change in morphology, from an amoeboid into a spindle shape (Figure 3A). Characteristically, the observed change in cell surface area induced by ibuprofen mainly affects the width of the treated cell whereas the length of the cell remains unchanged (Figure 3B-3D).

We next investigated whether the changes in morphology induced by ibuprofen correlated with changes in expression of markers of the differentiated state.^[34] HCASMCs cultured in the presence of serum, were either untreated (vehicle controls) or exposed to ibuprofen 500 μ M, and the protein levels of SM α -actin and SM22 α were then assessed by immunoblotting analysis. As shown in Figure 4A-C, the protein levels of SM α -actin and SM22 α were up-regulated following treatment with ibuprofen for 72 hours. When we analyzed the myofilament density of HCASMC, it revealed that treatment with ibuprofen increased the phalloidin staining intensity of F-actin, another marker of differentiation of VSMCs,^[34] as

compared with untreated, vehicle controls (Figure 4D), further supporting the possibility that ibuprofen induces a switch from a synthetic to a contractile phenotype in HCASMCs.

The effects of ibuprofen on HCASMCs are partially mediated by PPAR γ

Many effects of NSAIDs are mediated via cyclooxygenase (COX)-independent mechanisms;^[36] indeed, previous studies have demonstrated that ibuprofen and other NSAIDs are agonists of PPAR γ .^[21-23] On the other hand, the major metabolite of arachidonic acid via COX in VSMCs is PGF2 α .^[37] Interestingly, PGF2 α is a selective antagonist of PPAR γ .^[26, 27] To study the molecular mechanisms of the effects of ibuprofen in HCASMCs, we evaluated whether the selective natural antagonist of PPAR γ , PGF2 α ,^[26, 27] or the selective synthetic antagonist, GW9662,^[28] could prevent the effects of ibuprofen in HCASMCs.

Figure (5A) shows that the inhibitory effect of ibuprofen on proliferation of HCASMCs was almost completely abrogated by pre-incubation with either PGF2 α or GW9662. Similarly, analysis of migration by wound healing “scratch” assay and time-lapse video-microscopy in the presence of either PGF2 α or GW9662 in combination with ibuprofen, suggest that the PPAR γ pathway is also involved in mediating the effects of ibuprofen on HCASMC migration (Figures 5B and 5C). Thus, whereas incubation with either antagonist alone does not significantly affect the migration of HCASMCs compared to control conditions, incubation of HCASMCs with ibuprofen in the presence of either PGF2 α or GW9662 significantly, but not completely reverses the effects of ibuprofen (Figures 5B and 5C).

We then explored whether the effects of ibuprofen on morphology and expression of markers of differentiation in HCASMCs were also mediated via PPAR γ . As shown in Figures 3A and 3B, cell surface area in HCASMCs treated with ibuprofen in combination with GW9662 (3 μ M) was similar to that of medium alone-treated control cells, and was significantly higher than those treated with ibuprofen alone, suggesting that PPAR γ does,

indeed, play a role in the phenotype switch induced by ibuprofen. However, whilst GW9662 significantly inhibits the effects on morphology induced by ibuprofen, they do not return to levels seen in untreated, control cells (Figures 3A and 3B), suggesting that other pathways might also be involved in mediating these morphological effects. Indeed, troglitazone, a selective PPAR γ agonist, induces changes in the morphology of HCASMCs but these are qualitatively different to those induced by ibuprofen (Figure 3A). Thus, whilst ibuprofen induces a change in morphology resembling the characteristic spindle shape of differentiated VSMCs as described previously,^[34, 35] troglitazone affects both cell width and cell length in HCASMCs as opposed to ibuprofen that mainly affects cell width without affecting cell length (Figures 3A-3D). Finally, we evaluated the expression of protein markers of VSMC differentiation. Significantly, whilst ibuprofen induced expression of both SMA α -actin and SM22 α (Figures 4A-4C, 6A-6C), incubation with PGF2 α did not significantly inhibit these effects. In fact, SM22 α expression levels in the presence of PGF2 α alone, or in combination with ibuprofen, did not differ; the effect of troglitazone was comparable to that of ibuprofen (Figures 6A and 6C). In contrast, whereas PGF2 α had a trend to inhibit the effects of ibuprofen on SMA α -actin expression, this was not significant, and troglitazone failed to induce expression of this protein above control levels (Figures 6A and 6B).

Ibuprofen does not affect migration and proliferation of endothelial cells

Taken together, our results suggest that ibuprofen inhibits proliferation and migration of HCASMCs and this is partially mediated by the PPAR γ pathway. In clinical practice, the ideal DES would prevent restenosis and also late and very late in-stent thrombosis. It has been suggested that the currently available DES target VSMC migration and proliferation, but in doing so they also prevent re-endothelialisation of the stent struts, with the risk of very late in-stent thrombosis steadily occurring over time,^[6, 7] indicating that complete healing of the vessel wall is never achieved. Novel DES that prevent migration and proliferation of VSMCs whilst permitting the migration and proliferation of ECs, and thus allowing re-

endothelialization, could offer a significant advantage for DES stent therapy. Therefore, we performed “scratch” wound assays and time-lapse videomicroscopy analysis to evaluate the effects of ibuprofen on migration of HCAECs.

As shown in Figure 7A, ibuprofen at all concentrations tested (100 – 1000 μM) does not significantly affect migration of HCAECs as measured via a “scratch” wound assay. Similarly, ibuprofen at a concentration of 750 μM , which potently inhibits migration of HCASMCs (Figure 1B and Figure 2), fails to inhibit migration of HCAECs as demonstrated by time-lapse videomicroscopy analysis (Figure 7B). In contrast, troglitazone does affect migration of HCAECs as measured by wound healing assay and time lapse videomicroscopy (Figure 7A and Figure 7B). We also evaluated the effects of ibuprofen on proliferation of HPMECs. Whilst doses of 100 – 1000 μM had no significant effect on proliferation of these cells, there was significant inhibition of proliferation at doses of 2000 μM , with an estimated IC_{50} of 1500 ± 50 μM (Figure 7C). Interestingly, this IC_{50} is up to three times higher than that required to inhibit HCASMCs proliferation. Taken together, these experiments suggest that the effect of ibuprofen on migration and proliferation at doses below 2000 μM is cell type specific thus affecting only HCASMCs while sparing migration and proliferation of ECs.

We next compared the expression levels of PPAR γ in both HCASMCs and HCAECs. First, we were able to determine that PPAR γ , as a nuclear receptor protein, was present mainly in the nuclear fractions of both cell types (Figure 8A). To verify the loading of nuclear fractions, membranes were probed with ATF2 antibody, and, as shown in Figures 8B and 8C, densitometric analysis showed that PPAR γ was more abundant in HCASMCs than in HCAECs when levels were normalized to the loading control, ATF2 (Figures 8B and 8C). Interestingly the protein expressed in both cell types had a molecular mass of ≈ 53 kDa (see Discussion).

Discussion

Restenosis post stent implantation is a complex pathophysiological process, which involves proliferation and migration of VSMCs to the site of injury (e.g. following balloon angioplasty and stent deployment) and a change in VSMCs towards a synthetic/proliferative phenotype.^[33] Indeed, the restenotic vessel wall is mostly composed of extracellular matrix proteins synthesized by dedifferentiated proliferative VSMCs.^[14] Recent advances in stent technology, including DES containing antiproliferative agents (e.g. rapamycin and paclitaxel) have reduced significantly the rate of restenosis to less than 10% over the past decade.^[3, 4] However, despite the success of DES in reducing restenosis rates, there is a constant threat of late and very late in-stent thrombosis, particularly following abrupt discontinuation of dual antiplatelet therapy.^[5] Several factors appear to be implicated in the biology of late in-stent thrombosis, but most evidence suggests it is the lack of selectivity of DES targeting not only VSMCs but also endothelial cells, thus interfering with the process of re-endothelialisation and vessel wall repair.^[12, 13] Thus, novel compounds capable of selectively targeting VSMCs migration and proliferation without interfering with the process of re-endothelialization are urgently needed. Indeed, the ideal stent should be capable of maintaining low rates of restenosis, reducing the risk of early or late in stent thrombosis, and promoting vessel wall healing by modulating the inflammatory response within the vessel wall.^[15, 38] Several second generation DES, containing everolimus, or zotarolimus, share the molecular mechanisms of their predecessor, sirolimus (rapamycin), and therefore target VSMCs as well as endothelial cells. Whilst recent clinical trials suggest improved outcomes compared to first generation DES up to 2 years of follow up,^[39] their performance in terms of late complications such as late and very late (>12 months) stent thrombosis remain to be determined.

We, and others, have previously shown that certain NSAIDs, including the propionic acid derivative, ibuprofen, are capable of reducing rat VSMCs proliferation in a dose- and cell cycle-dependent manner.^[19, 20] Here, we studied the ability of ibuprofen to inhibit proliferation

and migration of HCASMCs. First, we demonstrated that ibuprofen was able to inhibit both proliferation and migration of HCASMCs in a dose-dependent manner. Of note, the concentration required to inhibit proliferation of HCASMCs (IC_{50} $680 \pm 120 \mu M$) is similar to the dose that inhibited A10 rat VSMCs proliferation in our previous study (IC_{50} $646 \mu M$).^[20] A similar concentration range is required for inhibition of HCASMC migration (IC_{50} $410 \pm 60 \mu M$) as demonstrated by wound healing “scratch” assay and also by time lapse video microscopy. Interestingly, we also found that the effects of ibuprofen on HCASMC migration and proliferation correlated with the induction of a phenotypic switch on HCASMCs towards a differentiated, contractile state, characterized by a spindle shape morphology and increased expression of known contractile protein markers of differentiation.^[33-35]

Accumulating evidence indicates that NSAIDs have pleiotropic effects in many cell types, and therefore may have a therapeutic role in several conditions including prevention and treatment of certain types of cancer,^[36] and in prevention and treatment of neurodegenerative diseases, such as Alzheimer disease.^[22] It has been demonstrated previously that Ibuprofen, as well as other NSAIDs, activate PPAR γ in several cell types.^[21, 40] Indeed, Ibuprofen at the dose range used in our studies, has been shown to activate the PPAR γ pathway.^[23, 41] In addition, ibuprofen reduces glial inflammation via a PPAR γ -dependent mechanism, thus explaining the beneficial effects of ibuprofen and other NSAIDs in Alzheimer’s disease.^[23, 42] The report of Weber et al (2000), suggests that the effects of ibuprofen on VSMCs proliferation are COX-independent.^[19] Significantly, PPAR γ appears to be protective in the cardiovascular system, and has important regulatory roles in vascular biology.^[43] Our data indicate that ibuprofen-induced inhibition of HCASMC proliferation appears to be mediated primarily by the PPAR γ pathway, since pre-incubation with the selective PPAR γ antagonists, PGF2 α and GW9662, almost completely restored the proliferative ability of HCASMCs to control levels. To our knowledge, only few studies have aimed to elucidate the mechanisms whereby NSAIDs inhibit VSMCs proliferation. One study found that several NSAIDs, including ibuprofen, inhibit A10 VSMCs proliferation via inhibition

of store operated Ca²⁺ entry (SOCE) channels, an important Ca²⁺ entry pathway involved in cell proliferation.^[44] Another study showed that aspirin inhibited VSMCs proliferation by inducing phosphorylation of AMP-activated protein kinase.^[45] The precise mechanisms whereby ibuprofen via activation of PPAR γ inhibit proliferation in VSMCs remain to be elucidated; further studies are needed to clarify this. Several agonists of PPAR γ have been described including thiazolidinediones (e.g. ciglitazone, troglitazone, pioglitazone, rosiglitazone), and natural ligands including prostanoids, such as 15d-PGJ₂, as well as n³-polyunsaturated fatty acids.^[43] These PPAR γ agonists appear to inhibit VSMCs proliferation via downstream mechanisms that include, inhibiting the expression of the transcription factor c-fos,^[46, 47] blockade of Angiotensin-II induced activation of extracellular signal-regulated kinase 1/2 (ERK1/2),^[18, 48, 49] and suppression of cell cycle signaling through decreased phosphorylation of the retinoblastoma protein (Rb),^[50] a mediator of G1/S progression, and prevention of mitogen induced degradation of p27(Kip1),^[50] an inhibitor of cdk and Rb phosphorylation.

The ibuprofen-induced phenotypic switch in HCASMCs also appears to be mediated by PPAR γ as pre-incubation with GW9662 in the presence of ibuprofen induces a dedifferentiated amoeboid morphology similar to that of control cells, whereas incubation with ibuprofen alone induces a differentiated spindle-shaped morphology. Similarly, PPAR γ plays an important role in the migratory activity of HACSMCs as suggested by the partial rescue of the migratory activity of these cells by incubation with the PPAR γ antagonists, PGF₂ α and GW9662, in the presence of ibuprofen.

Since the effect of PPAR γ inhibitors did not completely abrogate the migratory response, other pathways could be involved in this response in addition to PPAR γ . We speculate that one such pathway could be COX2. Indeed, one of the main metabolites of arachidonic acid in VSMCs is PGF₂ α ,^[37] and this metabolite is a natural selective antagonist of PPAR γ .^[26, 27] Similarly, *in vivo* studies have shown that there is increased expression and activity of COX2

in the intima following vascular injury.^[17, 51] Furthermore, the synthesis of PGF2 α via COX activity is increased by PDGF,^[52, 53] an important regulator of the differentiation state of VSMCs, which induces dedifferentiation of VSMCs towards a synthetic phenotype and promotes their migration and proliferation.^[54-56] In agreement with these observations, our preliminary data using the wound healing “scratch” assay, indicate that celecoxib, a COX2 selective inhibitor, at doses that are non-toxic to the cells (6.25 – 25 μ g), prevents migration of rat A10 VSMCs whilst sparing HCAECs migration (unpublished observations).

Of note, our data also indicate that migration and proliferation of ECs is not affected by ibuprofen, suggesting that the effect of ibuprofen is cell-type specific. This is an important novel finding from our study and to our knowledge this is the first evidence indicating that a molecular compound such as ibuprofen has the ability to selectively inhibit VSMCs proliferation and migration while sparing ECs proliferation and migration. The potential implications of this observation could translate into the development of novel DES that could have advantages over DES currently in use by not only preventing the development of restenosis but at the same time, by not interfering with migration and proliferation of ECs, facilitate the process of re-endothelialization of the stent struts. The reasons for the selectivity of ibuprofen are not clear. PPAR γ are ligand-activated transcription factors that belong to a nuclear receptor superfamily, of which two isoforms, PPAR γ 1 (53 kDa) and PPAR γ 2 (57 kDa), are formed from the same gene by alternative mRNA splicing.^[57, 58] Our results indicate that a single protein band of approximate 53 kDa is detectable in nuclear extracts of both HCASMCs and HCAECs (Figure 8), likely corresponding to the PPAR γ 1 isoform. This is in agreement with previous studies suggesting that only PPAR γ 1 is expressed in VSMCs and endothelial cells of different species and tissues.^[59, 60] We also observed that the relative expression levels of PPAR γ appear to be higher in HCASMCs compared to HCAECs, and this could partly explain the sensitivity of HCASMCs to the effects of ibuprofen.

Drugs currently used in DES including sirolimus, paclitaxel and zotarolimus inhibit proliferation of VSMCs in nano molar concentrations.^[61, 62] Thus, a potential limitation of our study is that IC₅₀ concentrations of ibuprofen for migration and proliferation appear to be very high which may difficult the feasibility of using ibuprofen in a DES.

Taken together, our results suggest that the *in vitro* effects of ibuprofen on migration and proliferation, coupled with the induction of a phenotype switch towards a differentiated state whilst sparing migration of HCAECs, could be exploited by the incorporation of ibuprofen into a DES to prevent restenosis post stent implantation and potentially reduce other complications of DES stent implantation by not interfering with migration and proliferation of ECs. Whether our *in vitro* observations could be translated clinically into a DES of increased performance remains to be demonstrated. Future studies will aim to evaluate the feasibility of ibuprofen-containing DES in *in vivo* animal models of vascular injury and restenosis.

Conclusion

In conclusion, this study demonstrates for the first time that ibuprofen induces a phenotypic change in HCASMCs, which is consistent with the reduced proliferative and migratory ability of these cells. The effects of ibuprofen on morphology and proliferation of HCASMCs are mediated mainly by the PPAR γ pathway. Migration of HCASMCs appears to be mediated in part by PPAR γ , since pre-incubation with PPAR γ antagonists significantly, but not completely, reverses the effects of ibuprofen; other pathways (e.g. via COX 2) could be involved in this response. Importantly, migration and proliferation of ECs is not affected by ibuprofen at concentrations that potently inhibit HCASMCs proliferation and migration, indicating that HCASMCs are targeted selectively by ibuprofen. The reasons for the selectivity of ibuprofen affecting HCASMCs whereas it spares migration of HCAECs remain unclear, but the relatively higher expression levels of PPAR γ in HCASMCs could be, at least in part, responsible for the sensitivity of HCASMCs to this drug. Our *in vitro* observations

could translate into reduced rates of restenosis post angioplasty with stent deployment of an ibuprofen containing DES, providing ibuprofen-induced inhibition of proliferation and migration of HCASMCs, and could potentially also lead to a reduced risk of other complications following stent implantation, as ibuprofen spares the migration and proliferation of ECs.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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Authorship contributions: (AD, AG, PD, JMG, KAB, GB) Participated in research design, (AD, AG, IP) conducted experiments, (AD, AG) performed data analysis, (AD, AG, GB) wrote or contributed to the writing of the manuscript.

Footnotes:

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Figure legends

Fig 1. Ibuprofen inhibits migration and proliferation of HCASMCs. A) HCASMCs were either untreated (vehicle control) or treated with increasing concentrations of ibuprofen (100 – 1000 μM). Trypan blue exclusion was used to determine the inhibitory effect of ibuprofen on HCASMC proliferation 72 hours after incubation. Ibuprofen causes inhibition of proliferation in a dose-dependent manner with an IC_{50} of $680 \pm 120 \mu\text{M}$. B) HCASMCs were either unstimulated (vehicle control), or stimulated with increasing concentrations of ibuprofen for 24h. Mitomycin C was added at 23 h (1 hour before “scratch” wound) to prevent unwanted proliferation. Wound healing “scratch” assay was then performed as described in Materials and Methods. Ibuprofen causes inhibition of migration in a dose-dependent manner with an IC_{50} of $410 \pm 160 \mu\text{M}$ (100 – 1000 μM). Data shown in A and B are representative of 3-4 independent experiments. C) Representative images of the effects of ibuprofen (500 μM and 750 μM) versus control on migration of HCASMCs. * $p < 0.05$, by one way ANOVA with Dunnett’s multiple comparison test.

Fig 2. Ibuprofen inhibits migration of HCASMCs. Time lapse video microscopy of the effects of ibuprofen (500 μM) compared to control. Data are presented as bar and whisker graphs and are representative of three independent experiments and are shown as mean \pm SEM of average migrated displacement (AMD). * $p < 0.05$ as compared to controls.

Fig 3. Ibuprofen induces a phenotypic switch in HCASMCs. A)-C) HCASMCs were untreated (control) or exposed to troglitazone (100 μM) or GW9662 (3 μM) alone or in combination with ibuprofen (500 μM) for 72 hours. A) Cells were immunostained; pictures are representative of three independent experiments. Scale bar = 50 μm . B) Areas; C)

lengths; and, D) widths, were measured for at least 40 cells in each experiment. Results are means \pm SEM for three independent experiments. * $p < 0.05$, ** $p < 0.01$ one way ANOVA with Tukey's post test.

Fig 4. Ibuprofen increases the expression of protein markers of differentiation. A) HCASMCs were unstimulated (control) or exposed to 500 μ M ibuprofen for 72 h and expression of SM α -actin and SM22 α protein levels were assessed by immunoblotting. Images are representative of three independent experiments. B) and C), densitometric analyses of immunoblots. Data presented as mean \pm SEM for three independent experiments. D) fluorescence intensity analysis of F-actin expression on HCASMCs untreated (control) or exposed to 500 μ M for 72 hours. F-actin fibers were stained with Alexa Fluor® 594 phalloidin. * $p < 0.05$ paired two-tailed student t test.

Fig 5. The effects of ibuprofen on proliferation and migration of HCASMCs is mediated via PPAR γ . A) HCASMCs were either untreated (vehicle controls) or exposed to either PGF2 α (100 nM) or GW9662 (3 μ M) in the presence or absence of ibuprofen (500 μ M). Trypan blue exclusion was used to evaluate HCASMC proliferation. B) Cells were untreated (vehicle controls) or exposed to either PGF2 α or GW9662 in the presence or absence of ibuprofen (500 μ M) for 72 h. Mitomycin C (0.5 μ g/ml) was added at 1 hour before the "scratch" wound to prevent unwanted proliferation. Wound healing "scratch" assay was then performed. C) Time lapse video microscopy of the effects of ibuprofen (500 μ M) on migration of HCASMCs in the presence or absence of GW9662 compared to vehicle controls and troglitazone (100 μ M). Data are presented as bar and whisker graphs and are representative of three independent experiments and are shown as mean \pm SEM of average migrated displacement (AMD). * $p < 0.05$ one way ANOVA with Tukey's post test.

Fig 6. Ibuprofen-mediated expression of protein markers of differentiation is not dependent on PPAR γ . A) immunoblotting analysis of markers of contractile proteins expressed in HCASMCs in response to troglitazone (100 μ M), or ibuprofen 500 μ M alone or in combination with PGF2 α (100 nM) versus vehicle control. B) and C), densitometric analyses of immunoblots relative to GAPDH. Data presented are mean \pm SEM from three independent experiments. * $p < 0.05$ one way ANOVA with Tukey's post test.

Fig 7. Ibuprofen does not affect migration and proliferation of endothelial cells. A) HCAECs were untreated, exposed to different concentrations of ibuprofen (100 μ M – 1000 μ M), or exposed to troglitazone (100 μ M) for 24 h. Results shown are mean \pm SEM for three independent experiments. * $p < 0.05$ one way ANOVA with Tukey's post test. B) Time lapse video microscopy on HCAECs treated with troglitazone (100 μ M) or GW9662 (3 μ M) alone or in combination with ibuprofen (500 μ M). Data are presented as bar and whisker graphs and are representative of three independent experiments and are shown as mean \pm SEM of average migrated displacement (AMD). C) Human pulmonary microvascular endothelial cells (HPMECs) were either untreated (vehicle control) or treated with increasing concentrations of ibuprofen (100 – 2000 μ M). Direct cell counting using Trypan blue exclusion was used to determine the inhibitory effect of ibuprofen on HPMECs proliferation 72 hours after incubation. Ibuprofen causes inhibition of proliferation in a dose-dependent manner with an IC₅₀ of 1500 \pm 50 μ M. * $p < 0.05$, by one way ANOVA with Dunnett's multiple comparison test.

Fig 8. PPAR γ is mainly a nuclear protein and is more abundantly expressed in HCASMCs relative to HCAECs. A) and B) immunoblotting analysis of PPAR γ expression in untreated HCASMCs vs HCAECs. A) PPAR γ is mainly expressed in the nucleus. B) Representative images of immunoblotting for PPAR γ in HCASMCs versus HCAECs. C) densitometric analysis showing relative abundance of PPAR γ normalised to ATF2 protein as loading control.

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Figure 1.

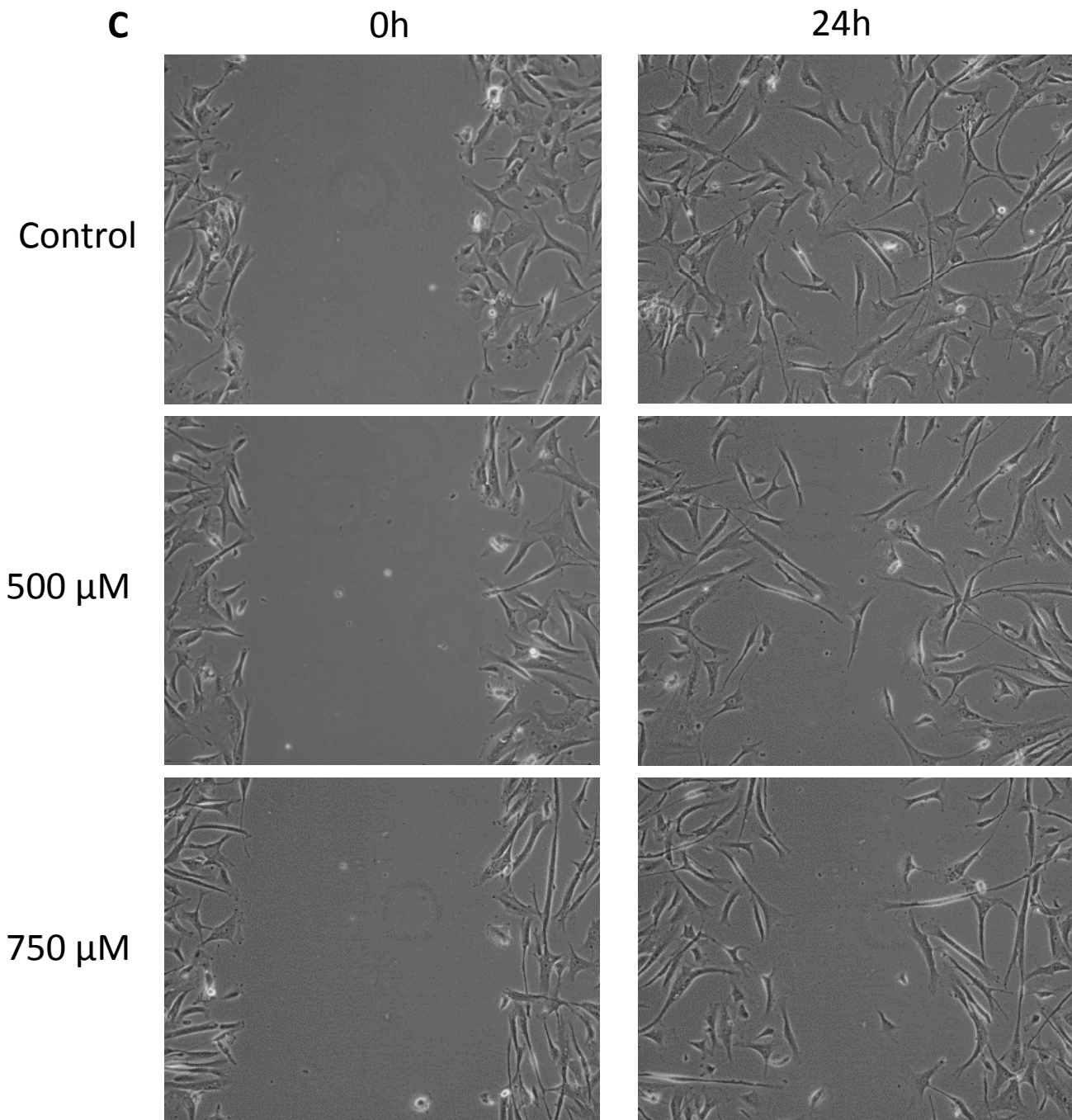
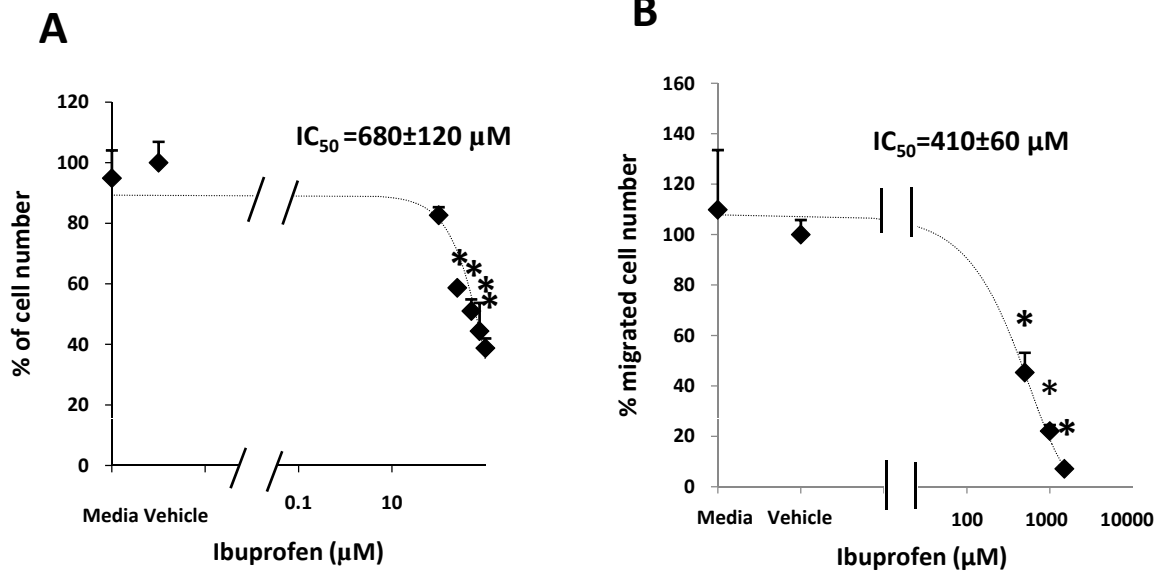


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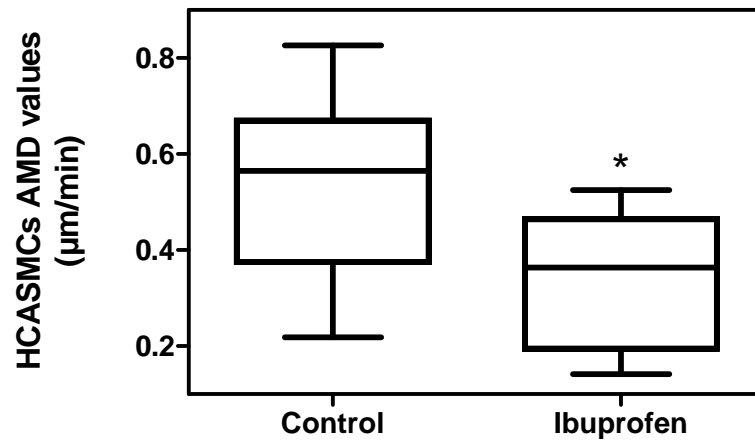
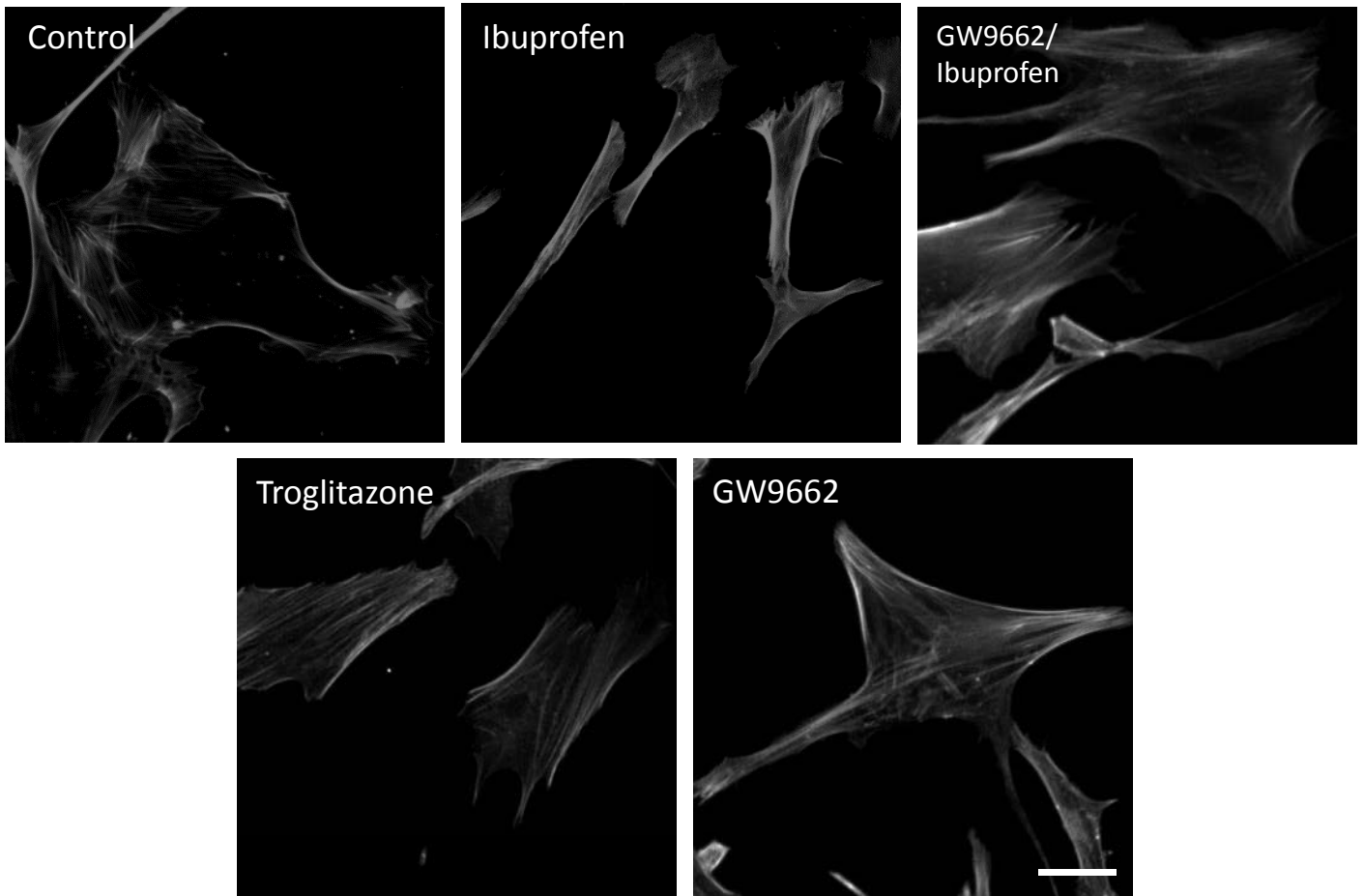
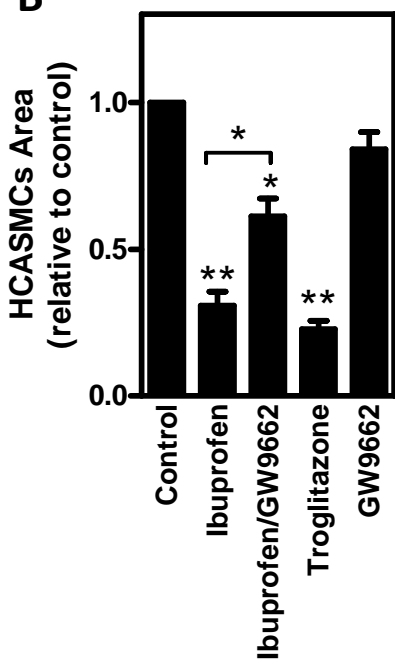


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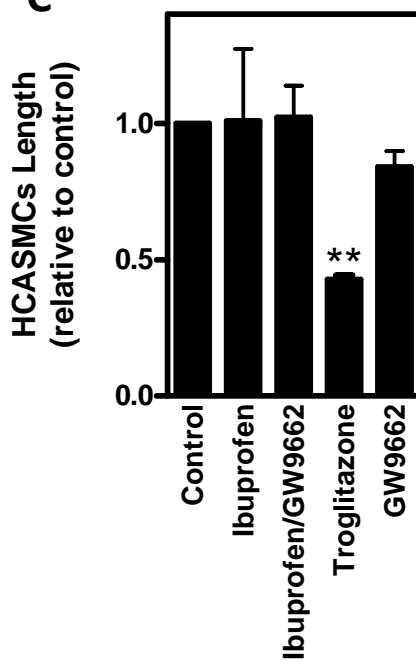
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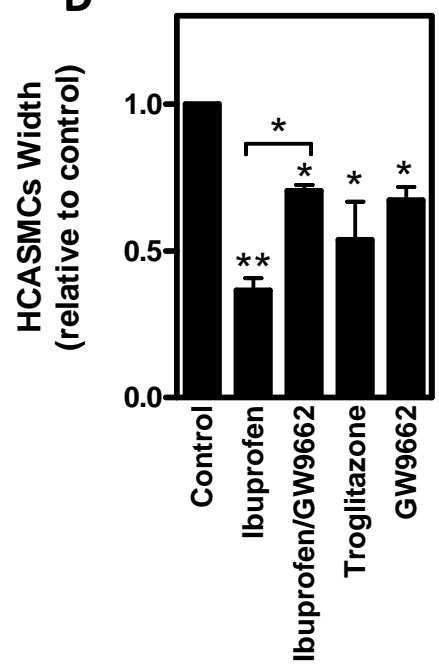


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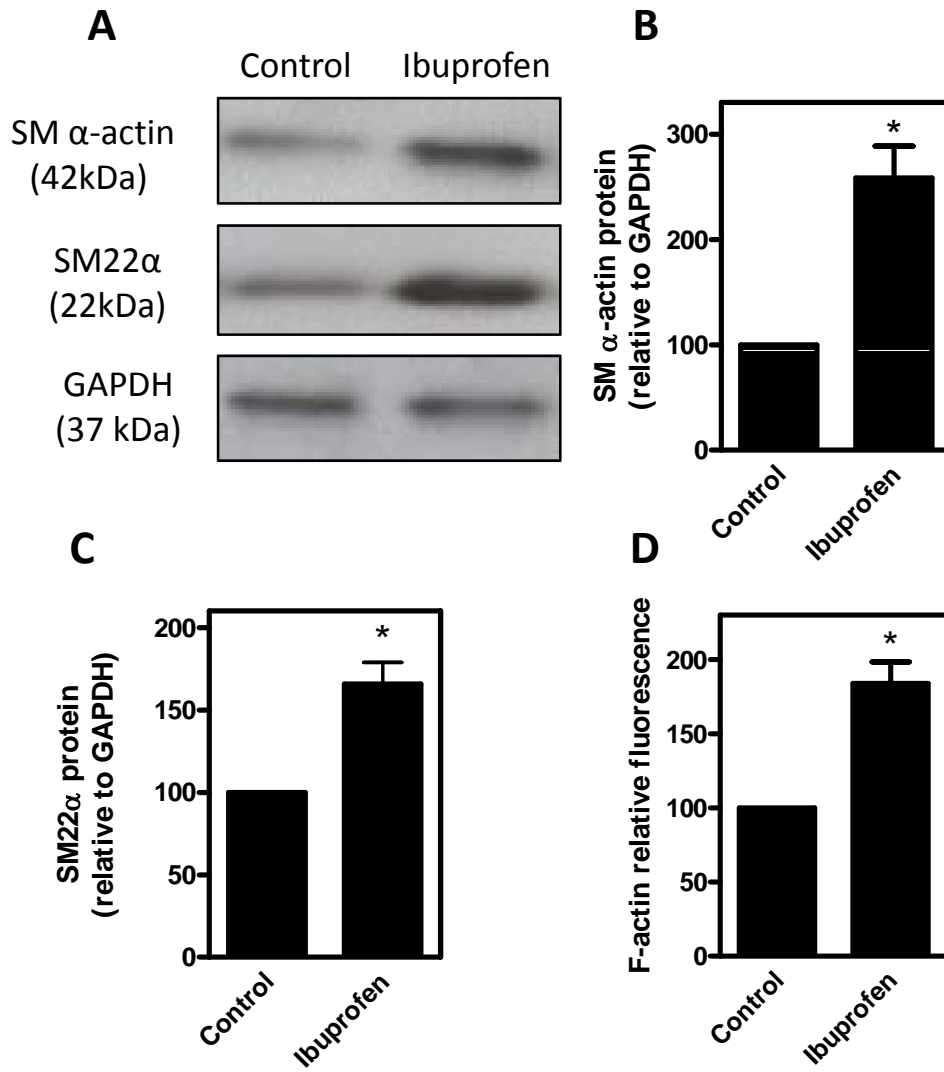


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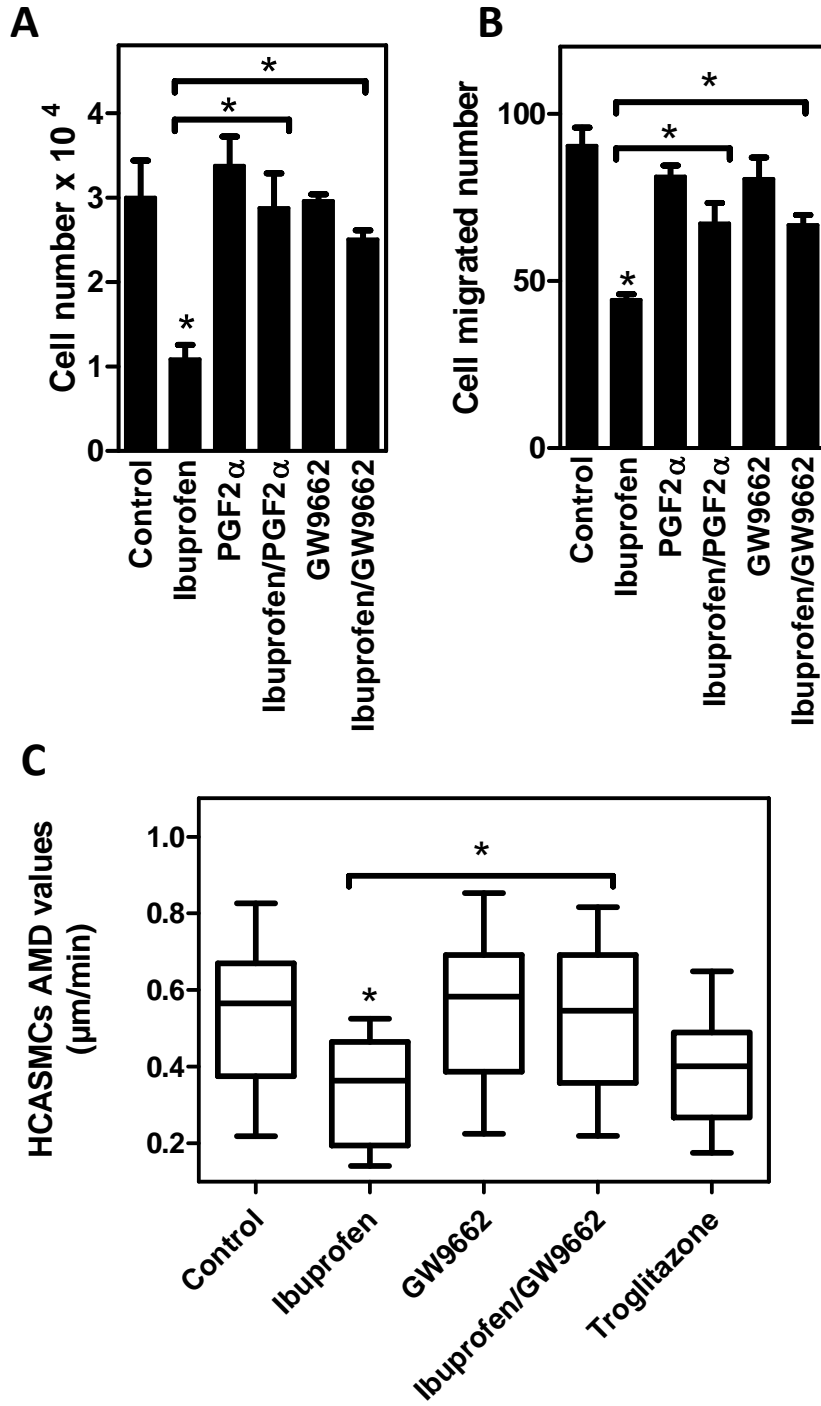


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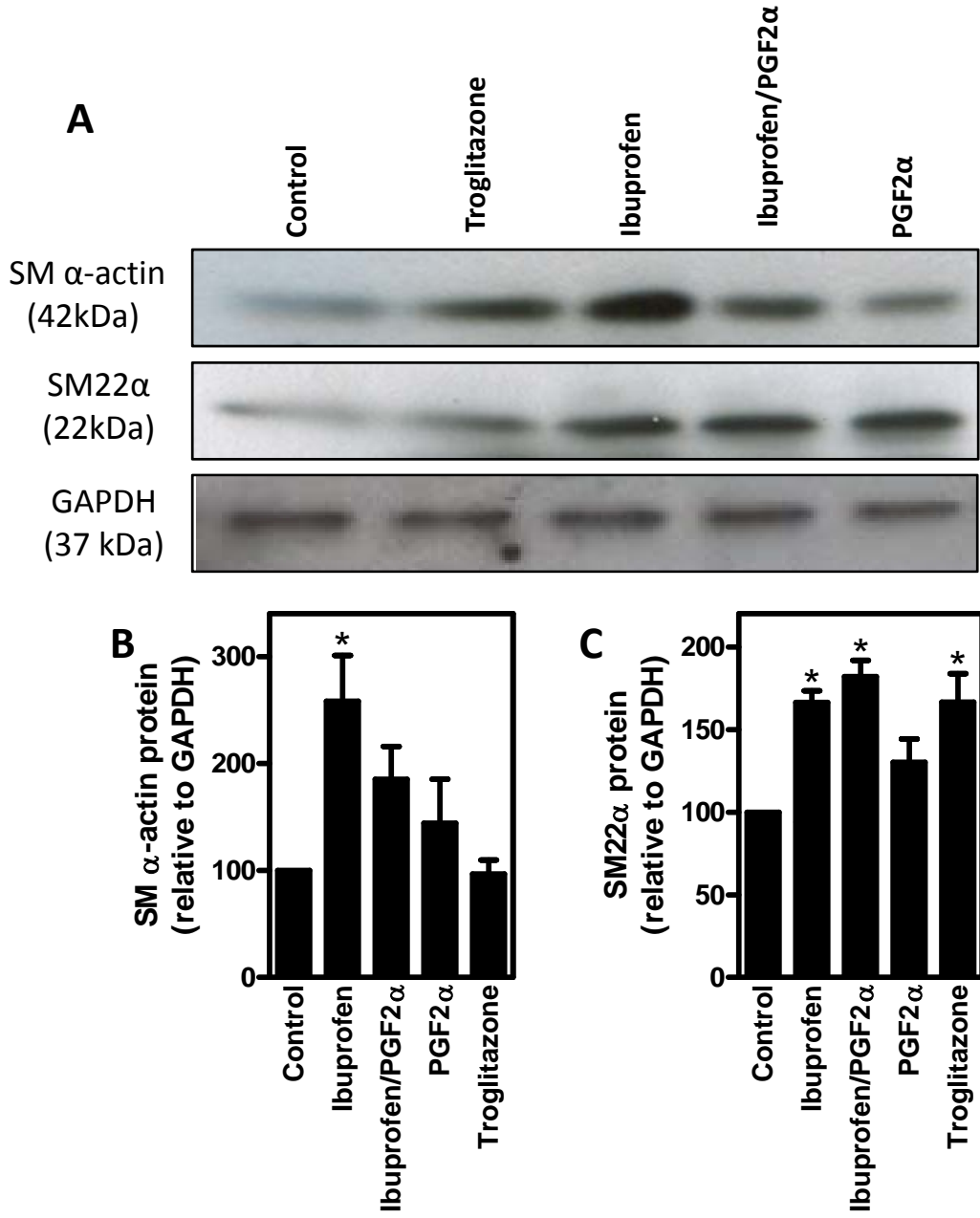


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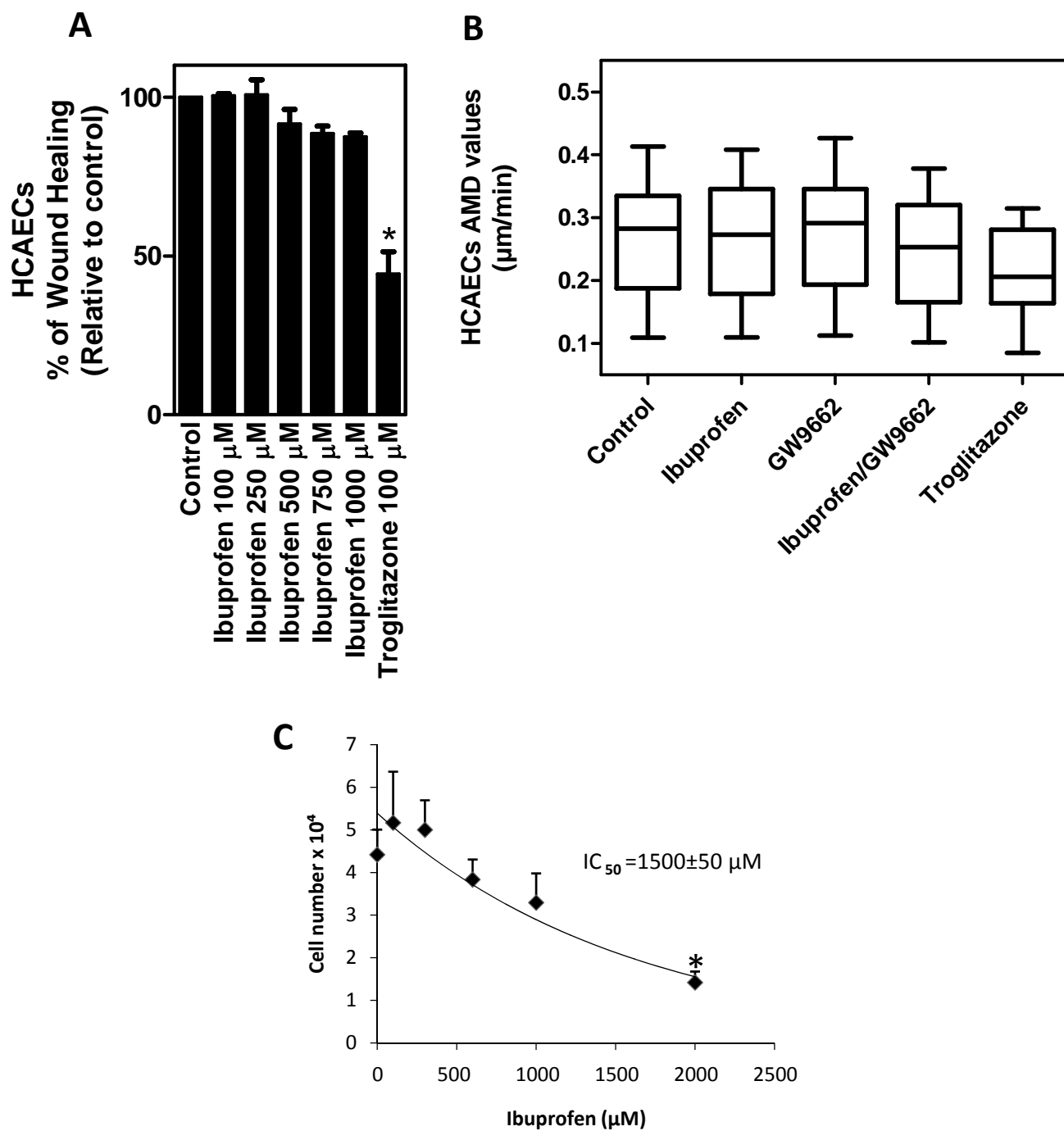


Figure 8.

