

# Novel *cis*-restricted $\beta$ -lactam combretastatin A-4 analogues display anti-vascular and anti-metastatic properties *in vitro*

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**Abstract.** Combretastatin A-4 (CA-4) is a naturally occurring microtubular-destabilising agent that possesses potent anti-tumour and anti-vascular properties both *in vitro* and *in vivo*. Clinical trials to date indicate that its water-soluble prodrug, combretastatin A-4 phosphate (CA-4P), is well tolerated at therapeutically useful doses. However, the stilbenoid structure of CA-4, consisting of two phenyl rings linked by an ethylene bridge, renders the compound readily susceptible to isomerisation from its biologically active *cis*-conformation to its more thermodynamically stable but inactive *trans*-isomer. To circumvent this problem, we synthesised a series of *cis*-restricted CA-4 analogues. Replacement of the ethylene bridge with a 1,4-diaryl-2-azetidinone ( $\beta$ -lactam) ring provided a rigid scaffold thus preventing *cis-trans* isomerisation. We previously documented that these tubulin-depolymerising  $\beta$ -lactam compounds potently induced cell cycle arrest and apoptosis in a variety of cancerous cell lines (including those displaying multidrug resistance) and *ex vivo* patient samples, whilst exerting only minimal toxicity to normal cells. The purpose of this study was to elucidate the effect of the  $\beta$ -lactam compounds on both tumour vascularisation and tumour cell migration, two critical elements that occur during the growth and metastatic progression of tumours. We established that two representative  $\beta$ -lactam compounds, CA-104 and CA-432, exerted both anti-endothelial effects [ $G_2/M$  arrest and apoptosis of primary human umbilical vein endothelial cells (HUVECs)] and anti-angiogenic effects [inhibition of HUVEC migration and differentiation and reduced vascular endothelial growth factor (VEGF) release from MDA-MB-231 breast adenocarcinoma cells]. In addition, we established that lead analogue, CA-432, abrogated the migration of MDA-MB-231 cells indicating an

anti-metastatic function for these compounds. In summary, our results to date collectively indicate that these *cis*-restricted  $\beta$ -lactam CA-4 analogues may prove to be useful alternatives to CA-4 in the treatment of cancer but with the added advantage of improved stability of the *cis*-isomer.

## Introduction

The combretastatins are a family of stilbenoids, some of which possess potent anti-tumour and anti-vascular properties. Originally isolated from the bark of the South African bush willow tree *Combretum caffrum*, these naturally occurring stilbenes consist of two phenyl rings, the A-ring and B-ring, linked by an ethylene bridge (1). The most extensively studied member of the family, combretastatin A-4 (CA-4) is well documented to function as a microtubule targeting agent (2). Microtubules are dynamic filaments composed of  $\alpha$ - and  $\beta$ -tubulin heterodimers that are key components of the mitotic spindle. Thus, agents that interfere with microtubule dynamics perturb mitotic cell division (3). CA-4 is a tubulin depolymerising agent that interacts with  $\beta$ -tubulin at or close to its colchicine binding site leading to the destabilisation of microtubules and preventing the formation of the mitotic spindle and hence results in mitotic arrest and subsequently cell death of tumour cells (2,4,5). Its water-soluble phosphate prodrug combretastatin A-4 phosphate (CA-4P) or fosbretabulin is readily cleaved *in vivo* by non-specific endogenous phosphatases to yield active CA-4 (6).

CA-4P induced rapid selective tumour vascular shutdown and tumour regression in both subcutaneous and orthotopic mouse tumour models at concentrations well below the maximum tolerated dose (7,8). It also reduced tumour blood flow in phase I clinical trials at well tolerated doses (9,10). Consequently, CA-4P has recently completed numerous phase II clinical trials including trials for the use of CA-4P in the treatment of advanced anaplastic thyroid cancer (11), CA-4P in combination with paclitaxel and/or carboplatin in the treatment of advanced solid tumours and combinations of CA-4P, anti-angiogenic bevacizumab, carboplatin and paclitaxel for chemotherapy naive non-small cell lung cancer (12). In addition, phase II trials for the treatment of neovascular age-related macular degeneration and polypoidal choroidal vasculopathy

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have also been undertaken (12). CA-4P in combination with bevacizumab is currently recruiting for phase II trials for reoccurring or persistent tumours of the ovarian epithelial, fallopian tube or peritoneal cavity and is about to enter phase I trials for the treatment of recurrent high grade gliomas (12).

While *in vitro* studies have demonstrated the anti-proliferative and cytotoxic effects of CA-4 on endothelial cells (7,13), the rapid vascular changes observed *in vivo* occur too early to be attributed to endothelial cell death. The rapid response of endothelial cells to CA-4 is thought to involve disruption to interphase microtubules triggering rapid remodelling of the actin cytoskeleton, assembly of actin stress fibres, actinomyosin contractility, formation of focal adhesions and disruption of cell-cell junctions (14).

Both the success and the limitations of CA-4 lie in its stilbene structure, illustrated in Fig. 1. Only the *cis*-configuration of CA-4 is biologically active (15). The spatial arrangement between its 3,4,5-trimethoxyphenyl A-ring and 3-hydroxy-4-methoxyphenyl B-ring are crucial to its functionality and ability to interact with tubulin (15-17). However, the *cis*-isomer is intrinsically unstable and readily isomerises to the more thermodynamically stable but inactive *trans*-configuration (15). *Cis-trans* isomerisation can be triggered by heat, light and protic media thus lowering the therapeutic efficacy of the agent.

We recently synthesised a series of CA-4 analogues that have been stabilised in their *cis*-configuration by the replacement of the usual ethylene bridge of CA-4 with a 1,4-diaryl-2-azetidinone ( $\beta$ -lactam) ring (18,19). The  $\beta$ -lactam ring provided a scaffold structure that retained a similar spatial arrangement between the two phenyl rings as the *cis*-conformation of CA-4. These compounds were either unsubstituted at position C-3 of the  $\beta$ -lactam ring or substituted with methyl groups (18) or aryl rings (19). Molecular docking studies indicated that representative compounds were capable of interacting with tubulin with similar positioning to CA-4. Several compounds inhibited tubulin polymerisation *in vitro* and demonstrated potent anti-mitotic potential in a selection of tumour cell lines derived of diverse origin including leukaemia, breast, non-small cell lung, colon, CNS, melanoma, ovarian, cervical, renal and prostate cancers (18-21).

Furthermore, low nanomolar concentrations of representative compounds caused tubulin depolymerisation, resulting in loss of cell viability mediated by G<sub>2</sub>M arrest and apoptosis of breast carcinoma ER-positive MCF-7 and ER-negative MDA-MB-231 cells whilst exerting no significant cytotoxicity to normal murine breast epithelial cells (IC<sub>50</sub> >10 mM) (19). Significantly, lead compound CA-432 also induced potent anti-tubulin, anti-proliferative and anti-mitotic effects in human promyelocytic leukaemia HL60 cells expressing multi-drug-resistant transporters P-glycoprotein or breast cancer resistance protein (BCRP) and in ovarian carcinoma A2780 cells also expressing P-glycoprotein. Molecular docking studies supported the notion that CA-432 was not a substrate for P-glycoprotein. Furthermore, CA-432 induced apoptosis in *ex vivo* samples from chronic myeloid leukaemia (CML) patients including those displaying resistance to imatinib mesylate, the frontline treatment for CML (20).

While our studies to date demonstrate the cytotoxic effects of these *cis*-restricted  $\beta$ -lactam CA-4 analogues on a variety of tumour cells derived from both the haematopoietic system

and from solid tumours, we have not investigated their effects on endothelial cells. Tumour vascularisation is essential for tumour growth and metastases. Therefore, the purpose of this study was to examine the anti-vascular, anti-angiogenic and anti-metastatic properties of these compounds through a series of *in vitro* tests. We selected two lead analogues, CA-104 and CA-432, for this purpose (Fig. 1). CA-104 was unsubstituted at position C-3 of the  $\beta$ -lactam ring (18), while, CA-432 was substituted with a 4-(3-hydroxy-4-methoxyaryl ring) (19). We established that both compounds displayed anti-endothelial properties *in vitro*. They induced tubulin depolymerisation in primary human umbilical vein endothelial cells (HUVECs). This effect was associated with a loss in endothelial cell viability mediated by G<sub>2</sub>M arrest and apoptosis. We also demonstrated both direct and indirect anti-angiogenic events. Both compounds prevented migration and *in vitro* capillary tubule formation by HUVECs whilst lead compound, CA-432, reduced the release of VEGF from breast adenocarcinoma MDA-MB-231 cells. Finally, we established that CA-432 abrogated migration of these highly metastatic MDA-MB-231 cells. Of note, these anti-angiogenic and anti-metastatic events preceded any cytotoxic effects attributed to the  $\beta$ -lactam analogues.

These findings indicate a novel function for these  $\beta$ -lactam CA-4 analogues. Our findings collectively demonstrated that these rigid *cis*-restricted analogues exhibited anti-tumour, anti-vascular, anti-angiogenic and anti-metastatic properties with minimal toxicity to normal cells. Replacement of the ethylene bridge with a  $\beta$ -lactam ring yielded compounds that retained the *in vitro* functionality of CA-4 but with the additional advantage of conformational stability.

## Materials and methods

Unless otherwise stated, chemicals were obtained from Sigma-Aldrich (Poole, Dorset, UK) and tissue culture vessels were sourced from Greiner Bio-One GmbH (Frickenhausen, Germany).

**Cell culture.** Pooled primary human umbilical vein endothelial cells (HUVECs) and their associated reagents were all obtained from Cascade Biologics (Invitrogen, Carlsbad, CA, USA). HUVECs were maintained between passages 1-4 in Medium 200 supplemented with LSGS (low serum growth factor supplement) and utilised for experiments at passage 4. Human breast adenocarcinoma MDA-MB-231 cells (ATCC, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) enhanced with GlutaMAX-I and supplemented with 10% foetal bovine serum, 50 U/ml penicillin and 50  $\mu$ g/ml streptomycin (all purchased from Gibco, Invitrogen). Cells were maintained in a humidified incubator at 37°C in 5% CO<sub>2</sub> and were subcultured by trypsinisation upon reaching 70-80% confluency.

**Reagents.** Two representative *cis*-restricted  $\beta$ -lactam combretastatin A-4 analogues were used in this study. Their structures are illustrated in Fig. 1. Analogue 4-(3-hydroxy-4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (CA-104) was synthesised as previously described in Carr *et al.* (18) where this compound was referred to as compound 12d.

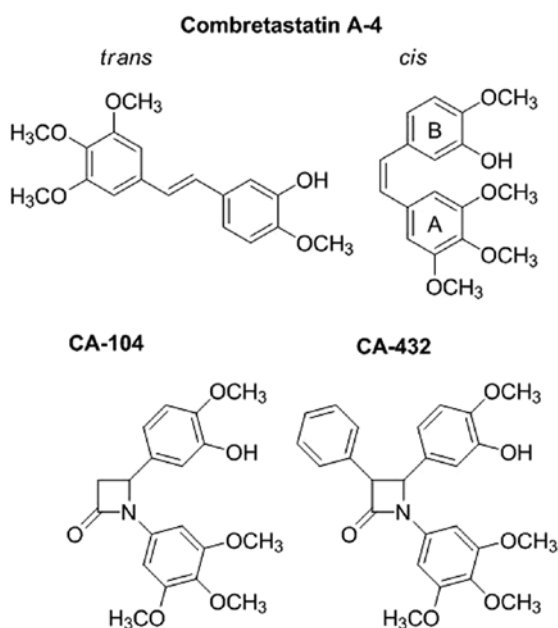


Figure 1. Chemical structures of combretastatin A-4 and its novel *cis*-restricted  $\beta$ -lactam analogues, CA-104 and CA-432.

Analogue 4-(3-hydroxy-4-methoxyphenyl)-3-phenyl-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (CA-432) was synthesised as described in O'Boyle *et al* (19) where it was referred to as compound 35. The analogues were dissolved in ethanol and stored in the dark at  $-20^{\circ}\text{C}$ . Human recombinant VEGF165 (R&D Systems Inc., Minneapolis, MN, USA) was reconstituted to  $1\text{ }\mu\text{g/ml}$  in 0.1% (w/v) bovine serum albumin (BSA) and also stored at  $-20^{\circ}\text{C}$ .

**Cell viability assays.** HUVECs (20,000 cells/well) or MDA-MB-231 cells (12,000 cells/well) were grown on 96-well plates. The cells were treated (24 h post-seeding) with a range of concentrations of the analogues for up to 72 h. Cellular metabolic activity and hence cell viability was monitored using AlamarBlue™ dye (BioSource, Invitrogen) which was added to each well [final concentration of 10% (v/v)] and incubated at  $37^{\circ}\text{C}$ . The change from an oxidized indigo blue non-fluorescent state to a fluorescent pink state in the reduced environment of living cells was measured at an excitation wavelength of 544 nm and an emission wavelength of 590 nm using a SpectraMax Gemini spectrofluorometric microtiter well plate reader (Molecular Devices, Sunnyvale, CA, USA). The results were expressed as the percentage of cell viability relative to the vehicle-treated control cells (100%). Dose-response curves were plotted, and  $\text{IC}_{50}$  values (the concentration of compound resulting in 50% reduction in cell viability) were obtained using Prism GraphPad 4.

**Determination of DNA content.** Following treatment, HUVECs were harvested by centrifugation at  $800 \times g$  for 10 min. Cell pellets were resuspended in  $200\text{ }\mu\text{l}$  PBS and fixed by a drop-wise addition of 2 ml of ice-cold 70% (v/v) ethanol/PBS while gently vortexing. Following overnight fixation at  $-20^{\circ}\text{C}$  the cells were again centrifuged to remove the ethanol and resuspended in PBS supplemented with 0.5 mg/ml RNase

A and 0.15 mg/ml propidium iodide. Cells were incubated in the dark at  $37^{\circ}\text{C}$  for 30 min. The fluorescence emitted from the propidium iodide was measured on a linear scale using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Data collections (10,000 events per sample) were gated to exclude cell debris and cell aggregates. Fluorescence was proportional to the amount of DNA present in each entity and therefore indicated the stage of the cell cycle it was in. Cells in  $G_0/G_1$  were diploid (2N DNA content), cells in  $G_2/M$  were tetraploid (4N DNA content), cells in the S phase had DNA contents between 2N and 4N, while apoptotic cells were hypoploid and contained  $<2\text{N}$  DNA. All data were recorded and analysed using the CellQuest software (Becton Dickinson).

**Microtubule staining.** HUVECs (60,000 cells/chamber) were cultured on BD Falcon™ 4-chamber glass slides (BD Biosciences, Bedford, MA, USA) for 24 h. Following treatment for 16 h, the cells were fixed in 100% methanol at  $-20^{\circ}\text{C}$  and the microtubular network was detected by indirect immunofluorescence as previously described (22). Briefly, the slides were sequentially incubated in a blocking solution [5% (w/v) BSA/0.1% (v/v) Triton X-100/PBS], monoclonal anti- $\alpha$ -tubulin antibodies (Merck Biosciences, Nottingham, UK), fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse antibodies (DakoCytomation, Glostrup, Denmark) and finally 0.2  $\mu\text{g/ml}$  propidium iodide (to stain DNA). An anti-quenching solution (2  $\mu\text{g/ml}$  *p*-phenylenediamine in 50:50 glycerol:PBS) was applied to the surface of the slides and coverslips were mounted. The organisation of the microtubule network (green) and the cellular DNA (red) was visualised under a  $\times 60$  oil-immersion lens using an Olympus IX81 fluorescent microscope (Olympus Corporation, Tokyo, Japan).

**Endothelial cell migration.** Costar® 8  $\mu\text{M}$ -pore Transwell inserts (Corning Incorporated, Corning, NY, USA) were coated overnight at  $4^{\circ}\text{C}$  with 10  $\mu\text{g/ml}$  human fibronectin. HUVECs (10,000 cells in  $100\text{ }\mu\text{l}$  medium) were seeded onto the transwell inserts, placed in 24-well plates containing 0.6 ml medium and incubated for 1 h. HUVEC migration was stimulated by addition of 10 ng/ml VEGF to the lower well. Vehicle or the indicated analogues were also added to the lower wells. After a period of 6 h the upper surfaces of the inserts were swabbed to remove non-migrated cells. Filters were incubated overnight in a solution containing 0.5% toluidine blue O and 0.5% sodium tetraborate to stain the migrated cells. Following solubilisation of the cells using 0.2% (w/v) SDS in 20 mM Tris-HCl, pH 7.7, the absorbance was determined at 650 nm in a spectrophotometer (Molecular Devices).

**In vitro tubule formation.** HUVECs ( $1.5 \times 10^6$  cells/well), were incubated on BD Biocoat™ Matrigel™-coated 6-well plates (BD Biosciences) for 6 h in the presence of vehicle or the indicated compounds. The ability of the HUVECs to spontaneously form capillary-like tubules on the Matrigel (basement membrane matrix preparation) was photographed under a Nikon Eclipse TE300 phase contrast microscope (Nikon Instruments Inc., Melville, NY, USA) at a magnification of  $\times 100$ .

**Detection of VEGF release.** MDA-MB-231 cells (60,000 cells/ $\text{cm}^2$ ) were grown for 24 h, then washed in PBS and incubated in

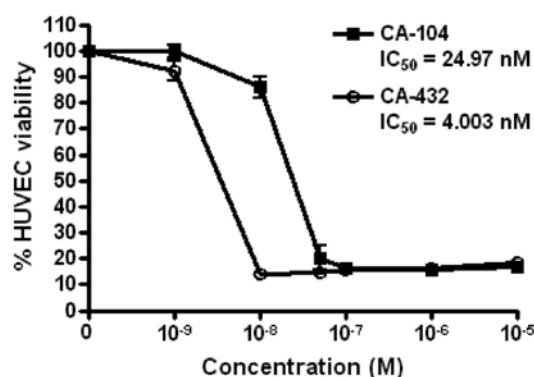


Figure 2. Treatment with *cis*-restricted  $\beta$ -lactam combretastatin A-4 analogues, CA-104 and CA-432, reduced viability of primary endothelial cells. HUVECs grown on 96-well plates were treated in triplicate with vehicle alone [1% (v/v) ethanol] or a range of concentrations of CA-104 or CA-432 for 72 h. The cells were then incubated in 10% (v/v) AlamarBlue™ and its reduction to a fluorescent state was measured at excitation 544 nm and emission 590 nm using a multi-well fluorimeter. The results were expressed as the percentage of cell viability relative to vehicle-treated control cells (100%). Values represent the mean  $\pm$  SEM for three separate experiments. IC<sub>50</sub> values (concentration of the compound resulting in 50% reduction in cell viability) were obtained using Prism GraphPad 4 software.

a low serum environment [DMEM supplemented with 1% (v/v) FBS] in the presence or absence of lead analogue CA-432 for 6 h. The conditioned medium was then centrifuged at 400  $\times$  g and the supernatants were collected while the cells were lysed in sample buffer [62.5 mM Tris (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol], sonicated briefly and denatured by boiling for 3 min. The concentration of VEGF in the supernatants was determined by ELISA using a Quantikine Human VEGF Immunoassay kit (R&D Systems Inc.) in accordance with manufacturer's handbook. The colour intensity (proportional to the concentration of VEGF) was determined by measuring the absorbance in a SpectraMax 340PC spectrophotometer (Molecular Devices) at wavelength 450 nm with correction at 540 nm. The readings were normalised to account for the total protein content of the cells which was determined from an aliquot of cell lysate using a BCA protein assay kit (Pierce, Thermo Fisher Scientific Inc., Rockford, IL, USA) in accordance with the manufacturer's instructions. Normalised VEGF-release was then expressed as a percentage of release from vehicle-treated control cells (100%).

**Tumour cell migration.** MDA-MB-231 cells were seeded at a density of 25,000 cells/well onto 24-well Falcon migration inserts (8- $\mu$ m pore size) (BD Biosciences) in serum-free medium. Inserts were placed into Falcon companion plates containing DMEM supplemented with 20% FBS in the presence or absence of CA-432. Following 6 h of incubation, the upper surfaces of the inserts were swabbed to remove non-migrated cells. Migrated cells on the underside of the membrane were fixed in methanol, stained with Mayer's hematoxylin, dehydrated in methanol and mounted on a glass slide. The cells in 5 fields at  $\times 10$  magnification were counted.

**Statistical analysis.** Results were presented as mean  $\pm$  SEM. Statistical analysis of the experimental data was performed

using the computer program Prism GraphPad 4. P-values were determined using a paired two-tailed Student's t-test. A value of  $P < 0.05$  was considered to be statistically significant.

## Results

*cis*-restricted  $\beta$ -lactam combretastatin A-4 analogues, CA-104 and CA-432, reduce endothelial cell viability. To determine the effect of *cis*-restricted  $\beta$ -lactam CA-4 analogues on endothelial cell viability, we tested a range of concentrations of the representative analogues, CA-104 and CA-432, on primary HUVECs for 72 h. We established that both agents led to a reduction in the metabolic activity and hence viability of HUVECs, with respective IC<sub>50</sub> values for CA-104 and CA-432 of 24.97 and 4.00 nM (Fig. 2). From these IC<sub>50</sub> values, it was deduced that analogue CA-432 was a more potent inhibitor of endothelial cell viability than CA-104. The concentrations of CA-104 and CA-432 used for the rest of this study were chosen to reflect the values obtained from this cell viability assay.

*CA-104 and CA-432 induce G<sub>2</sub>/M arrest and apoptosis in endothelial cells.* Cell cycle profiles were examined to define the mechanisms underlying the reduction in endothelial cell viability following exposure to CA-104 and CA-432. HUVECs were treated with vehicle [0.5% (v/v) ethanol], CA-104 (0.01–1  $\mu$ M) or CA-432 (1–100 nM) for 24 h. Subsequently, their DNA was fluorescently stained with propidium iodide and analysed by flow cytometry. Examination of their DNA profiles indicated that both CA-104 and CA-432 induced a dose-dependent increase in the percentage of HUVECs with 4N DNA content compared to vehicle-treated cells. Approximately 20% of HUVECs treated with the vehicle alone displayed tetraploid DNA contents compared to 38.1 $\pm$ 1.5% ( $P=0.0063$ ) or 37.0 $\pm$ 1.8% ( $P=0.0027$ ) of the HUVECs treated with CA-104 (100 nM) or CA-432 (50 nM) respectively (Fig. 3A and B). These statistically significant increases in 4N DNA content indicated that the compounds induced arrest in the G<sub>2</sub>/M phase of the cell cycle.

G<sub>2</sub>/M arrest was accompanied by significant increases in the number of entities presenting with hypodiploid (<2N) quantities of DNA as indicated by a sub G<sub>0</sub>/G<sub>1</sub> peak on the DNA profiles. While only approximately 5% of vehicle-treated HUVECs were found to be hypodiploid, treatment with CA-104 (100 nM) or CA-432 (50 nM) resulted in an increase in the amount of hypodiploid cells to 35.0 $\pm$ 2.6% ( $P=0.0064$ ) or 31.4 $\pm$ 1.3% ( $P=0.0019$ ) respectively (Fig. 3C and D). These statistically significant increases in hypodiploid cells represented an increase in the levels of apoptosis. Cell cycle analysis illustrated that both CA-104 and CA-432 inhibited proliferation and survival of endothelial cells through the induction of G<sub>2</sub>/M arrest and apoptosis.

*CA-104 and CA-432 cause destabilisation of the microtubule network in endothelial cells.* We next established the effect of CA-104 and CA-432 on the gross morphology of the microtubular network in endothelial cells. HUVECs, grown on glass 4-chamber slides, were treated for 16 h with vehicle [0.5% (v/v) ethanol], CA-104 (100 nM) or CA-432 (50 nM). Immunofluorescent staining was used to detect morphological changes in the tubulin cytoskeleton such as alterations in

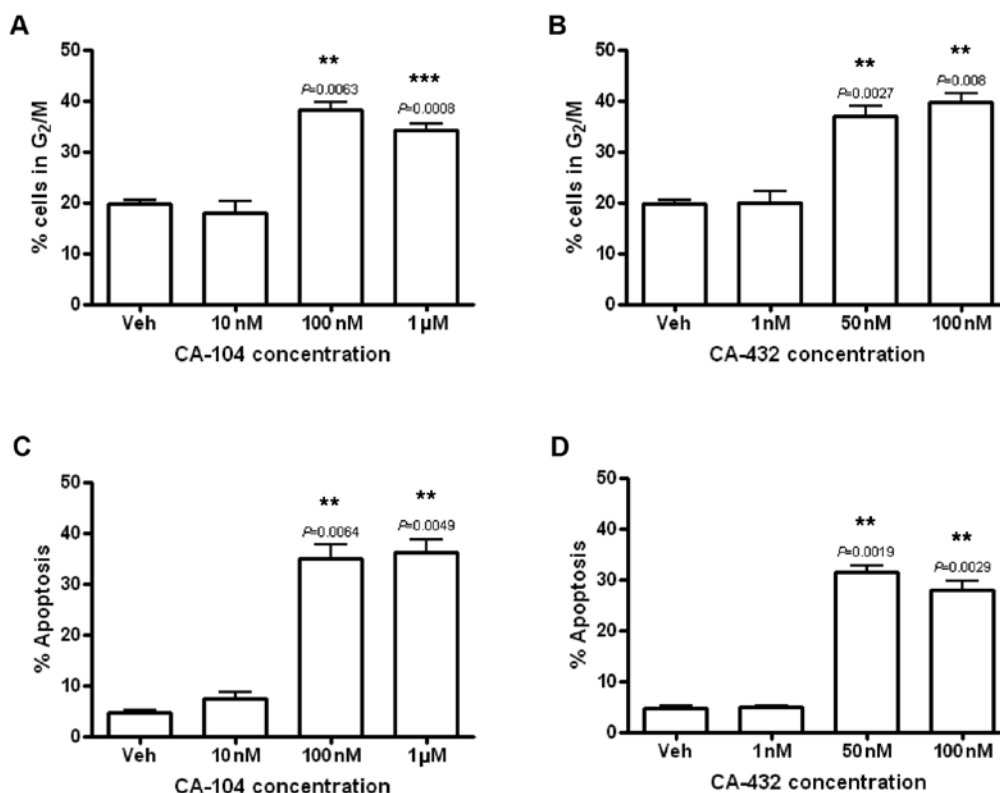


Figure 3. CA-104 and CA-432 induce G<sub>2</sub>/M arrest and apoptosis in primary endothelial cells. HUVECs were treated with vehicle (Veh) [0.5% (v/v) ethanol] or the indicated concentrations of CA-104 or CA-432 for 24 h, fixed in ethanol, stained with propidium iodide and their DNA content assessed by flow cytometry. Analysis of data was performed using the CellQuest software. Fluorescence was proportional to the amount of DNA present in each entity and therefore indicated the phase of the cell cycle. Cells in the subG<sub>0</sub>/G<sub>1</sub> phase (<2N DNA) were deemed apoptotic, while cells with 4N quantities of DNA were considered to be in the G<sub>2</sub>/M phase of the cell cycle. Values represent the mean  $\pm$  SEM for three independent experiments. A P-value <0.05 was considered to be statically significant (\*P<0.05, \*\*P<0.01, \*\*\*P<0.001).

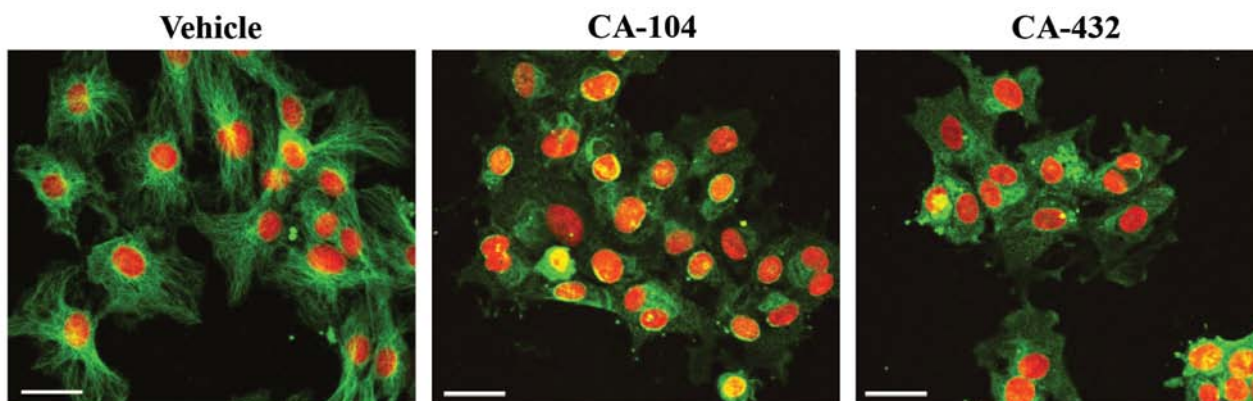


Figure 4. CA-104 and CA-432 disrupt the organisation of the microtubular network in endothelial cells. HUVECs grown on 4-chamber glass slides were treated with vehicle [0.5% (v/v) ethanol], CA-104 (100 nM) or CA-432 (50 nM) for 16 h. The cells were then fixed in methanol, incubated with monoclonal anti- $\alpha$ -tubulin antibodies, followed by FITC-conjugated anti-mouse antibodies and then briefly stained with propidium iodide. The organisation of the microtubule network (green) and the cellular DNA (red) was visualised using a fluorescence microscope at a magnification of x600 (scale bar, 30  $\mu$ m). Images illustrated are representative of three independent experiments.

organisation and arrangement. In normal cells, the microtubule network is organised into cytoplasmic tubulin filaments radiating from a central point to the periphery. HUVECs treated with vehicle alone (0.5% ethanol) displayed this typical tubulin morphology (Fig. 4). Treatment of cells with tubulin polymerising agents (for example, paclitaxel) results in a highly concentrated accumulation of filaments into dense

peripheral bundles indicative of microtubule stabilisation. In contrast, exposure of cells to tubulin depolymerising agents (such as vincristine) results in diffuse tubule staining with no definition of structure caused by microtubule disassembly. These typical morphological changes associated with tubulin depolymerising agents were observed when HUVECs were treated with either CA-104 or CA-432 (Fig. 4).

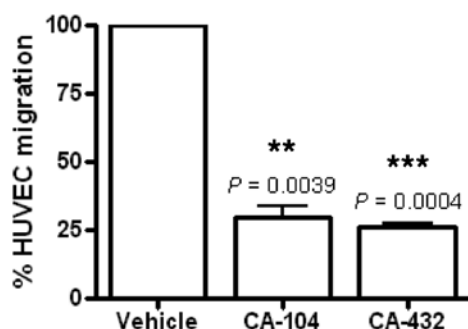


Figure 5. CA-104 and CA-432 significantly reduce VEGF-stimulated endothelial cell migration. HUVECs were seeded onto fibronectin-coated Transwell inserts and placed in medium-containing companion plates. HUVEC migration was stimulated by the addition of 10 ng/ml VEGF to the lower well in the presence of vehicle [0.5% (v/v) ethanol], CA-104 (100 nM) or CA-432 (50 nM). After 6 h, migrated cells were stained with 0.5% toluidine blue O and 0.5% sodium tetraborate and quantified as absorbance at 650 nm. The results were expressed as the percentage of migrated cells with vehicle-treated control cells representing 100% migration and displayed as mean  $\pm$  SEM of three experiments each carried out in duplicate. A P-value  $<0.05$  was considered to be statically significant (\* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ ).

**CA-104 and CA-432 reduce endothelial cell migration.** Having established that *cis*-restricted  $\beta$ -lactam combretastatin A-4 derivatives altered endothelial cell function, we then examined their implications in angiogenic processes *in vitro*. Firstly, their effect on HUVEC migration was evaluated using a modified Transwell migration assay. This chemotactic model representative of tumour-induced endothelial cell migration (23) consisted of an upper and a lower chamber separated by a membrane. Migration of HUVECs from the upper to the lower chamber was stimulated by the addition of VEGF to the lower chamber. The effect of CA-104 (100 nM) and CA-432 (50 nM) on this migration was determined by their addition along with VEGF into the lower chamber. Migration was expressed as a percentage of migration by vehicle-treated cells (100%). Incubation for 6 h with CA-104 or CA-432 significantly inhibited VEGF-stimulated HUVEC migration by  $70.3\pm4.4\%$  ( $P=0.0039$ ) and  $74.0\pm1.5\%$  ( $P=0.0004$ ) respectively (Fig. 5). This effect preceded endothelial cell toxicity since no loss of HUVEC viability was evident following a 6-h treatment with CA-104 or CA-432 (data not shown).

**CA-104 and CA-432 inhibit endothelial cell differentiation.** To further investigate the anti-angiogenic potential of CA-104 and CA-432, an endothelial tube formation assay was performed. The spontaneous formation of capillary-like structures by endothelial cells, when incubated on an extracellular basement membrane matrix preparation known as Matrigel, is a standard *in vitro* angiogenesis test (24). This process requires cell-matrix interaction, inter-cellular communication as well as cell motility and differentiation. HUVECs were seeded onto Matrigel in the presence of vehicle (0.5% ethanol), CA-104 (100 nM) or CA-432 (50 nM) for 6 h. The alignment of the cells on the 3D-Matrigel was assessed using a phase contrast microscope (Fig. 6). Vehicle-treated cells underwent alignment into capillary-like structures while treatment with either CA-104 or CA-432 reduced tubule formation. Again, as observed during the endothelial cell migration assay, inhibition of *in vitro* tubule formation preceded any cytotoxic effects attributed to CA-104 or CA-432.

**CA-432 reduces VEGF release from breast carcinoma cells.** The release of VEGF from tumour cells plays a key role in the stimulation of angiogenesis and promotion of endothelial cell survival (25). Therefore, we next investigated the effect of lead CA-4 analogue CA-432 (50 nM) on the release of VEGF from human breast adenocarcinoma MDA-MB-231 cells. VEGF release was stimulated by incubating the cells in a low serum environment. We found that the CA-432 (50 nM) reduced the release of VEGF from MDA-MB-231 cells to  $80.9\pm1.7\%$  ( $P=0.0080$ ) of that released from the vehicle-treated control cells (Fig. 7). This event preceded any cytotoxic effects due to CA-432, since at 6 h post-treatment, no loss in MDA-MB-231 cell viability was detected (data not shown). This finding suggested an indirect anti-angiogenic function for CA-432 through targeting of tumour cells.

**CA-432 prevents migration of breast carcinoma MDA-MB-231 cells.** The migration of tumour cells from the primary site is a critical step during tumour metastasis (26). We previously demonstrated that MDA-MB-231 cells display good migratory capabilities (27). Therefore, the migration of MDA-MB-231 cells across Transwell filters in the presence of vehicle [0.5% (v/v) ethanol] or CA-432 (50 nM) was compared. Migration was expressed as a percentage of the migration of vehicle-

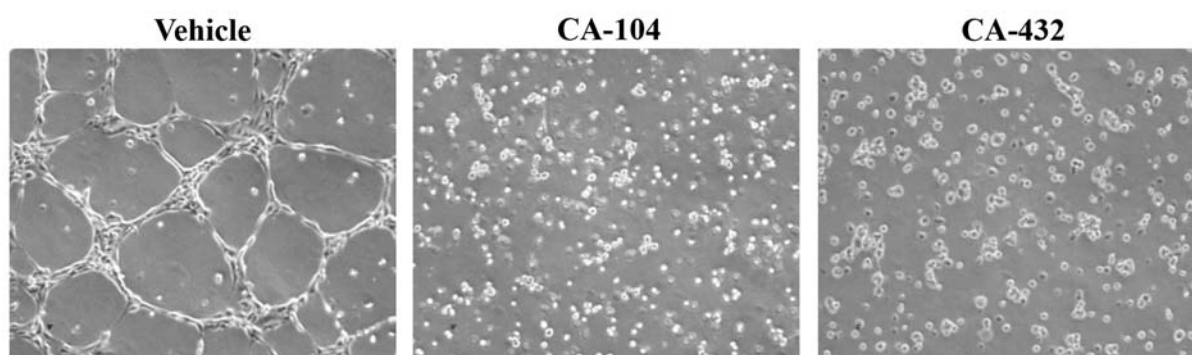


Figure 6. CA-104 and CA-432 inhibit the spontaneous *in vitro* tubule formation of endothelial cells. HUVECs were grown on Matrigel-coated 6-well plates for 6 h in the presence of vehicle [0.5% (v/v) ethanol], CA-104 (100 nM) or CA-432 (50 nM). The ability of the HUVECs to form capillary-like tubules on Matrigel was demonstrated using a phase contrast microscope at a total magnification of  $\times 100$ . Images shown are representative of three independent experiments.

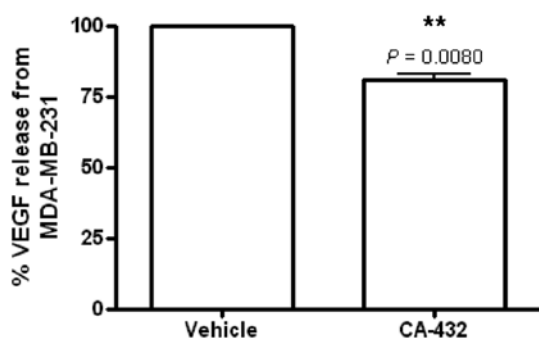


Figure 7. CA-432 reduces VEGF-release from human breast adenocarcinoma MDA-MB-231 cells. MDA-MB-231 cells were treated with vehicle [0.5% (v/v) ethanol] or CA-432 (50 nM) for 6 h in a low serum environment [DMEM supplemented with 1% (v/v) FBS]. The conditioned medium was then centrifuged at 400 x g and the supernatants were collected. The concentration of VEGF in the supernatants was determined by ELISA. The results were normalised for cellular protein concentration and expressed as the percentage of VEGF released in the CA-432-treated cells compared to release from vehicle-treated control cells (100%). Data represents the mean  $\pm$  SEM of three experiments each carried out in duplicate. A value of  $P < 0.05$  was considered to be statistically significant (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

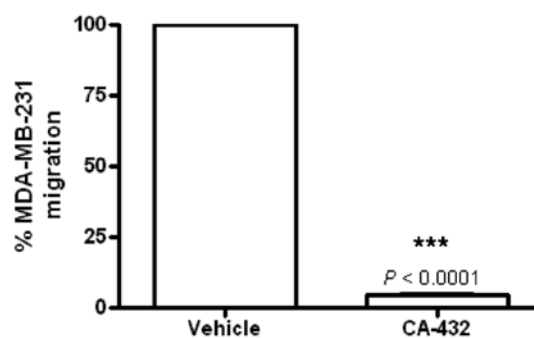


Figure 8. CA-432 inhibits the migration of human breast adenocarcinoma MDA-MB-231 cells. MDA-MB-231 cells were seeded onto Transwell inserts in serum-free medium and placed into companion plates containing 20% (v/v) FBS/DMEM and either vehicle [0.5% (v/v) ethanol] or CA-432 (50 nM) for 6 h. The upper surfaces of the inserts were swabbed to remove non-migrated cells. Migrated cells on the underside of the membrane were fixed in methanol, stained with Mayer's hematoxylin, dehydrated in methanol and mounted on a glass slide. The number of cells in 5 fields at x10 magnification was counted. The results were expressed as the percentage of migrated cells relative to vehicle-treated control cells (100%) and represent the mean  $\pm$  SEM of three independent experiments. A value of  $P < 0.05$  was considered to be statistically significant (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

treated cells (100%). Incubation for 6 h with CA-432 prevented MDA-MB-231 cell migration by  $95.7 \pm 0.7\%$  ( $P < 0.0001$ ) (Fig. 8). This event preceded any cytotoxic events, since following a 6 h treatment with CA-432, no loss in MDA-MB-231 cell viability was detected (data not shown).

## Discussion

Both tumour vascularisation and the migration of tumour cells are key events during the growth and metastatic progression of tumours. Therefore, agents that disrupt these events could potentially prove useful as anti-cancer therapies. Anti-vascular therapies can be divided into two main strategic subtypes: vascular-disrupting therapies that target the existing tumour vasculature and anti-angiogenic therapies that prevent the *de novo* synthesis of tumour blood vessels. CA-4 and its water soluble prodrug CA-4P fall into the first category (7) while microtubule-targeting agents such as the tubulin-stabilising taxanes (28) and the tubulin-destabilising vinca alkaloids (29) form the latter group. However, between these two subtypes there are overlaps, as depending on the dosing strategies, anti-angiogenic agents can also induce vascular disruption and the converse is also true (30). Therefore, the *in vitro* assays used to detect both strategies are similar. Anti-vascular therapies maximise their effects on the tumour vasculature while minimising damage to the systemic vasculature system by exploiting the fundamental differences between the normal mature vasculature and the immature tumour vasculature which tends to be disorganised, leaky and poorly associated with perivascular cells (31).

Endothelial cells are desirable targets for cancer therapies due to accessibility and genetic stability and unlike tumour cells, they do not readily acquire drug resistance (32). Hence, anti-vascular drugs can cause tumour regression even in drug-resistant tumours. For example, anti-tumour activity was reported with docetaxel in a xenograft tumour that had been established by inoculating mice with docetaxel-resistant

cancer cells, an effect that was attributed to the anti-angiogenic actions of docetaxel (33). After tumour vascularisation, several key events are critical for progression of a tumour to a metastatic phenotype. These events include migration of tumour cells from the primary site into the blood/lymph system followed by their invasion into a different site to begin the process of angiogenesis and the growth of a secondary tumour (26). Therefore, agents which target either the tumour vasculature or any of the steps that occur during tumour metastasis are potentially useful anticancer therapies, with agents that target both events the most desirable.

Naturally occurring stilbene, CA-4, is a potent inhibitor of tubulin polymerisation that displays potent anti-mitotic activities in both cancer and endothelial cells (2,4,5,7). *In vivo*, its water-soluble prodrug, CA-4P, induces a rapid and selective shutdown of tumour blood flow and subsequently tumour regression (7,8) and thus the compound is currently undergoing clinical trials as a vascular-disrupting agent. The therapeutic efficacy of CA-4 is limited by its intrinsic instability rendering it readily isomerisable to its inactive *trans*-conformation (15-17). Therefore, non-isomerisable CA-4 analogues have been developed to provide more stable alternatives to CA-4. Approaches to prevent isomerisation have included the replacement of the *cis*-double bond in CA-4 with heterocyclic rings such as tetrazole, imidazole and benzoxepin (34-36).

We previously developed and patented a series of CA-4 analogues that are conformationally *cis*-restricted. Replacement of the ethylene bridge of CA-4 with a  $\beta$ -lactam (2-azetidinone) ring provided a rigid scaffold that prevented *cis-trans* isomerisation and maintained a similar spatial arrangement between the two phenol rings as the *cis*-conformation of CA-4 (18). We have already established that some of these compounds displayed potent anti-proliferative activity in several cancer cell lines and *ex vivo* patient samples, including those displaying multidrug resistance (18-21). However, the anti-vascular and anti-metastatic properties of these *cis*-restricted analogues remained to be elucidated. The purpose



of this study was to perform a series of *in vitro* experiments to investigate the anti-vascular effects of the compounds directly on primary HUVECs and indirectly on the release of pro-angiogenic VEGF from tumour cells. Finally, we assessed the effect of the analogues on one of the critical events involved in metastasis, namely, tumour cell migration.

We selected two of the lead *cis*-restricted CA-4 analogues from our previous studies as representative compounds, CA-104 and its derivative CA-432, which contained an aryl ring substituent at position C-3 of the  $\beta$ -lactam ring (Fig. 1). We determined that both analogues potently inhibited HUVEC proliferation with  $IC_{50}$  values of 24.9 nM and 4 nM for CA-104 and CA-432, respectively. These values were either similar or lower than those we previously observed in tumour cells such as breast carcinoma MCF-7 and MDA-MB-231 cells, promyelocytic leukaemia HL60 cells, chronic myeloid leukaemia K562 cells and ovarian carcinoma A2780 cells, where  $IC_{50}$  values ranged between 17 and 60 nM for CA-104 and 7.5 and 28 nM for CA-432 (18-20). The magnitude of response obtained with CA-432 was similar to those reported for CA-4 and CA-4P which also demonstrated anti-proliferative effects on endothelial cells at lower concentrations than tumour cells (7,37). As we previously reported that exposure of normal breast epithelial cells to CA-432 induced only a minimal amount of cytotoxicity with an  $IC_{50}$  value of greater than 10 mM (19), it is interesting that the compounds had such a potent effect on non-cancerous endothelial cells. This phenomenon has been reported with CA-4P (38) and numerous other microtubule-targeting agents (22,39) and is postulated to be attributable to a variety of mechanisms including enhanced uptake mechanisms in endothelial cells (40) or differences in endothelial cell tubulin composition, its post-translational modifications or its microtubule-associated proteins (41,42).

The loss in endothelial cell viability following exposure to CA-104 and CA-432 was mediated by significant levels of  $G_2M$  arrest and apoptosis which was accompanied by depolymerisation of the microtubular networks in HUVECs. Tubulin depolymerisation and  $G_2M$  arrest are typical responses observed in tumour cells after exposure to the  $\beta$ -lactam analogues (19,20) and also parallels the effects observed with CA-4/CA-4P in endothelial cells (43). Studies suggest that the type of cell death culminating from CA-4(P)-induced  $G_2M$  arrest varies depending on the conditions and the type of cell. Modes of cytotoxicity reported include apoptosis (21,44), mitotic catastrophe (45) and autophagy (46,47). Recently we found that CA-432 induced mitotic catastrophe in breast carcinoma cells (48) and autophagy in adenocarcinoma-derived colon cancer cells (47). Mitotic arrest prevents the supply of endothelial cells required for angiogenesis and cytotoxicity leads to the destruction of existing tumour blood vessels, indicating a novel anti-vascular function for the  $\beta$ -lactam CA-4 derivatives.

Apart from the proliferation and survival of endothelial cells, angiogenesis requires several other critical events including the migration of endothelial cells into the extracellular matrix and their differentiation into new capillary networks (31,49). In addition, stimulation of these angiogenic events requires pro-angiogenic or vascular-survival signals such as the release of VEGF by tumour cells (50). Interference in these processes has been reported in endothelial and

tumour cells treated with MTAs such as paclitaxel, docetaxel, vinblastine and vincristine (28,29,51,52). We established that  $\beta$ -lactam CA-4 analogues were capable of directly interfering with angiogenic events since they completely abrogated VEGF-stimulated HUVEC migration and their spontaneous differentiation into capillary-like structures when grown on Matrigel, both standard tests for angiogenesis *in vitro*. Furthermore, the derivatives may also indirectly influence angiogenesis, as CA-432 significantly reduced the release of VEGF from metastatic breast carcinoma MDA-MB-231 cells by almost 20%. This is an interesting effect as in addition to its angiogenic activity, VEGF can protect endothelial cells from apoptosis by stimulating the activation of survival pathways such as phosphoinositol-3-kinase (PI3 kinase) and upregulation of anti-apoptotic Bcl-2 and in particular survivin which is an important microtubule-binding apoptosis inhibitor involved in mitotic spindle regulation (53-56).

These anti-angiogenic findings provided additional support to the anti-vascular profile of these CA-4 derivatives and it is interesting to note that these anti-angiogenic responses occurred at time points that preceded the onset of cytotoxicity indicating that the anti-vascular phenotype of these compounds cannot solely be attributed to endothelial cell death. This effect was also observed with CA-4P which can induce complete vascular shutdown within 20 min of drug exposure *in vivo* (57). As drug-induced effects on endothelial cell proliferation or cytotoxicity occur too slowly to account for this rapid response, it has been postulated that morphological and functional changes in endothelial cells are more likely to cause tumour vascular collapse (30,58). Such changes may include rounding-up of cells due to disruption of interphase microtubules leading to rapid remodelling of the actin cytoskeleton, assembly of actin stress fibres, actinomyosin contractility, formation of focal adhesions, disruption of cell-cell junctions, including those involving N- and VE-cadherin and an increase in monolayer permeability of macromolecules (14,59,60). It would therefore, be interesting in the future to investigate some of these mechanisms to extend our study of the  $\beta$ -lactam CA-4 analogues. It is important to note that our initial observations which illustrated inhibition of endothelial cell proliferation and cytotoxicity are still important therapeutically, since these mechanisms can play a role in the prevention of tumour re-growth in chronic dosing schedules.

Finally, we investigated the effect of CA-432 on one of the key events that occurs during metastasis, tumour cell migration. We chose breast adenocarcinoma MDA-MB-231 cells as a model since we previously found that these cells displayed good migratory potential (27). We determined that CA-432 abrogated MDA-MB-231 migration from a low to a high serum environment. We previously showed that CA-432 has an  $IC_{50}$  value of  $28.8 \pm 0.02$  nM in these cells (19), however, similarly to endothelial cell migration, interference with tumour cell migration preceded cytotoxicity.

In summary, these findings demonstrated novel anti-vascular and anti-metastatic functions for our *cis*-restricted  $\beta$ -lactam combretastatin A-4 analogues, CA-104 and CA-432. Collectively, this report along with our previous studies indicate that these  $\beta$ -lactam CA-4 analogues induced anti-tumour, anti-vascular and anti-metastatic events with minimal toxicity to normal quiescent cells *in vitro*. These events are analogous



with the functions of CA-4 *in vitro*. Therefore, these analogues should now be considered for further *in vivo* investigation of their anti-tumour and anti-vascular capabilities to further evaluate their potential as useful alternatives to the intrinsically unstable CA-4.

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