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Novel cis-restricted β-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 (β-lactam) ring provided a rigid scaffold thus preventing cis-trans isomerisation. We previously documented that these tubulin-depolymerising β-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 β-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 β-lactam compounds, CA-104 and CA-432, exerted both anti-endothelial effects [G2/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 β-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 α- and β-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 β-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 fosfobretulin 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.
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 (β-lactam) ring (18,19). The β-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 β-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 G2/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 (IC50 >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 multidrug-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 β-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 β-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 G2/M arrest and apoptosis. We also demonstrated both direct and indirect anti-angiogenic events. Both compounds prevented migration and in vitro capillary tube 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 β-lactam analogues.

These findings indicate a novel function for these β-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 β-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 (Fritzenhausen, 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 μg/ml streptomycin (all purchased from Gibco, Invitrogen). Cells were maintained in a humidified incubator at 37°C in 5% CO2 and were subcultured by trypsinisation upon reaching 70-80% confluency.

Reagents. Two representative cis-restricted β-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.
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°C. Human recombinant VEGF165 (R&D Systems Inc., Minneapolis, MN, USA) was reconstituted to 1 µg/ml in 0.1% (w/v) bovine serum albumin (BSA) and also stored at -20°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 for 10 min. Cell pellets were resuspended in 200 µl 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°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°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 G₀/G₁ were diploid (2N DNA content), cells in G₂/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 <2N DNA. All data were recorded and analysed using the CellQuest software (Becton Dickinson).

Endothelial cell migration. Costar® 8 µM-pore Transwell inserts (Corning Incorporated, Corning, NY, USA) were coated overnight at 4°C with 10 µg/ml human fibronectin. HUVECs (10,000 cells in 100 µl 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 overnight incubation the slides were sequentially incubated in a blocking solution [5% (w/v) BSA/0.1% (v/v) Triton X-100/PBS], monoclonal anti-α-tubulin antibodies (Merck Biosciences, Nottingham, UK), fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse antibodies (DakoCytomation, Glostrup, Denmark) and finally Alexa Fluor® 488 goat anti-rabbit (Molecular Probes, Eugene, OR, USA) for 24 h. Following treatment, the cells were fixed in 100% methanol at -20°C and the microtubular network was detected by indirect immunofluorescence as previously described (22). Briefly, the slides were incubated with a blocking solution [5% (w/v) BSA/0.1% (v/v) Triton X-100/PBS] for 6 h in the presence of vehicle or the indicated analogues were also added to the lower wells. 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 50 mM Tris-HCl, pH 7.7, the absorbance was determined at 560 nm using an Olympus IX81 fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Detection of VEGF release. MDA-MB-231 cells (60,000 cells/cm²) were grown for 24 h, then washed in PBS and incubated in

**Figure 1. Chemical structures of combretastatin A-4 and its novel cis-restricted β-lactam analogues, CA-104 and CA-432.**
Values represent the mean ± SEM for three separate experiments. IC₅₀ percentage of cell viability relative to vehicle-treated control cells (100%).

To reflect the values obtained from this cell viability assay, the IC₅₀ values for CA-104 and CA-432 used for the rest of this study were chosen to deduce 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.

Statistical analysis. Results were presented as mean ± 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 β-lactam combretastatin A-4 analogues, CA-104 and CA-432, reduce endothelial cell viability. To determine the effect of cis-restricted β-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 IC₅₀ values for CA-104 and CA-432 of 24.97 and 4.00 nM (Fig. 2). From these IC₅₀ 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 G₂/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 µM) 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±1.5% (P=0.0063) or 37.0±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 G₂/M phase of the cell cycle.

G₂/M arrest was accompanied by significant increases in the number of entities presenting with hypodiploid (<2N) quantities of DNA as indicated by a sub G₁/G₀ 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±2.6% (P=0.0064) or 31.4±1.3% (P=0.0019) respectively (Fig. 3C and D). These statistically significantly 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 G₂/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...
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).
CA-104 and CA-432 reduce endothelial cell migration. Having established that cis-restricted β-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 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 vehicletreated control cells representing 100% migration and displayed as mean ± SEM of three experiments each carried out in duplicate. A P-value <0.05 was considered to be statistically significant (P<0.05, "P<0.01, ""P<0.001).

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±1.7% (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-
treated cells (100%). Incubation for 6 h with CA-432 prevented MDA-MB-231 cell migration by 95.7±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 vivo 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 β-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 β-lactam ring (Fig. 1). We determined that both analogues potently inhibited HUVEC proliferation with IC₅₀ 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 IC₅₀ 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 expression of normal breast epithelial cells to CA-432 induced only a minimal amount of cytotoxicity with an IC₅₀ value of greater than 10 nM (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 G₂/M arrest and apoptosis which was accompanied by depolymerisation of the microtubular networks in HUVECs. Tubulin depolymerisation and G₂/M arrest are typical responses observed in tumour cells after exposure to the β-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 G₂/M 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 β-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 β-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 β-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 IC₅₀ value of 28.8±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 β-lactam combretastatin A-4 analogues, CA-104 and CA-432. Collectively, this report along with our previous studies indicate that these β-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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