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ORIGINAL ARTICLE

Dual targeting of tumour cells and host endothelial cells by novel microtubule-targeting agents, pyrrolo-1,5-benzoxazepines

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Abstract
Purpose Some members of a novel series of pyrrolo-1,5-benzoxazepines (PBOXs) are microtubule-targeting agents capable of inducing apoptosis in a variety of human cancerous cells, hence, they are currently being developed as potential anti-cancer agents. The purpose of this study was to first characterise the activities of a novel PBOX analogue, PBOX-16 and then investigate the anti-angiogenic potential of both PBOX-16 and its prototype PBOX-6.
Methods The effects of PBOX-6 and -16 on cancerous cells (chronic myeloid leukaemia K562 cells and ovarian carcinoma A2780 cells) and primary cultured human umbilical vein endothelial cells (HUVECs) were examined by assessing cell proliferation, microtubular organisation, DNA analysis of cell cycle progression and caspase-3/7 activity. Their anti-angiogenic properties were then investigated by examining their ability to interfere with HUVEC differentiation into capillary-like structures and vascular endothelial growth factor (VEGF)-stimulated HUVEC migration.
Results PBOX-6 and -16 inhibited proliferation of K562, A2780 and HUVEC cells in a concentration-dependent manner. PBOX-16, confirmed as a novel depolymerising agent, was approximately tenfold more potent than PBOX-6. Inhibition of cell proliferation was mediated by G_2/M arrest followed by varying degrees of apoptosis depending on the cell type; endothelial cells underwent less apoptosis than either of the cancer cell lines. In addition to the anti-tumourigenic properties, we also describe a novel anti-angiogenic function for PBOXs: treatment with PBOXs inhibited the spontaneous differentiation of HUVECs into capillary-like structures when grown on a basement membrane matrix preparation (Matrigel™) and also significantly reduced VEGF-stimulated HUVEC migration.
Conclusion Dual targeting of both the tumour cells and the host endothelial cells by PBOX compounds might enhance the anti-cancer efficacy of these drugs.

Keywords PBOX · Microtubule-targeting agents · HUVEC · Angiogenesis

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Introduction

Microtubules are dynamic polymers that form key components of the cytoskeleton. They are essential elements of many important cellular processes including cell division. Drugs that interfere with microtubule polymerisation dynamics such as the tubulin-stabilising taxanes or the tubulin-destabilising vinca alkaloids have proved to be effective in the treatment of cancer [1]. In addition to inducing apoptosis of tumour cells, taxanes [2–5] and vinca alkaloids [6, 7] have also been demonstrated to

exhibit anti-angiogenic properties. This is significant since the formation of new capillary blood vessels derived as extensions from the pre-existing vasculature (angiogenesis) is essential for the growth and progression of tumours [8]. More recently, several compounds with diverse chemical structure that bind to an additional site on tubulin, the colchicine binding site, have been shown to inhibit tumour growth in vitro and in vivo and exhibit anti-angiogenic behaviour and are now in clinical development (ABT-751 [9], STX140 [10] and IRC-083927 [11]. Therefore, drugs capable of dual targeting both tumour cells and tumour vasculature offer promising therapeutic strategies.

Our research group has demonstrated that some members of a novel series of pyrrolo-1,5-benzoxazepine (PBOX) compounds are microtubule depolymerising agents [12]. These compounds do not bind to the characterised vinblastine or colchicine binding sites on tubulin suggesting that they bind to, as yet, an uncharacterised novel site on tubulin [12]. These compounds potently induced apoptosis in a variety of human cancer cells derived from both the haematopoietic system and from solid tumours including promyelocytic leukaemia HL60 cells, Jurkat T-lymphoma cells, Hut-78 lymphoma cells, T cell leukaemia CEM cells, highly resistant CML K562 cells and breast carcinoma MCF-7 cells [13–17] while displaying no cytotoxic effect on normal peripheral blood mononuclear cells [17] or bone marrow cells (unpublished data). These results have been confirmed in vivo as PBOX-6 significantly impaired growth of tumours in a mouse 4T1 breast carcinoma tumour model [18].

To extend the investigation of PBOX compounds as a potential new class of anti-tumour drugs, the purpose of this series of experiments was firstly to evaluate the anti-neoplastic potential of the novel PBOX analogue, PBOX-16 and secondly to investigate if PBOX-6 or PBOX-16 (Fig. 1) could elicit any anti-angiogenic effects. In this report, we demonstrated that PBOX-6 and PBOX-16 reduced proliferation of cancerous cell lines (human ovarian carcinoma A2780 cells and CML K562 cells) and also HUVEC cells. This anti-proliferative effect was associated with microtubule depolymerisation. In all three cell types studied, the novel compound PBOX-16 was found to be the more potent analogue. The effects observed by the PBOX-6 and PBOX-16 were found to be mediated by G_2/M arrest, however notably while the PBOXs were more potent at arresting HUVECs compared to the tumour cell lines, progression to apoptosis (assessed by DNA profile analysis and caspase-3/7 activation) was higher in cancer cell lines than in the endothelial cells. Lastly, we established that the anti-proliferative effect of PBOXs on endothelial cells was accompanied by inhibition of both the ability of HUVEC cells to spontaneously differentiate into capillary tube-like structures and their migrational activity. As proliferation, migration and differentiation of host endothelial cells are critical parameters during tumour vascularisation, these results suggest a novel anti-angiogenic function for PBOX compounds.

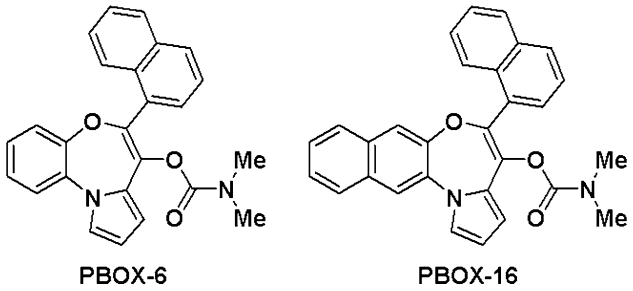


Fig. 1 Chemical structure of microtubule-targeting agent PBOX-6 and its novel analogue PBOX-16

The ability of PBOX compounds to target both the cancer cells, by directly inducing their apoptosis, and indirectly, by reducing the proliferation, differentiation and migration of endothelial cells should enhance their anti-cancer potential and therefore these novel compounds should be considered for further in vivo studies to advance their development as new class of anti-cancer agent.

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 (Portland, OR, 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 ovarian carcinoma A2780 cells and chronic myelogenous leukaemia K562 cells (European Collection of Cell Cultures, Salisbury, UK) were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium enhanced with GlutaMAX-I and supplemented with 10% foetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin (all from Gibco, Invitrogen, Carlsbad, CA, USA). All cells were maintained in a humidified incubator at 37°C in 5% CO_2. Adherent cells were subcultured by trypsinisation upon reaching 70–80% confluency.

Reagents

The pyrrolo-1,5-benzoxazepine compounds, 7-[(*N*,*N*-dimethylcarbamoyl)oxy]-6-(naphth-1-yl)pyrrolo[2,1-d][1,5]benzoxazepine (PBOX-6) and 4-[(*N*,*N*-dimethylcarbamoyl)oxy]-5-(naphth-1-yl)naphtho[2,3-b]pyrrolo[1,2-d][1,4] oxazepine (PBOX-16) were synthesised as described previously [19] and dissolved in ethanol. Paclitaxel and vincristine were dissolved in DMSO or sterile water, respectively. Human recombinant $VEGF_{165}$ (R&D Systems Inc., Minneapolis, MN, USA) was reconstituted to 1 μg/ml in 0.1% (w/v) bovine serum albumin (BSA). Once dissolved in the relevant solvent, all compounds were stored at –20°C

Cell proliferation

Cell proliferation was monitored using AlamarBlue™ dye (BioSource, Invitrogen, Carlsbad, CA, USA). This dye changes from an oxidised indigo blue non-fluorescent state to a fluorescent pink state in the reduced environment of living cells. A2780, K562 or HUVEC cells grown on 96-well plates (15,000–20,000 cells/well) for 24 h were treated with PBOX-6 or PBOX-16 for up to 72 h. AlamarBlue™ was added such that its final concentration in each well was 10% (v/v) and incubated at 37°C. Fluorescence 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). The results were expressed as the percentage cell viability relative to vehicle-treated control cells (100%). Dose–response curves were plotted and IC_{50} values (concentration of PBOX resulting in 50% reduction in cell viability) were obtained using Prism GraphPad 4.

Analysis of tubulin polymerisation by Western blot

The status of tubulin polymerisation in A2780, K562 and HUVEC cells was assessed using a modified version of the method employed by Verma et al. [20]. Briefly, cells treated with microtubule-targeting agents (MTAs) for 4 h were harvested into an ice-cold microtubule-preserving buffer containing 0.1 M Pipes (pH 6.9), 2 M glycerol, 5 mM $MgCl_2$, 2 mM EGTA, 0.5% (v/v) Triton X-100, 4 μM paclitaxel supplemented with 1 complete™ mini-EDTA-free protease inhibitor cocktail tablet per 10 ml buffer (Roche Diagnostics GmbH, Mannheim, Germany) to maintain stability of assembled microtubules. Polymerised and unpolymerised tubulin were separated by centrifugation at 20,000*g* for 45 min at 4°C. Supernatants, containing depolymerised tubulin, were diluted by the addition of an equal volume of 2× sample buffer [125 mM Tris (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 0.0025% (w/v) bromophenol blue and 100 mM DTT] while pellets containing sedimented polymerised tubulin were washed in microtubule-preserving buffer then resuspended in a buffer containing 62.5 mM Tris (pH 6.8), 6 M urea, 2% (w/v) SDS, 10% (v/v) glycerol, 0.00125% (w/v) bromophenol blue and 50 mM DTT. The samples were then denatured at 65°C for 10 min before separation of proteins on a polyacrylamide gel and transfer to PVDF membrane. The PVDF transfers were co-probed with anti-α-tubulin antibody (1:715 dilution) and anti-*β*-actin antibody (1:5,000 dilution), both purchased from Merck Biosciences (Nottingham, UK), followed by incubation with a horseradish peroxidase-conjugated anti-mouse antibody, 1:2,500 dilution (Promega, Madison, WI, USA). Expression of tubulin and *β*-actin were visualised by enhanced chemiluminescence.

Microtubule staining by indirect immunofluorescence

HUVECs (0.6×10^5 cell/ml), cultured on BD Falcon™ four-chamber glass slides (BD Biosciences, San Jose, CA, USA) for 24 h were treated for 16 h. The cells were fixed in 100% methanol at −20°C (10 min). The cells were then incubated in the following solutions, each incubation preceded by washing in PSB (Oxoid Ltd., Hampshire, UK): blocking solution [5% (w/v) BSA dissolved in 0.1% (v/v) Triton X-100 in PBS] (30 min), monoclonal anti-α-tubulin antibody (Merck Biosciences, Nottingham, UK) (1 h), fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse antibody (DakoCytomation, Glostrup, Denmark) (1 h) and 0.2 μg/ml propidium iodide (2 min). The chamber partitions were removed from the slides and anti-quenching solution (2 μg/ml *p*-phenylenediamine in 50:50 glycerol to PBS solution) was applied to the surface of each slide and coverslips mounted. The organisation of the microtubule network (green) and the cellular DNA (red) was visualised under a 60× oil-emersion lens using an Olympus IX81 Fluorescent Microscope (Olympus Corporation, Tokyo, Japan).

Determination of DNA content

Following treatment, cells were harvested by centrifugation at 800*g* 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 (PI). Cells were incubated in the dark at 37°C for 30 min. The PI fluorescence 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. PI 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 <2N DNA. All data was recorded and analysed using the CellQuest software (Becton Dickinson, San Jose, CA, USA).

Caspase-3/7 activity assay

A2780, K562 or HUVEC cells were seeded onto black 96-well plates (15,000–20,000 cells/well) and treated for 48 h with vehicle (0.5% EtOH), PBOX-6 or PBOX-16. Cell viability was then measured using AlamarBlue™ reagent as described above. Caspase-3/7 activity was then measured by the Apo-ONE® homogeneous caspase-3/7 assay (Promega, Madison, WI, USA). Briefly, the cells were lysed by the addition of an equal volume of Apo-ONE® reagent and incubation on an orbital plate shaker for 1 h at room temperature in darkness. This reagent also contained an artificial substrate (Z-DEVD-R110) which fluoresced when cleaved to rhodamine 110 by caspase-3 or -7. Fluorescence (caspase-3/7 activity) was detected at an excitation wavelength of 499 nm and emission wavelength of 521 nM using a SpectraMax Gemini fluorescent plate reader (Molecular Devices, Sunnyvale, CA, USA). Readings were corrected for cell viability and expressed on graphs as fold change in capase-3/7 activity (fluorescence) compared to the vehicle control.

In vitro tubule formation

HUVECs (1.5×10^6 cells/well) were incubated on BD Biocoat™ matrigel™-coated 6-well plates (BD Biosciences, San Jose, CA, USA) for 6 h in the presence of vehicle or the indicated drugs. 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 100×.

Transwell migration assay

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 the addition of 10 ng/ml VEGF to the lower well. Vehicle or the desired concentrations of PBOX-6 or PBOX-16 were also added to the lower well. 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, Sunnyvale, CA, USA).

Statistical analysis

Results were presented as mean ± SEM. The statistical analysis of experimental data was performed using the computer program Prism GraphPad 4. *P* values were determined using a two-tailed Student's paired *t* test. A value of $P < 0.05$ was considered to be significant.

Results

PBOX-6 and novel analogue PBOX-16 inhibited the proliferation of cancerous cells and endothelial cells

To determine the effect of PBOX compounds on cancer and endothelial cell proliferation, we tested a range of concentrations of a lead PBOX compound, PBOX-6 and its novel analogue PBOX-16 on ovarian carcinoma A2780 cells, CML K562 cells and primary HUVEC cells for 72 h. We established that both agents lead to a reduction in proliferation of both cancer and endothelial cells, with respective IC_{50} values for PBOX-6 and PBOX-16 of 3.58 μM and 0.23 μM in A2780 cells (Fig. 2a), 6.49 μM and 0.37 μM in K562 cells (Fig. 2b) and 0.697 μM and 64.8 nM in HUVECs (Fig. 2c). From these IC_{50} values, it was deduced that in all three cell types studied, the novel analogue PBOX-16 was found to be a more potent inhibitor of cell proliferation than parent compound PBOX-6 (A2780: 15.6-fold; K562: 17.5-fold; HUVEC: 10.8-fold). Furthermore, both PBOX-6 and PBOX-16 were capable of inhibiting HUVEC cell proliferation at slightly lower concentrations than those required in either of the two cancer cell lines studied. The concentrations of PBOX compounds used for the rest of this study were chosen to reflect the IC_{50} values obtained from this cell proliferation assay.

PBOX-6 and PBOX-16 resulted in depolymerisation of tubulin in cancer and endothelial cells

It was previously reported by this lab that PBOX-6 disrupted the assembly of purified tubulin in vitro and interfered with gross microtubule polymerisation dynamics in MCF-7 human breast carcinoma cells using fluorescence

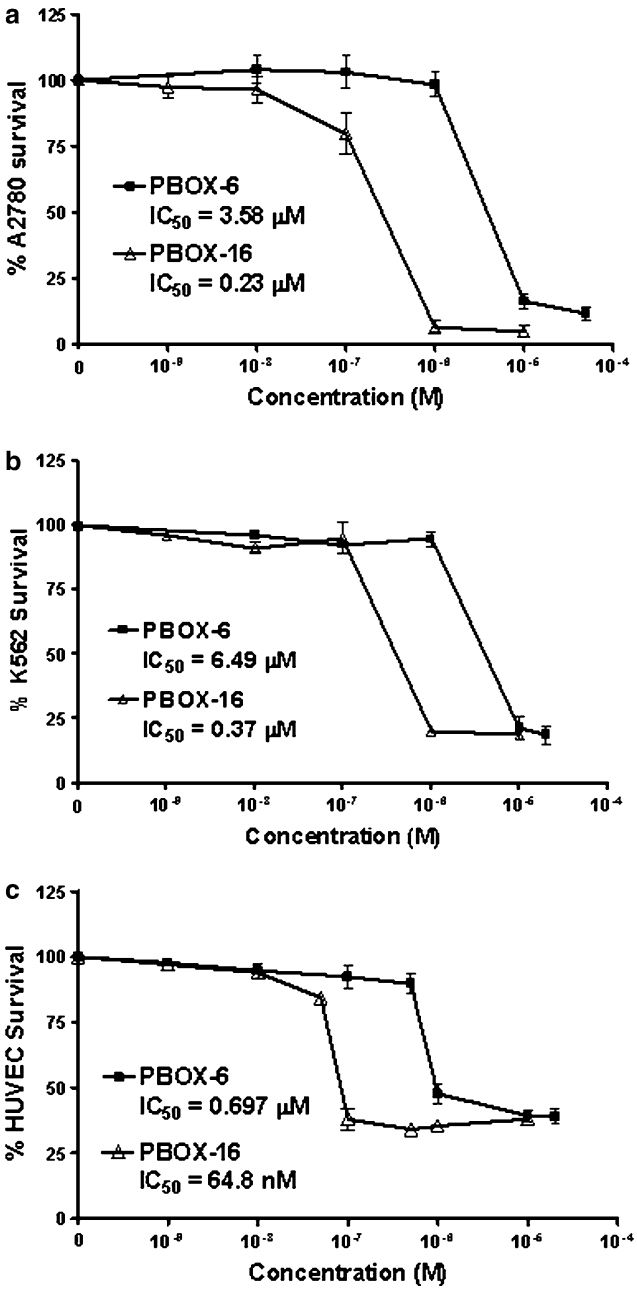


Fig. 2 PBOX-6 and PBOX-16 treatment reduced proliferation of ovarian carcinoma A2780 cells, CML K562 cells and primary endothelial HUVEC cells. A2780 (**a**), K562 (**b**) or HUVEC (**c**) cells were seeded onto 96-well plates and treated in triplicate with vehicle alone [1% (v/v) ethanol] or a range of concentrations of PBOX-6 or PBOX-16 for 72 h. The cells were then incubated in 10% (v/v) AlamarBlue™ and its reduction to a fluorescent state measured at excitation 544 nm and emission 590 nm using a multi-well fluorimeter. The results were expressed as the percentage cell proliferation relative to vehicle-treated control cells (100%). Values represent the mean ± the SEM for three separate experiments

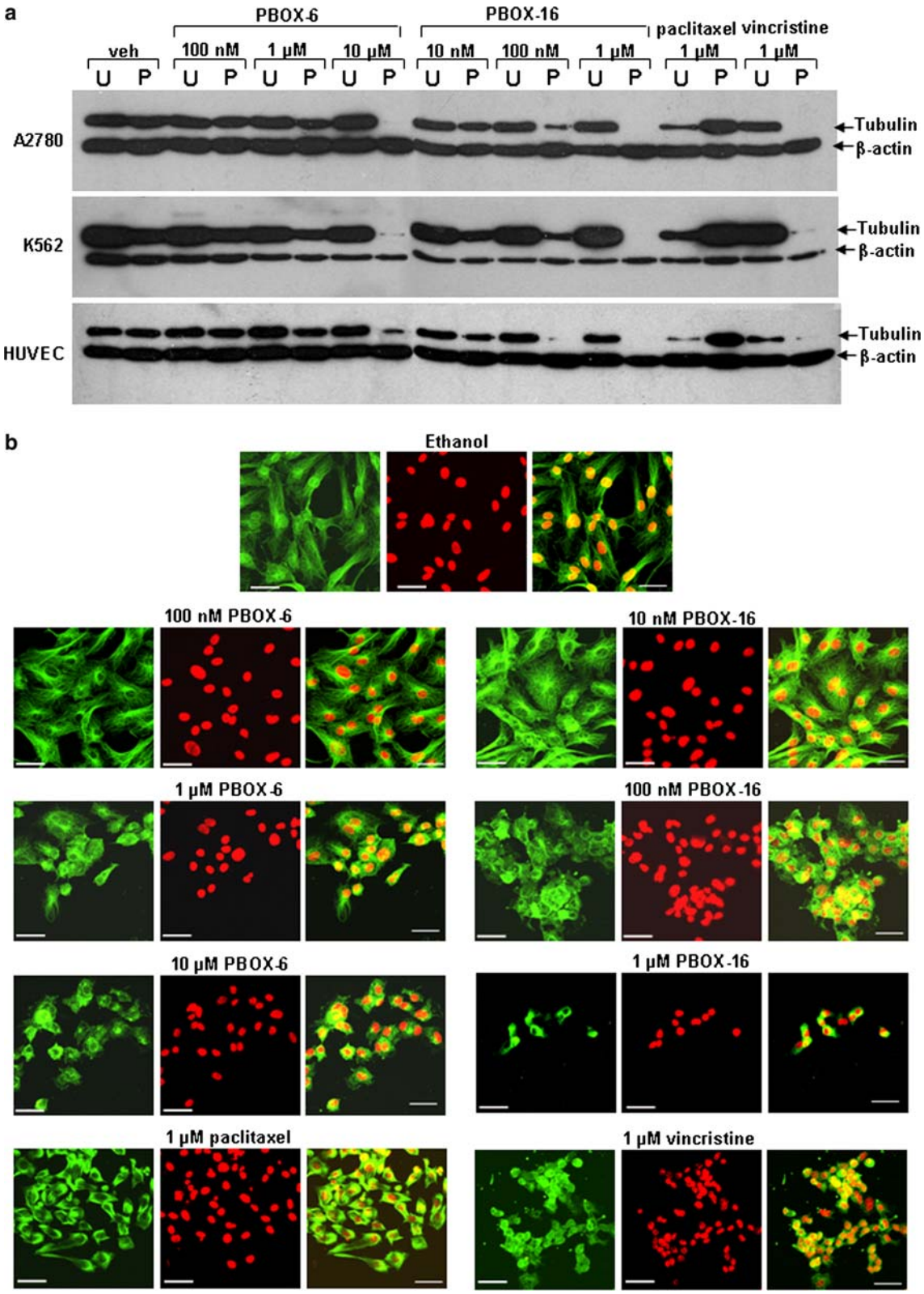
microscopy [12]. In this study we demonstrated by Western blot that treatment (4 h) with PBOX-6 and PBOX-16 led to a reduction in the amount of polymerised tubulin (P) found in A2780, K562 and HUVEC cells in a dose-dependent manner (Fig. 3a). PBOX-16 proved to be a more potent tubulin depolymeriser than PBOX-6, with concentrations of PBOX-16 as low as 100 nM resulting in a pronounced depolymerisation of tubulin in A2780 and K562 cells, while even 10 nM produced a slight depolymerisation effect in HUVEC, whereas 1 µM PBOX-6 was required to produce a noticeable depolymerisation in the cell types examined. Treatment with known tubulin destabiliser, vincristine, produced a similar pattern of tubulin depolymerisation to the PBOXs, as opposed to common tubulin stabiliser paclitaxel which caused an increase in the proportion of polymerised (P) to unpolymerised (U) tubulin (Fig. 3a).

PBOX-6 and PBOX-16 caused destabilisation of the microtubule network in endothelial cells

As the effect of PBOX compounds on the morphology of the microtubular structure in endothelial cells had not yet been examined, HUVEC cells, grown on glass 4-chamber slides, were treated for 16 h with PBOX-6, PBOX-16, paclitaxel or vincristine. Immunofluorescent staining was used to detect morphological changes in the microtubule network, such as alteration in organisation and arrangement (Fig. 3b). In normal cells the microtubule network is organised as cytoplasmic tubulin filaments radiating from a central point to the periphery. HUVEC cells treated with vehicle alone (0.5% ethanol) displayed this typical tubulin morphology. Exposure of cells to tubulin depolymerising agents, for example vincristine, typically resulted in exhibition of diffuse tubule staining with no definition of structure caused by microtubule disassembly. Tubulin polymerising agents such as paclitaxel result in highly concentrated accumulation of filaments into dense peripheral bundles indicative of microtubule stabilisation. While no change in microtubule structure was evident following treatment with the lower doses of 100 nM PBOX-6 and 10 nM PBOX-16, gross morphological changes in the tubulin cytoskeleton typical of depolymerising agents and visualised as diffuse tubule staining were observed when cells were treated with 1 µM PBOX-6, 10 µM PBOX-6, 100 nM PBOX-16 and 1 µM PBOX-16. In agreement with our tubulin Western blot results, the effect on microtubule organisation of HUVEC cells treated with 1 µM PBOX-6 is less pronounced than that observed with 100 nM PBOX-16.

PBOX-6 and PBOX-16 induced cell cycle arrest at the G_2/M phase followed by varying degrees of apoptosis in cancer and endothelial cells

To define the mechanism underlying how these PBOX compounds elicited their anti-proliferative effects, the cell cycle profiles of cancer and endothelial cells were



examined. A2780, K562 and HUVEC cells were exposed to PBOX-6 or PBOX-16 for up to 48 h, their DNA was fluorescently stained with propidium iodide and analysed by flow cytometry. The DNA profiles of vehicle-treated A2780, K562 and HUVEC cells (Fig. 4a–c, respectively) displayed only 1–2% of cells with hypodiploid (<2N) quantities of DNA as indicated by the subG_0/G_1 peak. These values represented the

◀ **Fig. 3** PBOX-6 and PBOX-16 resulted in tubulin depolymerisation in A2780, K562 and HUVEC cells and disrupted the organisation of the HUVEC microtubule network. **a** A2780, K562 or HUVEC cells were treated for 4 h with either vehicle [0.5% (v/v) ethanol] or the indicated concentrations of PBOX-6, PBOX-16, paclitaxel or vincristine. Polymerised (*P*) and unpolymerised (*U*) tubulin were then separated by centrifugation in a microtubule-preserving buffer. The quantity of polymerised compared to unpolymerised tubulin was assessed by Western blot using monoclonal antibodies directed against tubulin and loading control *β*-actin followed by incubation with a HRP-conjugated anti-mouse secondary antibody. **b** HUVECs grown on four-chamber glass slides were treated as described above for 16 h. The cells were then fixed in methanol, incubated with a monoclonal anti-α-tubulin antibody, followed by a FITC-conjugated anti-mouse antibody and then briefly stained with propidium iodide. The organisation of the microtubule network (*green*) and the cellular DNA (*red*) was visualised using a fluorescent microscope at a magnification of ×600 (*bar* 40 μm). *Blots* and *photographs* illustrated are representative of three independent experiments

amount of background apoptosis found in each cell type. The percentage of cells with 4N DNA content (i.e. cells in the G_2/M phase of the cell cycle) was between 15 and 20%. Treatment with 10 μM PBOX-6 (48 h) lead to apoptosis in A2780 (39%) and K562 (55%) cancer cells, while in HUVECs this concentration resulted in only 13% apoptosis with 62% of the HUVECs being sustained in a state of G_2/M arrest. The extent of progression from G_2/M arrest to apoptosis induced by PBOX-6 seemed to be cell-type-dependent. Similarly, PBOX-16 (1 μM) resulted in less apoptosis in HUVECs than in cancer cells. Interestingly, although the lower concentrations tested of PBOX-6 and PBOX-16 failed to alter the cell cycle profiles of cancer cells, 100 nM PBOX-16 induced a similar effect to that observed with 1 μM PBOX-16 in HUVECs. While PBOX-6 (1 μM) initially lead to G_2/M arrest of HUVECs (42% after 6 h, data not shown), this concentration of PBOX-6 was not capable of sustaining HUVECs in the G_2/M phase. Cell cycle profiles measured after 24 and 48 h indicated that the cells appeared to have re-entered into the G_0/G_1 phase (data not shown) accompanied by a small amount of apoptosis (8.5% after 48 h). These results indicated that both PBOX-6 and PBOX-16 arrested cancer and endothelial cells in the G_2/M phase of the cell cycle. G_2/M arrest is a common feature of MTAs. This arrest was followed subsequently by varying degrees of apoptosis depending on the cell type and the concentration used. Again PBOX-16 appeared the more potent analogue. Generally, although endothelial cells appeared more sensitive to the effects of PBOX-6 and PBOX-16 which altered DNA profiles at lower concentrations than in the tumour cells, the effect on HUVECs seemed to involve a more sustained G_2/M arrest and less apoptosis than in cancer cells.

PBOX-6 and PBOX-16 induced caspase-3/7 activation in cancer and endothelial cells

Activation of downstream effector caspases is a common feature during the apoptotic demise of a cell [21]. To further investigate the mechanism of PBOX-induced inhibition of cell proliferation in cancer and endothelial cells we performed an assay to detect the activity of caspase-3/7 in cells treated for 48 h with PBOX-6 (Fig. 5a) or PBOX-16 (Fig. 5b) compared to control cells. Effector caspase-3/7 activation in A2780 cells was increased by 12- or 40-fold following treatment with PBOX-6 (10 μM) or PBOX-16 (1 μM), respectively, compared to controls. This followed a similar pattern to the results obtained by A2780 cell cycle analysis where PBOX-16 caused more apoptosis (50%) than PBOX-6 (39%). Similarly, caspase-3/7 activity was also increased in K562 cells following treatment with PBOXs; however, PBOX-6 (31-fold) caused a larger induction of caspase-3/7 activity than PBOX-16 (19-fold). This corresponded to the results from cell cycle analysis where the percentages of K562 cells in the subG_0/G_1 peak were 55% (PBOX-6) and 27% (PBOX-16) because PBOX-16 sustained a higher percentage of K562 cells in the G_2/M phase. In contrast, treatment of HUVECs with PBOX-6 or PBOX-16 resulted in only a twofold increase in caspase-3/7 activity. Again the PBOXs appeared slightly more potent in endothelial cells than cancer cells with doses of 100 nM PBOX-6 and 1 μM PBOX-16 also culminating in a twofold increase in caspase-3/7 activity in HUVECs while these concentrations were ineffective in A2780 or K562 cells. Together with the results we obtained from the cell cycle profiles, these results indicated that while PBOX compounds were capable of altering cellular functionality at lower concentrations in endothelial cells, progression to apoptosis was not as pronounced as in cancer cells.

PBOX-6 and PBOX-16 inhibited endothelial cell differentiation

Having established that PBOX-6 and PBOX-16 altered HUVEC cell function, we then examined their implications on angiogenic processes in vitro. First, 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 [22]. 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), paclitaxel, PBOX-6 or PBOX-16 for 6 h. The alignment of the cells on the 3D-Matrigel was assessed using a phase contrast microscope (Fig. 6a). Vehicle-treated cells underwent alignment into the capillary-like structures while positive

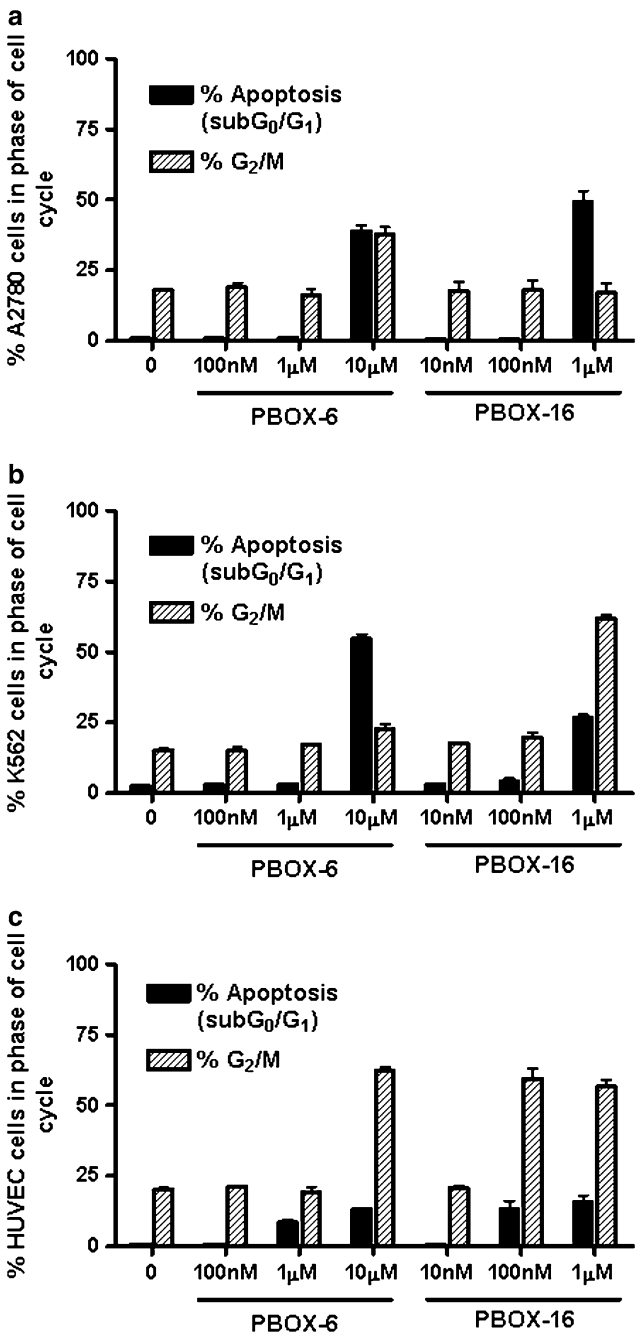


Fig. 4 PBOX-6 and PBOX-16 induced G_2/M arrest and varying degrees of apoptosis in A2780, K562 or HUVEC cells. A2780 (**a**), K562 (**b**) or HUVEC (**c**) cells were treated with vehicle [0.5% (v/v) ethanol] or the indicated concentrations of PBOX-6 or PBOX-16 for 48 h, fixed in ethanol, stained with propidium iodide and DNA content assessed by flow cytometry. Analysis of data was preformed using the computer program CellQuest. Cells in the subG_0/G_1 phase (<2N DNA) were deemed apoptotic, while cells with 4N quantities of DNA were considered to be in the G_2/M phase of the cell cycle. Values represent the mean ± SEM for three independent experiments

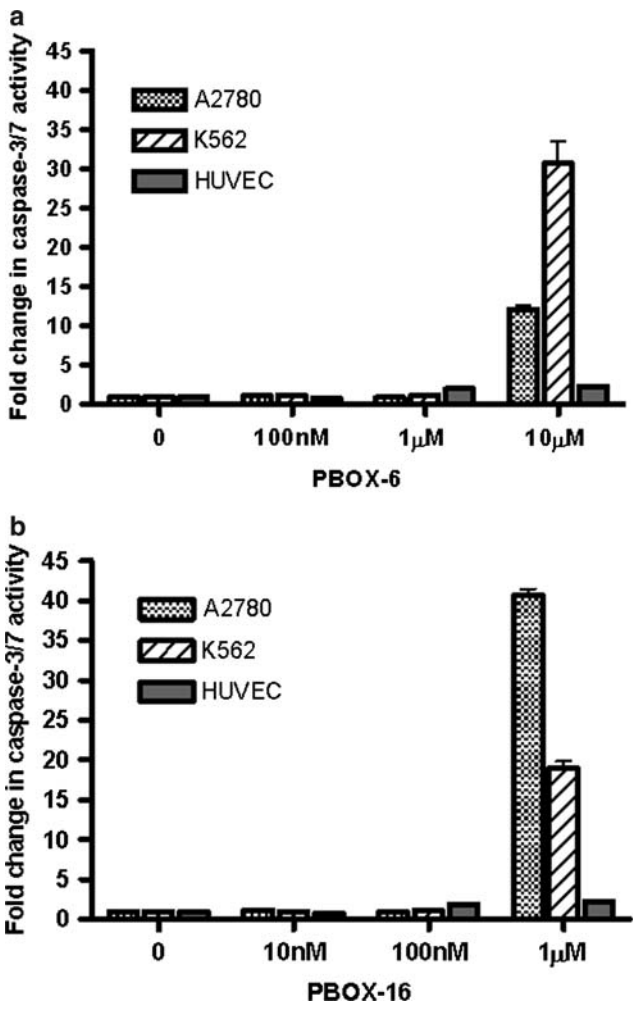


Fig. 5 PBOX-6 and PBOX-16 induced less caspase-3/7 activation in endothelial cells than in cancer cells. A2780, K562 or HUVEC cells were seeded onto to black 96-well plates and treated for 48 h with either PBOX-6 (**a**) or PBOX-16 (**b**). Caspase-3/7 activity was then quantified by incubation with Apo-ONE® homogenous caspase-3/7 reagent containing an artificial substrate which fluoresced when cleaved by caspase-3 or -7. Fluorometric analysis was carried out at excitation 499 nm and emission 521 nm. Results were corrected for cell viability and expressed as fold-change in caspase-3/7 activity compared to vehicle-treated controls. Values represent the mean ± SEM of three independent experiments

control, 1 μM paclitaxel, reduced tubule formation. Similarly, 1 μM PBOX-6 and 100 nM PBOX-16 reduced the formation of tubules in a concentration-dependent manner. This effect is likely to be associated with G_2/M arrest, because following 6 h treatment with vehicle or low doses of PBOX-6 (100 nM) or PBOX-16 (10 nM), the quantity of HUVECs in the G_2/M phase was ~24%, while in HUVECs treated with 1 μM PBOX-6 or 100 nM PBOX-16 the percentages rose to 42 or 45%, respectively (data not shown).

PBOX-6 and PBOX-16 reduced endothelial cell migration

To further confirm the anti-angiogenic potential of the PBOX compounds, we evaluated the effect of PBOX-6 and PBOX-16 on HUVEC cell migration using a modified

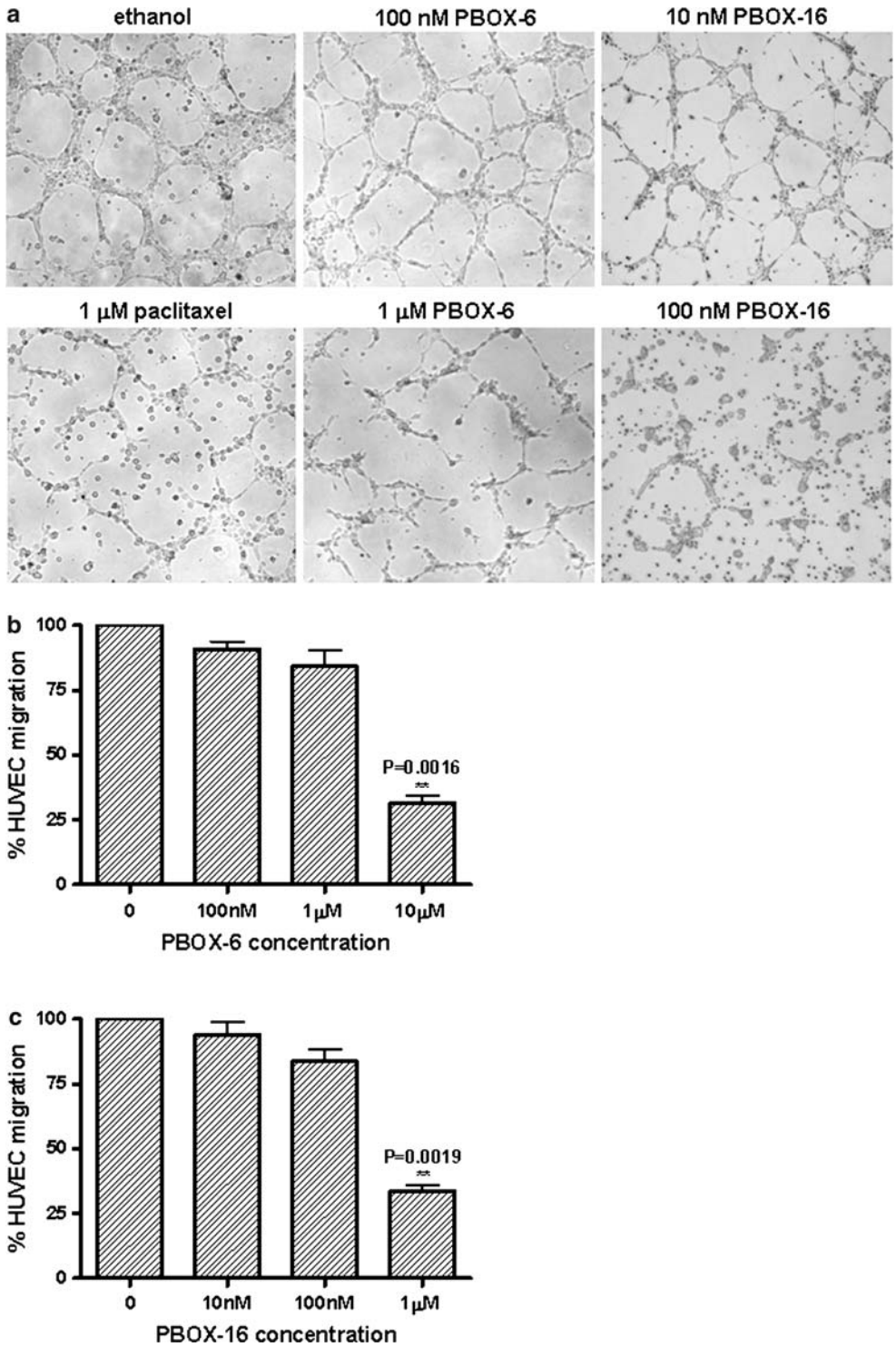


Fig. 6 PBOX-6 and PBOX-16 inhibited endothelial cell in vitro tubule formation and reduced endothelial cell migration. **a** HUVECs were grown on matrigel™-coated 6-well plates for 6 h in the presence of vehicle [0.5% (v/v) ethanol] or the indicated concentrations of paclitaxel, PBOX-6 or PBOX-16. The ability of the HUVECs to form capillary-like tubules was demonstrated using a phase contrast microscope at a total magnification of ×100. *Pictures* shown are representative of three independent experiments. **b**, **c** HUVECs were seeded onto fibronectin-coated 8 μM-pore transwell inserts in 24-well plates containing medium. HUVEC migration was stimulated by the addition of 10 ng/ml VEGF to the lower well. Vehicle [0.5% (v/v) ethanol] or the indicated concentrations of PBOX-6 (**b**) or PBOX-16 (**c**) were also added. After 6 h, migrated cells were stained with 0.5% toulidine blue O and 0.5% sodium tetraborate and quantified as absorbance at 650 nm. The results were expressed as the percentage migrated cells relative to vehicle-treated control cells (100%) and displayed as mean ± the SEM of three experiments each carried out in duplicate. *P* values were determined using the computer programme Prism GraphPad 4

Boyden chamber assay. This is a chemotactic model representative of tumour-induced endothelial cell migration [23]. It consists 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 PBOX-6 and PBOX-16 on this migration was determined by their addition along with VEGF into the lower chamber. Migration was expressed as a percentage of migration in control chambers treated with the vehicle (100%). Incubation for 6 h with either PBOX-6 or PBOX-16 inhibited VEGF-stimulated HUVEC migration in a concentration-dependent manner, 9.1, 15.9 and 68.5% when treated with 100 nM, 1 μM and 10 μM PBOX-6, respectively (Fig. 6b) and 6.2, 16.3 and 66.5% with 10 nM, 100 nM and 1 μM PBOX-16, respectively (Fig. 6c). Statistical significance was reached with 10 μM PBOX-6 (**$P = 0.0016$) and 1 μM PBOX-16 (**$P = 0.0019$).

Discussion

It has been well documented that tumours can survive for many years in an avascular, quiescent state up to a size of approximately 1–2 mm in diameter. At this point, a change to an angiogenic phenotype is necessary for further tumour progression [24]. Therefore, drugs which can interfere with these angiogenic events may provide useful therapeutic strategies to prevent the development of tumours.

All studies with PBOX compounds carried out to date have focused on targeting cancer cells; this study was conducted to explore the potential inhibitory effects of these novel agents on some of the key parameters critical for tumour angiogenesis. Through a series of in vitro experiments we determined that a representative PBOX compound, PBOX-6 and its novel analogue PBOX-16 had the ability to induce apoptosis in cancer cells and inhibit several of the steps essential for tumour angiogenesis, namely, endothelial cell proliferation, migration and differentiation.

First, we determined that both PBOX-6 and PBOX-16 reduced proliferation of human ovarian carcinoma A2780 cells, CML K562 cells and HUVEC cells. PBOX-16 was a more potent anti-proliferative agent than parent compound PBOX-6 in all three cell types examined. Both PBOXs inhibited proliferation of endothelial cells at slightly lower concentrations than those required in cancer cells. This is the first report documenting the activity of novel agent, PBOX-16 on cancer cells and the first report to examine the effect of any PBOX compound on endothelial cells.

Although we had previously shown that PBOX-6 interfered with tubulin polymerisation in vitro and microtubular structure in MCF-7 breast carcinoma cells [12], we had not yet investigated the effects of novel PBOX-16 on tubulin or the effect of any PBOX compound specifically on the microtubular structure in endothelial cells. We examined the status of tubulin polymerisation by performing a Western blot to compare the ratio of polymerised to unpolymerised tubulin found in samples taken from vehicle-treated and PBOX-treated cancer and endothelial cells. Both PBOX-6 and PBOX-16 caused a shift in balance towards the unpolymerised fraction of tubulin in A2780, K562 and HUVEC cells. This effect occurred in a concentration-dependent manner with higher concentrations leading to more depolymerisation. PBOXs, particularly PBOX-16, caused tubulin depolymerisation at lower levels in HUVEC than in either cancer cell line. These findings indicated that like PBOX-6, novel compound PBOX-16 also appeared to be capable of altering tubulin dynamics. To confirm these alterations in tubulin dynamics caused by PBOXs culminated in changes in the overall structure and organisation of endothelial cell microtubule networks, we then stained HUVECs with a fluorescently labelled tubulin antibody. Microtubule disorganisation was clearly evident in HUVECs treated with either PBOX-6 or PBOX-16 which exhibited features typical of microtubule depolymerisation, diffuse tubules with no definition of structure. This was in contrast to control cells where the microtubule network was organised into cytoplasmic tubulin filaments radiating from a central point to the periphery. In combination, these results confirm that PBOX-16 is a tubulin depolymerising agent. These findings are in keeping with those observed with PBOXs during an in vitro tubulin polymerisation assay and in PBOX-6-treated breast carcinoma MCF-7 cells [12].

We next sought to determine the mechanisms by which these representative PBOX compounds elicited the anti-proliferative response we observed in the cancer cells and HUVECs. Through the analysis of the DNA content present in control and treated cells, we found that PBOX-6 and PBOX-16 engaged their anti-proliferative activity by first inducing G_2/M arrest (indicated by tetraploid DNA content) followed by varying degrees of apoptosis (indicated by DNA hypoploidy) depending on the cell type. Again, PBOX-16 was found to be more potent than PBOX-6. Generally, both PBOXs caused a higher degree of apoptosis in the cancer cell lines than in the endothelial cells, probably because these agents seemed to be able to sustain HUVECs in G_2/M. Therefore, PBOX compounds display the potential to inhibit the cell proliferation needed to supply the number of cells required for de novo blood vessel synthesis and at the same time induce apoptosis of carcinogenic cells. This is a desirable response as it is preferable to inhibit the proliferation of endothelial cells required for de novo blood vessel synthesis without high levels of associated cytotoxicity. These results were supported by the caspase-3/7 activity assay, where substantially higher levels of caspase-3/7 activity (related to levels of apoptosis) were found to be

induced in cancer cells compared to endothelial cells by PBOX-6 or PBOX-16. We have previously shown that PBOX-6-induced apoptosis in K562 cells was accompanied by activation of both caspase-dependent and independent pathways (i.e. activation of trypsin-like serine proteases) [14, 25].

Having established that PBOX compounds can alter endothelial cell function, we next examined their effects on angiogenic processes in vitro. MTAs have been shown to reduce the capillary network formation of HUVECs cultured on Matrigel™ [7]. The ability of endothelial cells to form these tubes requires their motility and ability to differentiate. We confirmed, using the in vitro capillary differentiation assay that PBOX compounds possessed the ability to prevent formation of HUVECs into tube-like structures on Matrigel™. Similar to the endothelial function assays, even 1 μM PBOX-6 and 100 nM PBOX-16 were capable of interfering with HUVEC capillary-tube formation. These results correlated with G_2/M arrest, as 6 h post-treatment with these concentrations of PBOX-6 and PBOX-16, the percentage of cells in the G_2/M phase had increased from approximately 20% in vehicle-treated control cells to 42 and 45%, respectively. This rapid effect elicited by the PBOXs on the cell cycle is not unusual. In a study by Pasquier et al. [26], paclitaxel significantly slowed anaphase to metaphase transition of human microvascular endothelial cells within 4 h of drug treatment. Furthermore, a link between the cytostatic and anti-angiogenic effects of paclitaxel have previously been demonstrated by the same group where they showed that cytostatic concentrations of paclitaxel were able to inhibit morphogenesis of HUVECs on matrigel after a 6-hour incubation [27]. Our results provide further evidence arguing for a relationship between the cytostatic and antiangiogenic effects of microtubule targeting agents.

It has been reported that anti-angiogenic MTAs are capable of inhibiting chemotactic-induction of endothelial cell migration [3]. Similarly, we found that both PBOX-6 and PBOX-16 significantly inhibited VEGF-stimulated HUVEC migration. However, in contrast to our previous results for endothelial cell function and spontaneous in vitro tubule formation, the lower concentrations of 1 μM PBOX-6 and 100 nM PBOX-16 did not significantly reduce migration. This result may have occurred due to the high concentration of VEGF used as the chemotactic agent in the assay. The VEGF may have been capable of counteracting the effect of these lower concentrations of the PBOXs by providing strong survival signals and thereby altering the potency of the compounds. Indeed, in addition to its growth factor activity, VEGF can protect endothelial cells from apoptosis by stimulating the activation of survival pathways such as phosphoinositol-3-kinase (PI3 kinase) and up-regulation of Bcl-2 [28] and in particular survivin [29] which is an important microtubule-binding apoptosis inhibitor involved in mitotic spindle regulation [30, 31]. In this respect it is preferable for anti-angiogenic agents to also be capable of inducing cytotoxic effects on tumour cells as this will have an indirect anti-angiogenic consequence as damaging the tumour cells should reduce the amount of survival pro-angiogenic factors they produce and hence make the endothelial cells even more susceptible to anti-angiogenic therapies.

In previous studies, we reported that PBOXs displayed selectivity for cancer cells without inducing cytotoxic effects to normal cells [17]. Therefore, it may seem surprising that non-cancerous endothelial cells are susceptible to PBOXs. However, this phenomenon has also been observed with other MTAs. Wang et al. [5], for example, found that paclitaxel displayed increased selectivity for endothelial cells when compared to other non-cancerous human cell types. This effect is possibly a result of differences in composition of tubulin isotypes, tubulin post-translational modifications or alterations in proteins that regulate microtubule dynamics [26, 32]. The existence of enhanced uptake mechanism has also been suggested, for example, paclitaxel uptake was reported to be increased up to fivefold in endothelial cells compared to fibroblasts [33].

In combination, these results indicated that PBOX-6 and PBOX-16, which targeted the microtubules of cancer and HUVEC cells, possessed the ability to induce apoptosis of cancer cells and inhibit proliferation, migration and differentiation of endothelial cells in vitro. Previously we showed that PBOX-6 induced apoptosis in a variety of cancer cells without causing cytotoxic damage to normal human peripheral blood mononuclear cells [14] or donor blood marrow cells (unpublished data). Together with our previous observations, the findings obtained during the current study generate a highly desirable picture of the activities of the PBOX compounds. In vitro, these compounds appear to have the potential to exhibit both anti-tumour and anti-angiogenic activity, therefore, suggesting they are worthy of further evaluation of their anti-angiogenic potential in vivo. This approach leading to the dual targeting of both the tumour and its vasculature may improve the outcome of cancer treatment by increasing treatment efficacy.

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