Alkylphospholipids inhibit capillary-like endothelial tube formation *in vitro*: antiangiogenic properties of a new class of antitumor agents

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Synthetic alkylphospholipids (APLs), such as edelfosine, miltefosine and perifosine, constitute a new class of antineoplastic compounds with various clinical applications. Here we have evaluated the antiangiogenic properties of APLs. The sensitivity of three types of vascular endothelial cells (ECs) (bovine aortic ECs, human umbilical vein ECs and human microvascular ECs) to APL-induced apoptosis was dependent on the proliferative status of these cells and correlated with the cellular drug incorporation. Although confluent, nondividing ECs failed to undergo apoptosis, proliferating ECs showed a 3–4-fold higher uptake and significant levels of apoptosis after APL treatment. These findings raised the question of whether APLs interfere with new blood vessel formation. To test the antiangiogenic properties *in vitro*, we studied the effect of APLs using two different experimental models. The first one tested the ability of human microvascular ECs to invade a three-dimensional human fibrin matrix and form capillary-like tubular networks. In the second model, bovine aortic ECs were grown in a collagen gel sandwich to allow tube formation. We found that all three APLs interfered with endothelial tube formation in a dose-dependent manner, with a more than 50% reduction at 25 μmol/l. Interference with the angiogenic process represents a novel mode of action of APLs and might significantly contribute to the antitumor effect of these compounds. *Anti-Cancer Drugs* 19:65–75 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Synthetic alkylphospholipids (APLs) represent a group of membrane-permeable compounds with antineoplastic properties and a broad range of clinical applications. For example, edelfosine (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine) has been used as a purging agent in autologous bone marrow transplantation [1]. Topical application of miltefosine (hexadecylphosphocholine) was shown to be an effective therapy for skin metastases of breast cancer [2] and cutaneous lymphomas [3]. Oral administration of miltefosine is successfully used in the treatment of visceral leishmaniasis, a systemic protozoal infection [4]. The most recent derivative, perifosine (octadecyl-(1,1-dimethyl-piperidinio-4-yl)-phosphate), has been evaluated as an oral anticancer drug in clinical phase I [5,6] and II [7,8] studies, and as a potential radiosensitizer in a clinical phase I study that we recently concluded [9].

APLs differ from most of the currently used cytotoxic drugs with respect to their cellular targets. APLs primarily act on cell membranes where they accumulate in sphingolipid-enriched or cholesterol-enriched microdomains, known as lipid rafts [10]. Following raft-dependent internalization, these compounds interfere with the rapid and continuous phospholipid turnover that is essential for cell survival [11,12]. This interference occurs at different levels: edelfosine and miltefosine inhibit phosphoinositide-specific phospholipase C and the consequent formation of the second messengers, diacylglycerol and inositol 1,4,5-trisphosphate [13,14]. In addition, both APLs inhibit phosphatidylcholine (PC) turnover at the level of both PC degradation and PC resynthesis [11,15,16]. The latter inhibition occurs at the level of phosphocholine cytidylyltransferase [17,18], the rate-determining enzymatic step in PC biosynthesis. Signaling events, which occur downstream of these disturbing effects of APLs on lipid metabolism and signaling, include inhibition of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway [19,20], activation of proapoptotic stress-activated protein kinase/c-Jun N-terminal kinase

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signaling [21,22] and, as we and others reported more recently, inhibition of the Akt/protein kinase B (PKB) survival pathway [23,24]. These effects most likely contribute to a change in the balance between proapoptotic and antiapoptotic signaling. Indeed, APLs are potent inducers of apoptosis in a variety of tumor cell lines [25–27]. In addition, APLs enhance radiation-induced and chemotherapy-induced cytotoxicity, both *in vitro* [22,28–32] and *in vivo* [32].

Angiogenesis is the outgrowth of new blood vessels from preexisting ones and occurs during development, but normally stops at maturity. In the healthy adult, it is only found in the endometrium and ovaries during the menstrual cycle and in conditions associated with tissue repair and inflammation. Angiogenesis is increased in a number of diseases including rheumatoid arthritis, diabetic retinopathy and cancer. This increase is accompanied by changes in the behavior of endothelial cells (ECs), which are reflected as a large increase in their proliferation rate, increased migration and invasion into the extracellular matrix and the formation of new tubular structures. The increased vascular bed nourishes the malignant tissue and accelerates the growth of many tumors. In the last two decades, not only have the mechanisms and factors that underlie the angiogenic process become better known, but insight into its possibilities has also increased: the potential implication that inhibition of the angiogenic process can contribute to the treatment of solid tumors [33–38]. In this context, the recombinant humanized anti-vascular endothelial growth factor (VEGF) monoclonal antibody bevacizumab represents an apposite example of an antiangiogenic approach that increases the effectiveness of chemotherapy and radiotherapy [33].

In the current studies, we used three well characterized and clinically relevant APLs (edelfosine, miltefosine and perifosine) to evaluate their effects on endothelial integrity. We found that each compound induced apoptosis in ECs of both human and bovine origin, depending on the proliferative status of the cells. Confluent, quiescent ECs were relatively resistant, whereas proliferating ECs were highly sensitive to APL-induced apoptosis. In addition, we investigated whether APLs were capable of interfering with angiogenesis *in vitro*. For these studies, two experimental models were selected. In the first, human microvascular ECs were cultured on top of a three-dimensional fibrin matrix and allowed to migrate and form an invasive capillary-like tubular network [39,40]. In the second model, bovine aortic ECs were grown in a collagen gel sandwich to reorganize and form sustained tubular structures [41]. In both models, APLs inhibited the formation of endothelial tube-like structures. We therefore conclude that, besides the preferential apoptotic effect on malignant cells, interference with angiogenesis might contribute to the antitumor effect of these compounds.

Materials and methods

Reagents

Miltefosine was purchased from Sigma Chemical Co. (Zwijndrecht, The Netherlands). Edelfosine was from Biomol (Plymouth Meeting, Pennsylvania, USA) and platelet-activating factor-18 (PAF-18) from ICN Biomedical Inc. (Aurora, Ohio, USA). [^{3}H]Edelfosine (specific activity 58 mCi/mmol) was synthesized by Moravek Biochemicals (Brea, California, USA). [^{14}C]Miltefosine (specific activity 42 mCi/mmol), perifosine and [^{14}C]perifosine (specific activity 31 mCi/mmol) were kindly provided by Zentaris GmbH (Frankfurt, Germany). These compounds were diluted in serum-free culture medium. Thrombin was purchased from Leo Pharmaceutic Products (Weesp, The Netherlands) and human fibrinogen from Chromogenix AB (Mölndal, Sweden). Factor XIII was generously provided by Dr H. Metzner and Dr G. Seemann (Aventis Behring, Marburg, Germany); basic fibroblast growth factor (bFGF) was obtained from PeproTech Inc. (London, UK); human recombinant tumor necrosis factor (TNF)-α was obtained from Biogent (Gent, Belgium) and human recombinant VEGF-A_{165} from ReliaTech (Braunschweig, Germany). A crude preparation of EC growth factor (ECGF) was prepared from bovine hypothalamus as described [42]. Collagen was purchased from Vitrogen 100 (Cohesion, Palo Alto, California, USA).

Cell culture

The human squamous carcinoma cell lines, A431 and HeLa, and human fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum, penicillin (50 units/ml) and streptomycin (50 μg/ml). Human monoblastic leukemia U937 cells and human T lymphoid leukemic Jurkat cells (J16; kindly provided by Professor J. Borst, The Netherlands Cancer Institute, Amsterdam, The Netherlands) were grown at a density between 0.1×10^{6} and 1×10^{6} cells/ml in Iscove's modified Dulbecco's medium (GIBCO-BRL), supplemented with 10% heat-inactivated fetal calf serum, penicillin (50 U/ml) and streptomycin (50 μg/ml). Before APL treatment, cells were resuspended in serum-free medium (RPMI-1640 or DMEM) and kept overnight. ECs from human umbilical vein ECs (HUVECs; kindly provided by Dr J.A. van Mourik, Sanquin, Amsterdam, The Netherlands) were cultured in plastic six-well plates, precoated with human fibronectin (2 mg/ml). The medium consisted of an equal mixture of RPMI-1640 and M199 (GIBCO-BRL), 20% (v/v) heat-inactivated pooled human serum, 2 mmol/l glutamine (Merck, Darmstadt, Germany), penicillin (100 U/ml), streptomycin (100 U/ml) and fungizone (2.5 μg/ml; GIBCO-BRL). When human serum had to be omitted from the medium,

0.5% human serum albumin (Sanquin, Amsterdam, The Netherlands) and human transferrin (20 μg/ml; Sigma) were added. Confluent monolayers were harvested by trypsinization, resuspended in medium and subcultured. Subcultured cells from passages 1 and 2 were used. The medium was replaced every 3 days. ECs of bovine aortic origin (BAECs; kindly provided by Dr Haimovitz-Friedman, Memorial Sloan-Kettering Cancer Center, New York, USA) were grown to confluence in low-glucose (1 g/l) DMEM (GIBCO-BRL), supplemented with 10% bovine calf serum, penicillin (50 U/ml) and streptomycin (50 μg/ml). For serum-free conditions, medium containing 0.5% bovine calf serum (BCS) was used. Confluent monolayers were either used for experiments or were further subcultured at a plating density of 0.75×10^5 cells/cm^2. Confluence (cell density of 6×10^5/cm^2 and $> 90\%$ of cells in G_0–G_1) was reached 4–5 days after plating. Human foreskin microvascular ECs (HMVECs) were isolated, cultured and characterized as previously described [43]. HMVECs were cultured on gelatin-coated dishes in M199 supplemented with 20 mmol/l HEPES (pH 7.3), 10% heat-inactivated pooled human serum, 10% heat-inactivated newborn bovine calf serum (NBCS), 150 μg/ml crude ECGF, 2 mmol/l glutamin, 5 U/ml heparin, 100 U/ml penicillin and 100 U/ml streptomycin. Cells were used after they had reached confluence and had been cultured without growth factor for at least 24 h. In some experiments proliferating ECs were used. For these studies, cultures were harvested at 1–2 days after plating, that is, during the exponential phase of cell growth (cell density 1.5×10^5/cm^2).

Apoptosis assay

Apoptosis was determined by either staining with the DNA-binding fluorochome bisbenzimide (Hoechst 33258; Sigma) [44], to detect morphological nuclear changes, or by propidium iodide staining and fluorescence-activated cell-sorting analysis [45], to determine the percentage of subdiploid apoptotic nuclei.

For the bisbenzimide staining, APL-treated cells were harvested at the indicated time points, washed once with phosphate-buffered saline (PBS) and resuspended in 3.7% (v/v) paraformaldehyde/PBS solution. After 10 min at room temperature, the fixative was removed and the cells were resuspended in 15 μl of PBS containing 16 μg/ml bisbenzimide. Following a 15-min incubation at room temperature, a 10-μl aliquot was placed on a glass slide, and 400 cells/slide were scored in duplicate for the incidence of apoptotic nuclear changes under an Olympus AH2-RFL fluorescence microscope using a BH2-DMU2UV exciter filter (Olympus Nederland BV, Zoeterwoude, The Netherlands).

For the propidium iodide staining, cells were seeded at 2×10^5 cells/ml, 100 μl/well in round-bottomed, 96-well microtiter plates in serum-free RPMI medium. Cells were lysed overnight in 200 μl of Nicoletti Buffer (0.1% sodium citrate, 0.1% Triton X-100 and 50 μg/ml propidium iodide) and the percentage apoptotic nuclei, recognized by their subdiploid DNA content, was determined on a FACScan (Becton Dickinson, San Jose, California, USA) using Lysys II software (Becton Dickinson).

Incorporation of alkylphospholipids

Cultures of confluent or proliferating BAECs were incubated in low-serum (0.5%) culture medium containing 15 μmol/l APL, traced with 0.05 μCi/ml radiolabeled compound. At various time intervals up to 2 h, the medium was removed and cells were washed three times with ice-cold PBS and subsequently lysed in 0.1 N NaOH. The incorporated radioactivity was quantified by liquid scintillation counting and normalized for total cell number.

In-vitro angiogenesis models

Two in-vitro angiogenesis models were used for studying the formation of tubular structures, as previously described [39–41]. For the first model, human fibrin matrices were prepared by addition of 0.1 U/ml thrombin to a mixture of 2.5 U/ml factor XIII (final concentrations), 2 mg/ml fibrinogen, 2 mg/ml sodium citrate, 0.8 mg/ml NaCl and 3 μg/ml plasminogen in M199 without indicator; 300-μl aliquots of this mixture were added to 48-well plates. After clotting at room temperature, the fibrin matrices were soaked with 0.5 ml M199 supplemented with 10% heat-inactivated pooled human serum and 10% heat-inactivated NBCS for 2 h at 37°C to inactivate the thrombin. Highly confluent HMVECs (0.7×10^5 cells/cm^2) were seeded in a 1.25 : 1 split ratio on the fibrin matrices and cultured for 24 h in M199 without indicator, supplemented with 10% heat-inactivated pooled human serum, 10% heat-inactivated NBCS and penicillin/streptomycin. Confluent monolayers of HMVECs were then stimulated with the indicated mediators (2.5 ng/ml TNF-α and 10 ng/ml bFGF or 25 ng/ml VEGF) for 8 to 10 days in the absence or presence of APL. Every second day the culture medium was removed, and fresh medium containing appropriate mediators and test compounds was added. An important feature of this model is that it does not allow ECs to proliferate. Instead, cells migrate and invade the underlying matrix. The formation of capillarylike tubular structures of ECs in the three-dimensional fibrin matrix was analyzed by phase-contrast and dark-field microscopy. The total length of capillary-like tubular structures of six randomly chosen microscopic fields (7.3 mm^2/field) was measured using a Nikon FXA (Nikon, Tokyo, Japan) microscope equipped with a monochrome CCD camera (MX5; Nikon) connected to a computer with Optimas image analysis software (Tokyo, Japan), and expressed as mm/cm^2.

For the second model, 0.25 ml of collagen solution (5 ml of collagen, 1 ml of 10 × DMEM, 1 ml of 0.1 M NaOH, 1 ml of 0.1 mol/l Na_2CO_3 and 2 ml of 1 × DMEM) was placed in a 24-well plate. After polymerization, 350 000 cells/0.5 ml of DMEM supplemented with 0.5% BCS were evenly distributed in the wells. After the cells were attached, the medium was aspirated, and the top layer of collagen was added. After polymerization, medium with APL, was added and incubated for 24–48 h at 37°C. After incubation, the cells were fixed with 3% paraformaldehyde. In this model, ECs reorganize themselves, mimicking the resolution phase of angiogenesis; the readout used is the total additive sprout length [41].

Statistical analyses

Statistical analyses of the data were performed by standard procedures, using Student's *t*-tests. Differences were considered significant when *P* values were smaller than 0.05.

Results

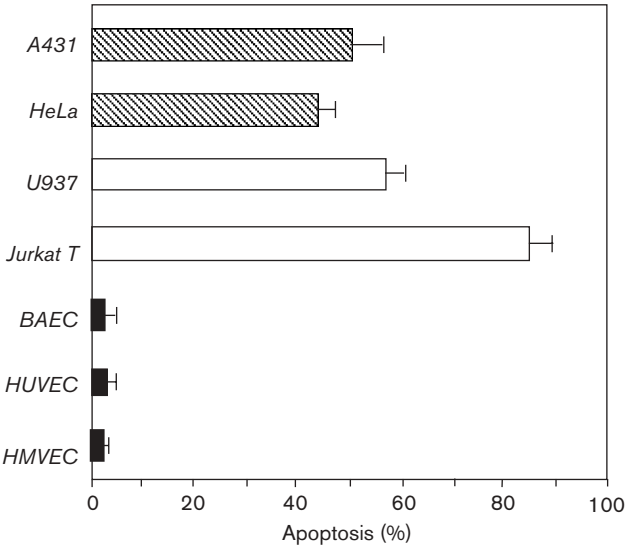
Alkylphospholipid-induced apoptosis in malignant versus normal cells

Three clinically relevant APLs (edelfosine, miltefosine and perifosine) were assayed for their capacity to induce apoptosis in a panel of cancer cell lines and a variety of normal cell types. As illustrated in Fig. 1, edelfosine induced apoptosis in all the human tumor cell lines tested, both of solid (A431 and HeLa) and leukemic origin (U937 and Jurkat T). In contrast, three types of confluent normal vascular ECs (BAECs, HUVECs and HMVECs) failed to undergo apoptosis after APL treatment. This resistance was observed in all three compounds, even after doses as high as 30 μmol/l (not shown). Table 1 shows the ED_{50} values in the different tumor cell lines for the three APLs used. The most potent APL was edelfosine, which is considered as the prototype for this group of compounds.

Alkylphospholipid-induced apoptosis in confluent versus proliferating endothelial cells

We observed a striking difference in the propensity to undergo APL-induced apoptosis between confluent and proliferating ECs (Fig. 2). As discussed above and consistent with our previous observations [22], APLs did not induce significant levels of apoptosis in confluent cultures of BAECs, HUVECs and HMVECs. Up to concentrations of 25 μmol/l for 48 h, APLs exerted no significant effect on EC viability in confluent culture (not shown). As shown in Fig. 2a, exponentially proliferating ECs, however, showed a dose-dependent increase in apoptosis after edelfosine treatment. Similar differences in apoptosis sensitivity between confluent and proliferating cells were observed in HUVECs and HMVECs (Fig. 2b), after treatment with the two other APLs (not shown).

Fig. 1



Alkylphospholipid-induced apoptosis in tumor cells (A431, HeLa, U937 and Jurkat T) and normal confluent endothelial cells [ECs; bovine aortic origin ECs (BAECs), human umbilical vein ECs (HUVECs) and human foreskin microvascular ECs (HMVECs)]. Apoptosis was determined at 16 h after treatment with 10 μmol/l edelfosine by FACScan analysis after propidium iodide staining. Data are expressed as mean ± SD from three independent experiments.

Table 1 Alkylphospholipid-induced apoptosis in a panel of human carcinoma and leukemic cell lines

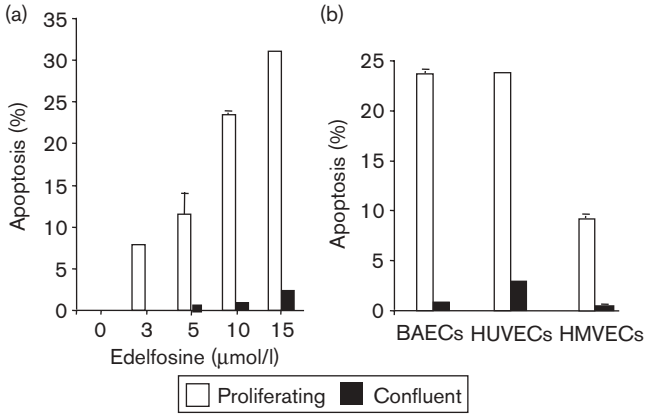
	ED_{50} (μmol/l)		
Cell type	Edelfosine	Miltefosine	Perifosine
A431	15.4 ± 2.9	17.2 ± 3.0	23.1 ± 2.7
HeLa	5.1 ± 1.6	8.1 ± 0.4	9.2 ± 1.8
U937	6.2 ± 0.3	7.9 ± 2.3	10.3 ± 1.2
Jurkat T	5.0 ± 1.3	8.0 ± 1.9	8.2 ± 0.6

ED_{50} values were calculated from full dose–response curves at $t = 16$ h. Apoptosis was quantified by *bis* benzimide staining. Data are expressed as mean ± SD from three independent experiments.

Incorporation of alkylphospholipids in confluent versus proliferating endothelial cells

As it has been shown that the cytotoxic effect of APLs correlates with its cellular uptake [25,46], we measured the incorporation of [^{3}H]edelfosine, [^{14}C]miltefosine and [^{14}C]perifosine in cultures of confluent and proliferating ECs. We found that proliferating BAECs incorporated much larger amounts of APLs than confluent BAECs, namely by a factor of approximately 3–4 at 2 h (Fig. 3a). The kinetics of APL uptake was also different, with a more rapid and prolonged uptake in proliferating BAECs. Figure 3b shows the uptake of edelfosine over a period of 2 h. The kinetics observed for miltefosine and perifosine was similar (not shown). In confluent BAECs, the uptake reached its maximum at about 30 min after addition. It should be noted that the incorporation of APLs in

Fig. 2



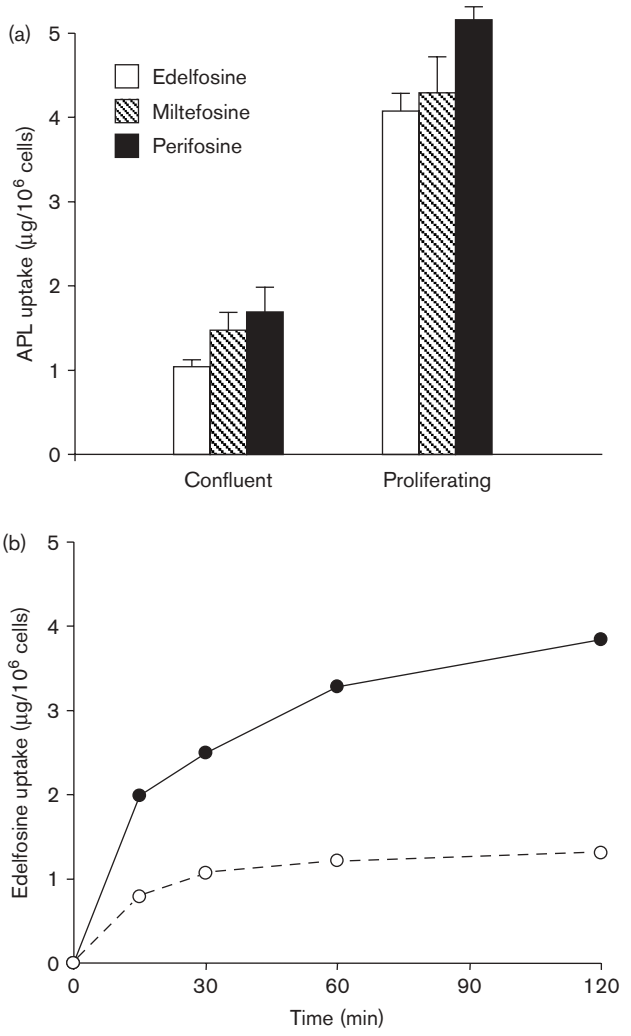
Alkylphospholipids induce apoptosis in proliferating but not in confluent endothelial cells (ECs). (a) Dose–effect relationship of edelfosine-induced apoptosis at 24 h, in proliferating and confluent bovine aortic origin ECs (BAECs). (b) Apoptosis induced by edelfosine (10 μmol/l) in proliferating and confluent BAECs, human umbilical vein ECs (HUVECs) and human foreskin microvascular ECs (HMVECs) at 24 h. For (a) and (b), apoptosis was determined by FACScan analysis after propidium iodide staining. Data are expressed as mean ± range from two independent experiments.

proliferating BAECs preceded the appearance of apoptotic morphology that was detected after 4–6 h (not shown).

Effect of alkylphospholipids on endothelial tube formation *in vitro*

As endothelial proliferation and apoptosis are both major determining factors in angiogenesis, we reasoned that APLs might interfere with new vessel formation. To test this hypothesis, we employed two in-vitro angiogenesis models. It is important to note that both models do not allow endothelial proliferation, thereby excluding an antiproliferative effect of APLs as the main cause of their antiangiogenic properties. In the first model, described by Koolwijk *et al.* [39,40], HMVECs are seeded on a three-dimensional human fibrin matrix to form a confluent monolayer. In the continuous presence of the combination of an angiogenic factor (VEGF or bFGF) and TNF-α, outgrowth of capillarylike tubular structures in the fibrin matrix is observed over a period of 8–10 days. The total length of these tubular networks is quantified by computer-assisted image analysis [39]. Figure 4 shows a set of phase-contrast microscopy images of a representative experiment. In the unstimulated cultures, the confluent monolayer of HMVECs remained on top of the three-dimensional fibrin matrix. Invading ECs and tubular structures could not be observed (Fig. 4a). The addition of bFGF or TNF-α alone was not sufficient to induce tube formation (not shown).The simultaneous addition of bFGF and TNF-α, however, resulted in the outgrowth of tubular structures invading the fibrin matrix and forming a capillary network (Fig. 4b). The number of ECs on top of the fibrin matrix was not significantly

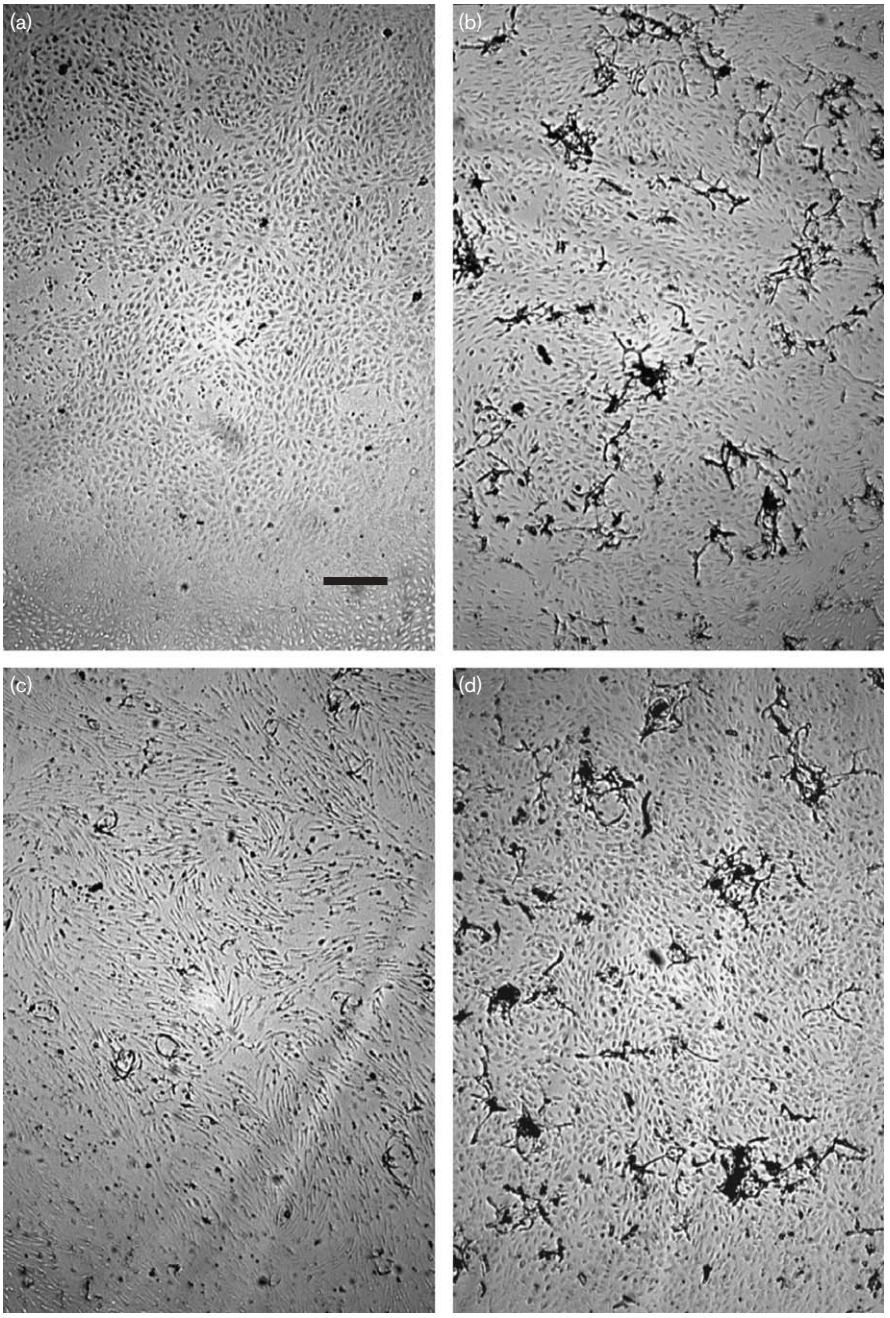
Fig. 3



Incorporation of radiolabeled alkylphospholipid (APL) in proliferating (●) and confluent (○) bovine aortic origin endothelial cells (BAECs). At the indicated time points after addition of 0.05 μCi/ml [^{3}H]edelfosine, [^{14}C]miltefosine or [^{14}C]perifosine (final APL concentration 15 μmol/l), the incorporation of the compound was measured by liquid scintillation and normalized for total cell number. (a) APL uptake at 2 h. Data are expressed as mean ± range from two independent experiments. (b) Time course of [^{3}H]edelfosine uptake. Data shown are representative of three experiments performed.

changed compared with unstimulated cultures (95% of control; not shown). In the presence of APL, a significant inhibition in the formation of tubular structures was observed. Figure 4c shows the effect of 100 μmol/l edelfosine. The morphology of the endothelial monolayer covering the fibrin matrix was slightly altered, but no significant detachment of cells was observed. The specificity of APL-induced interference with tube formation was demonstrated by the use of PAF-18, a structurally related but ineffective counterpart of edelfosine [25]. The addition of PAF-18 up to 100 μmol/l did not significantly affect the outgrowth of tubular structures (Figs. 4d and 5).

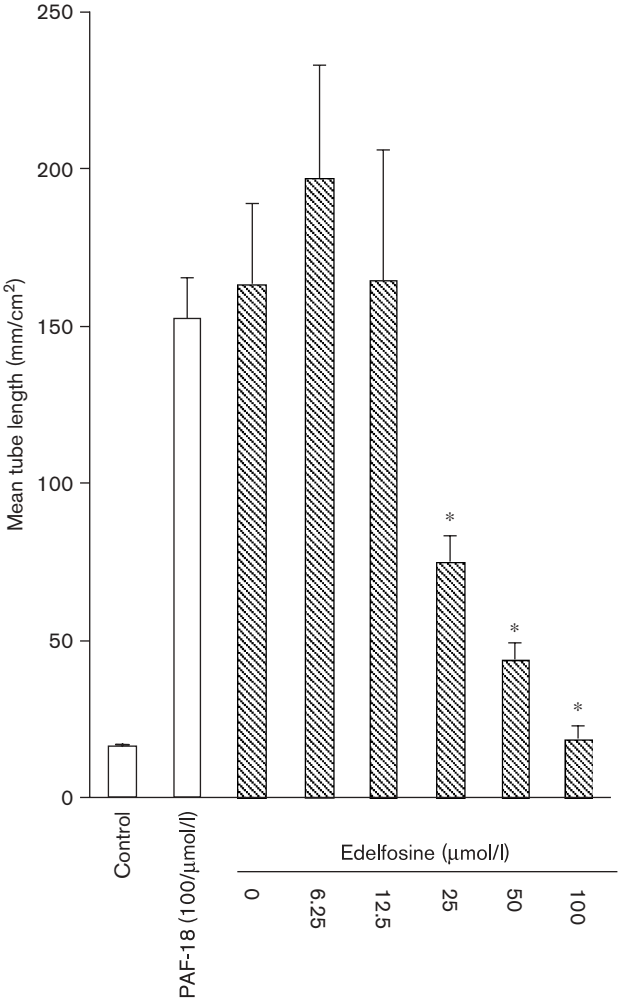
Fig. 4



Capillary-like tube formation is inhibited by alkylphospholipids. Human foreskin microvascular endothelial cells cultured on top of a three-dimensional fibrin matrix were either not stimulated (a), or stimulated with 10 ng/ml basic fibroblast growth factor (bFGF) and 2.5 ng/ml tumor necrosis factor (TNF)-α (b), or with bFGF and TNF-α in the presence of 100 μmol/l edelfosine (c) or with bFGF and TNF-α in the presence of 100 μmol/l platelet-activating factor-18 (d). After 8 days of culture, representative phase-contrast photographs were taken (bar: 300 μm). Similar results were obtained in three independent experiments.

The inhibitory effect of edelfosine on bFGF/TNF-α-induced tube formation was dose-dependent, as shown in Fig. 5. At 25 μmol/l, this inhibition was 54% of controls and reached statistical significance. At 100 μmol/l, the inhibition was complete. We note that the final concentration of APL in the angiogenesis studies was kept higher than in the apoptosis assays, as the higher serum concentration in the former type of experiments sequesters APLs and thus diminishes the effective concentration by a factor of 2–3. To assure that the cell membrane integrity was not impaired under these conditions, we performed a separate set of standard

Fig. 5



Dose-dependent inhibition by alkylphospholipids of capillary-like tube formation. Human foreskin microvascular endothelial cells seeded on top of a three-dimensional fibrin matrix were either not stimulated (control) or stimulated with 10 ng/nl basic fibroblast growth factor and 2.5 ng/ml tumor necrosis factor-α in the presence of increasing amounts of edelfosine or 100 µmol/l platelet-activating factor-18 (PAF-18). After 8 days of culturing, total tube length/cm^2 ± SD of triplicate wells was measured (*$P < 0.005$ compared with 0 µmol/l edelfosine). Similar results were obtained in three independent experiments.

culture experiments in which endothelial lactate dehydrogenase release and Trypan blue exclusion were measured after APL treatment. No significant changes in either parameter was found in APL-treated EC cultures compared with controls, confirming the viability of the cells (data not shown).

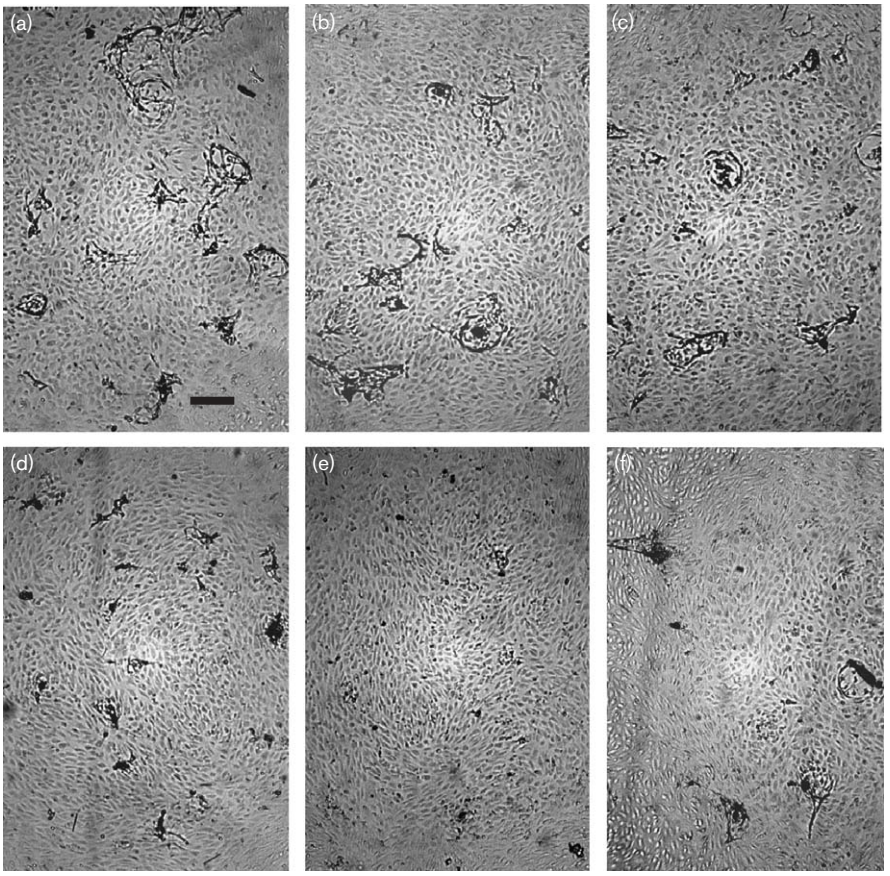
Next, we introduced another angiogenic factor into this system and investigated the effect of other APLs on tube formation. Just as we had observed in the case of bFGF (Fig. 6a), VEGF added along with TNF-α to the endothelial monolayers induced the formation of tubular structures (Fig. 6d). Furthermore, similar to edelfosine, miltefosine (Fig. 6b and e) and perifosine (Fig. 6c and f) interfered with the outgrowth of endothelial tubes by both bFGF/TNF-α and VEGF/TNF-α.

To confirm these inhibitory effects of APLs on tube formation, we employed a second model [41] using ECs of bovine origin. In this model, BAECs are seeded between a collagen sandwich and allowed to reorganize and rapidly form tubular structures within 48 h (Fig. 7a and e). Similar to its action on tube formation by the HMVECs grown on a fibrin matrix, APL inhibited the tube formation by BAECs in a collagen sandwich as well. Figure 7b–d shows the dose-dependent interference with tube formation by edelfosine. At 20 µmol/l and higher, no tubular structures could be discerned; quantification of the total sprout length was therefore not possible. Miltefosine (Fig. 7f) and perifosine (Fig. 7g) inhibited this process in a comparable fashion.

Discussion

This study was undertaken to investigate the effect of three clinically relevant APLs on normal vascular ECs, in comparison with a panel of tumor cell lines, and to study the antiangiogenic properties of these compounds *in vitro*. Edelfosine, miltefosine and perifosine induced a time-dependent and dose-dependent increase in apoptosis in a variety of human leukemic and solid tumor cell lines. Importantly, the ED_{50} values we found here fall within the same micromolar range as those obtained in plasma from APL-treated patients [5,6,9]. In our phase I study, we measured a dose-dependent steady-state plasma concentration of perifosine between 4 and 23 µmol/l, which was maintained throughout the 4-week treatment period [9]. In contrast to the effect on tumor cells, three types of normal quiescent ECs were insensitive to APL-induced apoptosis. This differential cytotoxic effect of APLs is consistent with data obtained from other cell systems [25,27,47], and offers a solid basis for further clinical evaluation of these compounds as selective anticancer drugs. Another attractive biological property of APLs is their capacity to strongly enhance radiation-induced apoptosis of tumor cells *in vitro* and *in vivo*, as we described recently [22,28,32]. In these studies we observed a remarkable difference in APL-induced apoptosis between confluent, resting versus actively proliferating ECs. These findings are consistent with those of Araki *et al.* [48], who reported on apoptosis induced by the structurally related compound Et-16-OCH_3 (edelfosine = Et-18-OCH_3) in subconfluent cultures of HUVECs. We found that APL-induced endothelial apoptosis correlated with the cellular uptake of the compound. Proliferating ECs incorporated large amounts of APL, resulting in significant levels of apoptosis. In contrast, in quiescent ECs, the uptake of APL was only one third of that of the proliferating cells, and was insufficient to induce significant apoptotic cell death. The relationship

Fig. 6

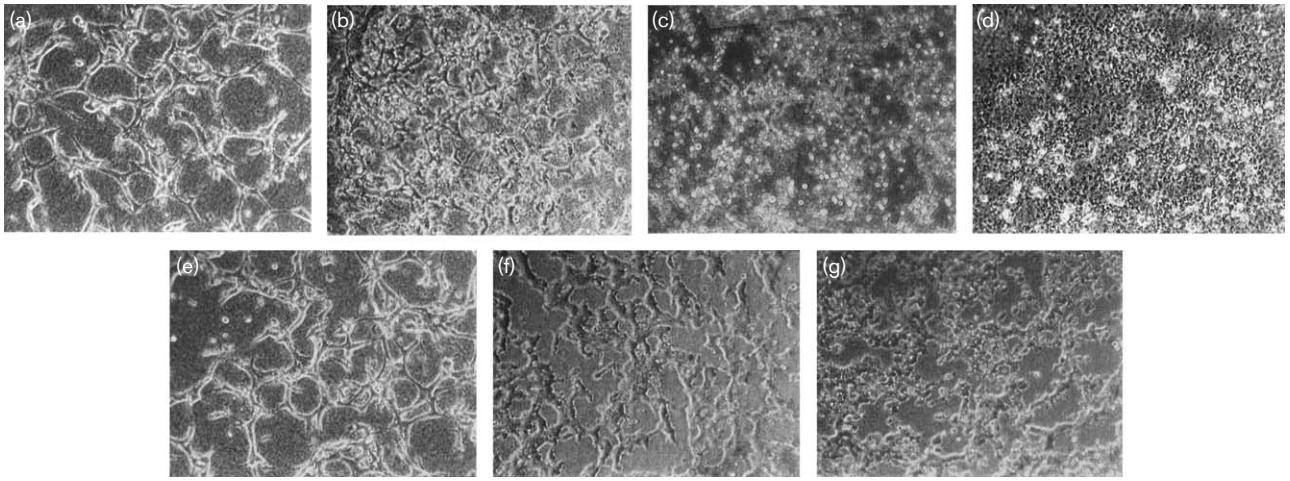


Different alkylphospholipids (APLs) inhibit basic fibroblast growth factor (bFGF)/tumor necrosis factor (TNF)-α-mediated and vascular endothelial growth factor (VEGF)/TNF-α-mediated tube formation. Human foreskin microvascular endothelial cells cultured on top of a three-dimensional fibrin matrix were stimulated either with 10 ng/nl bFGF and 2.5 ng/ml TNF-α (a–c) or with 25 ng/ml VEGF and 2.5 ng/ml TNF-α (d–f). No APL was added (a,d); 50 μmol/l miltefosine was added (b,e) and 50 μmol/l perifosine was added (c,f). After 8 days of culture, representative phase-contrast photographs were taken (bar: 300 μm). Similar results were obtained in three independent experiments.

between APL uptake and apoptosis sensitivity is emerging as a more general phenomenon. Mollinedo *et al.* [25] demonstrated that upon transformation with SV40, 3T3 fibroblasts became sensitive to edelfosine and incorporated high amounts of the lipid. Similarly, apoptosis sensitivity was restored in human T lymphocytes after activation with mitogens [49]. The amount of APL incorporated by the EC, most likely in combination with the enhanced metabolic activity of the cell, thus apparently dictates the biological effect. As endothelial apoptosis has been identified as an important determinant in tumor angiogenesis [50–52], these observations prompted us to study the antiangiogenic properties of APLs *in vitro*.

Angiogenesis is a complex and tightly regulated process of new blood vessel formation from preexisting vasculature. Its role in tumor growth and metastases has now clearly been established, and several strategies of antiangiogenic therapy have been developed and tested clinically [33–35]. During angiogenesis, several phases can be distinguished: (i) degradation of the basement membrane, (ii) endothelial migration and invasion in the extracellular matrix, (iii) endothelial proliferation and (iv) the formation of capillary-like tubes [36]. A large number of angiogenic factors have been identified in recent years, including VEGF and bFGF [37,38]. The formation of capillary-like structures can be studied *in vitro*, using different model systems. For our experiments, we employed two well characterized models. The first consists of a three-dimensional human fibrin matrix covered by human microvascular ECs [39,40]. This model mimics the in-vivo situation in which fibrin is a common component of the matrix that is present at sites of chronic inflammation and tumor stroma [53]. Both an angiogenic factor (bFGF or VEGF) and a factor to induce urokinase-type plasminogen activation (e.g. TNF-α) are required in this in-vitro model, to induce endothelial migration and the formation of capillary-like tubular structures without endothelial proliferation [39,40].

Fig. 7



Alkylphospholipid (APL)-induced interference with endothelial tube formation. Bovine aortic origin endothelial cells were seeded between a collagen sandwich and allowed to rapidly reorganize and form capillary-like tubular structures within 48 h. No APL added (a,e); 10, 20 and 50 μmol/l edelfosine added (b–d), 10 μmol/l miltefosine added (f) and 10 μmol/l perifosine added (g). Similar results were obtained in three independent experiments. Magnification 100 ×.

These studies demonstrate that APLs are efficient inhibitors of both VEGF/TNF-α-induced and bFGF/TNF-α-induced tube formation from preexisting monolayers of confluent HMVECs. Moreover, the structurally related but ineffective compound PAF-18 failed to interfere with this process. In addition, in a second reorganization model using a collagen sandwich and ECs of bovine origin, APLs interfered with tube formation (Fig. 7). The antiangiogenic action of APLs cannot be explained by an antiproliferative effect, as both models do not allow endothelial proliferation. Instead, these models study endothelial migration and reorganization. It is also unlikely that extensive cytotoxic effects can account for the inhibition of angiogenesis, as the endothelial monolayer remains intact throughout the observation period (Figs. 4 and 6).

It remains to be established which of the signal-transduction pathway(s) are important for the apoptotic and antiangiogenic effects of APLs. In this context, we have previously shown that APLs activate the proapoptotic stress-activated protein kinase/c-Jun N-terminal kinase pathway. In addition, APLs efficiently prevent MAPK/ERK signaling induced by serum and growth factors, both in tumor cells and ECs [19,20,22,54]. More recently, we found that APLs also inhibit the Akt/PKB survival pathway [23]. These signaling systems are not only important for cell death and survival, but have been implicated in angiogenesis as well [55–58]. In different in-vitro and in-vivo angiogenesis models, it has been shown that the blockade of the MAPK/ERK or Akt/PKB pathway by pharmacological or molecular approaches induces apoptosis and inhibits angiogenesis [55,56,59]. Our current line of research is focused on the identification of additional, critical (intra)cellular targets of APLs [10].

In conclusion, our data show that not only tumor cells, but also normal ECs can be targets for APLs. The cytotoxic effect, however, depends on the proliferative status, with actively dividing cells incorporating more APL and thus being apoptosis sensitive. Furthermore, we demonstrated that APLs are effective inhibitors of endothelial capillary-like tube formation *in vitro*. Taken together, these results support the concept that APLs exert their antitumor effect both directly through apoptosis and indirectly, through interference with the angiogenic process.

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