Cancer Chemother Pharmacol (1998) 41: 326–332

ORIGINAL ARTICLE

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The antineoplastic ether lipid, s-phosphonate, selectively induces apoptosis in human leukemic cells and exhibits antiangiogenic and apoptotic activity on the chorioallantoic membrane of the chick embryo

Received: 10 March 1996 / Accepted: 14 July 1997

Abstract *Introduction*: We investigated the cytotoxic and antiangiogenic activity of the ether lipid, 2′-(trimethylammonio)ethyl 4-(hexadecyloxy)-3(*S*)-methoxybutane-phosphonate (termed s-phosphonate). *Method*: Cytotoxicity was determined using an XTT bioassay. Apoptosis was measured by either DNA fragmentation or immunolabelling techniques. Angiogenesis was measured using the in vivo chorioallantoic membrane (CAM) of the chick embryo. *Results*: s-phosphonate was selectively cytotoxic towards the human leukemic cell lines, HL-60 and AML-14, whereas leukemic K-562 cells and the murine mast cell line, MC-9, were resistant to this agent at concentrations as high as 50 μ*M*. This selectivity resulted from the induction of apoptosis (or programmed cell death) by s-phosphonate in HL-60 and AML-14 cells but not in resistant K-562 or MC-9 cells. S-phosphonate induced localized antiangiogenic effects and membrane thinning in the CAM. This concentration-dependent antiangiogenic effect was associated with apoptosis in the CAM as measured by DNA fragmentation in extracted CAM tissue. The localized areas of membrane thinning and antiangiogenesis on the CAM caused by s-phosphonate were also the only areas of the membrane in which apoptosis occurred. *Conclusion*: We conclude that s-phosphonate selectively induces apoptosis in human leukemic cells and exhibits antiangiogenic and apoptotic activity on the CAM.

Key words S-phosphonate · Apoptosis · Antiangiogenesis

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Introduction

Synthetic ether lipids such as 1-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3) are a new class of antineoplastic agent. Cytotoxic ether lipids do not interact directly with DNA but accumulate in the plasma membrane of cells where they exert a variety of effects such as enzyme inhibition, membrane fluidization and intracellular signalling events [1–3]. ET-18-OCH_3 has been recently reported to inhibit tubule formation in the human microvasculature endothelial cell line HMEC-1, indicating antiangiogenic activity in vitro [4]. Angiogenesis, the process of new blood vessel growth, is fundamental in various physiological conditions and is induced by many pathological states such as tumor development, rheumatoid arthritis and neovascular diseases of the eye [5, 6]. Since angiogenesis is the basis of such conditions, many researchers are developing antiangiogenic agents as a treatment for malignancy or as an adjunct to standard chemotherapeutic regimens.

The mechanisms of action by which ET-18-OCH_3 acts as an antineoplastic or antiangiogenic agent are unknown. The cytotoxic activity of ET-18-OCH_3 has been investigated in many studies using sensitive cell lines such as leukemic HL-60 cells and resistant cell lines such as K-562 cells. ET-18-OCH_3 is reported to induce apoptosis in HL-60, Jurkat T lymphoid, U937 and HELA cells, but not in K-562 or fibroblast 3T3 cells [1, 3, 7–9]. This variation in the induction of apoptosis in different cells by ET-18-OCH_3 may explain the selective toxicity of this ether lipid.

Recently, we have described the potent antineoplastic effects of a novel isosteric analogue of ET-18-OCH_3,

s-phosphonate, that contains a methylene group substituted for an oxygen atom connecting phosphorus to glycerol at the sn-3 position. [2′-(trimethylammonio) ethyl 4-(hexadecyloxy)-3(*S*)-methoxybutane-phosphonate]. This substitution may render s-phosphonate immune to degradation by phospholipases that hydrolyze agents such as ET-18-OCH_3 at the sn-3 position [2]. In colony-forming assays, s-phosphonate has been shown to have an antineoplastic effect equal to that of ET-18-OCH_3 with an IC_{50} value (the concentration required to produce 50% inhibition of cell growth) in the 2–5 μ*M* concentration range [2, 10].

In this study, we demonstrated that s-phosphonate selectively induces apoptosis in HL-60 and AML-14 cells but not in K-562 or MC-9 cells, using DNA fragmentation analysis. Furthermore, by encapsulating s-phosphonate into a slow-release polymer (polycaprolactone, PCL) we investigated the antiangiogenic and apoptotic activity of the compound using an in vivo bioassay, the chorioallantoic membrane of the chick embryo (CAM). We demonstrated that s-phosphonate was a potent inhibitor of angiogenesis and induced apoptosis in the cells of the CAM after a 2-day exposure. This activity was localized at the site of s-phosphonate/PCL application on the CAM and was accompanied by pronounced membrane thinning.

Materials and methods

Cells

All cells were grown in a humidified incubator in an atmosphere containing 5% CO_2 in RPMI-1640 medium (Gibco Burlington, Oreg.) supplemented with 10% fetal bovine serum albumin (Intergen) with 2.5 units/ml penicillin, 2.5 μg/ml streptomycin (Sigma, St. Louis, Mo.) and 40 μ*M* 2-mercaptoethanol. HL-60 is a human promyelocytic leukemia cell line; K-562 is also a human leukemic cell line; AML-14 cells were derived from a human patient with acute myeloid leukemia [11] and were kindly provided by Drs. Cassandra Paul and Michael Baumann. MC-9 is a murine mast cell line that is cytokine-dependent and was grown in the presence of 5–10% WEHI-3-conditioned medium.

DNA fragmentation assay

Assays to detect fragmented DNA indicative of cells undergoing apoptosis were performed as described previously [12]. Briefly, cells or excised CAM tissue sections were solubilized in 400 μl lysis buffer (0.6% SDS and 10 m*M* EDTA, pH 8.0). Following the addition of 100 μl 5 *M* NaCl, the samples were incubated overnight at 4 °C, then pelleted by centrifugation. Supernatants containing DNA fragments were transferred to clean tubes. RNase A (1 μl, 1 mg/ml) was added and samples were incubated for 20 min at 37 °C. To the tubes was added 500 μl of a 1:1 mixture of Tris-buffered phenol, pH 8.0 and chloroform. The samples were briefly vortexed and the aqueous layer retained. To this layer were added 55 μl 3 *M* sodium acetate and 1 ml ice-cold absolute ethanol. After 10 min at −20 °C, the DNA was pelleted, washed with 70% ethanol and separated by electrophoresis on a 2% agarose/tris/boric acid/EDTA(TBA) gel. Visualization of DNA bands was performed by staining with ethidium bromide and viewing on a UV transilluminator. Video images of the stained gels were obtained using a Gelprint 2000 system (Biochem Scientific, Missisauga, Oreg.).

Bioassay

The XTT bioassay was performed essentially as described previously [13]. Cells were washed three times and used at 5×10^5 per ml in 96-well plates. Following incubation for 24 h in 100 μl per well, 25 μl of medium containing 1.0 mg/ml XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) and 25 μ*M* phenazine methosulfate was added and incubation continued for 4 h. Plates were shaken briefly and absorbance read in a plate reader at 450 nm. For cytokine-dependent cells, wells containing cells incubated in the absence of cytokines were used as the blank. For cytokine-independent cells, wells containing medium alone were used as the blank.

Polymer–drug encapsulation

PCL (Birmingham Polymers, Birmingham, Ala.) and s-phosphonate were levigated in the appropriate proportions at 55 °C for 2 min. The molten mixture was then pipetted into 3 mg semispherical pellets and allowed to set at 4 °C.

CAM bioassay

Fertilized, domestic chick embryos (Fitzsimmons Consulting & Research Services, Vancouver, B.C.) were incubated for 4 days and then windowed as described previously [14] with slight modifications. Briefly, a small hole (measuring approximately 2 cm in diameter) was formed by removing the shell and inner shell membrane from the blunt end of the egg (air space site) and then the exposed area was sealed with sterilized Parafilm wax. The egg was then placed into an incubator at 37 °C for an additional 2 days with the window upright. On day 6 of incubation, 3-mg pellets of s-phosphonate-loaded PCL or PCL with no drug (control) was placed on the surface of the growing CAM vessels. After a 2-day exposure (day 8 of incubation), the vasculature was examined using a stereomicroscope fitted with a Contax camera system. To increase the contrast of the vessels and to mask any background information, the CAM was injected with 1 ml of intralipid solution (Clinter-Baxter, Deerfield, Ill.) prior to imaging.

Light microscopy

Treated CAM and control tissue were fixed in situ, excised and processed using conventional light microscopy techniques. The tissue was stained with toluene blue and imaged using a light microscope fitted with a Contax camera system (Yashica, Somerset, NJ).

Apoptosis immunolabelling technique

Apoptosis was detected in the CAM using an Apoptag immunolabelling kit (Oncor, Gaithersburg, Md.). Briefly, treated CAM and control tissue were fixed in situ using 4% formalin. The tissues were then excised and sectioned using standard cryostat methods. These sections were tested for positive apoptosis labelling using the Apoptag immunolabelling kit and then viewed and imaged with a fluorescent microscope (Zeiss Axiophot).

Results

The ability of s-phosphonate to induce apoptosis was compared in several cell lines. HL-60 cells underwent apoptosis in the presence of 10 μ*M* s-phosphonate (Fig. 1), while another leukemia cell line, AML-14, was even more sensitive to the drug, with a maximal effect being observed with 5 μ*M* s-phosphonate. Both of these cell lines also underwent apoptosis in the presence of the

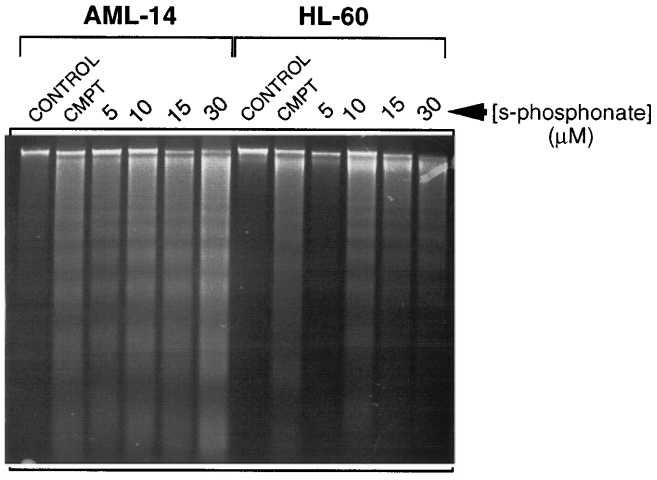

Fig. 1 Effect of s-phosphonate on AML-14 or HL-60 cells. Cells at 5×10^5/ml were incubated with vehicle (0.5% v/v ethanol), camptothecin (*CMPT*, 1 μg/ml) or s-phosphonate at the concentrations shown for 12 h and DNA fragmentation was measured

DNA topoisomerase inhibitor, camptothecin. Another human leukemic line, K-562, was resistant to the addition of up to 50 μ*M* s-phosphonate (results not shown). Interestingly, the same concentration of camptothecin used in HL-60 and AML-14 cell incubations was unable to induce DNA fragmentation in K-562 cells.

A murine cytokine-dependent cell line, MC-9, was also resistant to s-phosphonate at concentrations up to 50 μ*M* (Fig. 2). Withdrawal of cytokine from these cells resulted in DNA fragmentation, but incubation in the presence of either interleukin-3 (IL-3) or granulocyte-macrophage colony-stimulating factor (GM-CSF) inhibited apoptosis. In the presence of either cytokine, s-phosphonate had no effect.

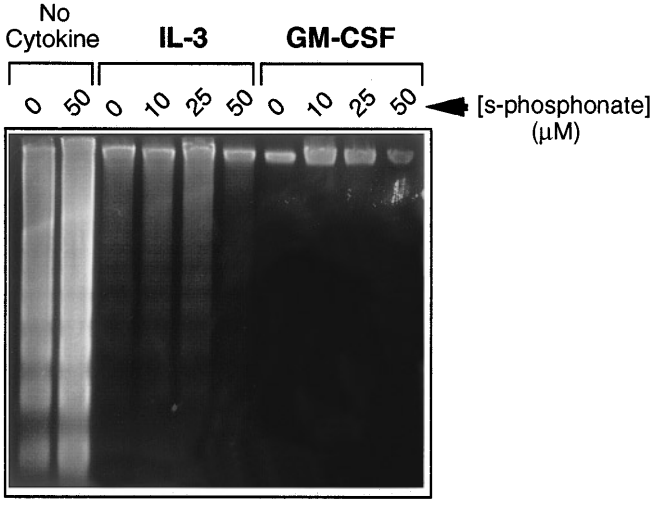

Fig. 2 Effect of s-phosphonate on MC-9 cells. Cells at 5×10^6/ml were washed three times in Hank's buffered salt solution pH 7.4 and resuspended in fresh medium containing no cytokine, 500 ng/ml IL-3 or 60 U/ml GM-CSF. Cells were then treated with vehicle (0.5% v/v ethanol) or s-phosphonate at the concentrations shown for 12 h, and DNA fragmentation was measured

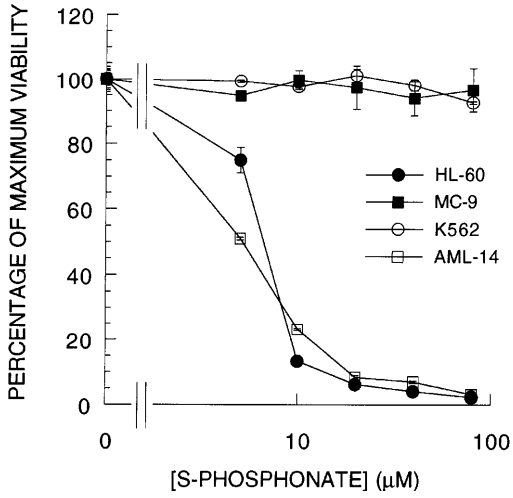

Fig. 3 Bioassay of cells incubated with various concentrations of s-phosphonate. Readings of absorbance at 450 nm were normalized to 100% for cells in the absence of drug. Values are the average +/− SD of samples done in triplicate. Cytokine-dependent MC-9 cells were incubated in the presence of 1 μg/ml synthetic IL-3. Other cells were incubated in RPMI with 10% fetal calf serum. The absolute values of the absorbance readings used as 100% viability for the different cell lines were: MC-9 0.561, K-562 1.558, HL-60 1.093, AML-14 0.457

Figure 3 shows the results of a bioassay in which the various cells were incubated in the presence or absence of increasing concentrations of s-phosphonate. Clearly, the sensitivity of the cells to s-phosphonate correlated with the induction of apoptosis by the drug. Similar to the effects observed with MC-9, two other human cytokine-dependent cell lines, UT-7 and TF-1, were also resistant to the effects of s-phosphonate in a bioassay, as well as in assays of apoptosis (results not shown).

Figure 4 (A, B, C, D and E, respectively) shows that PCL pellets loaded with s-phosphonate at 0%, 1%, 2%, 4% and 8% induced a dose-dependent antiangiogenic reaction in the CAM. The antiangiogenic reaction was characterized by the absence of blood vessels in the region directly below the s-phosphonate/PCL pellet. The normal growth of the dense capillary network seen in the control CAM (Fig. 4A) was clearly inhibited in the s-phosphonate-treated CAMs. At higher concentrations (4% and 8%), the treated CAMs were structurally altered in the vicinity of the s-phosphonate/PCL pellet. This alteration included a pronounced thickening of the CAM immediately adjacent to the s-phosphonate/PCL pellet and membrane thinning subjacent to the pellet. In all of the CAMs treated with s-phosphonate, an avascular zone was apparent after a 2-day exposure; this was defined as an area devoid of a capillary network measuring approximately 3 mm^2 in area.

Histologically, s-phosphonate at high concentrations (4% and 8%) induced significant structural alterations in the CAM (Fig. 5). These alterations were characterized by pronounced cell proliferation at the periphery of

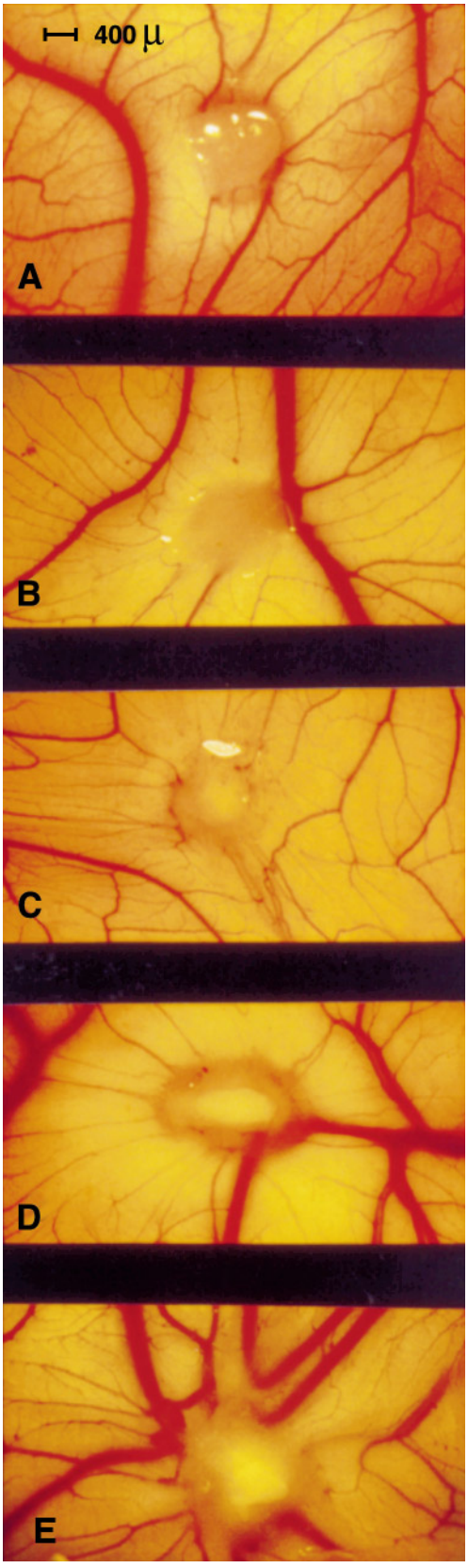

Fig. 4A–E Effect of s-phosphonate on angiogenesis in the CAM. Membranes were treated with (**A**) PCL alone (PCL pellet still in position on the CAM), (**B**) 1% s-phosphonate/PCL (pellet still on the CAM), (**C**) 2% s-phosphonate/PCL, (**D**) 4% s-phosphonate/PCL and (**E**) 8% s-phosphonate/PCL. **C**–**E** show CAMs with the s-phosphonate/PCL pellet removed

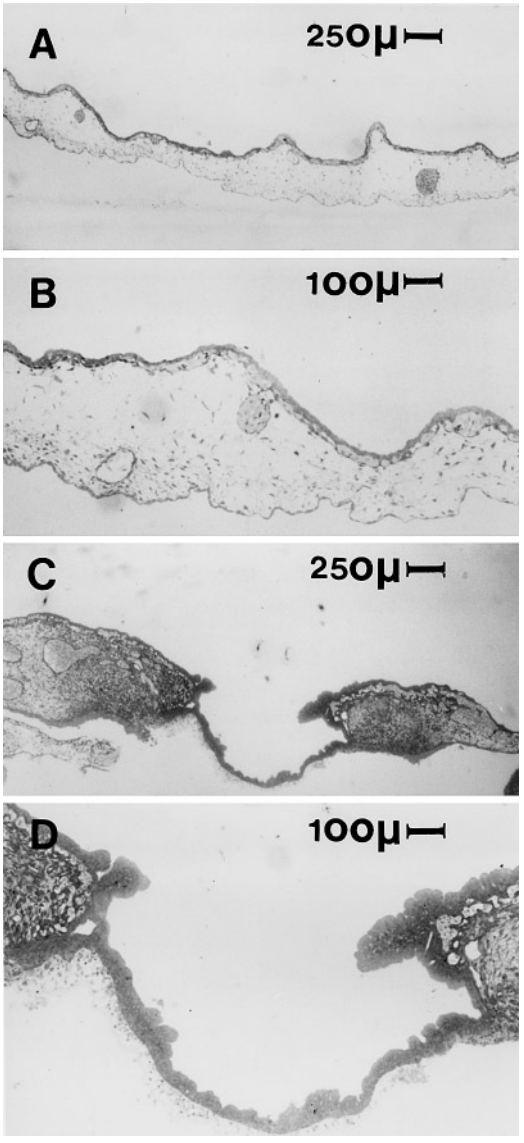

Fig. 5A–D Effect of s-phosphonate on CAM morphology. A–D Toluene blue-stained transverse sections of excised membranes subjacent to the position of PCL pellet application. **A**, **B** Control membranes (PCL alone); **C**, **D** membranes treated with 4% s-phosphonate/PCL

the treatment and significant thinning of the membrane immediately subjacent to the s-phosphonate/PCL matrix. At lower concentrations (1% and 2%) these alterations were significantly reduced or absent.

The CAM tissue directly subjacent to the s-phosphonate/PCL pellet was excised and analyzed for evidence of apoptosis. At higher concentrations (>2% s-phosphonate in PCL), extensive apoptosis was observed in the CAM, as shown in Fig. 6. Each lane in Fig. 6 represents DNA extracted from PCL-treated CAMs from separate embryos. No apoptosis was ever observed in membranes treated with PCL alone. Since extensive morphological changes had been observed to be localized in the area where the s-phosphonate/PCL pellet was positioned, the possibility of localized apoptosis in that area was also investigated using an

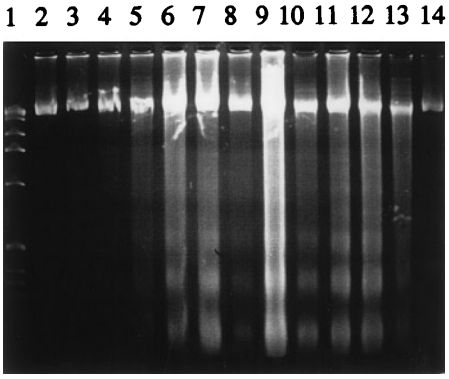

Fig. 6 Effect of s-phosphonate on the CAM showing DNA fragmentation in extracts from membrane sections excised from the region subjacent to s-phosphonate/PCL application. Each lane shows DNA extracted from a separate CAM: *lane 1* no extract (DNA marker ladder), *lanes 2–4* 1% s-phosphonate/PCL, *lanes 5–7*, 2% s-phosphonate/PCL, *lanes 8–10*, 4% s-phosphonate/PCL, *lanes 11–13*, 8% s-phosphonate/PCL, *lane 14* control membrane (PCL alone).

immunolabelling technique that pinpoints cells undergoing apoptosis in situ. Extensive apoptosis was observed in the thin part of the membrane subjacent to the application site of the s-phosphonate/PCL pellet as shown in Fig. 7. Nonapoptotic tissue was counterstained red by propidium iodide and apoptotic cells were stained yellow–green owing to the fluorescein-antidigoxin antibody binding to fragmented DNA labelled with digoxin. This experiment was repeated more than three times using CAMs treated with 4% s-phosphonate/PCL and always showed the same effect. A representative photomicrograph is shown in Fig. 7.

To investigate whether the inhibition of angiogenesis (Fig. 4) might specifically have resulted from s-phosphonate-induced apoptosis of endothelial cells, human umbilical vein endothelial cells (HUVEC) were grown in vitro and subjected to increasing concentrations of s-phosphonate. S-phosphonate was unable to induce apoptosis in either growing or quiescent-confluent HUVEC cells (data not shown). At high concentrations of s-phosphonate (100 μM) cytolysis of HUVEC cells was observed.

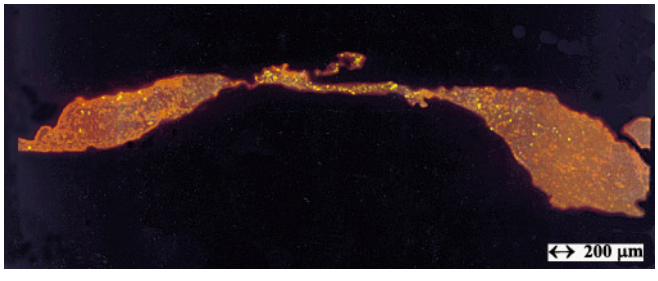

Fig. 7 Effect of s-phosphonate on the morphology and apoptotic activity of the CAM, showing a transverse section of an excised membrane subjacent to the position of 4% s-phosphonate/PCL application. Apoptotic cells are stained yellow–green against a red (propidium iodide) counterstain

Discussion

The mechanism of action of ether lipids as antineoplastic agents is poorly understood. It is now established that the action of ether lipids depends on the cellular accumulation of these compounds [15, 16], but the subsequent effects on membrane integrity and/or intracellular signalling and the connection to cytotoxicity remain unknown. The great potential of ether lipids as antineoplastic drugs is based on data showing that tumor and leukemic cells are more sensitive to the cytotoxic action of ether lipids than normal cells [1]. The most extensively studied cytotoxic ether lipid is ET-18-OCH_3. This compound has been shown to be selectively toxic to leukemic cells and has been successfully used to purge bone marrow in clinical trials [17]. The mechanism underlying the selectivity of ET-18-OCH_3 towards certain cancer cell lines has been suggested to be related to an accumulation of the compound in the plasma membranes of these cells as a result of a low enzymatic degradation rate [18, 19]. However, ET-18-OCH_3 has been shown to be susceptible to cleavage by phospholipase C and D [20] and the activities of these two cleavage enzymes have been found to be similar in both responsive and resistant cells [21].

S-phosphonate, which lacks the ester linkage at the sn-3 position, may be less susceptible to phospholipase degradation. This compound has been previously shown to be a potent antineoplastic agent as well as a potent cytolytic agent causing nonspecific perturbation of cell membranes by a mechanism similar to detergent-induced membranolysis. However the inclusion of serum proteins in the drug–cell incubation medium strongly inhibits this nonspecific lysis, so that the concentrations of s-phosphonate necessary to cause cell lysis are far greater than the antineoplastic concentrations [2, 10]. It is interesting to note that serum proteins have been shown to reduce the influx and antineoplastic effects of ET-18-OCH_3 in leukemic cells [22, 23]. Also, a maltosyl derivative of s-phosphonate has been previously shown to have a nonspecific cytolytic potential comparable to that of s-phosphonate without displaying any significant antineoplastic effects [10]. These reports indicate that the cytotoxic action of s-phosphonate is not related to its cytolytic action.

The results of this study show that the cytotoxic effect of s-phosphonate is a consequence of its ability to induce apoptosis in susceptible cells. In accordance with the results of studies by Diomede et al. [23], using ET-18-OCH_3, we found that HL-60 cells, but not K-562 cells, underwent apoptosis in the presence of s-phosphonate at a concentration of 10 μM. We also tested the effects of s-phosphonate on murine MC-9 cells, which are known to undergo apoptosis within as little as 6 h following withdrawal of the cytokine, as well as the human cell

line, UT-7. These cells were also resistant to the effects of the drug at concentrations as high as 50 μM. The cytotoxicity of the drug in a bioassay using these cells confirmed the dramatic differences observed in the assays of apoptosis. We have recently shown that both MC-9 and UT-7 cells undergo apoptosis in the presence of inhibitors of phosphatidylinositol 3-kinase; however, this was observed in cells incubated in the presence of IL-3, but not GM-CSF [12]. The results shown in Fig. 2 demonstrate that s-phosphonate did not induce apoptosis in cells incubated with either IL-3 or GM-CSF. Berggren et al. [24] have reported inhibition of phosphatidylinositol 3-kinase by ET-18-OCH_3, suggesting that inhibition of this enzyme may contribute to the cytotoxic effect. If the cytotoxic effects of s-phosphonate were via inhibition of phosphatidylinositol 3-kinase, MC-9 cells incubated with IL-3 might have been expected to be sensitive to the drug, but cells incubated with GM-CSF would not be sensitive. The results of this study show this not to be the case, suggesting that the mechanism by which these ether lipids induce apoptosis is much more complex.

This study provides further evidence of the antineoplastic potency of s-phosphonate and the cell-specific cytotoxicity similar to that shown by ET-18-OCH_3. A similar pattern of apoptotic selectivity of ET-18-OCH_3 towards HL-60 cells but not K-562 cells has been described by many groups [1, 3, 23]. S-phosphonate was also shown to induce apoptosis in another leukemic cell line, AML-14, whereas the murine mast cell line MC-9 was resistant, reinforcing results showing the specificity of the cytotoxic effects of s-phosphonate.

The CAM assay primarily assessed the antiangiogenic effects of s-phosphonate. However, this in vivo model was also used to assess the nonspecific membrane disruption caused by s-phosphonate by dissecting and analyzing the morphology of the membrane subjacent to the s-phosphonate/PCL matrix. The encapsulation of s-phosphonate in PCL, a biodegradable biocompatible polymer, allowed a pool of s-phosphonate to be localized on a 3 mm^2 area of the CAM. Since s-phosphonate diffused out of the PCL matrix slowly, the precise drug concentration on the CAM surface could not be determined. However, increased concentrations of s-phosphonate were achieved by loading the PCL matrix with increasing concentrations of s-phosphonate. After a 2-day exposure, s-phosphonate was shown to induce localized disruption of membrane morphology and organization as illustrated in Figs. 4 and 5.

Initially, the pronounced membrane thinning subjacent to the s-phosphonate/PCL matrix was assumed to be a result of nonspecific cytolysis of the membrane cells by s-phosphonate. Since ether lipids are known to accumulate in cell membranes, s-phosphonate may have accumulated locally in the surface cells of the CAM with little diffusion away from the site. In support of this concept are the previously reported results showing irreversible binding of ET-18-OCH_3 to leukemic cells [22]. Such an accumulation of s-phosphonate in the CAM might inevitably lead to nonspecific cell lysis as previously described for this compound [10]. Thus the antiangiogenic effects of s-phosphonate directly under the PCL matrix might simply be a result of cytolysis of the endothelial tissue of the capillaries. However, membranes dissected out from such disrupted CAMs showed evidence of extensive apoptosis, demonstrated by DNA laddering techniques (Fig. 6), providing the first evidence that membrane thinning and the antiangiogenic effects induced by s-phosphonate might occur by the induction of cellular apoptosis rather than by cytolysis. This results were subsequently confirmed by immunolabelling techniques that showed extensive apoptosis occurring only in the thin area of the CAM subjacent to the s-phosphonate/PCL matrix (Fig. 7).

To our knowledge, this is the first report of the use of immunolabelling techniques to demonstrate apoptosis in cryostat-sectioned CAM membranes. The localized apoptotic effects of s-phosphonate on the thin section of the CAM (Fig. 7) were confirmed by correspondence with Oncor Scientific (the manufacturers of the immunolabelling kit Apoptag). Although the Apoptag method showed apoptotic cells in the thin section of the CAM, it was unable to clearly distinguish which cells had undergone apoptosis. Indeed, in vitro tests using endothelial cells (HUVEC) failed to show any s-phosphonate-induced apoptosis at concentrations as high as 50 μM, whereas cytolysis was observed at higher concentrations (100 μM; (data not shown).

We have previously described the powerful antiangiogenic and apoptotic activity of the drug taxol on the endothelial cells of the capillaries in the CAM, (manuscript submitted for publication; [25]), but taxol (like s-phosphonate) was also unable to induce apoptosis in HUVEC cells when this drug was used as a positive control. Therefore HUVEC cells grown in vitro may not necessarily provide a good model for the growing endothelial cells of the CAM. Nevertheless, this failure of s-phosphonate to induce apoptosis in HUVEC cells and the observation of cytolysis in these cells at high concentrations of the drug (100 μM) may be evidence that the inhibition of angiogenesis on the CAM, as shown in Fig. 4, may have resulted from s-phosphonate-induced cytolysis of capillary endothelial cells and that the observed apoptosis may have arisen from the effect of s-phosphonate on other cells in the CAM.

Angiogenesis is dependent on the stimulation of endothelial cell proliferation. Cell proliferation is a fundamental process common to both angiogenesis and tumor development. Both CAM development and tumor growth (with associated angiogenesis) require rapid cell division controlled by growth factors/cytokines [26]. In this study we showed that the antineoplastic effects of s-phosphonate in various cell lines correlated with the induction of apoptosis in those cells. We also showed that s-phosphonate halted the progression of angiogenesis on the CAM via an apoptotic mechanism (in part at least). These apoptotic effects of s-phosphonate on proliferating cells indicate that s-phosphonate may

possess great potential as an anticancer drug via both its direct cytotoxic action on tumor cells and its antiangiogenic effects. This potential may depend in part on the ability to localize drug application close to the tumor site. Experiments are in progress to assess the antitumor effects of s-phosphonate using a murine tumor model. In these experiments implants of PCL with encapsulated s-phosphonate are implanted adjacent to the tumor site (or resected tumor site) so that the in vivo potential of s-phosphonate as an antiangiogenic/anti-tumor agent may be assessed.

Acknowledgements This research was funded by a grant from the Medical Research Council of Canada. Financial support was also provided from Angiogenesis Technologies Inc.

References

1. Paltauf F (1994) Ether lipids in biomembranes. Chem Phy Lipids 74: 101–139
2. Bittman R, Byun HS, Mercier B, Salari H (1993) 2′-(trimethylammonio)ethyl 4-(hexadecyloxy)-3(*S*)-methyoxybutane phosphonate: a novel potent antineoplastic agent. J Med Chem 36: 297–299
3. Diomede L, Piovani B, Re F, Principe P, Colotta F, Modest E, Salmona M (1994) The induction of apoptosis is a common feature of the cytotoxic action of ether-linked glycerophospholipids in human leukemic cells. Int J Cancer 57: 645–649
4. Candal FJ, Bosse DC, Vogler WR, Ades EW (1994) Inhibition of induced angiogenesis in a human microvascular endothelial cell line by ET-18-OCH_3. Cancer Chemother Pharmacol 34: 175–178
5. Auerbach R, Alby L, Morrissey LW, Tu M, Joseph J (1985) Expression of organ-specific antigens on capillary endothelial cells. Microvasc Res 29: 401–411
6. Folkman J, Hochberg M (1973) Self regulation of growth in three dimensions. J Exp Med 138: 745–753
7. Mollinedo F, Martinez-Dalmau R, Modelell M (1993) Early and selective induction of apoptosis in human leukemic cells by the alkyl-lysophospholipid ET-18-OCH_3. Biochem Biophys Res Commun 192: 603–609
8. Diomede L, Colotta F, Piovani B, Re F, Modest EJ, Salmona M (1993) Induction of apoptosis in human leukemic cells by the ether lipid 1-octadecyl-2-methyl-rac-glycero-3-phosphocholine. A possible basis for its selective action. Int J Cancer 53: 124–130
9. Mollinedo F, Gajate C, Modelell M (1993) The ether lipid 1-octadecyl-2-methyl-rac-glycero-3-phosphochloline induces expression of fos and jun proto-oncogenes and activates AP-1 transcription factor in human leukaemic cells. Biochem J 302: 325–329
10. Jackson JK, Burt HM, Mok W, Salari H, Kumar ER, Byun HS, Bittman R (1994) A comparison of the lytic properties of two ether linked lipids, one with and one without antineoplastic activity. Biochem Cell Biol 72: 297–303
11. Paul CC, Tolbert M, Mahrer S, Singh A (1993) Cooperative effects of interleukin-3 (IL-3), IL-5, and granulocyte-macrophage colony-stimulating factor: a new myeloid cell line inducible to eosinophils. Blood 81: 1193–1199
12. Scheid MP, Lauener R, Duronio V (1995) Role of phosphatidylinositol 3-OH-kinase activity in the inhibition of apoptosis in haemopoietic cells: phosphatidylinositol 3-OH-kinase inhibitors reveal a difference in signalling between interleukin-3 and granulocyte-macrophage colony stimulating factor. Biochem J 312: 159–162
13. Scudiero DA, Shoemaker RH, Paull KD, Monks A, Tierney S, Nofziger TH, Currens MJ, Seniff D, Boyd MR (1988) Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. Cancer Res 48: 4827–4833
14. Hamburger V (1960) A manual of experimental embryology. University of Chicago Press, Chicago
15. Modolell M, Andressen R, Pahlke W, Brugger U, Munder PG (1979) Disturbance of phospholipid metabolism during the selective destruction of tumor cells induced by alkyl-lysophospholipids. Cancer Res 39: 4681–4686
16. Berdel WE (1991) Membrane-interactive lipids as experimental anticancer drugs. Br J Cancer 64: 208–211
17. Vogler WR, Berdel WE, Olson AC, Winton EF, Heffner LT, Gordon DS (1992) Autologous bone marrow transplantation in acute leukemia with marrow purged with alkyl-lysophospholipid. Blood 80: 1423–1429
18. Andreeesen R, Modelell M, Weltzien HU, Eibl H, Common HH, Lohr GW, Munder PG (1978) Selective destruction of human leukemic cells by alkyl-lysophospholipids. Cancer Res 38: 3894–3899
19. Berdel WE, Greiner E, Fink U, Stavrou D, Reichert A, Rastetter J, Hoffman DR, Snyder F (1983) Cytotoxicity of alkyl-lysophospholipid derivatives and low-alkyl-cleavage enzyme activities in rat brain tumor cells. Cancer Res 43: 541–545
20. Wilcox RW, Wykle RW, Schmitt JD, Daniel LW (1987) The degradation of platelet-activating factor and related lipids: susceptibility to phospholipases C and D. Lipids 22: 800–807
21. Hoffman DR, Hoffman LH, Snyder F (1986) Cytotoxicity and metabolism of alkyl phospholipid analogues in neoplastic cells. Cancer Res 46: 5803–5809
22. Kelley EE, Modest EJ, Burns CP (1993) Unidirectional membrane uptake of the ether lipid antineoplastic agent edelfosine by L1210 cells. Biochem Pharmacol 45: 2435–2439
23. Diomede L, Piovani B, Modest EJ, Salmona M (1993) The effect of culture medium composition on ether lipid cytotoxic activity. Lipids 28: 189–192
24. Berggren MI, Gallegos A, Dressler LA, Modest EJ, Powis G (1993) Inhibition of the signalling enzyme phosphatidylinositol-3-kinase by antitumor ether lipid analogues. Cancer Res 53: 4297–4302
25. Dordunoo SK, Jackson JK, Arsenault LA, Oktaba AMC, Hunter WL, Burt HM (1995) Taxol encapsulation in poly(E-Caprolactone) microspheres. Cancer Chemother Pharmacol 36: 279–282
26. Folkman J, Klagsbrun M (1987) Angiogenic factors. Science 235: 442–447