Notes

Antitumor Activity of the *R*- and *S*-Enantiomers of *RS*-2-[[Hydroxy[[2-[(octadecyloxy)methyl]tetrahydrofuran-2-yl]methoxy]-phosphinyl]oxy]-*N*,*N*,*N*-trimethylethylaminium Hydroxide Inner Salt

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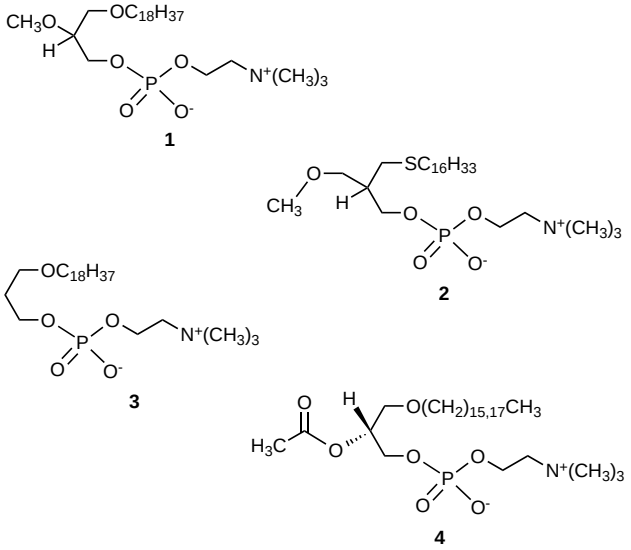
The *R*- and *S*-enantiomers of 2-[[hydroxy[[2-[(octadecyloxy)methyl]tetrahydrofuran-2-yl]methoxy]-phosphinyl]oxy]-*N*,*N*,*N*-trimethylethylaminium hydroxide salt (SRI 62-834) have been evaluated in several assays to determine potential antitumor activity. The *S*-enantiomer showed slightly greater cytotoxic activity than the *R*- or *RS*-forms against several murine tumor cell lines. In the mouse Meth A fibrosarcoma model, the *S*-enantiomer was ca. 4 times more effective than the *R*-isomer in controlling the size of tumor growth and increasing the number of survivors.

Introduction

Synthetic phospholipids are reported to be antagonists of platelet activating factor (PAF),[1] antitumor agents,[2] anti-HIV agents[3] and useful for the treatment of multiple sclerosis.[4] The antitumor activity of these agents differ from most currently used anticancer agents in that they do not interfere with DNA at the level of replication and transcription. Their precise mechanisms of action are unclear, but the antineoplastic action is believed to be membrane mediated.[5] Immunomodulating effects, particularly macrophage activation,[6] and interference with cell signaling enzymes such as protein kinase C (PKC)[5] and phospholipase C (PLC)[6] appear to be important. Promotion of tumor cell differentiation[7] and inhibition of tumor cell invasion[8] may also play a role in their tumorcidal action. Although the structure of phospholipids such as the clinically studied edelfosine[9] (ET-18-OCH_3, **1**), ilmofosine[10] (BM-41440, **2**), and miltefosine[11] (He-PC, **3**) are closely related to PAF (**4**), their in vitro toxicity does not correlate with their ability to bind to the PAF receptor.[12−15]

We reported[16] that (*RS*)-2-[[hydroxy[[2-[(octadecyloxy)methyl]tetrahydrofuran-2-yl]methoxy]phosphinyl]-oxy]-*N*,*N*,*N*-trimethylethylaminium hydroxide inner salt (SRI 62-834, **5**) has a profile of in vitro and in vivo antitumor activity similar to **1**. Compound **5** is effective on multidrug resistant tumor cell lines[17,18] and inhibits the mitogenic effect of PDGF,[16] and its cytotoxic activity is not blocked by WEB 2086, a potent PAF receptor antagonist.[19] It also elicits an elevation of intracellular Ca^{2+} in HL-60 and EMT6 tumor cells, probably the result of opening of a calcium channel the activity of which is regulated by PKC.[20,21] The antineoplastic activity of **5** is probably mediated via the intact molecule since the putative metabolites **6** and **7** exhibit weak cytotoxic effects on human and rat tumor cell lines.[22]



This paper describes the antitumor activity of the *R*-(**8**) and *S*-enantiomers (**9**) of **5**.[23−25] The synthesis and proof of sterochemistry for both enantiomers has been reported.[26]

Pharmacology

The direct cytotoxic effect of the *RS*-, *R*-, and *S*-phospholipids **5**, **8**, and **9** at 1, 3, and 5 μg/mL against the murine tumor cell lines Abelson 8.1, YAC-1, L1210, and P815 is given in Table 1. At the lower dose (1 μg/mL), the *S*-enantiomer (**9**) was more effective than the *R*- or *RS*-forms against all four tumor cell lines. At the highest dose (5 μg/mL), the cytotoxic activity of **5**, **8**, and **9** was very similar. The tumor cytotoxicity of **5**, **8**, and

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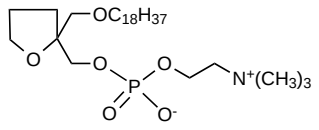

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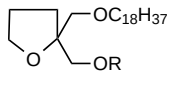
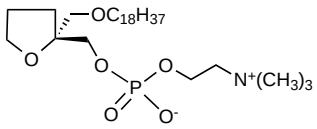


6: R = PO_3H_2
7: R = H



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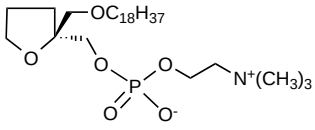

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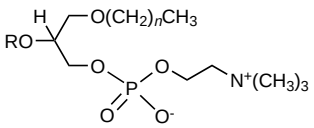
9, in the presence of macrophages, was considerably enhanced with all three compounds giving similar levels of activity (Table 1, AMC data).

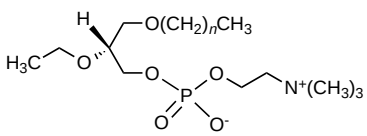
Evaluation of the phospholipids at 0.25 and 2.5 mg/kg po, respectively, in the mouse Meth A fibrosarcoma model over a 28 day period is given in Table 2. The *S*-enantiomer (**9**) was more effective than the *R*- (**8**) and *RS*-forms (**5**) in controlling the size of tumor growth and increasing the number of survivors. At the lower dose, **9** was ca. 4 times more effective than **8** in increasing the number of survivors.

Discussion

Conflicting results on the importance of chirality on the direct tumor cell toxicity of phospholipids have been reported. The D-isomer of **10a** is ca. 2.5 and 12 times more effective than the L-isomer at 10 and 20 μg/mL, respectively, in HL-60 cells[27] and ca. 4 times more active against Raji tumor cells.[28] Both the D- and L-isomers of **10b** have similar effects in four freshly explanted leukemia cell lines,[29] but the D is more active than the L against Raji tumor cells.[28] At a dose of 40 μM, the *R*- and *S*-isomers of **10c** have the same cytotoxicity in a variety of tumor cells.[30] The D- and L-isomers of **1** have similar cytotoxic activity in HL-60 cells,[31] and the (+)- and (−)-forms of **2** showed no difference in sensitivity in cell proliferation studies with several tumor cell lines.[31] The D-isomer of **11** is more effective than the L-isomer in inhibiting the growth of X-5563 tumor cells.[33] A recent report indicates that **8** and **9** have equipotent cytotoxicity against HT29 human colon carcinoma cells.[34]



10a: R = H, *n* = 15
10b: R = H, *n* = 17
10c: R = CH_3, *n* = 15



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In vivo studies with D- and L-**1** have shown that both enantiomers effect a similar increase in life-span (ILS) in the mouse S180 tumor model but only the D-isomer is effective in the mouse MM46 tumor model. Evidence suggests that the results found in the MM46 tumor may be due to a stereoselective interaction with macrophages.[31]

The in vitro results on direct cytotoxicity suggest the *R*- and *S*-enantiomer **8** and **9** exhibit a chiral preference on the tumor cell lines studied at the lower dose range (1 μg/mL). At the higher dose (5 μg/mL) there appears to be less chiral selectivity and more nonspecific cytotoxic effects. The in vivo studies in the Meth A fibrosarcoma model clearly indicate a chiral preference in decreasing tumor volume and increasing the number of survivors. This enantioselectivity is probably not due to a preferential effect on macrophages as with D- and L-**1** in the MM46 model since both **8** and **9** have similar effects on activating macrophages (Table 1). Our in vivo findings suggest that other factors not yet established may account for the difference with **8** and **9**. Additional studies with **8** and **9** and other chiral antitumor phospholipids are needed on a variety of tumors to clarify the effect of chirality in this class of antitumor agents.

Experimental Section

Cell Lines. The tumor cell line Abelson-8.1 was obtained from A. W. Harris (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) and the YAC-1 from G. Klein (Department of Tumor Biology, Karolinska Institute, Stockholm, Sweden). The cells were grown in stationary suspension culture in Dulbecco Modified Eagle's Medium (DMEM) and 10% heat-inactivated fetal calf serum (FCS) supplemented with 50 μM 2-mercaptoethanol, 100 units of penicillin, and 1 μg of streptomycin. The P815 and L1210 cells were from the Max-Planck-Institut für Immunbiologie cell line collection and were passed intraperitoneally (ip) in vivo every week. The MethA fibrosarcoma cells were originally induced in BALB/C mice by administering methylcholanthrene according to the procedure of Old et al.[35]

Macrophages were grown from the bone marrow cells of (Balb/C × C57/bl6)F1 mice. Cultivation was effected by placing a suspension of 3×10^6 bone marrow cells/50 mL, DMEM, 10% FCS, 5% horse serum, and 3% supernatant of L929 fibroblast culture containing stimulating factor into ethylene oxide-sterilized 30 × 5 cm Teflon bags (Biofolie, Heraeus, Hanau, FRG) as previously described. The cells are cultivated in the hydrophobic side of polymeric fluorocarbon film, and in 8−12 days the precursor cells develop into mature macrophages. Macrophages are brought into a single cell by rolling the cultivation suspension side of the bag slightly without pressure between two fingertips. After 12 min, the moderately attached macrophages came off. After sterilizing, the cell suspension was centrifuged on a Ficoll hypaque layer at 500*g* for 30 min, and the mononuclear cells were washed, first at 400*g* for 10 min and then at 250*g* for 10 min.

Tumor Cell Cytotoxicity. Abelson-8.1 tumor cells (1×10^3 cells/well) in DMEM and 10% FCS were placed in flat-bottom microtiter plastic plates (Nunc Roskieide, Denmark) and incubated with 1, 3, or 5 μg/mL of **5**, **8**, or **9** dissolved in water for 24−72 h. The number of tumor cells present was determined by measuring alkaline phosphatase activity by a modified procedure of Culvenor;[36] the tumor cell plates were centrifuged at 500*g* for 10 min, and the supernatant was flicked off. Without further washing, 100 μL of buffer containing 20 μL of diethanolamine, 2 μM of magnesium chloride hexahydrate, 2.5 μM of *p*-nitrophenyl phosphate, and 10 mg of Triton X-100 was added. The samples were incubated for 60 min at room temperature, and the enzymatic activity was terminated by the addition of 100 μL of 0.5 N sodium hydroxide. The absorbance was then measured at 405 μM using a Titertek Multiskan apparatus and compared to non-drug-treated cells. The same assay procedure was used to determine P815, YAC-1, and L1210 tumor cells.

Table 1. Direct and Activated Macrophage Cytotoxicity of **5**, **8**, and **9** on Various Murine Tumor Cell Lines

		% viable cells[a] at 72 h								
		5			**8**			**9**		
tumor cells	assay[b]	1 μg/mL	3 μg/mL	5 μg/mL	1 μg/mL	3 μg/mL	5 μg/mL	1 μg/mL	3 μg/mL	5 μg/mL
Abelson-8.1	DC	32	2.6	2.0	71	6.8	4.6	8.6	2.1	1.8
	AMC	0.4	0.2	0.1	0.5	0.3	0.2	0.8	0.3	0.4
YAC-1	DC	81	26	8.9	92	14	4.5	60	22	9
	AMC	16	2.5	1.2	11	1.6	0.9	11	2.3	0.9
L1210	DC	100	20	8.6	84	24	13	55	7.7	3.2
	AMC	9.4	1.6	0.9	5.3	1.4	1.1	3.4	0.6	0.2
P815	DC	60	13	5.3	68	20	2.8	16	7.0	2.6
	AMC	3.4	0.7	0.6	3.1	1.8	1.3	2.8	1.1	0.3

[a] Values are averages of quadruplicate assays and have an average error of ±4.2%. Viable cells in the DC assay are measured by alkaline phosphatase activity and by [^{3}H]thymidine incorporation in the AMC assay. [b] DC, direct cytotoxicity; AMC, activated macrophage cytotoxicity.

Table 2. Mouse Meth A Fibrosarcoma Assay of **5** (*RS*), **8** (*R*), and **9** (*S*)

	dose, mg/kg po	tumor volume % control (cm^3)[a]				survivors
compd	(n = 10)	day 7	day 14	day 21	day 28	on day 28
control		100 (14.23)	100 (19.58)	100 (35.67)	100 (101.16)	0/10
5 (*RS*)	0.25	82 (9.84)	47 (7.79)	20 (7.04)	32 (26.30)	3/10
8 (*R*)	0.25	90 (10.77)	52 (8.67)	21 (7.50)	37 (30.60)	2/10
9 (*S*)	0.25	66 (7.89)	19 (3.13)	6 (2.19)	11 (9.16)	8/10
5 (*RS*)	2.50	76 (9.12)	38 (6.24)	14 (4.91)	21 (17.57)	6/10
8 (*R*)	2.50	85 (10.15)	53 (8.72)	21 (7.59)	32 (26.54)	3/10
9 (*S*)	2.50	70 (8.36)	28 (4.65)	8 (2.93)	13 (10.89)	8/10

[a] Values are averages of all surviving animals and have an average error of ±10.5%.

Macrophage Cytotoxicity. Mouse macrophages (10^5/well) were incubated with 10 μg/mL of **5**, **8**, or **9** for 24 h in flat-bottom microtiter plates, after which they were centrifuged and washed once. Abelson-8.1 tumor cells (1×10^3/well) in DMEM and 10% FCS plus 1, 3, or 5 μg/mL of **5**, **8**, or **9** were added to the plates and incubated for 72 h. The same assay procedure was used to determine P815, YAC-1, and L1210 tumor cells.

Mouse Meth A Fibrosarcoma Assay. Ten CBF_1 mice, 10−12 wk of age, were implanted with 10^5 Meth A sarcoma cells to serve as a control. Ten other CBF mice were implanted with 10^5 Meth A sarcoma cells and on day 1 after implant were each treated per os (po) with daily drug treatment continued for 27 days. On days 7, 15, 21, and 28 after tumor implant, the entire tumor volume was calculated by the equation $V = {}^2/_3\pi AB(AB + B/2)$, where A and B are measured tumor diameters.

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