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Platelet-activating factor (PAF) antagonist WEB 2086 does not modulate the cytotoxicity of PAF or antitumour alkyl lysophospholipids ET-18-O-Methyl and SRI 62-834 in HL-60 promyelocytic leukaemia cells

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The ether lipids are a novel class of antitumour agents which do not interfere with the synthesis or structure of DNA, but rather exert their effects by acting at the cell membrane [1, 2]. Examples include 1-*O*-octadecyl-2-*O*-methyl-*rac*-3-phosphocholine (ET-18-O-Methyl) (1, 2) and (+/−) - 2 - {hydroxy[tetrahydro - 2 - (octadecyloxy)-methylfuran - 2 - yl]methoxyl} - phosphinyloxy - *N,N,N*-trimethylethaniminium hydroxide, inner salt (SRI 62-834, also designated CRC 86-05 and NSC 614383) [3]. Both are now undergoing clinical trial. As can be seen from Fig. 1, the two agents are related in structure to platelet activating factor or PAF (1-*O*-octadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine) [4, 5].

As well as causing immune effects such as macrophage activation, the ether lipids clearly do exhibit a direct cytotoxic effect against tumour cells *in vitro* [1, 2]. A key feature may be the ability of pharmacological concentrations of ET-18-O-Methyl and SRI 62-834 to raise the level of intracellular free calcium in cultured cells [6, 7]. Our own studies have demonstrated that similar concentrations of these ether lipids bring about a series of consecutive increases in membrane permeability [8, 9], and recent multiparameter flow cytometry data show that the calcium elevation precedes, and may therefore be a cause of, membrane permeabilization and subsequent cell death [10]. However, the precise molecular nature of the interaction of ether lipids in the cell membrane leading to calcium and permeability changes is unknown.

Perhaps the major outstanding question has been the possible role of a membrane PAF receptor, analogous to that in the platelet, as against other specific biochemical mechanisms or biophysical, detergent-like perturbations. In fact direct demonstration of a receptor for PAF has proved difficult even in the platelet, but the considerable evidence for such a receptor includes particularly [11]: (1) the low (nM) concentrations required; (2) the precise structure–function specificity; (3) the stimulus-specific desensitization or down-regulation of the response; (4) the inhibition of stimulation by anti-platelet antibodies; (5) the high affinity, saturable binding by PAF; and (6) the inhibition of the response by a variety of PAF antagonists. Clearly, similar criteria can be applied to elucidate the possible involvement of a PAF receptor in the *in vitro* cytotoxicity of PAF-related ether lipids.

Compared to the extremely potent effects of PAF in platelets, much higher concentrations, i.e. micromolar, are required for the pharmacological and cytotoxic action of ether lipids against *in vitro* cell lines, although this in itself does not rule out a low abundance or low affinity receptor. Radiolabelled PAF binding experiments are complicated by the hydrophobic nature of the ligand, leading to a significant non-specific binding component, as well as the metabolism of PAF [12]. Although ET-18-O-Methyl does exhibit weak PAF agonist activity in the rabbit platelet, the direct cytotoxicity of this and related ether lipids against HL-60 human promyelocytic leukaemia cells and erythrocytes did not exhibit PAF receptor-like stereospecificity [13].

Experiments using specific PAF antagonists can be extremely informative. Very recently Bazill and Dexter [14] reported that one such antagonist, the lignan analogue *trans*-2,5-bis(3,4,5-trimethoxy phenyl tetrahydrofuran) (L-652,731) was able to counteract the *in vitro* cytotoxicity of ET-18-O-Methyl and SRI 62-834 in WEH I-3B myelomonocytic leukaemia cells. On the other hand, Lazenby *et al.* [7] have shown that L-652,731 and other PAF antagonists did not block the calcium rise induced by SRI 62-834 in WEHI-3B cells. In this report we describe the comparative cytotoxic potencies of PAF, ET-18-O-Methyl and SRI 62-834 in HL-60 cells as measured using the MTT tetrazolium dye reduction assay, and demonstrate a complete lack of cytotoxicity modulation by various

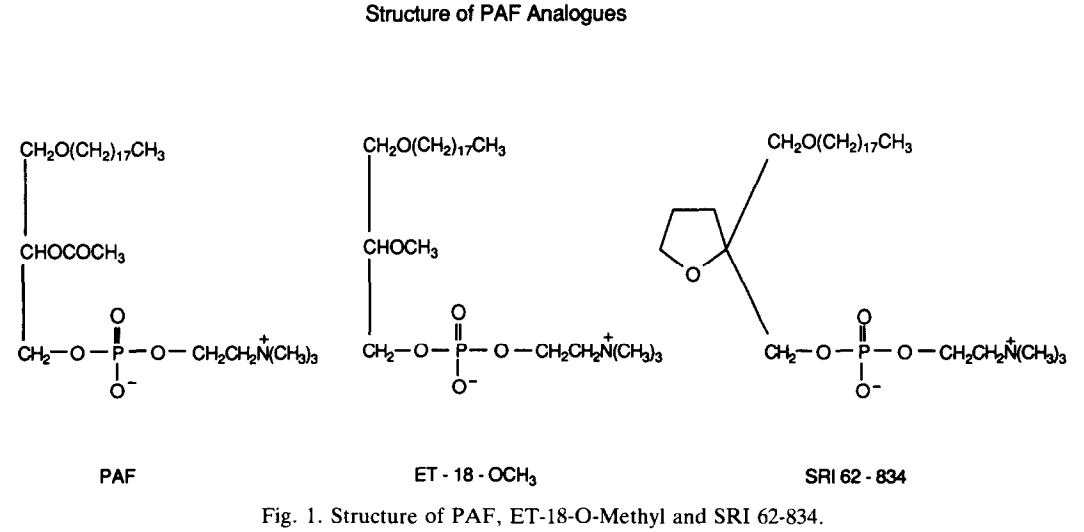

Fig. 1. Structure of PAF, ET-18-O-Methyl and SRI 62-834.

concentrations of the highly potent and specific PAF antagonist 3-[4-(2-chlorophenyl)-9-methyl-6*H*-thieno[3,2-f][1,2,4]triazolo- [4,3-*a*][1,4]- diazepin-2-yl]- 1- (4-morpholinyl)-1-propanone (WEB 2086) [15].

Materials and Methods

PAF was purchased from Peninsula Laboratories Europe (St Helens, Merseyside, U.K.). ET-18-O-Methyl was a generous gift from Dr Wolfgang Berdel (Technical University, Munich, F.R.G.). SRI 62-834 was kindly provided by Dr Bill Houlihan (Sandoz Research Institute, East Hanover, NJ, U.S.A.) via Professor Brian Fox (Paterson Institute for Cancer Research, Manchester, U.K.) and Professor John Hickman (CRC Experimental Chemotherapy Research Group, Aston University, Birmingham, U.K.) under the auspices of the CRC Phase 1/2 Clinical Trials Committee. WEB 2086 was obtained from Dr Karl-Heinz Weber, Boehringer Ingelheim, Ingelheim am Rhein, F.R.G.). All four agents were dissolved in phosphate-buffered saline.

HL-60 human promyelocytic leukaemia cells were obtained from Professor John Hickman. They were grown routinely in suspension culture in RPMI medium containing 10% foetal calf serum without antibiotics in an atmosphere of 8% CO_2 92% air at 37°. Cytotoxicity was determined using the MTT dye reduction assay in which the yellow water soluble tetrazolium salt is reduced to the purple insoluble formazan by dehydrogenases present in viable cells [16, 17]. For cytotoxicity assays, cells in log phase were placed into 96-well microtitre plates at 2×10^3 cells/well in a volume of 200 μL full medium. After 4 hr test agents were added in a volume of 20 μL PBS to give the appropriate concentration. In experiments to determine the effects of WEB 2086 as a response modifier, PAF antagonist was added to the cells 30 min before the lipid agents.

Cells were incubated at 37° without medium change for 4 days, during which time control cells increased logarithmically by a factor of 10-fold. At the end of this period the MTT tetrazolium dye (Sigma Chemical Co., Poole, U.K.) was added at a concentration 5 mg/mL in PBS and the plates were incubated for a further 2 hr at 37°. The plates were then centrifuged (200 *g*, 5 min, 4°) and medium removed by careful aspiration. The crystalline deposit of the purple MTT formazan reduction product was then dissolved in 200 μL dimethylsulphoxide and the solution agitated gently for 10 min on a plate shaker. Absorbance was read on a Titertek Multiscan MCC ELISA plate reader (Flow Laboratories, Helsinki, Finland) using a test wavelength of 540 nm and a reference of 690 nm. Experiments were carried out to show that optical density was proportional to viable cell number and increased linearly during the 2 hr incubation in the presence of MTT substrate. Mean optical densities for treated wells were expressed as a percentage of the mean for control wells. Eight replicate wells were used at each individual drug concentration plus the control. All experiments were replicated independently.

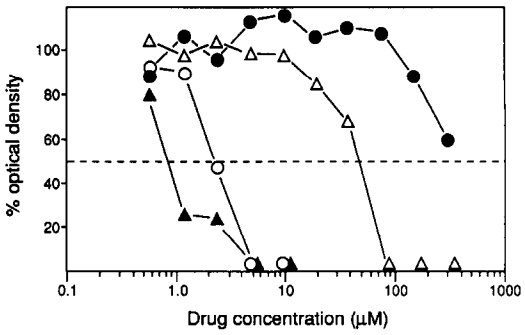

Fig. 2. Cytotoxicity of SRI 62-834 (▲), ET-18-O-Methyl (○), PAF (△) and WEB 2086 (●) against HL-60 human promyelocytic leukaemia cells *in vitro*. Results shown are for a typical experiment using the MTT dye reduction assay. The broken line indicates the 50% response level.

Results

Figure 2 shows typical dose–response curves for cytotoxicity of PAF, ET-18-O-Methyl, SRI 62-834 and WEB 2086 as single agents against HL-60 cells. The results for the entire series of experiments are summarized in Table 1. It can be seen that ET-18-O-Methyl and SRI 62-834 exhibit similar cytotoxic potencies with IC_{50} values of about 3 μM. PAF is also cytotoxic but about 20 times less so. The PAF antagonist WEB 2086 was very weakly active indeed.

In the next series of experiments we investigated the effects of WEB 2086 on the cytotoxicity of PAF, ET-18-O-Methyl and SRI 62-834. It was important to establish that WEB 2086 did not itself exhibit significant toxicity at the concentrations used for modulation. To complement the IC_{50} results in Table 1, the percentage reductions in control optical density are summarized in Table 2 for the same series of experiments. It is clear that the PAF antagonist shows a 20–25% reduction in viable cell number at 100–150 μM and essentially no effect at 75 μM and below. WEB 2086 has been shown to inhibit PAF-induced human platelet and neutrophil aggregation *in vitro* with IC_{50} values of 0.17 and 0.36 μM, respectively. Figure 3 shows the results of experiments in which the modulatory effects of WEB 2086 at 1 and 100 μM were investigated in

Table 1. Cytotoxic effects of PAF, alkyl lysophospholipids ET-18-O-Methyl and SRI 62-834, and the PAF antagonist WEB 2086 on HL-60 human promyelocytic leukaemia cells *in vitro*

	IC_{50} (μM)							
Drug	1	2	3	4	5	6	7	Median
PAF	50	45		63	66			57
ET-18-O-Methyl	5.5	2.5		2.6				2.6
SRI 62-834	3.0	0.8	3.7	2.6	<7.8			3.0
WEB 2086	>300	>300		>100		350	290	320

Cytotoxicity was measured using the MTT tetrazolium dye reduction assay. IC_{50} = concentration to reduce the control optical density by 50%.

Results are shown for 7 independent experiments, each including 8–11 concentrations, total dose range 5 nM to 500 μM, with 8 replicates per concentration.

Table 2. Cytotoxic effect of WEB 2086 on HL-60 human promyelocytic leukaemia cells *in vitro*

	% Control optical density			
Concentration	1	2	6	7
1	90	107		
10	104	116	89	
75	104	100	89	
100				80
150			75	75
200				66
300	64	60	56	48
400			44	38
500				33

Cytotoxicity was measured using the MTT dye reduction assay.

The mean optical density for treated wells was expressed as a percentage of the untreated control.

Results are shown for 4 independent experiments, with 8 replicates per concentration.

terms of cytotoxic response to PAF, WEB 2086 and SRI 62-834. It is clear that the PAF antagonist was completely without effect on the cytotoxicity of these agents. Identical results were also obtained with 10 μM WEB 2086, but these are not shown for reasons of clarity. Closely similar results were obtained in repeat experiments with all three lipids. In addition, two direct comparative experiments showed lyso PAF (IC_{50} values 45 and 37 μM) to exhibit similar potency to PAF itself (IC_{50} 61 and 51 μM). There was no modulation of lyso PAF activity by WEB 2086.

Discussion

The results reported here show clearly that the potent PAF antagonist WEB 2086 has no effect on the cytotoxicity of PAF or the related developmental antitumour ether lipid analogues ET-18-O-Methyl and SRI 62-834 against the HL-60 human promyelocytic leukaemia cells. PAF itself did exhibit cytotoxicity but was about 20-fold less potent than ET-18-O-Methyl and SRI 62-834 as single agents. These last two agents gave IC_{50} values of 3 μM, and >90% reduction in cell number was achieved at concentrations below 10 μM. WEB 2086 had very little effect below 100 μM.

The concentrations of WEB 2086 used to look for modulation (1, 10 and 100 μM) were similar to or much greater than those causing 50% inhibition of PAF-induced aggregation of human platelets and neutrophils (0.17 and 0.36 μM, respectively, Ref. 15). We believe that our results provide clear evidence that a PAF receptor is not involved in the mechanism of direct cytotoxic action of the ether lipid drugs (and PAF) in HL-60 cells. We have also obtained similar results in other cell lines, including EMT6 mouse mammary tumour cells (not shown). The negative findings are in keeping with our observation of a lack of effect of WEB 2086 on membrane permeabilization by SRI 62-834 and PAF in both HL-60 and EMT6 cells *in vitro* [8]. They also agree with the failure of PAF antagonists including L-652,731 to block the SRI 62-834-induced calcium rise in WEHI-3B cells [7]. Although L-652,731 was able to antagonize the cytotoxicity of SRI 62-834 and ET-18-O-Methyl in WEHI-3B myelomonocytic cells [14] this may have involved an alternative mechanism. Since L-652,731 reduced the uptake of tritiated ET-18-O-Methyl [18], it may be that the effect of the PAF antagonist is on cellular handling of the ether lipids rather than via a specific PAF receptor. It should also be noted that L-562-731 by itself had a growth inhibitory effect on the cells by inhibiting

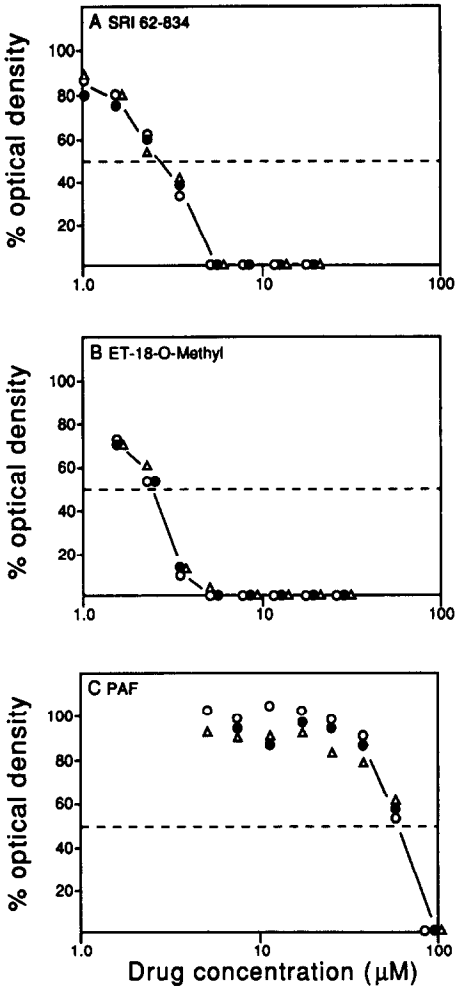

Fig. 3. Lack of modulation by non-toxic concentrations of WEB 2086 on the cytotoxicity of SRI 62-834, ET-18-O-Methyl and PAF against HL-60 human promyelocytic leukaemia cells *in vitro*. (△) control, no WEB 2086; (●) plus 1 μM WEB 2086; (○) plus 100 μM WEB 2086. A similar lack of effect was also seen for 10 μM WEB 2086 (not shown). Results shown are for a typical experiment using the MTT dye reduction assay. The broken line indicates the 50% response level.

thymidine transport [16]. In fact more recent data from the same laboratory show that this and certain other PAF antagonists exhibit a different modulatory mechanism not involving a PAF receptor but an endocytotic pathway [19]. Evidence against a PAF receptor also comes from the absence of PAF-like stereospecificity in structure–activity studies of the cytotoxic effects of ether lipids against tumour cells including in HL-60 [13, 20].

The precise mechanism involved in the ether lipid effects on calcium homeostasis, membrane permeability and cell killing remain to be established. Higher concentrations of ether lipid drugs undoubtedly cause biophysical effects on membranes, ranging from changes in fluidity to pore formation [21, 22]. In addition PAF itself has been shown to act as a general membrane perturbant due to its detergent-like properties at concentrations above 4 μM [23]. On the other hand, both SRI 62-834 and ET-18-O-Methyl are much more active than PAF against tumours cells *in vitro* (e.g. as shown here) and it is possible that

more specific membrane effects may occur at lower concentrations. In support of this it has been shown that the calcium rise induced by these agents can be attenuated by protein kinase-C-active phorbol ester but not by an inactive analogue [6, 24], implicating the involvement of this important signal transduction enzyme at some stage in the calcium cascade.

Although the exact molecular mechanisms remain to be elucidated, what is clear is that ether lipids such as ET-18-O-Methyl and SRI 62-834 can exert substantial differential cytotoxic responses across a range of *in vitro* cell lines, providing optimism for achievement of biochemical selectivity against tumour versus normal tissue and supporting their continuing clinical evaluation.

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