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Lack of enantio-selectivity in the *in vitro* antitumour cytotoxicity and membrane-damaging activity of ether lipid SRI 62-834: further evidence for a non-receptor-mediated mechanism of action

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Abstract—SRI 62-834 ([tetrahydro-2-(octadecycloxy)methylfuran-2-yl]methoxylphosphocholine; CRC 86-05; NSC 614383) is a cyclic antitumour ether lipid (AEL) with a novel, but ill-defined, mechanism of action. AELs are believed to act on membranes and cell signals, but the precise mechanisms of selectivity are unclear. Receptor-mediated mechanisms can often be identified by the differential activity of the individual stereoisomers of a drug. We have therefore compared the *R*- and *S*-enantiomers of SRI 62-834 for: (1) cytotoxicity against the human HT29 colon carcinoma cell line using a tetrazolium dye reduction assay and (2) membrane-damaging effects monitored by ^{51}Cr radiolabel release. The tetrazolium assay revealed near-identical mean ID_{50} values around of 2–3 μM for the *R*- and *S*- isomers as well as for the racemic mixture. Moreover, pre- and co-incubation of the cells with the potent platelet-activating factor (1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine; PAF) receptor antagonist WEB 2086BS (3-[4-(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) had no effect on the cytotoxicity of either isomer or the racemate. Short-term membrane damage was not evident at low micromolar concentrations and between 139 and 163 μM ether lipid was required to release 50% of the incorporated ^{51}Cr label. Again, there was no difference in potency between the enantiomers and the racemate. Coincubation with WEB 2086BS also failed to modulate the membrane-lytic potency of the AELs. These results indicate that the site(s) of cytotoxic action of SRI 62-834 is (are) not stereospecific and also appear to rule out the involvement of a conventional PAF receptor in the mechanism of action of SRI 62-834.

Antitumour ether lipids (AELs*) such as ET18-OMe and SRI 62-834 are promising novel antineoplastic agents, some of which have already progressed to phase I and II clinical trials [1, 2]. These alkylphospholipids are not DNA interactive and are believed to exert their selective antitumour effects solely by interacting with components of the plasma membrane [3]. This is in contrast to most currently used anticancer agents, whose main activity is focused on intervention at the level of DNA replication and transcription. As evident from Fig. 1, both ET18-OMe and SRI 62-834 are structurally related to platelet-activating factor (PAF). PAF has been widely studied as the intercellular lipid mediator of platelet aggregation, bronchoconstriction and hypotension, to name but a few of the established cellular PAF responses (for a comprehensive review, see Ref. 4).

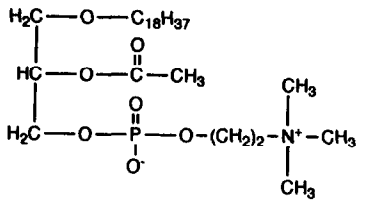
The antineoplastic action of AELs is believed to be membrane mediated, but the precise mechanism(s) of selectivity are unclear [2, 5]. Early studies showed that AELs can activate cytotoxic host macrophages *in vitro* [6–9]. However, these indirect antitumour effects are probably not the main mechanism of toxicity, since potent direct antitumour reactivity has also been established [10–12]. As would be expected from their phospholipid structure, ether lipids are incorporated into tumour cell plasma membranes, leading to alterations in membrane fluidity [13, 14]. The lipid nature of the agents has prompted the hypothesis that AELs are toxic simply because exposure of the plasma membrane to high concentrations of these phosphocholine lipids induces permeability changes and cell lysis [15–18].

However, AELs also affect protein kinase C activity [19–21] and are among the most potent known inhibitors of phospholipase C [22] (G. Powis, personal communication). Both of these ubiquitous enzymes have a number of different isoforms and are crucial mediators of intracellular signal transduction [23]. Protein kinase C is the integrator at the crossroads of a number of cell signalling pathways and phospholipase C initiates the important inositol-1,4,5-trisphosphate/diacyglycerol signalling cascade [24, 25]. The fact that AELs can inhibit these important signal transduction enzymes begs the question as to whether these enzymes might feature a specific binding site for the AELs.

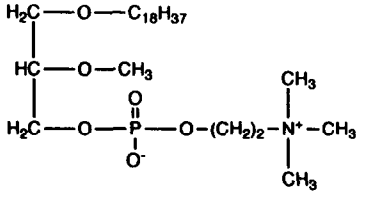
Furthermore, the structural similarity between AELs and PAF suggests that AELs may be able to bind specifically to the recently cloned cellular PAF receptor [26, 27]. Such an interaction might interfere pathologically with PAF receptor-mediated cell signalling. This question was partially addressed in an earlier communication in which we showed that the potent and specific PAF receptor antagonist WEB 2086BS does not modulate the cytotoxicity of AELs against HL60 human promyelocytic leukemia cells [28]. However, it was conceivable that a WEB 2086-insensitive PAF receptor might be involved. We now present further evidence which argues against a role for the PAF receptor in the mechanism of the cytotoxic action of SRI 62–834.

Because of the presence of a chiral centre at the *sn*-2 carbon, the AELs are routinely used in the form of racemic mixtures. In the case of PAF, only the *R*-enantiomer is able to activate the PAF receptor. The *S*-enantiomer has virtually no activity [29]. We decided to investigate the potential stereoselective activities of the *R*- and *S*-enantiomers of SRI 62-834 (Fig. 2) with respect to their cytotoxicity and direct membrane-damaging potency. Since

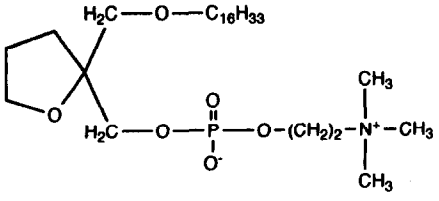
* Abbreviations: AEL/AELs antitumour ether lipid/lipids; ET18-OMe, 1-*O*-alkyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine, edelfosine; SRI 62-834, [tetrahydro-2-(octadecyloxy) methylfuran-2-yl]methoxylphosphocholine, CRC 86-05, NSC 614383; PAF, platelet-activating factor, 1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine; WEB 2086BS, 3-[4-(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; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.



Platelet-activating factor (PAF) C-18



ET18-OMe



SRI 62-834

Fig. 1. Molecular structures for PAF, ET18-OMe and SRI 62-834.

the PAF receptor is stereospecific for PAF, the involvement of this and possibly other related receptors should become apparent through the differential activity of the *R*- and *S*-enantiomers. Comparison of the *in vitro* cytotoxic potencies in HT29 human colon carcinoma cells, using the MTT tetrazolium dye reduction assay, revealed that the *R*- and *S*- enantiomers were equitoxic with similar potencies to the racemate. Direct membrane damaging potencies assayed by release of preloaded [^{51}Cr]chromate were also identical. In addition, coincubation with the highly potent and specific PAF antagonist WEB 2086BS failed to modulate the cytotoxic and membrane-damaging potencies of either the enantiomers or the racemate.

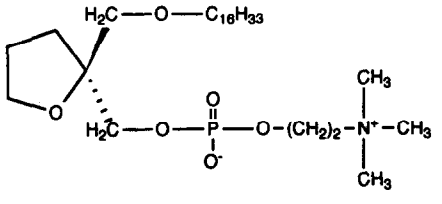
Materials and Methods

Racemic SRI 62-834 and the purified *S*- and *R*-enantiomers (SDZ-266336 and SDZ 266337, respectively) were synthesized and kindly donated by Dr Bill Houlihan (Sandoz Research Institute, East Hanover, NJ, U.S.A.). Enantiomeric purity was at least 95% as determined by standard techniques. WEB 2086BS was a gift from Dr Karl-Heinz Weber (Boehringer Ingelheim, Ingelheim am Rhein, F.R.G.). MTT was purchased from the Sigma Chemical Co. (Poole, U.K.) and sodium [^{51}Cr]chromate from Amersham International (Amersham, U.K.). All agents were dissolved in phosphate-buffered saline (PBS).

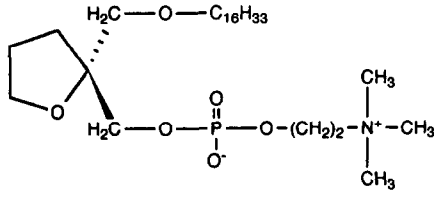
The human colon carcinoma cell line HT29 was cultured routinely in antibiotic-free Eagle's MEM medium and supplemented with 10% foetal calf serum and 1 mM glutamine. Stock cultures were maintained in 75-cm^2 plastic tissue culture flasks at 37° in a humidified atmosphere of 92% air and 8% CO_2. Cells were mycoplasma free and used in log-phase throughout.

The cytotoxic potencies of racemic SRI 62-834 and the enantiomers were determined by the MTT tetrazolium dye reduction assay, using a protocol adapted for the HT29 cell line from Coley *et al.* [30]. Cells (10^4/mL) were seeded in a volume of 200 μL into 96-well plastic microtiter plates. Four hours later, drug was added to the wells in a volume of 20 μL to give the required final concentrations. The cells were given a nominal 4-day drug exposure under normal cell culture conditions, during which control cells increased in number by a factor of 12–16. On day 4, 20 μL of MTT solution (5 mg/mL in PBS) was added to each well and the cells were incubated for a further 3–4 hr to allow dye reduction to occur. The formazan crystals were dissolved in 200 μL dimethylsulfoxide, aided by gentle agitation on a plate shaker for 10 min. Absorbances were read on a Titertek Multiskan MCC MKII ELISA plate reader (Flow Laboratories, Helsinki, Finland) using a test wavelength of 540 nm and a reference of 690 nm. The values from four or eight replicate wells per treatment dose were averaged. Results were expressed as a percentage of untreated vehicle controls to establish the concentration at which MTT to formazan conversion was reduced to 50% of control (IC_{50}). Experiments were carried out in order to establish that absorbance was linear with viable cell number. Neither SRI 62.834 nor WEB 2086BS affected MTT dye reduction acutely.

Membrane damage was measured independently by monitoring the release of [^{51}Cr]chromate ion from previously loaded cells using a standard procedure [31],



(S) - SRI 62-834
(SDZ 266336)



(R) - SRI 62-834
(SDZ 266337)

Fig. 2. Structural diagrams of the two enantiomers *R*- and *S*-SRI 62-834.

which we adapted for use with cytotoxic drugs. Briefly, 2–5 × 10^6 cells were harvested, pelleted and resuspended in 100–200 μL of sodium [^{51}Cr]chromate in PBS (total activity = 100 μCi). The cells were incubated in the presence of [^{51}Cr]chromate for 1 hr, then washed and incubated in full medium for a further hour to avoid analysis during the initial period of rapid spontaneous release. After this stabilization period, the cells were washed twice, counted and seeded into 96-well plastic microtiter plates at a density of 10^4 cells/well. The test agents were added immediately thereafter. Following a 3–4-hr incubation, the plates were spun and 100-μL aliquots of supernatant were drawn from each well for analysis on a γ counter. The activity in counts per minute (cpm) was calculated for each well from duplicate 1-min counts with background and half-life correction. The results from four replicate wells per treatment dose were then averaged and the concentration to cause 50% release of the isotope (R_{50}) was calculated as a fraction of control according to the formula: R_{50} = (EXR − SR)/(TU − SR), where EXR is the observed experimental release, SR is the spontaneous release and TU represents total uptake of the ^{51}Cr-label, all in cpm [31].

Results

Figure 3 illustrates a typical dose–response curve for the cytotoxicity of racemic SRI 62-834 and its pure enantiomers against HT29 human colon carcinoma cells. This and similar repeat experiments revealed almost identical IC_{50} values of 2.4 ± 0.6 and 2.7 ± 0.8 μM for the *R*- and *S*-isomers, respectively (mean ± SD of eight independent experiments). This result is similar to that of 3.2 ± 1.2 μM obtained for the racemic mixture.

To establish whether inhibiting cellular PAF receptors can modulate the cytotoxicity of SRI 62-834 in HT29 cells, we tested the racemic mixture and two enantiomers for toxicity in the presence and absence of the potent PAF receptor antagonist WEB 2086BS. The PAF antagonist alone had no effect on cell viability up to concentrations of 100 μM. Pretreating cells with 45 μM WEB 2086BS before addition of AEL failed to modulate the cytotoxicity of either isomer or the racemate. Mean IC_{50} values all ranged between 2.6 and 3.1 μM (Table 1).

Table 1. The cytotoxicity of SRI 62-834 to HT29 cells in the presence and absence of 49 μM WEB 2086

	Without WEB 2086 (μM)	With WEB 2086 (μM)
rac-SRI 62-834	3.2 ± 1.2 (8)	3.1 ± 0.5 (5)
S-SRI 62-834	2.7 ± 0.8 (8)	3.0 ± 0.8 (4)
R-SRI 62-834	2.4 ± 0.6 (8)	2.6 ± 0.8 (4)
WEB 2086		⩾ 100 (4)

The data represent the average IC_{50} concentrations ± SD of 4–8 independent experiments. The number of experiments is indicated in brackets.

The [^{51}Cr]chromate release studies were carried out using 3–4-hr drug exposures to quantify direct, short-term membrane-lytic potency. Figure 4 shows a dose–response curve for a typical [^{51}Cr]chromate experiment. Drug concentrations causing 50% of incorporated label to be released (R_{50}) were 153 ± 42 and 163 ± 55 μM for the *R*- and *S*-enantiomers, respectively (mean ± SD; N = 3). These compare well with the average R_{50} concentration of 139 ± 14 μM (N = 4) obtained for the racemate. In line with our findings for cytotoxicity, coincubation with 49 μM WEB 2086BS did not modulate the membrane-lytic potency of any of the AELs (Table 2).

Discussion

Earlier studies on the importance of stereochemistry for biological activity of ether lipids have yielded conflicting results. Kudo *et al.* [32] reported *R*-ET18-OMe to be more

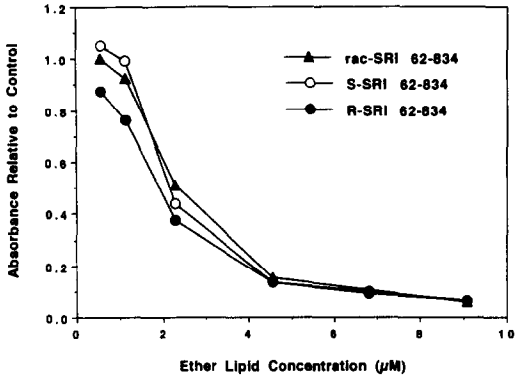


Fig. 3. Cytotoxicity dose–response profiles for SRI 62-834 and its pure enantiomers in HT29 cells. The data are representative of eight independent experiments. The IC_{50} concentrations are those at which MTT dye absorbance was reduced to 50% of control values. Error bars have been omitted as standard deviations of the four replicate wells were routinely below 15% of the mean. Means and standard deviations for replicate experiments are given in Table 1.

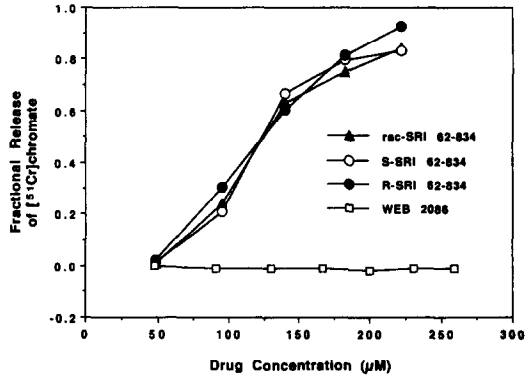


Fig. 4. The comparative membrane-damaging potencies of WEB 2086, SRI 62-834 and its pure enantiomers. Fractional radiolabel release was calculated as described in Materials and Methods, where the R_{50} concentrations are those at which [^{51}Cr]chromate release reached 50% of total [^{51}Cr]-chromate incorporation. The data shown are from one of three independent experiments and symbols represent the average of four replicate wells. Error bars have been omitted for clarity since the per cent error, as calculated by the γ counter software, was routinely well below 10%. Means and standard deviations for replicate experiments are given in Table 2.

Table 2. The membrane toxicity of SRI 62-834 to HT29 cells in the presence and absence of 49 μM WEB 2086

	Without WEB 2086 (μM)	With WEB 2086 (μM)
rac-SRI 62-834	139 ± 14 (4)	130 ± 33 (3)
S-SRI 62-834	163 ± 55 (3)	150 ± 43 (3)
R-SRI 62-834	153 ± 42 (3)	159 ± 49 (3)
WEB2086		« 260(3)

The data represent the average R_{50} concentrations ± SD of 3–4 independent experiments. The number of experiments is indicated in brackets.

effective than S-ET18-OMe in retarding the subcutaneous growth of sarcoma cells, while no stereoselectivity was evident against HL-60 cells grown in culture. In contrast, Melchior *et al.* [33] have reported the *S*-enantiomer to be more active in inhibiting sugar transport in red blood cells. Similarly, a roughly 6-fold increase in potency of the *S*-enantiomer was reported by Hoffman *et al.* [34], investigating the cytotoxic potencies of *S*- and *R*-PAF against HL-60 cells.

Our results indicate that the target of cytotoxic action of SRI 62-834 in HT29 human colon carcinoma cells is not stereospecific. The *R*- and *S*-enantiomers were equipotent in both assays used. Thus, there was no evidence of stereospecificity either for cytotoxicity after prolonged exposure or for acute membrane damage. The PAF receptor was an obvious candidate for mediating the pharmacological effects of SR 62-834 and other ether lipids. However, since the various pathophysiological effects of PAF exhibit a high degree of enantio-specificity [29], the lack of differential activity between the enantiomers of SRI 62-834 argues strongly against the involvement of a PAF receptor. The participation of a conventional PAF receptor in the mediation of the toxic effect of SRI 62-834 can also be discounted in view of the failure of the specific and highly potent PAF receptor antagonist WEB 2086BS to modulate the toxicity of either enantiomer of the racemic mixture. Other structure–activity data are also consistent with this view [32, 35] (Lohmeyer and Workman, (manuscript in preparation).

AELs are reported to be potent and specific inhibitors of a number of membrane-bound or membrane-associated cell signalling enzymes [23]. Our findings show that the observed inhibitory activities are either not of significance for the mechanism of AEL cytotoxicity or are not mediated by stereospecific receptor sites on the affected proteins. To clarify this point, it would be interesting to evaluate the enantio-selectivity of the inhibitory effects on signal transduction enzymes in cell-free systems. If the inhibition of cell signalling enzymes is involved in the mechanism of cytotoxicity, the observed lack of enantio-selectivity might be explained either by a non-stereospecific interaction with the protein or by alterations of the biophysical properties of the lipid bilayer structure. Alterations of membrane state are known to be able to modulate the activities of enzymes located in or on the plasma membrane [36] and various changes in membrane properties have been observed in response to AEL exposure [13, 14].

In contrast to our results with the stereoisomers of SRI 62-834 *in vitro*, recent studies have reported a therapeutic advantage for the *S*-enantiomer using the Meth A fibrosarcoma model in mice (W. Houlihan and P. Munder, personal communication). Given the lack of stereoselectivity in our *in vitro* experiments, this finding is indicative of differential metabolic stability and/or additional mechanism(s) of action operating *in vivo*. Indeed, it is thought that part of the selective cytotoxic action of AELs in this *in vivo* tumour model is mediated by immunological mechanisms, especially those involving AEL-activated macrophages.

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