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Is metabolism an important arbiter of anticancer activity of ether lipids? Metabolism of SRI 62-834 and hexadecylphosphocholine by [^{31}P]-NMR spectroscopy and comparison of their cytotoxicities with those of their metabolites

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Summary. Antineoplastic ether lipids have entered phase I clinical trial and, although their mechanism of action remains unclear, it is widely believed that the plasma membrane is the primary cellular drug target. In the present study the hypothesis was tested that metabolism of ether lipids acts as a detoxification process. [^{31}P]-nuclear magnetic resonance (NMR) spectroscopy was used to study the metabolism of the ether lipid SRI 62-834 (SRI) and the phosphate ester hexadecylphosphocholine (HPC) in the presence of both isolated phospholipases C and D and post-mitochondrial rat liver homogenate. Both SRI and HPC were slowly metabolised by phospholipase D to their alkyl phosphates and choline, and the alkyl phosphates were subsequently metabolised by phosphatase to yield the alcohols and inorganic phosphate. These studies failed to detect any metabolism of either SRI or HPC by phospholipase C, and the metabolism of platelet-activating factor (PAF) by this enzyme was not inhibited by the addition of either compound. The cytotoxicity of SRI, the related compound HPC and their metabolites was determined in vitro using three cell lines. Cytotoxicity was measured by analysis of cell growth kinetics, MTT assay and lactate dehydrogenase release. Closely similar results were obtained in the JB1 rat hepatoma cell line, in the non-transformed BL8 rat hepatocyte cell line, and in A549 human lung adenocarcinoma cells. SRI was the most toxic of the compounds analysed, the concentration required to produce 50% toxicity or growth inhibition (IC_{50}) being 6–9 μM. The putative metabolite of SRI, 2,2′-bis(hydroxymethyl)tetrahydrofuran, and the known metabolites [2′-(octadecyloxymethyl)tetrahydrofuran-2′-yl]methyl phosphate and 2-hydroxymethyl-2-octadecyloxymethyltetrahydrofuran exhibited IC_{50} values of >200, >100 and 40–70 μM, respectively, consistent with metabolic detoxification. HPC was more cytotoxic (IC_{50}, 37 μM) than its phosphate metabolite (IC_{50}, 140 μM), but its toxicity was similar to that of its metabolite hexadecanol (IC_{50}, 28 μM), suggesting that only the former metabolic route leads to detoxification.

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Abbreviations: BnP, benzylphosphonate; ET18O CH_3, 2-methyl-1-octadecyl-glycero-3-phosphocholine; HPC, hexadecylphosphocholine; LDH, lactate dehydrogenase; MES, morpholinoethanesulphonate; MTT,3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; NMR, nuclear magnetic resonance; PAF, platelet-activating factor, 2-acetyl-1-octadecyl-glycero-3-phosphocholine; PBS, phosphate-buffered saline; SRI 62-834, SRI, [2′-(octadecyloxymethyl)tetrahydrofuran-2′-yl]methyl 2-[*N,N,N*-trimethylammonio]ethyl phosphate.

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Introduction

[2′-(Octadecyloxymethyl)tetrahydrofuran-2′-yl]methyl 2-(*N,N,N*-trimethylammonio)ethyl phosphate (SRI 62-834, SRI) and hexadecylphosphocholine (miltefosine, HPC) are experimental anticancer agents that are analogues of platelet-activating factor (PAF) and possess a phosphocholine moiety in their structures (Figs. 1, 2). They are members of a class of antineoplastic agents collectively named ether lipids [5]. Since DNA is not the target of treatment with ether lipids [30], these compounds may not suffer from the same limitations as many currently used anticancer drugs. In particular, SRI has been shown to be selectively toxic towards leukaemic but not normal bone marrow cells [2] and has been useful in purging bone marrow in patients with leukaemia [19]. SRI has recently entered phase I clinical trial in the United Kingdom. The dose-limiting toxicity

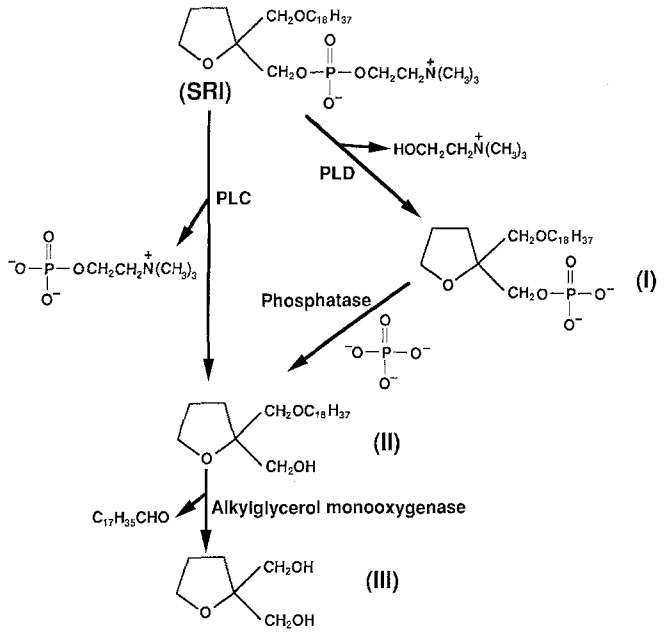

Fig. 1. Proposed route of metabolism of SRI

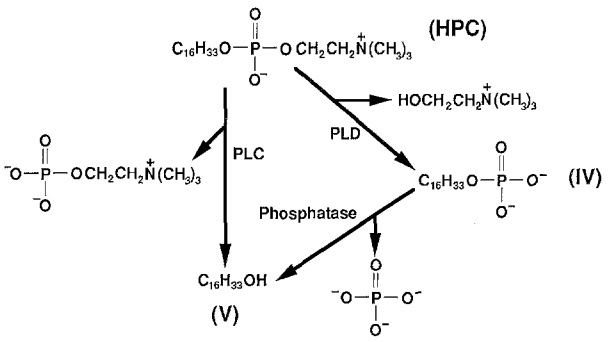

Fig. 2. Proposed route of metabolism of HPC

of HPC after oral administration is nausea and vomiting [27].

The mechanism of action of the ether lipids and their apparent selectivity towards tumour cell populations has been the subject of much research in recent years [30]. They appear to have both direct and indirect actions. The indirect toxicity of ether lipids appears to be mediated via the activation of macrophages [4]. Their direct cytotoxicity is thought to involve perturbation of the plasma membrane [30]. At micromolar concentrations, the mechanisms implicated are acute changes in membrane fluidity and permeability [18]. SRI has been shown to increase cytosolic calcium and this increase was modulated by protein kinase C (PKC)-activating phorbol esters [15]. These effects are heavily dependent on both temperature and the amount of serum present. More recently, in long-term incubations (18 h) with Swiss 3T3 fibroblasts, ether lipid 2-methyl-1-octadecyl-glycero-3-phosphocholine (ET18O CH_3) at 1- to 5-μM concentrations interfered with inositol-trisphosphate-mediated Ca^{2+} release [22, 31]. These studies imply that ET18O CH_3-induced modulation of signaling pathways could abrogate the actions of growth factors and mitogens on cellular proliferation. HPC has also been reported to inhibit PKC activity in NIH3T3 cells [25]. The selective toxicity seen in leukaemic cells has been shown to result from differences in the uptake kinetics of the ether lipid via endocytosis [3].

This report addresses the role of metabolism in the activity of ether lipids. Both SRI and HPC possess a phosphocholine group and may therefore be susceptible to metabolism mediated by phospholipases C and D as described for PAF [9, 13, 29] (Figs. 1, 2). The primary products of the metabolism of SRI and HPC mediated by phospholipase D would be the alkyl phosphates I and IV, respectively. Alcohols II and V would result either from metabolism catalysed by phospholipase C or from the reaction of the alkyl phosphates I and IV with phosphatase. We tested the hypothesis that [^{31}P]-NMR spectroscopy can be used to study the metabolism of SRI and HPC by phospholipases C and D. Reaction of alcohol II with alkylglycerol monooxygenase should yield diol III; this route of metabolism was not considered in the present study.

The cytotoxicity of SRI, HPC and their metabolites was determined in three cell lines, two malignant lines (A549 from human lung and JB1 aflatoxin-induced rat hepatoma) and one non-transformed line (BL8 from rat liver). Three assays were employed in this study: cell growth kinetics were examined to study drug or metabolite effects on cell proliferation, the MTT assay was used to detect changes in enzyme activity within the intact cell and LDH release was used to assess cell membrane integrity. Severe biophysical damage of the cell membrane would be expected to result in changes in the MTT assay concurrently with LDH release. More subtle biochemical effects such as interference with cellular signaling pathways might be identified by the MTT assay without the occurrence of such membrane degeneration that LDH would be released. Biophysical damage would be expected to result in cytotoxicity, whereas biochemical perturbation via signaling pathways might be equally likely to cause cytostasis.

Materials and methods

Compounds. SRI and HPC were kindly supplied by Dr. W. J. Houlihan, Sandoz Research, New Jersey (USA) and Dr. P. Hilgard, Asta Pharm, Bielefeld (FRG), respectively. 2-Hydroxymethyl-2-(octadecyloxymethyl)tetrahydrofuran II was made from III using a method previously described elsewhere [14]. Gentamicin was obtained from Sigma Chemicals (UK); PAF, from Cascade Biochemicals, Reading University (UK); and other chemicals, from Aldrich or Sigma Chemicals (UK). Tissue-culture medium was purchased from Gibco (UK), and JB1 and BL8 cells were kindly provided by Dr. R. F. Legg, MRC Toxicology Unit, Carshalton (UK).

2,2-Bis(hydroxymethyl)tetrahydrofuran III. A solution of furfural diacetate (18 g, 0.09 mol) [7] in ethanol (60 ml) was hydrogenated and maintained at 100 psi for 6 h under agitation in the presence of 10% palladium on activated charcoal (0.3 g). After 6 h, the suspension was filtered through celite and concentrated to give tetrahydrofurfural diacetate. This diacetate was deprotected to yield tetrahydrofuran-2-carboxaldehyde using the method of Amouroux et al. [1]. A solution of the aldehyde (1.5 g, 15 mmol) in methanol (30 ml) was added to a stirred solution of aqueous formaldehyde (37%, 40 ml, 0.6 mol) and NaOH (7.8 g, 0.2 mol) in water (25 ml). The mixture was stirred in an ice bath for 15 min, after which time formic acid (98%, 1 ml) was added. After

15 min the mixture was concentrated under vacuum. Dichloromethane (30 ml) was added and the mixture was stirred for 15 min. The organic layer was decanted and the slurry was extracted with more dichloromethane (3 × 15 ml). The organic phases were combined and concentrated under vacuum to yield 2,2-bis(hydroxymethyl)tetrahydrofuran III, which was purified by distillation under vacuum (40%, 0.75 g, 6 mmol): bp, 112° C (0.5 mmHg) {lit bp, 110° C (0.47 mmHg) [11]}; mp, 51° C (lit mp, 51° – 53° C [11]); [^{1}H]-NMR (60 MHz, $CDCl_3$), δ 4.6 – 4.4 [2H, m, (-CH_2OH)$_2$], 4.0 – 3.7 [2H, m, -C(5)H_2], 3.5 [4H, s, (-CH_2OH)$_2$] δ 2.3 – 1.7 [4H, m, -C(3)H_2, -C(4)H_2]; ν_{max} (thin film), 3400 cm^{-1} (OH).

Hexadecyl phosphate IV. Hexadecyl phosphate IV was prepared by a method similar to that described for other phosphates [21]. [IH]-Tetrazole (0.76 g, 3 mmol) was added to a stirred solution of di-*tert*-butyl *N,N*-diethylphosphoramidite (0.75 g, 3 mmol) [17] and hexadecanol (0.8 g, 3.3 mmol) in dry tetrahydrofuran. After 5 min, the mixture was cooled to –40° C using a dry ice/isopropanol bath, and a solution of 50% meta-chloroperoxy benzoic acid (1.38 g, 4 mmol) in dichloromethane (4 ml) was added. The stirred mixture was then allowed to warm up to room temperature. After 5 min, an aqueous solution of 10% sodium bisulphite (10 ml) was added and the solution was stirred for 10 min. The aqueous layer was discarded and the organic phase was washed with 10% aqueous sodium bisulphite (2 × 20 ml) and 5% aqueous sodium bicarbonate (2 × 20 ml). The dried filtrate (Na_2SO_4) was concentrated to give hexadecyl di-*tert*-butyl phosphate. This triester was purified by flash column chromatography eluting with ethyl acetate-hexane (1 : 1, v/v) [23] in a yield of 73% (1.0 g, 2.2 mmol).

The triester (1 g, 2.2 mmol) was stirred in a solution of 6 M HCl (2 ml) and dioxane (2 ml) at room temperature for 48 h, after which time the solvent was evaporated. The solid residue was resuspended in water (2 ml) and the phosphate (IV) was extracted first into ether and then into NaOH (10 M, 4 ml). The aqueous layer was concentrated and the residue was resuspended in water (1 ml), acidified to pH 1 with concentrated hydrochloric acid and back-extracted into chloroform. The chloroform solution was evaporated under vacuum to yield 0.4 g phosphate IV as a colourless waxy solid (1.2 mmol, 55%): [^{1}H]-NMR (250 MHz, $CDCl_3$), δ 5.4 – 5.2 [2H, br, -P(OH)$_2$], 4.0 – 3.8 [2H, br, -OCH_2CH$_2$(CH$_2$)$_{13}$CH$_3$], 1.8 – 1.7 [2H, m, -OCH$_2$CH_2(CH$_2$)$_{13}$CH$_3$], 1.25 [26H, brs, -OCH$_2$CH$_2$(CH_2)$_{13}$CH$_3$], 0.9 [3H, brt, -OCH$_2$CH$_2$(CH$_2$)$_{13}$CH_3]; [^{31}P]-NMR (101.3 MHz, $CDCl_3$), δ_p 1.15 (s, ^{1}H decoupled), 1.2 (t, J_{PH} = 5.5 Hz, ^{1}H coupled); m/z (CI, ammonia), 340 (M+NH_4, 100%), 323 (M+H, 55%). Observed accurate m/z on (M+NH_4) gave 340.2617; $C_{16}H_{35}O_4P.NH_4$ requires 340.2617. Phosphate IV was back-extracted into NaOH, which was neutralised for use in tissue-culture experiments.

Dihydrogen [2′-(octadecyloxymethyl)tetrahydrofuran-2′-yl]-methyl phosphate I. This compound was prepared using the same method described for the preparation of phosphate IV using alcohol II. Phosphate I was a colourless sticky solid (0.30 g, 0.5 mmol, 45%): [^{1}H]-NMR (250 MHz, $CDCl_3$), δ 4.00 – 3.90 [(2H, brs, -P(OH)$_2$], 3.50 – 3.30 [8H, m, (-CH_2O-)$_2$, -OCH_2CH$_2$(CH$_2$)$_{15}$CH$_3$, -C(5)H_2], 2.00 – 1.80 [4H, brs, -C(3)H_2, -C(4)H_2], 1.60 – 1.45 [2H, brs, -OCH$_2$CH_2(CH$_2$)$_{15}$CH$_3$], 1.34 – 1.14 [30H, brs, -OCH$_2$CH$_2$(CH_2)$_{15}$CH$_3$], 0.85 (3H, t, J_{HH} = 6.5 Hz, -OCH$_2$CH$_2$(CH$_2$)$_{15}$CH_3]; [^{31}P]-NMR (101.3 MHz, $CDCl_3$), δ_p -0.17 (s, ^{1}H decoupled); m/z (CI, ammonia), 465 (M+H, 10%), 403 (M–PO_3+NH_4, 5%), 385 (M–PO_3+H, 30%), 97 (100%). Observed accurate CI m/z on (M+H) gave 465.3345; $C_{24}H_{50}O_6P$ requires 465.3345.

Preparation of post-mitochondrial rat liver homogenate. Male Sprague-Dawley rats (weight around 100 g) obtained from Bantin and Kingman, Hull (UK), were killed by cervical dislocation. The liver was removed, minced and suspended in 4 parts of TRIS buffer (0.05 M) with sucrose (0.25 M) at pH 7.6. The protease inhibitors pepstatin and phenylmethylsulphonyl fluoride were added to the buffer in acetone (500 µl) prior to homogenisation. The tissue was homogenised and centrifuged at 9000 *g* for 22 min (4° C) and the supernatant was decanted and used in the metabolism studies.

Metabolism studies using phospholipase C and D. Studies were performed using a Bruker FT AC 250 MHz NMR spectrometer. [^{31}P]-NMR spectra were recorded at 121.5 MHz and are reported δ_p ppm downfield of 85% phosphoric acid. SRI, HPC or PAF (10 µmol) was dissolved in 900 µl buffer (0.1 M). TRIS buffer of pH 7.3 was used to study metabolism catalysed by phospholipase C from *Clostridium perfringens*, type XIV, and buffer of pH 8.0 was employed to examine that mediated by phospholipase D from *Streptomyces chromofuscus*, type VI. D_2O (100 µl) was added to each sample to give a deuterium lock for the spectrometer. To allow the time course of the metabolism of SRI to be followed, a solution of benzylphosphonate (BnP; 5 µmol, 0.9 mg) in D_2O (100 µl) was added as an internal standard. The chemical shift of BnP (18.8 ppm) is significantly different from that of the substrates or any putative metabolites and its carbon-phosphorus bond is resistant to chemical or enzymic cleavage.

A [^{31}P]-NMR spectrum was recorded immediately after the addition of 25 units of the enzyme diluted with buffer of appropriate pH (10 µl). The NMR tubes were incubated in a water bath at 37° C and analysed at regular intervals by [^{31}P]-NMR spectroscopy. The temperature of the NMR probe was kept at 37° C and spectra were recorded at appropriate intervals. In all reactions with phospholipase D, metabolism was quenched by the addition of two drops of 10 M NaOH, which was necessary to sharpen the peaks in the [^{31}P]-NMR spectrum. Products of metabolism were identified by adding reference compounds. Alkaline phosphatase was added to confirm the presence of alkyl phosphate monoesters, as these were cleaved to yield inorganic phosphate. Metabolites formed from the incubations of SRI and HPC with phospholipase D were isolated by making the sample acidic, extracting with $CDCl_3$ (1 ml) and then back-extracting into 10 M NaOH (1 ml).

The presence of phosphatase activity in samples reacted with phospholipase D was confirmed by incubating 25 units of enzyme in TRIS buffer of pH 8 (1 ml) with the disodium salt of *p*-nitrophenyl phosphate (10 mg), which gave a bright yellow solution due to the formation of *p*-nitrophenolate. SRI and HPC were also incubated with heat-inactivated phospholipase D in TRIS buffer (pH 8). Metabolism was not observed, confirming that production of phosphates I and IV was due to enzyme catalysis and not to chemical breakdown.

To establish whether HPC and SRI were inhibitors of phospholipase C, SRI or HPC (10 µmol) was dissolved in TRIS buffer (500 µl) and the respective [^{31}P]-NMR spectra were recorded. The samples were incubated for 30 min at 37° C with phospholipase C from *C. perfringens* (12.5 units). [^{31}P]-NMR spectra were recorded after the incubation period and then immediately following the addition of PAF (10 µmol in 400 µl TRIS buffer of pH 7.3). The rate of metabolism of PAF was compared with that in a control sample without the ether lipid. For this control sample, [^{31}P]-NMR spectra were run prior to and immediately after the addition of phospholipase C (12.5 units).

Metabolism studies using rat liver homogenate. The [^{31}P]-NMR spectrum of rat liver homogenate gave weak peaks at δ_p 2.77, 3.93 and 4.71 ppm. After incubation for 24 h there was only one peak at 2.61 ppm, which on the addition of NaOH shifted to δ_p 7.66 ppm, corresponding to inorganic phosphate. PAF, SRI or HPC (10 µmol) was dissolved in 500 µl TRIS buffer (pH 7.3) and D_2O (100 µl). A [^{31}P]-NMR spectrum was run prior to the addition of liver homogenate (400 µl), after which another spectrum was recorded, the samples were incubated in a water bath at 37° C and spectra were then taken at regular intervals.

Cell culture. JB1 and BL8 cells were cultured in William's E medium supplemented with 5% foetal calf serum as described by Manson et al. [16]. A549 cells were obtained from the American Type Culture Collection, Rockville, Maryland (USA) and cultured in Hams F12 medium. Compounds were added after dissolution in medium or in dimethylsulphoxide (DMSO). The final concentration of DMSO in the cultures (<1%) did not affect growth or viability.

MTT assay. The method used was based on those previously described [17, 24], but conditions were optimised for each cell line. A549 cells were detached using trypsin, counted and resuspended at 6 × 10^3/ml. Aliquots (200 µl) were added to individual wells of a 96-well plate. Cells were allowed to adhere for 1 h prior to treatment. Test compounds dissolved in 20 µl solvent were added to the wells to give a final concen-

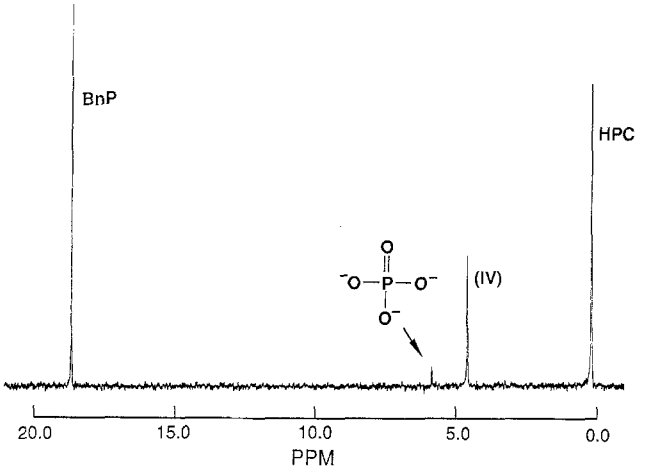

Fig. 3. [^{31}P]-NMR spectrum of a sample of an incubate of HPC with phospholipase D, following the addition of NaOH (t = 24 h)

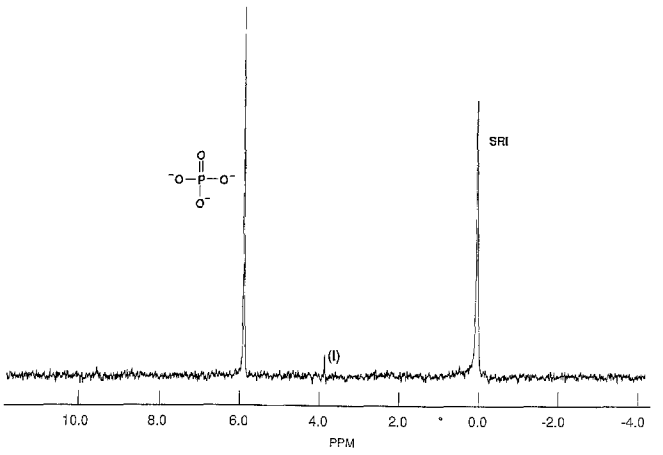

Fig. 4. [^{31}P]-NMR spectrum of a sample of an incubate of SRI with rat liver homogenate, following the addition of NaOH (t = 72 h)

tration of 0.1–200 μM. Control incubates contained medium with solvent. Determinations were conducted in quadruplicate and the plates were then incubated at 37° C for 72 h. The stock solution of MTT (5 mg/ml) was passed through a 0.2-μm filter. To each well an aliquot of this solution (20 μl) was added and the plates were incubated for a further 6 h. Medium was removed carefully to avoid disruption of the formazan product in the cells. Cells were washed with PBS (200 μl). DMSO (200 μl) was added and the plates were agitated for 5 min on a Titertek plate shaker. The optical density was read in a Titertek multiscan spectrophotometer at 550 nm. The results were expressed as a percentage of the values obtained in incubates without drugs.

In the case of JB1 and BL8 cells, the cells were initially suspended at 9×10^3/ml; incubation with MTT was carried out for 5 h, and the cells were not rinsed with PBS.

Cell growth kinetics and LDH release assay. Cells were treated with SRI, alcohol II or diol III (1, 10 or 100 μM) and were counted at 12, 24, 48 and 72 h using a haemocytometer. Medium was aspirated and assayed for LDH activity using a method based on that described by Bergmeyer and Bernt [6]. LDH activity was also measured in the medium of cells treated with SRI (100 μM) for 5, 15, 30 and 60 min. The results were expressed as a percentage of the maximal LDH activity released by Triton X-100 (1% v/v) per 10^5 cells.

Graphs were prepared using the mean values obtained in the experiments. IC_{50} values (the concentration required to produce 50% toxicity or growth inhibition) were calculated at 72 h.

Results

Metabolism by phospholipase C

A solution containing 10 μmol PAF [δ_p 0.13 (s, ^{1}H decoupled; pent, J_{PH} = 7 Hz, ^{1}H coupled)] was completely metabolised within 30 min by phospholipase C from *Clostridium perfringens* (25 untis) to phosphocholine [δ_p 3.86 (s, ^{1}H decoupled; t, J_{PH} = 5 Hz, ^{1}H coupled)]. On the addition of alkaline phosphatase, phosphocholine was converted into inorganic phosphate (δ_p 2.28 ppm). In contrast, SRI and HPC were not substrates for phospholipase C, as the [^{31}P]-NMR spectra of their incubates did not contain any metabolite peaks after 48 h. It is estimated that 5% metabolism would have been detected. To establish whether HPC and SRI were inhibitors of phospholipase C, SRI (δ_p 0.16 ppm) and HPC (δ_p 0.28 ppm) were incubated with phospholipase C from *C. perfringens* at pH 7.3. After 30 min, PAF (δ_p 0.00 ppm) was added to this mixture. In both reactions the [^{31}P]-NMR spectra showed the formation of a peak at δ_p around 3.9 ppm, which corresponded to phosphocholine, and complete metabolism of PAF occurred at a rate similar to that observed in the absence of SRI or HPC.

Metabolism by phospholipase D

A solution containing 10 μmol PAF [δ_p -0.13 ppm (s, ^{1}H decoupled; pentet, J_{PH} = 7 Hz, ^{1}H coupled)] was incubated with phospholipase D from *Streptomyces chromofuscus* (25 units) at pH 8. After 24 h, 50% metabolism had occurred, giving a broad peak at 3.7–2.5 ppm, which after the addition of NaOH gave four singlets. The peak at 0.00 ppm was due to PAF, and the metabolite resonating at 4.73 ppm (s, ^{1}H decoupled; t, J_{PH} = 5 Hz, ^{1}H coupled) was the phosphate of PAF, obtained by cleavage of choline. With time, the peaks at 4.73 and 0.00 ppm were converted into those at 5.17 and 0.45 ppm, respectively. Control experiments showed that these were products of base-catalysed hydrolysis. The [^{1}H]-NMR spectrum of this sample included a peak at δ_H 1.9 ppm for sodium acetate. These results and control experiments using PAF indicate that sodium hydroxide catalyses ester hydrolysis at the 2-acetyl group, suggesting that the peak at 0.45 ppm represents lyso-PAF and the peak at 5.17 ppm, lyso-PAF without the choline group.

SRI was incubated with phospholipase D from *S. chromofuscus*, and a broad metabolite peak at δ_p 6.00–5.40 ppm appeared within 10 min. After 21 h, the reaction was quenched by the addition of NaOH, by which time approximately 70% of the SRI had been metabolised. The metabolite peak at δ_p 5.08 ppm was shown to be alkyl phosphate I by three methods. Firstly, the mixture was incubated with alkaline phosphatase, which converted I into inorganic phosphate (δ_p 6.15 ppm). Secondly, a standard of phosphate I displayed a chemical shift similar to

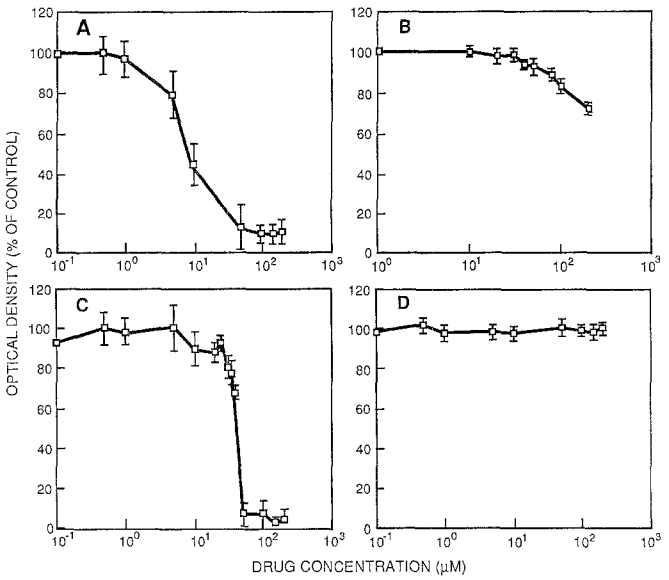

Fig. 5 A – D. Dependency of cytotoxicity on the concentration of SRI (**A**) and its metabolites I (**B**), II (**C**) and III (**D**) in JB1 rat hepatoma cells. Cell number and viability were assessed by MTT assay at 72 h after seeding. Data represent mean values ± SE for 3 – 4 determinations, each performed in quadruplicate

Table 1. Growth inhibition and cytotoxicity of SRI and its metabolite II after incubation for 72 h as determined by three assays in three cell lines

Agent	Cell line	IC_{50} (50 μM)		
		Growth	MTT	LDH
SRI	JB1	0.6	9.0	2.0
	BL8	1.5	7.0	2.5
	A549	0.1	6.0	2.8
Metabolite II	JB1	56	45	24
	BL8	28	40	27
	A549	60	27	29

Values were derived from graphs in which the SE for individual data points (from 3 – 4 experiments) at each concentration did not exceed 23% for the growth curves, 13% for the MTT assay and 22% for the LDH assay

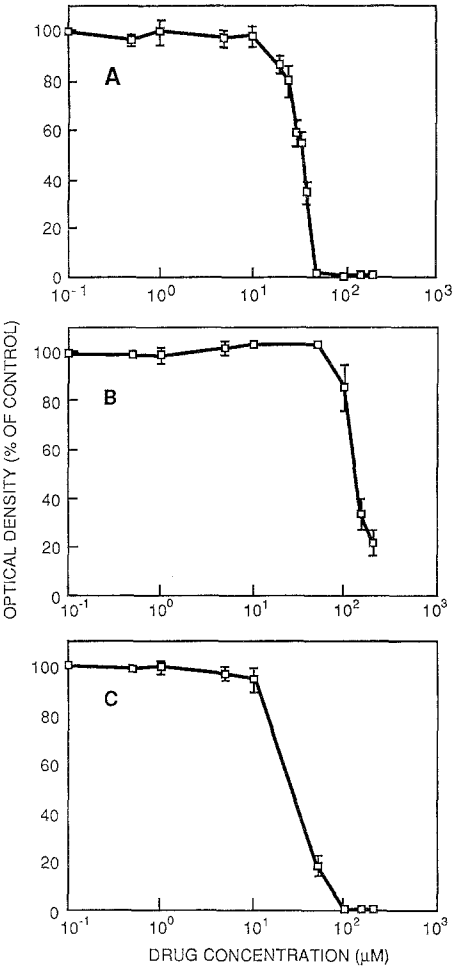

Fig. 6 A – C. Dependency of cytotoxicity on the concentration of HPC (**A**) and its metabolites IV (**B**) and V (**C**) in JB1 rat hepatoma cells. Cell number and viability were assessed by MTT assay at 72 h after seeding. Data represent mean values ± SE for 3 – 4 determinations, each performed in quadruplicate

that shown by the metabolite and produced inorganic phosphate on incubation with alkaline phosphatase. Thirdly, after isolation by the addition of hydrochloric acid and subsequent extraction into chloroform, the metabolite was compared with phosphate standard I by thin-layer chromatography and NMR.

HPC [δ_P 0.20 ppm (s, ^1H decoupled; pentet, J_{PH} = 6 Hz, ^1H coupled)] was incubated with phospholipase D isolated from *S. chromofuscus*. The pattern of metabolism was similar to that observed for SRI, although HPC was metabolised more slowly, with a decrease in substrate of only 40% being observed within 24 h. A broad metabolite peak (δ_P 2.6–2.1 ppm) was observed, which on the addition of NaOH gave a peak at δ_P 4.67 ppm (t, J_{PH} = 5.5 Hz, ^1H coupled; 35%) for hexadecyl phosphate IV and a smaller peak at 6.18 ppm (5%) for inorganic phosphate (Fig. 3).

Metabolism by rat liver homogenate

PAF (δ_P -0.11 ppm) was incubated with liver homogenate for 72 h, after which time NaOH was added and left for 30 min to allow hydrolysis of the 2-acetyl ester. The [^{31}P]-NMR spectrum showed that 50% of the PAF remained with the formation of lyso-PAF at δ_P 0.37 ppm. The metabolite peaks represented inorganic phosphate (δ_P 5.95 ppm, 34%), the phosphate of lyso-PAF (δ_P 5.60 ppm, 6%) and phosphocholine (δ_P 4.09 ppm, 10%). These results suggest that metabolism of PAF in rat liver homogenate is catalysed by both phospholipase C and phospholipase D.

SRI (δ_P 0.01 ppm) was incubated with rat liver homogenate, and 50% metabolism had occurred by 72 h. After the addition of NaOH, two metabolite peaks were observed, one due to inorganic phosphate (δ_P 5.84 ppm, 45%) and the other, to phosphate I (3.90 ppm, 5%; Fig. 4). These results are consistent with the notion that metabolism of SRI by phospholipase D yields alkyl phosphate I, which is subsequently metabolised by phosphatase to inorganic phosphate and alcohol II. Phosphocholine was not ob-

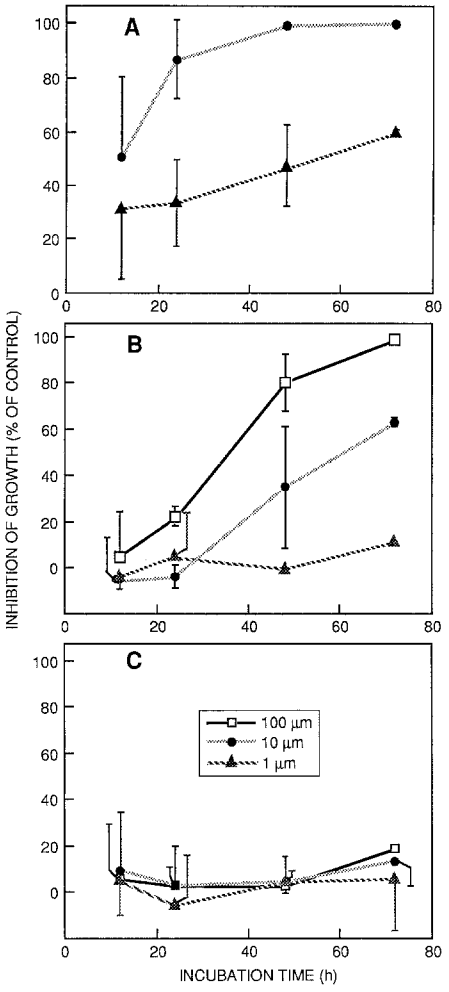

Fig. 7 A – C. Effects of SRI (**A**) and its two metabolites II (**B**) and III (**C**) on the growth of JB1 rat hepatoma cells. Cells were counted using a haemocytometer. Data represent mean values ± SE for 3–4 determinations, each performed in quadruplicate

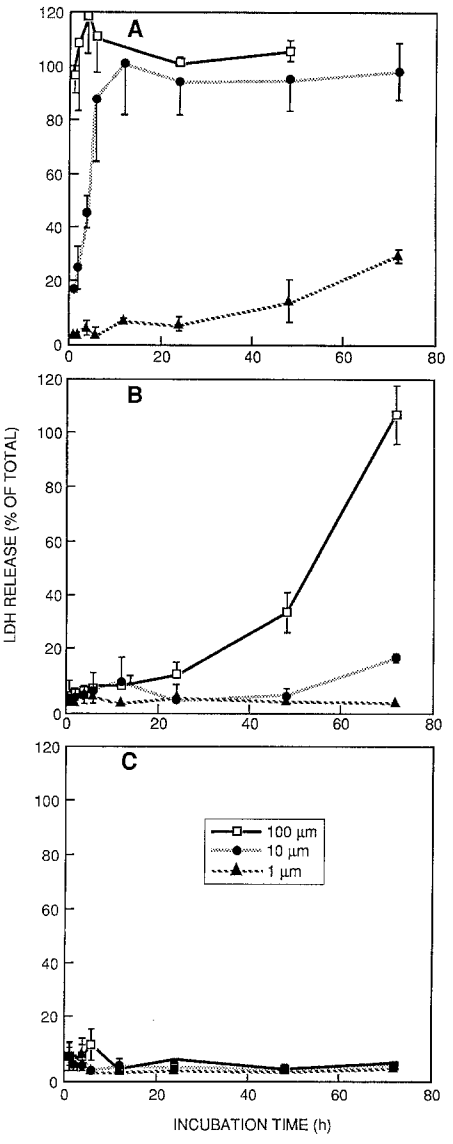

Fig. 8 A – C. Dependency of the cytotoxicity of SRI (**A**) and its metabolites II (**B**) and III (**C**) on the duration of incubation with JB1 rat hepatoma cells. Toxicity was assessed by determination of LDH levels in the cell supernatant. Data represent mean values ± SE for 3 experiments, each performed in triplicate, expressed as a percentage of the LDH released by Triton X-100

served, which is commensurate with a lack of metabolism by phospholipase C.

After 72 h incubation with rat liver homogenate, 25% of the HPC (δ_p 0.07 ppm) was metabolised to a broad peak at δ_p 3.0–2.4 ppm. On the addition of NaOH this peak sharpened into two peaks, one at δ_p 5.88 ppm (20%) for inorganic phosphate and the other at 4.05 ppm (5%) for hexadecyl phosphate IV. By analogy with SRI, HPC seems to be metabolised by phospholipase D to give hexadecyl phosphate IV, which is then metabolised by phosphatase to give inorganic phosphate. Again, metabolism by phospholipase C was not observed.

Cytotoxic properties of SRI, HPC and their metabolites

The cytotoxicity of SRI, HPC and their metabolites was elucidated in JB1, BL8 and A549 cells using the MTT assay. Figure 5 shows the effects of SRI and its metabolites on JB1 cells after 72 h incubation. All three cell lines responded in a similar manner as shown by the IC_{50} values given in Table 1. SRI was more toxic than its metabolites, the IC_{50} values being 6–9 μM for SRI, 40–70 μM for alcohol II and >200 μM for diol III. The phosphate metabolite of SRI (I) was also studied in JB1 cells and proved to be devoid of substantial toxicity (Fig. 5B). The effects of HPC and its metabolites on the growth and viability of cells after 72 h incubation are shown in Fig. 6. HPC was less toxic than SRI, its IC_{50} value being 37 μM in JB1 cells (Fig. 6B). However, alcohol V produced toxicity similar to that of HPC (IC_{50}, 28 μM; Fig. 6C).

The results of studies in which the effect of SRI and its metabolites on cells were evaluated using growth curves support the findings obtained in the MTT assay. SRI was more toxic to JB1 cells than were its metabolites; indeed, diol III did not have a significant effect on cell growth (Fig. 7). As seen in the MTT assay, similar results were also observed in A549 and BL8 cells (data not shown). At 1 μM SRI there was detectable inhibition of cell growth

(Fig. 7A). Alcohol II was less toxic than SRI and required 72 h at 100 μM to produce 100% inhibition of cell growth (Fig. 7B). On inspection under the haemocytometer, cells treated with SRI and alcohol II (100 μM) appeared swollen prior to death and those treated with SRI (100 μM) were occasionally seen to rupture.

Figure 8 demonstrates the effects of SRI and its metabolites on viability as measured by LDH release in JB1 cells. At a concentration of 100 μM, SRI produced its effect rapidly and 100% LDH release was observed within 15 min (Fig. 8A). A similar pattern of toxicity was observed in A549 and BL8 cells (data not shown). Table 1 demonstrates the IC_{50} values calculated from the LDH assay for the three cell lines. SRI (10 μM) produced 100% LDH release within 24 h (Fig. 8A). Alcohol II (100 μM) required 72 h to produce this level of toxicity (Fig. 8B), whereas diol III had only a minimal effect on LDH release (Fig. 8C).

Discussion

The aim of this study was to elucidate the metabolism of the ether lipids SRI and HPC and to test the hypothesis that either their metabolism leads to detoxification or an increased cytotoxic effect is observed on the addition of their metabolites to cells in vitro.

In the metabolism studies, SRI and HPC were apparently not metabolised by phospholipase C under conditions for which PAF was a substrate. However, the method used in these experiments was not sensitive enough to detect less than 5% metabolism. For a compound to serve as a substrate for phospholipase C, the polar trimethylammonio head and the fatty acid groups are essential for substrate recognition, and the enzyme favours substituents with chains of 12–16 carbons in length for the hydrophobic binding site [8]. HPC has a C_{16} chain, but it is a simple alkyl phosphocholine and lacks a fatty acid group. SRI has an ether linkage to the C_{18} chain, and it has been shown that substitution of ester linkages of fatty acids with ether moieties yields poor substrates for this enzyme [12]. The absence of crucial enzyme-recognition moieties in either HPC or SRI can be used to explain the lack of their metabolism by phospholipase C.

In contrast to these findings, HPC radiolabeled with tritium in the choline group has been reported to give choline, phosphocholine and 1,2-diacylphosphatidylcholine as metabolites when given to mice [9]. However, it is possible that the phosphocholine was not formed by direct metabolism of HPC by phospholipase C. Ether lipids, including HPC, have been postulated to inhibit phosphatidylinositol-specific phospholipase C from fibroblasts [22, 25]. Therefore, it was considered that SRI and HPC may be inhibitors of phospholipase C. However, the addition of either SRI or HPC did not alter the rate of metabolism of the endogenous phospholipase C substrate PAF [20, 29].

[^{31}P]-NMR spectroscopy was used to monitor the metabolism of PAF to the alkyl phosphate by phospholipase D [29]. Similarly, SRI and HPC were shown to be substrates for phospholipase D, giving rise to phosphates I and IV. When PAF was incubated with rat liver homogenate, both alkyl phosphate and phosphocholine were formed, consistent with metabolism by both phospholipases C and D. Inorganic phosphate was also observed, presumably generated by metabolism of either the alkyl phosphate or phosphocholine by phosphatase.

Analysis of incubates of SRI and HPC with rat liver homogenate by [^{31}P]-NMR spectroscopy showed the formation of alkyl phosphates I and IV, consistent with metabolism by phospholipase D. Phosphocholine was not formed, commensurate with the results obtained using phospholipase C. Inorganic phosphate was the major metabolite, and it is likely to arise via the reaction of the alkyl phosphates I and IV with phosphatase.

The cell lines chosen for the cytotoxicity study should allow the preliminary detection of differences between organs and species as well as between transformed and non-transformed phenotypes. The results presented herein should be considered in the light of recent evidence concerning the possible mechanism(s) of action of ether lipids. It is believed that ether lipids elicit different effects depending on the drug concentration, the exposure period and the presence of serum [10]. They are thought to cause biophysical membrane damage at 10- to 100-μM concentrations and to modulate proliferation via a biochemical mechanism of action involving cell signaling pathways at lower concentrations (<5 μM). Selective uptake of ether lipids into tumour cells via endocytosis as described by Bazill and Dexter [3] may also have important ramifications with regard to the metabolism of these compounds.

All three cell lines responded similarly to the compounds under study. Treatment of cells with 100 μM SRI caused toxicity in all cells within 15 min as observed in the LDH assay. The speed of onset of toxicity is consistent with the notion that ether lipids in the 10^{-4} M concentration range exhibit a detergent-like effect [10]. At 10 μM, SRI induced a complete release of cellular LDH that took 24 h to occur. Thus, the LDH assay seems to be capable of detecting the "rapid" cytotoxic potential that SRI exerts at concentrations exceeding 10 μM.

Cell number appeared to be the most sensitive indicator of the cytotoxicity of SRI at the more pharmacologically relevant concentrations in the 10^{-7} to 10^{-6} M range (Table 1). This method presumably detects the consequences of SRI-induced interference with proliferation via effects on cellular signal transduction. A comparison of the IC_{50} values obtained for SRI in the MTT or LDH assays after 72 h incubation (Table 1) suggests that of the two, the LDH assay is the more sensitive toxicity indicator. Both SRI and HPC caused cells to swell and become rounded, consistent with previous observations in the mouse mammary-tumour cell line EMT6 [10].

Unger et al. [26] have suggested that the toxicity of the ether lipid ET 18 OCH_3 is mediated by the primary alcohol formed by metabolic cleavage of the phosphocholine moiety, although this contention has been disputed [28]. In the three cell lines used in the present study, SRI was more toxic than its metabolites. HPC was slightly more toxic than its metabolite phosphate IV, but its toxicity was very similar to that of hexadecanol V. It appears that only one of the identified routes of HPC metabolism is a detoxification pathway. Metabolites of both ether lipids produced equiv-

alent or lower degrees of cytotoxicity as compared with their parent molecules, and this finding implies that metabolic activation does not occur. However, one cannot exclude the possibility that differences in lipophilicity between the parent molecules and the metabolites with an alcohol structure (II and V) may mask or counteract genuine toxicity differences. The phosphate metabolites I and IV are also less toxic than their metabolic precursors, perhaps due to their poor ability to penetrate cell membranes associated with their dianionic and hydrophilic nature. Similarly, diol III is very hydrophilic and relatively non-toxic. An alternative hypothesis to explain these data would be that the metabolites may be less readily endocytosed than the parent ether lipid molecules.

In summary, three different cell lines showed a closely similar degree of cytotoxicity when exposed to SRI and HPC, and metabolism to the species described above does not appear to be a prerequisite for their cytotoxicity against these cells.

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