A lysophosphatidic acid analogue is revealed as a potent inhibitor of phosphatidylcholine synthesis, inducing apoptosis

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A previous study demonstrated that cross-desensitization experiments performed with the lysophosphatidic acid (LPA) analogues (*R*)- and (*S*)-*N*-palmitoyl-norleucinol 1-phosphate (PNPAs) inhibited LPA-induced platelet aggregation without any stereospecificity. Here we report opposite biological effects of the two enantiomers on mitogenesis of IMR-90 fibroblasts in relation to their respective metabolism. (*R*)PNPA was proliferative, while (*S*)PNPA induced apoptosis by specifically inhibiting phosphatidylcholine biosynthesis at the last step of the CDP-choline pathway controlled by cholinephosphotransferase. This effect was not direct but required dephosphorylation of PNPAs by ecto-lipid phosphate phosphatase before cellular uptake of the generated *N*-palmitoyl-norleucinols (PNOHs). Inhibition of cholinephosphotransferase by the derivative (*S*)PNOH was

confirmed by an *in vitro* assay. (*S*)PNPA proapoptotic effects led us to clarify the mechanism linking cholinephosphotransferase inhibition to apoptosis. Three proapoptotic responses were observed: the activation of caspase-3, the production of ceramides from newly synthesized pools (as demonstrated by the inhibitor Fumonisin B1) and finally the activation of stress-activated protein kinase, p38 and c-Jun N-terminal kinases 1/2, as a result of ceramide increase. Thus our data demonstrate that synthetic analogues of LPA might display stereospecific effects leading to apoptosis independently of classical LPA-activated pathways.

Key words: ceramide, cholinephosphotransferase, stress-activated protein kinase.

INTRODUCTION

Lysophosphatidic acid (LPA; 1-acyl-*sn*-glycerol 3-phosphate) is structurally one of the simplest phospholipids, which has acquired the status of extracellular mediator in the past decade. It induces a wide array of biological responses such as growth factor-like activities, smooth muscle cell contraction, platelet aggregation, neurite retraction, closure of gap junctions and tumour invasion [1,2]. LPA is found in serum at micromolar concentrations (1–20 μ M), where it is mainly produced by stimulated platelets. Interestingly, LPA was reported to accumulate to high concentrations in ovarian cancer ascitic fluids, where it is used as a marker of tumour progression and shows mitogenic activity on ovarian cancer cells [3]. It was described as a progression factor in cells from mesenchymal origin [4,5], whereas it can act as an inhibitor of cell division in other cell types such as rat myeloma or human lung tumoral cells [6,7]. Moreover, LPA displays dual activity on the same cell type depending on the concentrations used in incubations [8,9]. LPA anti-proliferative effects have been related to its ability to increase cAMP [6]. To some extent, LPA was even described as an apoptosis inducer for neuronal cells [10,11].

Recently, G-protein-coupled receptors specific for lysophospholipid mediators were identified. To date, a cluster of

eight receptors, termed Edg (for endothelial differentiation gene), have been described in several species. Edg-2, -4 and -7 are specific for LPA.

It was recently demonstrated that the stereospecificity of Edg receptors as well as LPA degradation could not totally account for the mitogenic response, suggesting the existence of alternative signalling pathways to trigger those effects [12]. Indeed, while receptors for sphingosine metabolites, especially sphingosyl-phosphorylcholine, display a tight stereoselectivity for their ligand [13], no stereospecificity for LPA receptors was observed in platelets [14], A431 cells [15] and or *Xenopus* oocytes, even with an analogue of LPA, *N*-acyl-phosphoserine, displaying inhibitory activity [16,17]. Interestingly, *N*-acyl-phosphoserine developed a stereospecific response in the human breast cancer cell line MDA MB231 [18].

In order to further characterize LPA receptors, we synthesized two enantiomers of an LPA analogue, *N*-palmitoyl-norleucinol 1-phosphate (PNPA). Both enantiomers, (*R*)PNPA and (*S*)PNPA, were equally potent at inducing platelet aggregation. In cross-desensitization experiments, PNPAs specifically inhibited LPA-induced aggregation, suggesting they were acting on the same receptor(s) [14]. These data fully confirmed the lack of stereospecificity of platelet receptors. Because platelet responses differ from other biological events in terms of rapidity

Abbreviations used: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CPT, cholinephosphotransferase; CT, phosphocholine cytidyltransferase; DAG, *sn*-1,2-diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; Edg, endothelial differentiation gene; ERK, extracellular signal-regulated protein kinase; FCS, fetal calf serum; fura-2/AM, fura-2 acetoxymethyl ester; JNK, c-Jun N-terminal kinase; LDH, lactate dehydrogenase; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPP, lipid phosphate phosphatase; MAPK, mitogen-activated protein kinase; PARP, poly(ADP-ribose) polymerase; PC, phosphatidylcholine; PNacyl, *N*-palmitoyl-norleucinol 1-acyl; PNOH, *N*-palmitoyl-norleucinol; PNPA, *N*-palmitoyl-norleucinol 1-phosphate; PNPC, palmitoyl-norleucinol-phosphocholine; PNPE, palmitoyl-norleucinol-phosphoethanolamine; RT, reverse transcriptase; S1-P, sphingosine 1-phosphate; SAPK, stress-activated protein kinase.

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and sensitivity, this study was initiated to evaluate the impact of the analogues on long-term effects such as mitogenicity. Our data on IMR-90 fibroblasts highlighted different metabolic pathways leading to opposite effects of the two enantiomers. The natural (*R*) isomer mimicked the action of LPA, probably by acting on common receptors, while (*S*)PNPA induced cell death by inhibiting phosphatidylcholine (PC) biosynthesis. The last effect is of major interest because it unravels the potential of (*S*)PNPA as an anti-tumoral agent.

EXPERIMENTAL

Cell culture

Human lung fibroblasts IMR-90 (ATCC CCL-186), colorectal adenocarcinoma Caco-2 cells (ATCC HBT-27) and hepatoma HepG2 cells (ATCC HB-8065) were routinely grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Rockville, MD, U.S.A.) supplemented with 10% (v/v) fetal calf serum (FCS) at 37 °C in a 5% CO₂ humidified incubator. All the experiments were performed between passages 2 and 4 for IMR-90. When required, cells were treated with the p38 inhibitors SB203580 (15 µM) and SB202190 (30 µM; Calbiochem, La Jolla, CA, U.S.A.) and the caspase inhibitor Z-VAD-fmk (100 µM; Calbiochem) for 6 h. Then inhibitors were removed to avoid non-specific toxicity. Treatment with the inhibitor of ceramide synthesis, Fumonisin B1 (Alexis Biochemicals, San Diego, CA, U.S.A.), was carried out for 20 h at the indicated concentrations.

Synthesis of *N*-palmitoyl-norleucinol (PNOH) derivatives

(*R*)- and (*S*)-PNPA were synthesized as described previously [14]. [³H]PNPAs were synthesized using [9,10-³H]palmitic acid (434.5 Ci/mol). Synthesis of the acylated forms of PNOH, (*R*)- and (*S*)-*N*-palmitoyl-norleucinol 1-acyl (PNacyl), was achieved by esterification of the free alcohol function of the PNOH intermediate with palmitoyl chloride (Sigma, St. Louis, MO, U.S.A.). The reaction was carried out at 40 °C for 4 h in a mixture containing 1.1 mM PNOH/1.3 mM palmitoyl chloride in chloroform/pyridine (50:50, v/v). The organic phase was then washed three times with water, then dried. PNacyls were then separated by TLC in the solvent system chloroform/methanol (95:5, v/v) and extracted according to Bligh and Dyer [19]. Purity was confirmed by measurement of ¹H-NMR spectra on a Bruker 250 MHz spectrometer.

Mitogenesis assay

Subconfluent cells (1.5 × 10⁴ cells/cm²) were deprived of serum for 48 h and treated with the indicated doses of agonists for 24 h. [³H]Thymidine (0.25 µCi/ml) was added for the last 15 h. Cells were then washed twice with cold PBS and incubated for 15 min at room temperature with 0.5 ml of ethanol. The cells were solubilized in 0.5 M NaOH and incorporated radioactivity quantified by liquid scintillation counting.

Cytotoxicity assay

Cytotoxic effect of the analogues was determined by the release of lactate dehydrogenase (LDH) into the culture medium using an LDH enzymic assay according to the manufacturer's instructions (Cytotox 96 kit; Promega, Madison, WI, U.S.A.).

Flow cytometry analysis

Cells (1.5 × 10⁴ cells/cm²) were incubated at 37 °C in DMEM supplemented with 2% FCS. After treatment with the agonist

for 20 h, cells were trypsinized and resuspended in 70% cold ethanol. Nuclear DNA was labelled with propidium iodide and analysed by flow cytometry (Coulter ELITE). The fraction of cells with sub-G₁ DNA content was assessed using Cellfit software (Becton Dickinson, Mountain View, CA, U.S.A.).

Immunoblotting

Serum-starved IMR-90 cells (1.5 × 10⁴ cells/cm²) were stimulated with 5 µM (*S*)PNPA at 37 °C for the indicated times. Cells were scraped into Laemmli buffer (62.5 mM Tris/HCl (pH 6.8), 2% SDS, 10% glycerol, 5% mercaptoethanol and 0.001% Bromophenol Blue). Lysates were briefly sonicated. Total proteins were resolved by SDS/PAGE. The phosphorylation of extracellular signal-regulated protein kinase (ERK) 1/ERK2 was evaluated by immunoblotting with anti-phosphorylated mitogen-activated protein kinase (MAPK) (Sigma), anti-phosphorylated p38 stress-activated protein kinase (SAPK; New England Biolabs, Beverly, MA, U.S.A.), anti-phosphorylated p46/54 c-Jun N-terminal kinase (JNK) 1/2 (New England Biolabs) and anti-poly(ADP-ribose) polymerase (PARP; Clontech, Palo Alto, CA, U.S.A.) antibodies. Immunoreactive bands were revealed with horseradish peroxidase-conjugated secondary antibodies and the West-Pico chemiluminescent detection system (Pierce, Rockford, IL, U.S.A.).

Reverse transcriptase (RT)-PCR detection of lipid phosphate phosphatase (LPP) or Edg receptor mRNA expression

Total RNA was isolated using the Trizol reagent method (Life Technologies). cDNAs were obtained by reverse transcription using the Reverse Transcription System (Promega). The oligonucleotide primers used were as follows. β-Actin, forward primer, 5'-CTGGAACGGTGAAGGTGACAC-3'; reverse primer, 5'-GGTCTCAAGTCAGTGTACAGG-3'; LPP1, forward primer, 5'-CCCAGACTGGTCAAAAATCAA-3'; reverse primer, 5'-ACTCGAGAAAGGCCACAATA-3'; LPP2, forward primer, 5'-GCTGCCATCTACAAGGTGCT-3'; reverse primer, 5'-CATGCAATACATGCCAAAGG-3'; LPP3, forward primer, 5'-TCATCTGCCTGGACCTCTTC-3'; reverse primer, 5'-CCTGTAATGATCGCCAGGAT-3'; Edg 1, forward primer, 5'-CCTCTTCTGCTAATCAGCG-3'; reverse primer, 5'-ACAGGTCTTCACCTTGCAGC-3'; Edg 2, forward primer, 5'-CGGAGACGACTGACTGTCAGCAC-3'; reverse primer, 5'-GGTCCAGAACTATGCCGAGA-3'; Edg 3, forward primer, 5'-TCAGCCTGTCTCCCACGGTC-3'; reverse primer, 5'-ACGGCTGCTGGACTTCACCA-3'; Edg 4, forward primer, 5'-CCCAACACAGGACTGACT-3'; reverse primer, 5'-GAGCCCCTATCTCTCCCCAC-3'; Edg 5, forward primer, 5'-CATTGCCAAGGTCAAGCTGT-3'; reverse primer, 5'-ACGATGGTGACCGTCTTGAG-3'; Edg 6, forward primer, 5'-ACGGGAGGGCCTGCTCTTCA-3'; reverse primer, 5'-AAGGCCAGCAGGATCATCAG-3'; Edg 7, forward primer, 5'-GGACACC-CATGATCACTAATG-3'; reverse primer, 5'-TCTGGGTTCT-CCTGAGAGAA-3'.

Detection of intracellular calcium

Serum-starved IMR-90 cells (1 × 10⁴ cells/cm²) seeded on to glass coverslips were loaded with 5 µM fura-2 acetoxymethyl ester (fura-2/AM; Sigma) for 40 min at 37 °C. Cells were washed with PBS and stimulated by the indicated agonist at 5 µM in Ca²⁺-free PBS. Variations in intracellular Ca²⁺ concentration ([Ca²⁺]_i) were assessed by imaging single cells with a Zeiss Axiovert 100M inverted microscope. The cells were excited at 340 nm (complexed Ca²⁺) or 380 nm (free Ca²⁺) and 510 nm

emission of fura-2/AM was recorded for analysis. Results were plotted as the ratio of complexed Ca^{2+} to free Ca^{2+} (F_{340}/F_{380}).

Metabolism of PNPAs

Cells were treated with [^3H]PNPAs (4.0×10^6 d.p.m./ 2.0×10^4 cells) for the indicated times. To evaluate phosphatases involvement, cells were preincubated with $100 \mu\text{M}$ Na_3VO_4 for 20 min prior to PNPA treatment. Cells were then washed twice in PBS and scraped into methanol/water (2:1, v/v). Lipids were extracted according to Bligh and Dyer [19], then separated by TLC on silica gel plates (Merck, Darmstadt, Germany). They were developed after two consecutive migrations. First, a migration in chloroform/methanol (9:1, v/v), then half a migration in butan-1-ol/acetic acid/water (6:2:2, by vol.). Radioactive spots were then detected with a TLC linear analyser (Berthold, Wildbad, Germany), scraped off, and the radioactivity measured by scintillation counting. The different metabolites were identified by comparison with cold standards and by analogy to the migration of the respective natural lipids.

[^3H]Choline incorporation analysis

IMR-90 cells (2.0×10^5) were cultured in DMEM supplemented with 2% FCS. They were then treated with LPA, (R)PNPA or (S)PNPA at $5 \mu\text{M}$ for the indicated times. For the pulse experiments, $2 \mu\text{Ci/ml}$ [methyl- ^3H]choline chloride was added for the last 3 h. For the chase experiments, cells were prelabelled with $2 \mu\text{Ci/ml}$ [methyl- ^3H]choline chloride for 12 h, washed with PBS and then incubated in the presence of $5 \mu\text{M}$ of the agonists for the indicated times. Cells were then washed twice in PBS and scraped into methanol/water (2:1, v/v). After a brief sonication, an aliquot was taken for protein quantification. Lipids were extracted according to Bligh and Dyer [19]. Choline, phosphocholine and CDP-choline of the aqueous phase were separated by TLC, developed in the solvent ethanol/2.4% NaCl/water/ NH_3 (50:12.5:37.5:5, by vol.). Lipids of the chloroform phase were separated by TLC in the solvent chloroform/methanol/acetic acid/water (75:45:12:6, by vol.). The spots were revealed by iodine vapour, scraped off and the radioactivity measured by scintillation counting.

Determination of cellular ceramides

IMR-90 cells (2.0×10^5) were incubated with $5 \mu\text{M}$ LPA or (S)PNPA in 2% FCS supplemented DMEM for the indicated times. Determination of cellular ceramides was carried out using *sn*-1,2-diacylglycerol (DAG) kinase. Briefly, after stimulation, cellular lipids were extracted, then DAG was hydrolysed by treatment with 0.08 M NaOH in chloroform/methanol (2:0.5) for 1 h at 37°C . Remaining lipids were extracted and treated with 1 unit/ml *Escherichia coli* DAG kinase (Sigma) with [γ - ^{33}P]ATP ($2 \mu\text{Ci/sample}$) as described previously [20]. Labelled lipids were extracted [19] and resolved by TLC in the solvent chloroform/methanol/acetone/acetic acid/water (10:2:4:2:1, by vol.). Radioactive spots were visualized with a Phosphor-Imager (Molecular Dynamics, Piscataway, NJ, U.S.A.), and the spots corresponding to [^{33}P]ceramide 1-phosphate were scraped off and quantitated by scintillation counting.

Cholinephosphotransferase (CPT) activity assay

CPT activity was assayed in a mixture of 125 mM Tris, pH 8.5, 12 mM MgCl_2 , 0.5 mM EGTA, $2 \mu\text{M}$ 1,2 dioleoyl-*sn*-glycerol, 0.004% Tween 20, 0.1 mM CDP-[methyl- ^{14}C]choline

(14.28 Ci/mol) and $50 \mu\text{g}$ of proteins. Reactions were carried out at 37°C for 30 min and stopped by the addition of chloroform/methanol (1:2, v/v). The lipids were then extracted according to Bligh and Dyer, and the lower phase was washed with a theoretical upper phase (methanol/water/chloroform/acetic acid, 4.8:4.7:0.3:0.1, by vol.). Lipids of the chloroform phase were separated by TLC. They were developed after two consecutive migrations. First, a migration in chloroform/methanol (9:1, v/v) followed by half a migration in butan-1-ol/acetic acid/water (6:2:2, by vol.). Spots corresponding to PC were scraped off and the radioactivity measured by scintillation counting.

RESULTS

(R)- and (S)PNPA have opposite effects on cellular proliferation

Figure 1(A) shows the structure of the analogues used in this study. (R)- and (S)PNPA met the criteria defined by previous studies for LPA receptor agonists, which was a long aliphatic chain and a free phosphate group [21]. Indeed, PNPAs bear a palmitic acid linked to the amino group on the asymmetric carbon of norleucinol equivalent to the *sn*-2 position of LPA. Interestingly, whereas both PNPA enantiomers were equally potent at inducing platelet aggregation [14], only (R)PNPA, which displayed the configuration of natural phospholipids, showed growth factor-like activity on IMR-90 cells (Figure 1B). (R)PNPA (up to $5 \mu\text{M}$) induced a 5-fold increase in [^3H]thymidine incorporation, whereas (S)PNPA had no effect at any tested doses. Microscopic observation of the cultures demonstrated that (S)PNPA treatment was toxic for the cells (results not shown). To discriminate between non-specific cytotoxicity of lysophospholipid-like molecules and apoptosis, we evaluated (S)PNPA's ability to alter cell cycle distribution (Figure 1C). Whereas most of the control cells (2% FCS) were in the G_0/G_1 phase, $5 \mu\text{M}$ (S)PNPA induced up to 58% of cells with a sub- G_1 DNA content (Figure 1C), suggesting that cell death occurred by apoptosis. This was confirmed by monitoring the activation of caspase-3 (a major apoptosis executioner), evaluated by the cleavage of the PARP, a specific target for this caspase [22]. Indeed, when IMR-90 cells were incubated with $5 \mu\text{M}$ (S)PNPA, the 89 kDa fragment of cleaved PARP appeared as soon as 2 h after treatment and increased in intensity up to 20 h (Figure 1D). By contrast, incubation of cells with up to $10 \mu\text{M}$ LPA or $10 \mu\text{M}$ (R)PNPA did not result in PARP cleavage even after 20 h. These data indicated that (S)PNPA was able to induce apoptosis of fibroblasts in a caspase-3-dependent way.

There is no correlation between the expression of Edg receptors and (S)PNPA biological effects

In an attempt to relate differential effects of PNPAs to a set of Edg receptors, we investigated the effect of LPA and PNPAs in relation to the presence of different receptors in various cell types, namely human IMR-90 fibroblasts, human colorectal adenocarcinoma Caco-2 cells and human hepatoma HepG2 cells. Total RNAs were extracted and RT-PCR performed with primers specific to the human sequences of Edg receptors. IMR-90 and Caco-2 cells displayed the same expression pattern and expressed the seven Edg receptors cloned in human. Only Edg-5 and -6 were detected in HepG2 cells (Figure 2A). [^3H]Thymidine incorporation demonstrated that Caco-2 cells were responsive to LPA and (R)PNPA. It was previously reported that HepG2 cells were not responsive to LPA. Accordingly, both LPA and (R)PNPA failed to induce proliferation (Figure 2B). In contrast, (S)PNPA treatment resulted in a drop in [^3H]thymidine

Figure 1 The LPA analogue (S)PNPA causes apoptosis of IMR-90 fibroblasts

(A) The structure of (R)PNPA is shown. (B) LPA and (R)PNPA stimulate [3 H]thymidine incorporation. Mitogenicity was determined by measuring the incorporation of tritiated thymidine into serum-starved IMR-90 cells (1.5×10^4 cells/cm 2) upon treatment with the indicated doses of compounds for 24 h. The data are representative of at least three experiments; error bars reflect S.E.M. (C) Cells were treated with 2% FCS without or with 5 μ M (S)PNPA. After 24 h of incubation, the number of cells in different phases of the cell cycle was determined by flow cytometry after nuclear propidium iodide staining. (D) (S)PNPA induces caspase-3 activation. IMR-90 in 2% FCS were incubated with 5 μ M (S)PNPA, 10 μ M LPA or 10 μ M (R)PNPA for the indicated times. C, control (no additions). Cells were then scraped into Laemmli buffer and analysed after SDS/PAGE by immunoblotting with anti-PARP antibodies. * indicates a non-specific band used as an internal loading control.

incorporation of IMR-90 and HepG2 cells (Figure 2B). Interestingly, (S)PNPA did not induce cell death in Caco-2 cells; however, they expressed the same receptors as IMR-90 (Figure 2). Therefore, no correlation could be made between a pattern of expression of Edg receptors and the effects of PNPAs.

Cross-desensitization experiments demonstrated that both PNPAs induced platelet aggregation via LPA and sphingosine 1-phosphate (S1-P) receptors, demonstrating that they actually could interact with Edg receptors in some cell types [14]. Human platelets express several LPA receptors (Edg-2, -4 and -7) and at least one S1-P receptor (Edg-6), which are all present in IMR-90 cells. Proliferation usually requires several hours of stimulation, whereas interaction of an agonist with a receptor can lead to a rapid and transient response, such as intracellular calcium mobilization that peaks within 30 s. LPA and S1-P have been shown to induce intracellular Ca^{2+} mobilization, which is dependent on Edg receptors in many cell types [23]. Therefore, we investigated whether LPA, S1-P, and (R) and (S)PNPA were able to affect intracellular calcium in IMR-90. Fura-2/AM was used as a Ca^{2+} -sensitive fluorescent probe and changes in fluorescence were monitored in single cells (Figure 2C). While LPA and S1-P were both potent at inducing an increase in $[\text{Ca}^{2+}]_i$, as demonstrated by the increase in F_{340}/F_{380} , neither (R) nor

(S)PNPA were able to mobilize intracellular calcium (Figure 2C). Furthermore, a calcium response was observed when cells were stimulated with LPA or S1-P after a pre-treatment with PNPAs, demonstrating that no cross-desensitization could be observed in IMR-90 (Figure 2C) and therefore suggesting that PNPAs were not able to interact with LPA or S1-P receptors in this model.

Taken together, these data failed to demonstrate a relationship between LPA or S1-P receptors and (S)PNPA-induced apoptosis, as was observed for platelet aggregation, suggesting that the mechanism involved might not be receptor-dependent.

(S)PNPA derivatives inhibited PC biosynthesis

Both PNPAs happened to have the same non-stereospecific effects when checked for receptor-dependent responses such as platelet aggregation [14] or calcium mobilization in IMR-90 (Figure 2C). Because the apoptotic enantiomer displayed the non-natural configuration of phospholipids, and because many enzymes of phospholipid metabolism are stereospecific, we investigated the impact of PNPA treatment on lipid metabolism.

Thus we investigated whether treatment of cells with PNPAs affected general biosynthesis of PC, the major phospholipid class

Figure 2 Lack of relationship between Edg receptors and (S)PNPA effects

(A) Total RNAs were extracted from IMR-90 fibroblasts, Caco-2 colorectal adenocarcinoma or HepG2 hepatoma cells and transcription of the genes encoding Edg receptors was assessed by RT-PCR with primers to the indicated receptors. PCR products were visualized by ethidium bromide staining after migration on agarose gels. (B) Serum-starved IMR-90, Caco-2 or HepG2 cells were incubated for 24 h with 5 μ M of either LPA, (R)PNPA or (S)PNPA. O, control (no additions). Mitogenic response was followed by measuring the incorporation of tritiated thymidine. Results are reported as the fold increase over the incorporation of untreated cells. Data are means $\pm$ S.E.M. from three or four independent experiments. (C) Cells loaded with fura-2/AM were exposed to 5 μ M of the indicated agonists and changes in the F_{340}/F_{380} ratio were recorded in single cells as an indicator of changes in $[Ca^{2+}]_i$. The arrows indicate when the lipids were added, which then remained present throughout the recording. The curves are representative of 6–12 cells. The x axis shows time (s).

of mammalian cells. As shown in Figure 3(A), when (S)PNPA was added to the culture medium simultaneously with [3 H]choline, a strong inhibition of choline incorporation into PC was detected, by contrast with what was observed with (R)PNPA (Figure 3A). As a consequence, the rate of subsequent incorporation into sphingomyelin, a downstream product of the PC pathway, was affected as well (Figure 3A). Analysis of the soluble intermediates of PC biosynthesis according to the Kennedy pathway [24] indicated that radiolabel accumulated in the CDP-choline pool upon (S)PNPA treatment, suggesting inhibition of CPT, the enzyme that catalyses the transfer of the choline phosphate moiety from the CDP-choline to DAG (Figure 3B). Labelling of the two other soluble compounds, choline and phosphocholine, was not differentially affected (results not shown and Figure 3B).

To rule out the hypothesis of apparent changes in the labelling of PC from isotope dilution due to the differences in the pool sizes of intermediate metabolites, pulse-chase experiments were performed. They confirmed the accumulation of CDP-choline only in the presence of (S)PNPA (Figure 3C). Again, no change in labelling of phosphocholine was observed, in agreement with the inhibition of CPT (Figure 3C).

Rescue of (S)PNPA-induced apoptosis by lysophosphatidylcholine (LPC)

It was previously reported that a drop in the cellular level of PC, because of inhibition of PC metabolism, induced apoptosis in many cell types, such as Chinese hamster ovary cells, fibroblasts

Figure 3 (S)PNPA alters PC biosynthesis

(A) Cells were labelled with 2 μ Ci/ml [methyl- 3 H]choline chloride without or with 5 μ M (R)PNPA (■) or (S)PNPA (▲). Radioactive choline incorporation in the organic phase was determined as described in the Experimental section. The left-hand panel shows the incorporation into PC and the right-hand panel the incorporation in sphingomyelin over time. (B) Cells were treated as described above and the radioactive choline incorporation into soluble precursors of the Kennedy pathway quantified. Left, incorporation into phosphocholine; right, incorporation into CDP-choline. (C) Pulse-chase experiments. Cells were prelabelled for 15 h with [methyl- 3 H]choline chloride. The radioactive medium was then replaced by fresh medium containing 2% FCS (◇) or supplemented with 5 μ M (R)PNPA (■) or (S)PNPA (▲). At the indicated times radioactivity in phosphocholine (left-hand panel) or CDP-choline (right-hand panel) was measured.

or human lung adenocarcinoma A549 cells [25,26]. Addition of LPC to the culture medium was shown to restore cell survival [27,28]. Therefore, we studied the ability of increasing concentrations of LPC to restore cell viability of IMR-90 treated with (S)PNPA (Figure 4). Cell lysis was assessed by measuring the release of the cytosolic enzyme LDH in the culture medium. After 20 h of incubation, over 90% of the cells were lysed when incubated with 5 μ M (S)PNPA (Figure 4A). However, addition of LPC in the culture medium totally restored cell survival at 40 μ M. Since the effect of (S)PNPA correlated with an increase in sub-G₁ DNA, we checked whether LPC incubation affected DNA content (Figure 4B). As expected, incubation of cells with LPC blocked DNA fragmentation in a dose-dependent manner,

Figure 4 Addition of LPC prevents (S)PNPA-induced apoptosis

IMR-90 were treated with 2% serum or 5 μ M (S)PNPA for 24 h, with the indicated doses of LPC. (A) Cell lysis was assessed by measuring the release of LDH activity into the culture medium. Results are presented as the percentage of total LDH release induced by 0.1% Triton X-100. Data are the means from two independent experiments. (B) DNA fragmentation was followed by the appearance of sub-G₁ DNA measured by flow cytometry after propidium iodide staining. Results are presented as the percentage of (S)PNPA-induced apoptosis; data are means from two independent determinations.

with a maximal effect at 40 μ M (Figure 4B). These observations strongly confirmed that (S)PNPA apoptotic effect was the result of perturbation of the PC biosynthetic pathway.

Differential metabolism of (R)- and (S)PNPA

To identify the metabolite responsible for the inhibition of CPT, we first followed cellular metabolism of PNPAs as the time course of transformation of [3 H]palmitate-labelled PNPAs in IMR-90 cells. Figures 5(A) and 5(B) demonstrated that only the dephosphorylated forms of the compounds, [3 H]PNOH derivatives, accumulated rapidly in cells, suggesting that the action of phosphatases has a role in the regulation of PNPAs bioactivity. Maximal dephosphorylation was achieved between 30 min and 1 h of incubation, which matched the kinetics of LPP action [29,30] and was inhibited by 100 μ M Na₃VO₄, a potent phosphatase inhibitor [31]. The cellular uptake of [3 H]PNPAs was not affected by the treatment and remained extremely low (Figures 5A and 5B). Analysis of the culture medium revealed a decrease

Figure 5 Dephosphorylation of both PNPA is required for cellular uptake

Cells were treated with $[^3\text{H}]\text{PNPAs}$ (4.0×10^6 d.p.m.) for the indicated times, without (closed symbols) or with (open symbols) 20 min preincubation with $100 \mu\text{M Na}_3\text{VO}_4$. Then cells or the culture medium were recovered and phospholipids extracted and separated by TLC as described in the Experimental section. (**A, B**) Radioactivity in the derivatives of (*R*)PNPA (left-hand panel) or (*S*)PNPA (right-hand panel) from the cells. (**C, D**) Radioactivity in the derivatives of (*R*)PNPA (left-hand panel) or (*S*)PNPA (right-hand panel) from the culture medium. (**E**) Only LPP1 is expressed in IMR-90 fibroblasts. Total RNAs were extracted and transcription of the genes encoding the ectophosphatases LPP1, LPP2 and LPP3 was assessed by RT-PCR with specific primers. PCR products were visualized by ethidium bromide staining after migration on agarose gels. β -Actin amplification was used as a control. (**F**) Control RT-PCRs with primers specific to LPP2 and LPP3 were performed on total RNAs extracted from human umbilical vein endothelial cells.

in $[^3\text{H}]\text{PNPAs}$, which paralleled the increase in $[^3\text{H}]\text{PNOHs}$ (Figures 5C and 5D), suggesting the presence of an ecto-LPP activity. LPPs exist as three subtypes (1–3) with different activities towards LPA, S1-P, ceramide 1-phosphate or DAG pyrophosphate [32]. Interestingly, the non-stereospecific LPP1 is highly expressed at the plasma membrane, the catalytic site being exposed on the extracellular side of the cell, where it acts as an ectophosphatase. Under our conditions, RT-PCR performed on RNAs extracted from IMR-90 cells demonstrated that only LPP1 was expressed, suggesting that it could be responsible for PNPA dephosphorylation (Figure 5E). The lack of detection of

LPP2 and LPP3 transcripts was not due to a defect in the RT-PCR since a control experiment performed on RNAs extracted from human umbilical-vein endothelial cells demonstrated the presence of the corresponding PCR products (Figure 5F). Interestingly, both PNPA were dephosphorylated at the same rate and to the same extent, which demonstrated the lack of stereospecificity of the phosphatase. Moreover, it indicated that dephosphorylation itself could not account for the differential biological effects of (*R*) and (*S*) derivatives.

To further investigate the metabolism of PNPA, we conducted experiments over 20 h, when dephosphorylated products could

Figure 6 Long-term metabolism of PNPAs in IMR-90 fibroblasts

Cells were treated with [^3H]PNPA for the indicated times and then collected. After lipid extraction and TLC, radioactivity incorporated in PNPAs metabolites was analysed by scintillation counting. ■, (*R*)PNPA; ▲, (*S*)PNPA. (A) Conversion to phosphocholine derivatives. (B) Conversion to phosphoethanolamine derivatives. (C) Evolution of the dephosphorylated forms over time. (D) Acylation of the dephosphorylated metabolites.

enter the general lipid metabolism (Figure 6). (*R*)PNPA derivatives accumulated as [^3H]palmitoyl-norleucinol-phosphocholine (PNPC) and [^3H]palmitoyl-norleucinol-phosphoethanolamine (PNPE), demonstrating that derivatives of the natural configuration could enter the Kennedy pathway for phospholipid metabolism (Figures 6A and 6B). Interestingly, derivatives of (*S*)PNPA accumulated as [^3H]PNPE in the same range as (*R*) derivatives, but the conversion into [^3H]PNPC was almost nil (Figures 6A and 6B), strengthening the hypothesis that a metabolite of (*S*)PNPA was responsible for the blockade of PC biosynthesis. Accordingly, (*S*)PNOH accumulated in large amounts after 12 h (Figure 6C). Triacylglycerols are commonly considered as a storage form of intracellular lipids. In agreement with this process, an acylated form of (*S*)PNOH, (*S*)PNacyl, accumulated in cells after 6 h, reflecting the cellular response to the accumulation of (*S*)PNOH that did not enter PC metabolism. Such a conversion failed to take place with (*R*)PNOH (Figure 6D). Taken together, these data confirmed a difference in the metabolism of intracellular derivatives of PNPAs, mostly at the level of the conversion to phosphocholine derivatives. They also suggest that CPT but not ethanolamine phosphotransferase is stereospecific for (*R*) derivatives.

In vitro inhibition of CPT by (*S*)PNOH

In order to investigate whether (*S*)PNPA derivatives were able to directly inhibit CPT, we synthesized the two major products of

PNPA metabolism by IMR-90 cells, the dephosphorylated forms (*R*- and (*S*)PNOH, and analogues of the acylated forms (equivalent to the triacylglycerol-like products accumulating in Figure 6D) by esterification of the free alcohol function of the PNOH by palmitic acid to give the PNacyls. *In vitro* assays of CPT activity performed on untreated IMR-90 homogenates demonstrated that only (*S*)PNOH was able to inhibit the enzyme, while (*R*)PNOH was without effect, confirming the stereospecificity of CPT activity (Figure 7A). Both acylated forms, (*R*- and (*S*)PNacyl, were without effect (Figure 7A). The inhibition of (*S*)PNOH was dose-dependent with an IC_{50} of $2.5 \mu\text{M}$ (Figure 7B), which matched the *in vivo* observation. These data identified (*S*)PNOH as the active metabolite of (*S*)PNPA and confirmed that it was acting by inhibiting CPT.

Treatment of IMR-90 with $5 \mu\text{M}$ (*S*)PNOH induced the same morphology as cells treated with (*S*)PNPA after 24 h: mostly dead cells were present in the culture (Figure 7C). As expected, if (*S*)PNOH was the active compound, cell survival was restored by incubation with $25 \mu\text{M}$ LPC (Figure 7C), demonstrating that (*S*)PNOH had a similar effect to (*S*)PNPA.

Inhibition of CPT by (*S*)PNPA induces ceramide production

The relationship between the decline in PC level and apoptosis is still poorly understood. We took advantage of (*S*)PNPA being an inhibitor of CPT to investigate the signalling involved in the apoptosis induced by the blockade of this enzyme. Many reports

Figure 7 (S)PNOH inhibits choline phosphotransferase *in vitro*

Untreated IMR-90 cells were collected and lysed by sonication. Total lysate was used in CPT *in vitro* assay with DAG as substrate. Results are presented as the percentage of CPT activity without addition of any compound. **(A)** Activity was measured in the presence of 25 μM of either (*R*)- or (*S*)-PNOH or (*R*)- or (*S*)-PNacyl. Data are means $\pm$ S.E.M. from three determinations. **(B)** *In vitro* CPT activity was measured in the presence of increasing concentrations of (*S*)-PNOH. Data are means from two independent experiments. **(C)** Microscopic observation of IMR-90 fibroblasts stimulated with 5 μM (*S*)-PNOH for 24 h, with or without 25 μM LPC.

documented the role of ceramides in apoptosis. Thus we analysed the level of ceramides in IMR-90 cells upon (*S*)PNPA treatment. The amount of ceramides was assessed by measuring phospho-ceramides produced *in vitro* by phosphorylation of cellular ceramides by DAG kinase in the presence of [γ - ^{32}P]ATP. A strong ceramide accumulation was observed in cells treated with (*S*)PNPA (Figure 8A). When cells were incubated with increasing doses of Fumonisin B1, a potent cell-permeable inhibitor of ceramide synthase [33], cellular lysis was inhibited by 80 % as demonstrated by the decrease of LDH release (Figure 8B). In parallel, a clear inhibition of DNA fragmentation was observed, which confirmed that ceramides were key actors in the apoptotic process. The effect of Fumonisin B1 was in the same range as the effect of the caspase inhibitor Z-VAD-fmk, a broad specificity and irreversible inhibitor of caspases (Figure 8C).

Involvement of the SAPK, p38 and JNK1/2

We further investigated the signalling pathways involved in (*S*)PNPA-induced apoptosis. Activation of the MAP kinases ERK1/ERK2 (reflected by their phosphorylation status) is a key element of proliferative response and often corresponds to a double wave of phosphorylation. (*S*)PNPA only induced a rapid

and transitory phosphorylation that peaked after 5 min and was back to the basal level after 2 h (Figure 9A). Phosphorylated SAPK, p38 and JNK1/2 are required for induction of apoptosis in many cell types [34]. (*S*)PNPA treatment resulted in a strong and sustained activation of p38 and JNK1/2 after 30 min (Figure 9A). Interestingly, analysis of the phosphorylation of SAPK upon Fumonisin B1 incubation demonstrated a concomitant inhibition of p38 and JNK1/2 activation when cells were treated with the drug. No similar abolition was seen when ERK1/ERK2 phosphorylation was evaluated (Figure 9B). Thus our data supported a central role for ceramides in the apoptosis induced by (*S*)PNPA. Furthermore, they demonstrated that activation of SAPK was a consequence of ceramide production.

To evaluate the importance of these kinases in the apoptotic process driven by CPT inhibition, cells were treated with a set of inhibitors and viability was assessed by microscopic observation (Figure 9C). Only Z-VAD-fmk (100 μM) was able to keep the cells alive after 24 h of treatment with 5 μM (*S*)PNPA, in agreement with the early activation of caspase-3 shown in Figure 1(D). By contrast, pre-incubation with 15 μM SB203580 or 30 μM SB202190 (specific for p38) were unable to restore cell survival. Therefore, SAPK activation appeared not to be a limiting step of apoptosis in our model.

Figure 8 (S)PNPA-induced apoptosis requires ceramides production

(A) IMR-90 cells were treated with 5 μM (S)PNPA for the indicated times. Cells were then scraped, lipids extracted and ceramide production measured as [³³P]phosphoceramides after *in vitro* phosphorylation by DAG kinase. (B) Cells were pretreated with the indicated doses of Fumonisin B1 for 20 h prior to (S)PNPA incubation. LDH release into the culture medium was measured after 24 h of stimulation. Results are presented as the percentage of total LDH release induced by 0.1% Triton X-100. Data are means from two similar experiments. (C) Cells were pretreated with 2% FCS, 25 μM Fumonisin B1 or 100 μM Z-VAD-fmk for 24 h, then stimulated with 5 μM of (S)PNPA. DNA fragmentation was followed by the appearance of sub-G₁ DNA measured by flow cytometry after propidium iodide staining. Results are presented as a percentage of (S)PNPA-induced apoptosis.

DISCUSSION

In this work we aimed to study long-term effects of two enantiomers, (R)PNPA and (S)PNPA, that proved to act as LPA receptor agonists in platelet activation [14]. Whereas the three lipids, (R)PNPA, (S)PNPA and LPA, induced platelet aggregation in a similar way, data obtained from IMR-90 fibroblasts

Figure 9 Differential activation of signalling pathways by (S)PNPA

Serum-starved IMR-90 cells were stimulated by 5 μM (S)PNPA for the indicated times. (A) Phosphorylation of ERK1/ERK2, p38 or p46/54 JNK1/2 was followed by immunoblotting on total lysate, after SDS/PAGE, with antibodies to the phosphorylated forms of the kinases. Phosphorylation profiles representative of three to five experiments are shown. (B) IMR-90 cells were incubated with 25 μM Fumonisin B1 for 24 h prior to stimulation with 5 μM (S)PNPA for the indicated times. Phosphorylation of ERK1/ERK2, p38 or p46/54 JNK were followed by immunoblotting as described above. (C) Microscopic observation of IMR-90 fibroblasts preincubated with 100 μM of the caspase inhibitor Z-VAD-fmk, 30 μM of the p38 inhibitor SB202190 or 15 μM of the p38 inhibitor SB203580, and then stimulated with 5 μM (S)PNPA for 24 h.

demonstrated that PNPA had opposite effects on proliferation. Analysis of DNA content as well as observation of the nuclei stained with Hoechst dye (results not shown) demonstrated that cells treated with (S)PNPA displayed the features of apoptotic cells. Accordingly, (S)PNPA induced caspase-3 activation, while pre-treatment with the caspase inhibitor Z-VAD-fmk restored cell viability.

Although various bioactive lipids such as LPA and S1-P signal through Edg receptors to mediate their cellular effects [23], our studies failed to demonstrate a correlation between the pattern of receptors and the ability of (S)PNPA to evoke cell death in three human cell lines, IMR-90, Caco-2 and HepG2. Moreover, PNPAs were not able to induce a typical receptor-dependent response, i.e. intracellular Ca^{2+} mobilization in IMR-90 cells loaded with fura-2/AM, whereas cells responded to stimulation by LPA or S1-P, even when they were previously treated with 5 μM PNPAs. If they do not rule out the involvement of unknown stereospecific receptor(s) in the observed apoptotic effect, our data strongly suggested that another pathway was responsible for apoptosis [12,21,35].

Because PNPAs are phospholipids, they could integrate the phospholipid bilayer of the plasma membrane to be metabolized by the cell and may affect general lipid metabolism. The use of radioactive [^3H]PNPAs demonstrated that both enantiomers were equally dephosphorylated within the first hour of incubation, probably by non-stereospecific ectophosphatases of the LPP family. Actually, only dephosphorylated derivatives entered the cell. Three members of the LPP family were reported to be active on lysophospholipids, LPP1 (or PAP2A), LPP2 (or PAP2B) and LPP3 (or PAP2C) [32,36]. LPP1 has the broadest specificity and dephosphorylates LPA, S1-P, ceramide 1-phosphate and DAG pyrophosphate with similar efficiencies. Recent reports documented the major role of LPP in regulating the mitogenic effects of lysophospholipids [29,37,38]. In particular they highlighted the crucial role of LPP1 as a negative regulator of proliferation of fibroblasts [38]. Our data demonstrating that only LPP1 was expressed in IMR-90 cells are in agreement with those observations. An explanation for the differential effects of PNPA stereoisomers could have been that (R)PNPA was proliferative because it is a poor substrate for phosphatases, leading to a stronger association with receptors. However, this hypothesis was ruled out because the rate of dephosphorylation and cellular uptake of metabolites of (R)- and (S)PNPA were identical. The fact that both PNPAs were equally potent at inducing a receptor-dependent response in platelets could be explained by a lower ecto-LPP activity in this cell type compared with IMR-90 cells, allowing PNPAs to associate with receptors.

Interestingly, following the metabolites for longer times showed that (R)PNOH, which displayed the configuration of natural phospholipids, was processed by the enzymes of lipid metabolism, since it was converted to (R)PNPC and (R)PNPE. Surprisingly, while (S)PNPE accumulated to the same extent as (R)PNPE, no (S)PNPC was recovered. Slight production was detected at later time points (after 12–20 h), probably as a result of an alternative pathway of PC biosynthesis coming from the conversion of phosphatidylethanolamine to PC by base exchange [39].

Several studies demonstrated that a drop in cellular PC resulted in programmed cell death. The observation that (S)PNPA could not enter PC metabolism confirmed that PC biosynthesis could be the target of this phospholipid. Our data clearly demonstrated that (S)PNPA treatment dramatically affected the CDP-choline pathway. This inhibition was not the result of a deficiency of choline intake since the intracellular pools of choline were identical in cells treated with any of the tested lipids (results not shown). Moreover, the accumulation of radiolabelled CDP-choline in (S)PNPA-treated IMR-90 cells indicated that the derivative inhibited CPT, which catalyses the last step of PC biosynthesis through the Kennedy pathway [24]. It is generally assumed that PC biosynthesis is regulated mainly at the level of CTP/phosphocholine cytidylyltransferase (CT). In our experiments no accumulation of phosphocholine, which is the

substrate of CT, was detected, demonstrating that (S)PNPA treatment had no effect on CT activity. Moreover, a similar inhibition of CPT upon geranylgeraniol or farnesol treatment, also leading to apoptosis, was demonstrated in human lung carcinoma (A549) and leukaemia (CEM-C1) cells [26,40]. However, there is controversy about the direct effect of isoprenoids as inhibitors of CPT. Voziyan et al. [40] suggested that a cytosolic metabolite of farnesol or a secondary agent induced in response to farnesol treatment could be the specific CPT inhibitor. In their hands, *in vitro* CPT assays performed on cellular extracts with or without addition of farnesol resulted in no effect on the enzymic activity, while cellular treatment decreased it, supporting the idea that farnesol was not the active compound [40]. However, in a recent study by Miquel et al. [26], *in vitro* CPT assays performed on microsomes demonstrated that farnesol treatment inhibited the enzyme by acting as a competitive inhibitor. In our studies, both *in vitro* activity assays on cellular extracts as well as cellular treatment resulted in a very strong effect on CPT activity when the dephosphorylated product (S)PNOH was added to the reaction or to the culture. Because CPT assays were not performed on recombinant or purified CPT, we cannot strictly describe (S)PNOH as a specific inhibitor and totally rule out the possibility of (S)PNOH affecting an intermediate that would be the real inhibitor. However, the structure of (S)PNOH being very similar to the structure of the natural substrate of CPT, i.e. DAG, favoured the hypothesis of a direct interaction and inhibition. In any case, our data identified the derivative (S)PNOH as the active compound.

Under our conditions, restoration of PC biosynthesis by supplementation of the medium with LPC, which is acylated to generate PC, prevented apoptosis. Such an observation was also made on cell death induced by choline-containing lipid analogues such as hexadecyl phosphocholine analogues [28,41]. This indicates that the block of PC biosynthesis is the primary event of the apoptosis induced by those compounds. Although it is now well admitted that modulation of membrane phospholipid content plays a pivotal role in the induction of programmed cell death, the mechanisms linking phospholipid metabolism to apoptosis remain elusive.

Interestingly, (S)PNPA led to a strong increase in the level of intracellular ceramides. Ceramides can be produced by two pathways [42]. One comes from sphingomyelin breakdown by the action of sphingomyelinases, which generate phosphocholine and ceramides. The other results from the condensation of serine and palmitoyl-CoA, which, after several steps involving conversions of sphingosine derivatives, leads to the production of dihydroceramides, converted finally to ceramides. In this study, no higher levels of phosphocholine were observed in (S)PNPA-treated cells when compared with the control. Incubation of IMR-90 with Fumonisin B1, an inhibitor that specifically blocks the neo-synthesis of ceramides [33], abrogated (S)PNPA-induced apoptosis, suggesting that ceramides originated mainly from newly synthesized pools.

To better understand the mechanism that was driving apoptosis upon CPT block, we studied the signalling pathways activated by (S)PNPA. We first investigated the MAPK and SAPK pathways. ERK1/ERK2 have been largely described as major effectors of mitogenesis [43,44]. However, it was reported that the bioactive lipid sphingosylphosphorylcholine had anti-mitogenic activities that were dependent on MAPK activation. Indeed, treatment with PD98059 [an inhibitor of MAPK/ERK kinase 1 (MEK1)] restored proliferation [45]. In our model, both PNPAs activated ERK1/ERK2 in a rapid and transient way, but PD98059 treatment did not restore viability (results not shown). Con-

versely, (S)PNPA induced a sustained phosphorylation of SAPK, p38 and JNK1/2, suggesting that they might play a role in programmed cell death in IMR-90 cells. Interestingly, Fumonisin B1 treatment abrogated SAPK phosphorylation, demonstrating that the increase in ceramide level was responsible for their activation. To evaluate the importance of SAPK in this phenomenon, we used inhibitors described to restore survival in various apoptotic events such as PC12 neuroblastoma death upon serum removal or T-cell receptor-induced cell death in lymphocytes [46–48]. Again, neither of the two inhibitors, SB203580 nor SB202190, was able to save (S)PNPA-treated cells, suggesting that SAPK does not represent a general commitment point for the induction of apoptosis.

Altogether, our data support the following metabolic scheme. Dephosphorylation of (S)PNPA at the plasma membrane by ecto-LPP leads to the production of (S)PNOH, further internalized by cells. There, it inhibits CPT, leading to a drop in PC level which triggers the subsequent apoptosis. Then ceramides are produced by *de novo* synthesis, caspases activated and programmed cell death engaged. It has to be noted that the activation of SAPK results from the production of ceramides.

Our findings are of importance when we consider that alkylphospholipids possess anti-neoplastic activity *in vitro* as well as *in vivo*. Hence, the anti-proliferative properties of these phospholipids led to the development of another class of analogues, the alkylphosphocholines, a prototype of which is hexadecylphosphocholine (HePC) [49–51]. Because of its good cellular penetration, HePC was proposed as a drug for treatment of some cancers and is actually on clinical trial for treatment of skin metastases of breast cancer [52]. In addition, it was recently reported that HePC was acting on PC biosynthesis by inhibiting the CTP/CT, one step upstream of (S)PNPA in the pathway. Experiments are underway to evaluate the general toxicity of (S)PNPA and to what extent it could affect the growth of subcutaneous human tumours grafted into nude mice.

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