JOURNAL OF THE ROYAL SOCIETY Interface

Testing the hypothesis that amphiphilic antineoplastic lipid analogues act through reduction of membrane curvature elastic stress

Marcus Dymond, George Attard and Anthony D Postle

J. R. Soc. Interface 2008 **5**, 1371-1386
doi: 10.1098/rsif.2008.0041

JOURNAL OF THE ROYAL SOCIETY Interface

J. R. Soc. Interface (2008) **5**, 1371–1386
doi:10.1098/rsif.2008.0041
Published online 15 April 2008

Testing the hypothesis that amphiphilic antineoplastic lipid analogues act through reduction of membrane curvature elastic stress

Marcus Dymond[1], George Attard[1,*] and Anthony D. Postle[2]

[1]*School of Chemistry, University of Southampton, Highfield SO17 1BJ, UK*
[2]*School of Medicine, University of Southampton, Southampton General Hospital, Hampshire SO16 6YD, UK*

The alkyllysophospholipid (ALP) analogues Mitelfosine and Edelfosine are anticancer drugs whose mode of action is still the subject of debate. It is agreed that the primary interaction of these compounds is with cellular membranes. Furthermore, the membrane-associated protein CTP: phosphocholine cytidylyltransferase (CCT) has been proposed as the critical target. We present the evaluation of our hypothesis that ALP analogues disrupt membrane curvature elastic stress and inhibit membrane-associated protein activity (e.g. CCT), ultimately resulting in apoptosis. This hypothesis was tested by evaluating structure–activity relationships of ALPs from the literature. In addition we characterized the lipid typology, cytotoxicity and critical micelle concentration of novel ALP analogues that we synthesized. Overall we find the literature data and our experimental data provide excellent support for the hypothesis, which predicts that the most potent ALP analogues will be type I lipids.

Keywords: alkylphosphocholines; anticancer; type I lipids; CTP: phosphocholine cytidylyltransferase; curvature elastic stress; alkyllysophospholipids

1. INTRODUCTION

Since the discovery of the cytotoxic and antitumour properties of lysophosphatidylcholine analogues (Munder 1982; Brachwitz *et al.* 1987; Brachwitz & Vollgraf 1995), a number of alkylphosphocholine (APC) and alkyl-lysophospholipid (ALP) analogues have been developed for clinical use. Two compounds in particular have emerged as important chemotherapeutic agents: ET-18-OMe from the ALP series of materials (**1**) and hexadecylphosphocholine (HDPC) (**2**) from the APC series. These compounds have elicited considerable interest because while it is clear that their mode of action does not involve intercalation or direct interaction with DNA, the details of the mechanisms that underlie their potent biological activity remain unclear. Over the past few years a consensus has emerged that CTP: phosphocholine cytidylyltransferase (CCT; Geilen *et al.* 1996; Jackowski & Boggs 1998; Jimenez-Lopez *et al.* 2002) is the initial cellular target of the ALP and APC agents. CCT, a translocation protein, is a rate-determining enzyme in the biosynthesis of phosphatidylcholine (PtdCho) phospholipids (Kent 1997; Jackowski & Baburina 2002). In most mammalian cells, CCT catalyses the only route for introducing choline into the pathway that manufactures PtdCho lipids; regulation of CCT activity appears to be critical for cell membrane homeostasis and cell survival, especially during mitosis (Jackowski 1994, 1996). The key experimental evidence that points to CCT being the target for ALP and APC compounds can be summarized as follows.

— ET-18-OMe and HDPC inhibit CCT activity and both are cytotoxic agents *in vitro* (Vogler *et al.* 1996; Jimenez-Lopez *et al.* 2002).
— Choline incorporation is blocked by both compounds *in vitro* (Boggs *et al.* 1995); choline-deficient cells enter into a state of stasis and then undergo apoptosis, as do cells treated by ALP analogues (Konstantinov *et al.* 1998*a*).
— Both HDPC and ET-18-OMe inhibit PtdCho biosynthesis (Jackowski & Boggs 1998), leading to a rise in ceramide, which marks the onset of apoptosis (Wieder *et al.* 1998).
— The cytotoxic and cytostatic effects of HDPC and ET-18-OMe on HL-60 cells are attenuated by exogenous lysophosphatidylcholine, an alternate precursor for PtdCho production, which bypasses CCT (Boggs *et al.* 1998).

There is agreement in the literature that CCT activity is regulated by the lipid composition of the membranes to which it is attached (Jamil *et al.* 1993; Attard *et al.* 2000). Recently, it has been suggested that

*Author for correspondence (gza@soton.ac.uk).

Received 29 January 2008
Accepted 26 March 2008

the mechanism through which CCT is regulated involves membrane curvature elastic stress (Attard *et al.* 2000). More specifically, it has been postulated that type II lipids within a membrane lead to an increase in the membrane curvature elastic stress and that this, in turn, leads to increased partitioning of CCT onto the membrane. Since cytosolic CCT is essentially inactive, the net effect of type II lipids in the membrane is to increase the concentration of enzymatically active CCT. The converse situation applies in the case of type I lipids. These decrease the curvature elastic stress in a membrane, leading to decreased partitioning of CCT onto the membrane and hence lower quantities of active CCT. We use the terms type I, type 0 and type II lipids to describe the self-assembly of amphiphiles into aggregates and lyotropic liquid crystalline phases. Type I lipids are non-bilayer lipids that form aggregates whose polar–apolar interface curves away from the aqueous domains, while type II lipids are non-bilayer-forming lipids that form aggregates in which the interface curves towards these domains. Type 0 lipids are bilayer-forming lipids.

Since ALP and APC analogues are generally type I amphiphiles and inhibit CCT, we hypothesize that the principal and universal mode of action of all metabolically stable antineoplastic homologues of ALPs and APCs is the inhibition of CCT through the reduction of membrane curvature elastic stress, with a subsequent decrease in the biosynthesis of PtdCho leading to cytostasis, an increase in ceramide levels and ultimately apoptosis.

Membrane curvature elastic stress arises from the summation of the lateral stresses in a membrane (see Marsh (2006) for a detailed explanation). The magnitude of the curvature elastic stress in a monolayer, g_c, can be represented by the Helfrich Hamiltonian (Helfrich 1973)

$$g_c = \frac{1}{2}\kappa A(c_1 + c_2 - 2c_0)^2 + \kappa_G A c_1 c_2. \qquad (1.1)$$

In this Hamiltonian A is the cross-sectional area per molecule; c_1 and c_2 are the principal curvatures at the interface (with the convention that an interface with negative curvature curves towards water); c_0 is the spontaneous curvature of the monolayer; κ is the bending rigidity; and κ_G is the Gaussian curvature modulus. Stored curvature elastic energy arises from non-zero spontaneous curvature in each of the monolayers that compose a bilayer lipid membrane such that each monolayer wishes to bend, but is prevented from doing so by being apposed to the other monolayer. To a first-order approximation, the curvature elastic stress is governed by a combination of the two material parameters c_0 and κ.

The likelihood that an amphiphile can reduce the stored curvature elastic energy when it partitions into a membrane can be predicted qualitatively by considering the interactions that contribute to the lateral stress profile within an aggregate (figure 1; Seddon 1990). In general, the lateral stress profile of a monolayer can be divided into three distinct regions (α, β, γ). The spontaneous mean curvature (c_0) of the monolayer is dependent on the balance of the areas under the curve in these regions. When the areas are such that $\alpha < \gamma$, then

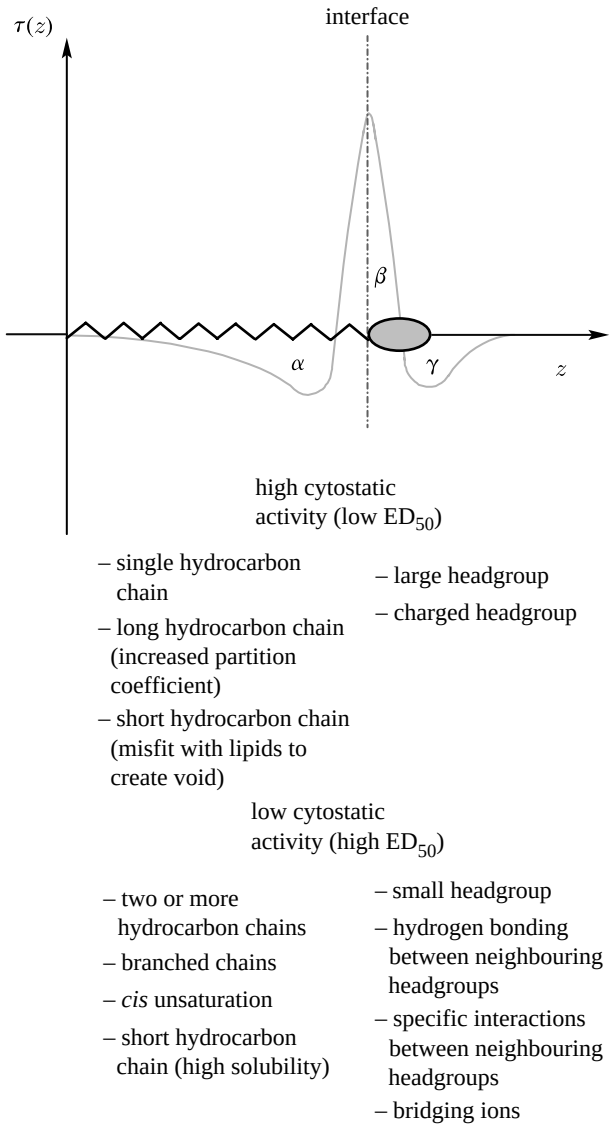


Figure 1. The lateral stress profile of an amphiphilic molecule, where z is the distance from the interface, $\tau(z)$ is the stress at z, where stress is the negative of pressure. Annotations describe the 'rules of thumb', derived from the lateral pressure profile, which we have used to predict the structure activity relationships of ALP and APC analogues.

type I behaviour is expected and the monolayer will seek to curve away from the polar–apolar interface (i.e. c_0 is positive). Conversely, if $\alpha > \gamma$ then type II behaviour is expected and the monolayer will want to curve towards the polar–apolar interface (i.e. c_0 is negative). Typically, the area associated with the hydrocarbon region (α) shows big changes if a saturated hydrocarbon chain is replaced by a branched chain, or by an unsaturated chain containing one or more *cis* double bonds. Changes in the area γ are caused by hydration, electrostatic repulsion, specific chemical interactions between neighbouring headgroups or interactions with counterions. Area β is dependent on the interfacial tension, and its magnitude must be equal and opposite to the sum of areas α and γ in order to have a stable monolayer. These considerations lead to a set of qualitative predictors of amphiphile typology which are widely used in surfactant science through the semi-quantitative concept of the 'critical packing parameter'.

Broadly speaking, type I amphiphiles tend to have larger cross-sectional headgroup areas and smaller hydrocarbon chain cross-sectional areas (type I molecular shape). Examples include lipids with bulky, or charged headgroups, and with saturated and relatively short alkyl chains. Conversely, type II amphiphiles tend to have larger hydrocarbon cross-sectional areas than headgroup cross-sectional areas (type II molecular shape), generally resulting from *cis* unsaturation, or have attractive interactions between neighbouring headgroups.

These qualitative predictors of amphiphile aggregation behaviour have serious limitations. In particular, the effective area per headgroup can change significantly as a function of the concentration of the amphiphile or lipid; consequently, classification of an amphiphile as type I or type II requires experimental data, and specifically an analysis of its phase behaviour. Strongly type I amphiphiles have phase diagrams that are dominated by micellar solutions at low concentrations and normal topology lyotropic liquid crystalline phases (e.g. micellar cubic, hexagonal) at higher concentrations. Type II amphiphiles have phase diagrams that are dominated by complex aggregates at low concentrations (e.g. vesicles, hexosomes or cubosomes) and inverse topology liquid crystal phases at higher concentrations. Although phase behaviour can provide unequivocal confirmation of type I behaviour, there are situations where it can lead to erroneous conclusions. These situations arise because the effect of an amphiphile on stored curvature elastic energy is dependent on the lipid composition of the target membrane. This means that even amphiphiles whose phase diagrams exhibit inverse topology phases can still lead overall to the inhibition of CCT if they are less strongly type II than the constituents of the target membranes.

The cytotoxic or cytostatic potency of an amphiphile that acts by inhibiting CCT through a reduction in the stored elastic energy of membranes can be described semi-quantitatively by

$$E = E_0 \exp\left\{ n\kappa A \left(2c_1 c_0 - \frac{1}{2} c_1^2 \right) / k_B T \right\}, \qquad (1.2)$$

where n is the number of molecules—comprising both lipids and type I amphiphiles—surrounding the binding domain of CCT, and E_0 is a constant. According to this equation, an increase in the fraction of molecules that have a positive spontaneous curvature, c_0, leads to a decrease in E (the degree of cell survival), since c_1 is negative. The quantity E is analogous to the ED_{50} that is measured in experiments on cytotoxicity.

The extension of this analysis to the relationships between the molecular structure of cytotoxic amphiphiles, their effects on stored curvature elastic energy in a lipid membrane and their ED_{50} values may be achieved using a series of qualitative 'rules of thumb' that are derived from the profile of interactions along the length of an amphiphile. These are summarized schematically in figure 1. In general, amphiphiles that form normal topology phases would be expected to be highly cytotoxic because they would result in a significant reduction of the stored elastic energy in a membrane. In particular we would expect that amphiphiles with charged headgroups would be potent cytotoxic agents. We would also expect that amphiphiles with sterically bulky headgroups will be effective at reducing stored curvature energy and hence exhibit cytotoxic properties. Furthermore, we predict that the closer the steric bulk is to the polar–apolar interface, the more effective the amphiphile will be at inhibiting the growth of cancer cells.

We have tested these simple predictions using literature data as well as data on compounds we have synthesized and evaluated in our laboratories and have found a remarkable degree of agreement covering a very wide diversity of amphiphile chemical structures. In particular we have found that cytotoxic analogues of ALPs and APCs are type I lipids.

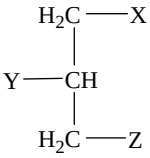
The wide range of biological effects that ALP analogues have on normal and malignant cells has been reviewed extensively (Brachwitz & Vollgraf 1995; Principe & Braquet 1995; Berkovic 1998). Brachwitz (Brachwitz & Vollgraf 1995) provides details of a variety of ALP analogue structures that have been reported to be cytotoxic or cytostatic. When the qualitative amphiphile classification scheme outlined earlier is applied to this extensive set of compounds, it becomes clear that type I amphiphiles dominate. For example; the *O*-alkylglycerophosphocholines, of which ET-18-OMe (**1**) is the best known homologue, bear a single saturated hydrocarbon chain and have a zwitterionic headgroup. These features conform to a type I molecular shape, corresponding to a hydrocarbon cross-sectional area that is smaller than the cross-sectional area of the headgroup. Experimentally, ET-18-OMe prevents the formation of inverse hexagonal phases in model membranes (Torrecillas *et al.* 2006) and the analogous ET-16-OMe forms normal typology micelles at low concentration in water (Dick & Lawrence 1992), confirming that it is a type I amphiphile. Table 1 lists selected examples of cytotoxic *O*-alkylglycerophosphocholines. Compounds **4**–**6** and **7**–**9** have been shown to have cytotoxic activities independent of the position of the main structural elements (Brachwitz *et al.* 1979, 1982, 1984), an observation that supports the hypothesis that it is the type I molecular shape that is critical for activity. Furthermore, branching in the alkyl chain, for example by introduction of phytanyl groups (**10** and **11**), causes a 3 to 10-fold decrease in cytotoxic activity when compared with ET-18-OMe (**1**) (Hoffman *et al.* 1984).

Compounds such as BM 41.440 (**12**) which retain the type I characteristic of a single, unbranched and saturated hydrocarbon chain are also highly cytotoxic (Fromm *et al.* 1987; Schick *et al.* 1987; Hanauske *et al.* 1992). SRI 62.834 (**13**), which differs substantially from ET-18-OMe through its cyclic headgroup, still retains a type I molecular shape and is also highly cytotoxic (Houlihan *et al.* 1987; Dive *et al.* 1991). Similarly, the *O*-alkylethyleneglycophosphocholine homologues have type I molecular shape and are also cytotoxic (Honma *et al.* 1983).

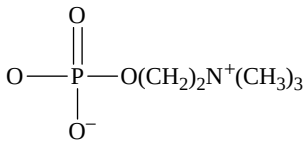
HDPC (**2**) is a type I amphiphile as confirmed by the phase diagram presented in this study; by analogy other APC analogues will also be strongly type I amphiphiles. Structure–activity studies show that shortening the

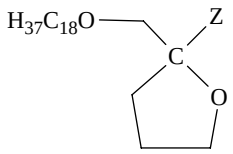
Table 1. A selection of ALP and APC analogues from the literature with type I molecular shape.

glycerol backbone structure



headgroup structure



compound number	X	Y	Z	comments
1	$OC_{18}H_{37}$	OCH_3	headgroup	ET-18-OMe
2	na	na	headgroup	$C_{16}H_{33}$-Z (HDPC)
3	$OC_{14}H_{29}$	OCH_3	headgroup	ET-14-OMe
4	$OC_{16}H_{33}$	Cl, F	headgroup	rac
5	$OC_{16}H_{33}$	headgroup	Cl, F	rac
6	Cl, F	$OC_{16}H_{33}$	headgroup	rac
7	$OC_{16}H_{33}$	OCH_2CF_3	headgroup	rac
8	$OC_{16}H_{33}$	headgroup	OCH_2CF_3	rac
9	OCH_2CF_3	$OC_{16}H_{33}$	headgroup	rac
10	$OC_2H_4(C\text{-}C_3H_6)_3\text{-}CH(CH_3)_3$	OCH_3	headgroup	sn
11	headgroup	OCH_3	$OC_2H_4(C\text{-}C_3H_6)_3\text{-}CH(CH_3)_3$	sn
12	$SC_{16}H_{33}$	CH_2OCH_3	headgroup	rac, BM41.440
13	na	na	headgroup	SRI 62.834 

alkyl chain of HDPC or introducing unsaturation within it reduces cytotoxicity (Unger *et al.* 1992). The relative cytotoxic effects of a homologous series of amphiphiles, containing a single hydrocarbon chain, in which only the length of this chain is varied, provide a challenging test of the hypothesis that is embodied by equation (1.2). In this equation both n and c_0 are dependent on the length (λ) of the amphiphile's hydrocarbon chain. Furthermore, in equation (1.2) the composition of the amphiphiles that surround the CCT-binding domain is dependent on the extent to which the antineoplastic amphiphiles partition into the membrane. To a first approximation, we would expect $n(\lambda)$ to be a linear function of λ for amphiphiles that contain a single hydrophobic chain. The functional dependence of $c_0(\lambda)$ on λ is not known, but phenomenologically it is reasonable to assume a power law dependence, λ^γ, in which the exponent $\gamma<1$. This means that equation (1.2) assumes the general form

$$E = E_0 \exp\{\zeta(A + B\lambda)(C + D\lambda^\gamma)\}, \qquad (1.3)$$

where the constants $\zeta\geq0$, $A\geq0$, $B<0$, $C>0$ and $D>0$ depend on the chemical structure of the amphiphile. The first part of equation (1.3) accounts for the observation that amphiphiles with longer hydrocarbon chains partition more readily into membranes than amphiphiles with shorter chains. While this should result in an enhanced cytotoxicity, the second part of equation (1.3) highlights the fact that as λ increases, the spontaneous curvature of the membrane will become less positive, thereby activating CCT and counteracting cytotoxicity. Conversely, for amphiphiles with short chains, equation (1.3) predicts that their lower partitioning will be counteracted by their higher type I characteristics, which drive the spontaneous curvature to become more positive. Correlations between alkyl chain length and cytotoxicity have been observed in a variety of cell lines; generally, the ED_{50} decreases as the length of the alkyl chain increases, but the rate of decrease is slower than expected from a simple exponential dependence. This correlates well with the 'stretched exponential' component of equation (1.3). In several studies it appears that activity goes through a maximum (minimum in ED_{50}) at a chain length of 16–18 carbon units (Andreesen *et al.* 1978; Morris-Natschke *et al.* 1986; Vogler *et al.* 1993; Geilen *et al.* 1994; Konstantinov *et al.* 1998*a*,*b*). Furthermore, Geilen *et al.* found that activity went through a maximum as chain length increased, but the amount of active compound in the membrane increased with chain length (Geilen *et al.* 1994), giving excellent experimental support for equation (1.3).

Equation (1.3) also explains why short-chain dialkyl (typically dioctyl) PtdCho analogues (Tarnowski *et al.* 1978) and the didecyl CP-46 665 prepared by Pfizer show similar cytotoxic activity to ET-18-OMe and BM41.440 (Danhauser *et al.* 1987). Introduction of the short-chain dialkyl analogues into a membrane leaves an area of 'free space' below the alkyl chains that the longer chain exogenous lipids will need to fill; consequently, there is a decrease in the curvature elastic stress within the membrane. The chain length versus

activity relationship of these compounds will also go through a pattern of activity as that of the single chain derivatives but the greater hydrophobicity of the dialkyl compounds will cause maximum potency to occur at a shorter chain length before the larger hydrocarbon cross-sectional area dominates and decreases potency as observed (Kudo *et al.* 1987).

It is interesting to note that hexadecylphosphonocholine has cytotoxic activity similar to its analogue HDPC (Langen *et al.* 1992). Exchange of the phospholipid headgroup from choline to *N*,*N*-dimethylethanolamine (Brachwitz *et al.* 1982) and then to serine (Langen *et al.* 1992) and phosphoinositol (Noseda *et al.* 1987) does not abrogate activity, although some decrease is observed. Furthermore, in the case of ALP analogues, the order of substituents on the glycerol backbone does not affect activity significantly (Langen *et al.* 1992). This is expected since such isomerism has minimal effects on the aggregation properties of many lipids.

As the study of the cytotoxicity of ALP and APC analogues developed, compounds with structures very different from the basic phosphocholine form of the ALP and APCs were reported in the literature as cytotoxic. Examples include the *N*-acyl derivatives of *O*-alkylglycerophospho- and alkylphospho-L-serines, nucleoside-5′-diphosphate and nucleoside-5′-phosphonophosphate-alkylglycerols prepared by Brachwitz (Brachwitz & Vollgraf 1995), *O*-alkylglycero-myo-inositols (Noseda *et al.* 1987; Ishaq *et al.* 1989) and a range of non-phosphorus-containing analogues. Within the group of non-phosphorus-containing analogues, glycolipid analogues of ET-18-OMe have been reported to be active (Weber & Benning 1988). Neutral lipids such as 1-*O*-hexadecyl-2-chloro-2-deoxyglycerol are also cytotoxic (Langen *et al.* 1979) as are the alkyl ether glycerolipids (Honma *et al.* 1991). An analysis of the structures of these compounds shows that they share the common characteristic of being amphiphilic with type I molecular characteristics.

Several issues were encountered when evaluating the literature on cytotoxic amphiphiles that limit the extent to which published data can be used to test our hypothesis. The two most significant issues are the wide diversity of experimental procedures and protocols employed to determine the ED_{50}, which poses serious challenges when comparing data from different studies, and the sparseness of information on the aggregation or phase behaviour of the novel compounds. In view of these limitations, we designed and synthesized in our laboratories a range of analogues of ALPs and APCs which would test predictions resulting from our hypothesis. These compounds included glycolipid analogues and some non-phosphorus-containing amphiphiles. In this way we were able to collect cytotoxicity data and classify the bioactive amphiphiles by investigating their aggregation behaviour, specifically their lyotropic liquid crystal properties.

2. EXPERIMENTAL PROCEDURES

RPMI medium, foetal calf serum and antibiotic–antimiotic solution were purchased from Invitrogen; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypan blue, ethanol and isopropanol were purchased from Sigma-Aldrich. Octaethylene glycol ethers (**42**–**46**) and quaternary ammonium amphiphiles (**39**–**41**) were purchased from Fluka.

HDPC was purchased from Alexa Biochemicals. Syntheses of the phosphoramide phospholipid analogues (Mackenzie 1995; **14**–**27**, table 2), of the heterocyclic ALPs (Wan 1997; **28**–**38**, table 3), of the classic type I amphiphiles (Wan 1997; **39**–**46**, table 4) and of the glycolipid compounds (Blackaby 1997; **47**–**55**, table 5) have been published previously.

2.1. Cytotoxicity assay protocol

ED_{50} values were determined on the HL-60 cell line. HL-60 cells were cultured at 37°C in 5% CO_2 in RPMI medium with Glutamax-1 and HEPES (25 mM) supplemented with 10% foetal calf serum and 5% antibiotic–antimiotic solution (10 000 units ml^{-1} penicillin G sodium, 10 000 µg ml^{-1} streptomycin sulphate and 25 µg ml^{-1} amphotericin B as Fungizone in 0.85% saline). Live cell counts were performed using trypan blue staining.

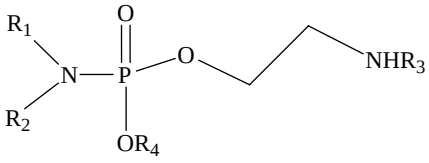
Cytotoxicity was determined using the MTT assay (Marks *et al.* 1992) or by thymidine incorporation, compounds (**14**–**27**) as previously reported (McGuigan *et al.* 1994). For the MTT assay, compounds were dissolved into 11 mM stock solutions in ethanol or culture medium depending on solubility. These solutions were further diluted with culture medium to give 1, 5, 10, 50 and 100 µM solutions. Cells were seeded at 6×10^4 per well in 100 µl of medium and compound solutions were added in 10 µl aliquots. Cultures were incubated at 37°C for 96 hours, MTT was added to each well and plates were incubated for a further 4 hours; 100 µl of isopropanol acidified with 0.04 M HCl was added to dissolve the formazan product and the absorbance of each well was measured using a Titertek Multiscan R plus 96-well plate reader at 492 nm. Assays were conducted in triplicate and the ED_{50} was determined from plots of average cell number against amphiphile concentration.

2.2. Lyotropic phase studies

The morphology of the lyotropic liquid crystalline phases formed by amphiphiles and lipid analogues was determined using an Olympus BH-2 polarizing optical microscope equipped with a Linkham Scientific Instruments THM600 hot stage. The accuracy of the temperature of the hot stage was ±0.2°C. Phases were assigned by their characteristic optical textures (Hyde 2002). Two techniques were employed to identify phases, depending on the quantity of material available. For compounds only available in small amounts, contact preparations were used to qualitatively map the phase diagram (Hyde *et al.* 1954). For compounds that were available in larger amounts, binary mixtures were prepared and the phase diagram was mapped quantitatively. In the contact preparation technique, a small amount of material was melted on a microscope slide and covered by a cover-slip, which was gently pressed to squash the material into a thin film. After

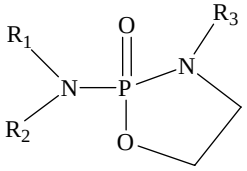
Table 2. The structure, cytotoxicity and lipid typology of phosphoramide analogues.

phosphoramide general structure



compound number	R_1	R_2	R_3	R_4	$EC_{50}/\mu M$[a]	lipid typology[b]
14	C_6H_{13}	H	CH_3	CH_3	1000	undetermined
15	$C_{12}H_{25}$	H	CH_3	CH_3	230	*type I*
16	$C_{16}H_{33}$	H	CH_3	CH_3	110	type I
17	$C_{18}H_{37}$	H	CH_3	CH_3	140	*type I*
18	oleyl	H	CH_3	CH_3	130	type I
19	$C_{18}H_{37}$	H	CH_3	C_2H_5	40	type I
20	C_6H_{13}	C_6H_{13}	CH_3	CH_3	140	undetermined
21	C_3H_7	$C_{15}H_{31}$	CH_3	CH_3	23	type I
22	C_6H_{13}	$C_{12}H_{25}$	CH_3	CH_3	77	undetermined
23	$C_{12}H_{25}$	H	H	CH_3	250	*type I*

cyclic phosphoramide general structure



compound number	R_1	R_2	R_3	$EC_{50}/\mu M$[a]	lipid typology[b]
24	$C_{16}H_{33}$	H	CH_3	750	type 0
25	C_6H_{13}	H	H	700	undetermined
26	$C_{12}H_{25}$	H	H	20	type 0
27	$C_{16}H_{33}$	H	H	60	*type 0*

[a] A standard error of $\pm 10\%$ should be applied when interpreting these data.
[b] Lipid typologies were determined by polarizing optical microscopy; those in italics were deduced because the typology of structurally similar compounds is known; some typologies were undetermined because the solubility of the compounds in aqueous solution was too low.

cooling the sample to room temperature, water was added which on coming in contact with the sample diffuses into it establishing a concentration gradient. Phases occurring at different degrees of hydration could be observed as successive bands from the external edge of the sample inwards. By changing the temperature of the sample, it was possible to observe temperature-induced changes in phase behaviour across the range of hydration. Phase diagrams were prepared from calculated compositions of lipid and water. Samples were thoroughly mixed and heated between a microscope slide and cover-slip.

2.3. Critical micelle concentration determinations

Critical micelle concentrations (CMCs) were determined in pure water from surface tension measurements using a CSC-Du Nouy Precision tensiometer 70535 (CSC Scientific Company, Inc.) fitted with a custom-made glass heating jacket. Solutions of the lipid were prepared in pure water and approximately 25 ml of each solution was poured into the heating jacket and allowed to equilibrate to 37 ± 0.5°C. Surface tension measurements and CMC determinations were made using a standard method (Sharma *et al.* 2003).

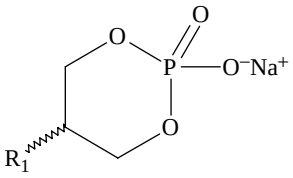
3. RESULTS

3.1. Evaluation of phosphoramide phospholipid analogues

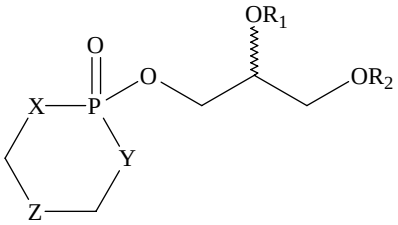
Most of the phosphoramide analogues (**14**–**27**) prepared show significant cytotoxicity towards the HL-60 cell line (table 2). The lyotropic liquid crystalline phase behaviour, obtained from penetration studies, of a representative selection of these compounds is shown in figure 2.

Table 3. Structure, cytotoxicity, lipid typology and CMC of lysophospholipid analogues with heterocyclic headgroups. (Definitions of terms a and b are the same as given in table 2.)

heterocyclic headgroup general structure



compound number	R_1	EC_{50} μM	lipid typology[b]	CMC/μM[a]
28	$C_{12}H_{25}$	287±21	type I	83
29	$C_{14}H_{29}$	73.4±47	type I	14
30	$C_{16}H_{33}$	43.4±37	type I	3.2
31	$C_{18}H_{37}$	12.6±7.4	type I	0.6

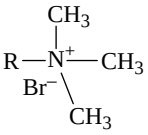


compound number	X	Y	Z	R_1	R_2	EC_{50} μM	lipid typology[b]	CMC μM[a]
32	O	O	CH_2	Me	$C_{16}H_{33}$	19.7±1.3	*type I*	
33	NH	NH	CH_2	Me	$C_{16}H_{33}$	4.5±2.1	*type I*	
34	O	NH	CH_2	Me	$C_{16}H_{33}$	2.8±1.2	*type I*	
35	O	O	CMe_2	H	$C_{14}H_{29}$	20±10	*type I*	
36	O	O	CMe_2	H	$C_{16}H_{33}$	16.5±8.5	*type I*	40
37	O	O	CMe_2	H	$C_{18}H_{37}$	19.7±1.3	*type I*	
38	O	O	CMe_2	H	oleyl	6.67±2.7	*type I*	100

3.1.1. Acyclic phosphoramides. Compound **16** is clearly a type I lipid, as evidenced by its phase behaviour being dominated by a normal topology hexagonal phase, which is stable up to 42°C. Compounds **15** and **17** are also type I lipids, since they are close homologues of compound **16**. It is not possible to classify compound **14** as it showed no liquid crystal properties at, or above, room temperature. This is most probably due to its high solubility; however it has all the characteristics of a type I amphiphile. Within experimental error, the ED_{50} decreases monotonically on ascending the series **14**–**17**. However the rate of decrease in the ED_{50} slows significantly for the higher homologues, with a minimum in ED_{50} occurring at compound **16**. The alkyl chain length at this minimum is 16 carbon units, a value consistent with earlier observations (Andreesen *et al.* 1978; Vogler *et al.* 1993; Wieder *et al.* 1999). This behaviour, which is characteristic of amphiphilic cytotoxic compounds, is consistent with the predictions from equation (1.3) and illustrates the competition between the type I characteristics of the amphiphile and the hydrophobic effect-driven partitioning of the amphiphile. Changing the hydrocarbon chain from octadecyl (**17**) to oleyl (**18**) gives a small change in activity, which is probably insignificant within error. Compounds **17** and **19** are both type I lipids as shown by optical microscopy (see figure 2). Although both compounds are close homologues, at least in terms of hydrophobicity, the increase in headgroup size on going from a methyl to an ethyl substituent on the phosphate triester headgroup suggests that compound **19** might be expected to be the more active. In view of the proximity of this substitution site to the polar–apolar interface, it might be expected that there would be a big difference in the activity between compounds **17** and **19**. The cytotoxicity data do indeed show a dramatic difference between the two compounds, with compound **19** having an ED_{50} that is nearly an order of magnitude lower than compound **17**. Compounds **17**, **21** and **22** provide an interesting test of the hypothesis. In this homologous series, the hydrophobic part of the amphiphile has the same number of carbon atoms, but while in compound **17** all **18** carbon atoms are part of a single alkyl chain, compounds **21** and **22** have two alkyl chains. All the three homologues are type I amphiphiles, as highlighted by the phase behaviour of the representative compound **21**.

Table 4. Structure, cytotoxicity, lipid typology and CMC of classic type I amphiphiles. (Definitions of terms a and b are the same as given in table 2.)

trimethylammonium series



octaethylene glycol series

R-$(OCH_2CH_2)_8OH$

compound number	headgroup	R	ED_{50}/μM	lipid typology[b]	CMC/μM[a]
39	trimethylammonium	$C_{12}H_{25}$	4.75±2.9	type I	1.00×10^4
40	trimethylammonium	$C_{14}H_{29}$	3.56±2.1	type I	3.20×10^3
41	trimethylammonium	$C_{16}H_{33}$	1.9±1.0	type I	1000
42	octaethylene glycol	$C_{10}H_{21}$	47.5±24	type I	560
43	octaethylene glycol	$C_{12}H_{25}$	22.6±13	type I	79
44	octaethylene glycol	$C_{14}H_{29}$	7.25±3.4	type I	13
45	octaethylene glycol	$C_{16}H_{33}$	4.51±2.6	type I	5.6
46	octaethylene glycol	$C_{18}H_{37}$	3.39±2.0	type I	4

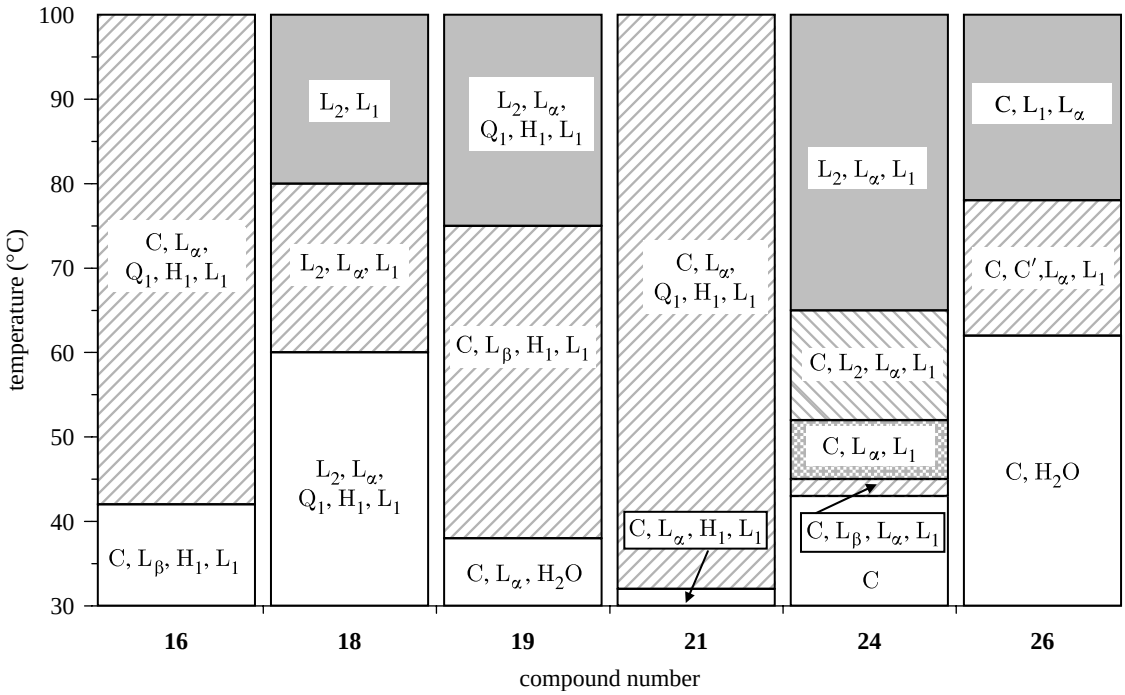


Figure 2. Lyotropic liquid crystal contact preparations of phosphoramide phospholipid analogues, where L_1, micellar solution; L_2, inverse micellar solution; I_1, micellar cubic phase; H_1, hexagonal phase; H_{II}, inverse hexagonal phase; Q_1/Q_2, cubic phases; L_α, fluid lamellar phase; L_β, solid lamellar phase; C′, hydrated crystal and C, crystal. Divisions show the phases present within the temperature range.

On the basis of the rules of thumb outlined in figure 1, it is predicted that compound **21** should exhibit the highest activity (lowest ED_{50}). This is because the propyl substituent on the phosphoramide nitrogen is close to the polar–apolar interface and leads to an amphiphile that has a larger headgroup cross section than the other two members of this series. In addition, the shorter alkyl chain means that this amphiphile is more effective at reducing stored elastic energy than compound **17**. These predictions are in agreement with the experimental observations. A similar argument would suggest that compound **20** should be more active than compound **15** and this is supported by the data.

Compounds **20**–**22** are difficult to assess as type I lipids; from a structural point of view it is difficult to determine the effect of a second alkyl chain. Classically, two long alkyl chains will be type 0 if saturated and type II if unsaturated. However, starting with a single alkyl chain and then increasing the length of a second alkyl chain, from 1 to 10 carbon units, a situation arises where firstly the short chain increases the headgroup volume and then at some critical length it switches to the alkyl chain region increasing the hydrocarbon volume, as demonstrated for a series of quaternary ammonium amphiphiles (Hertel & Hoffmann 1989). Behaviour in the region of six carbon units is hard to

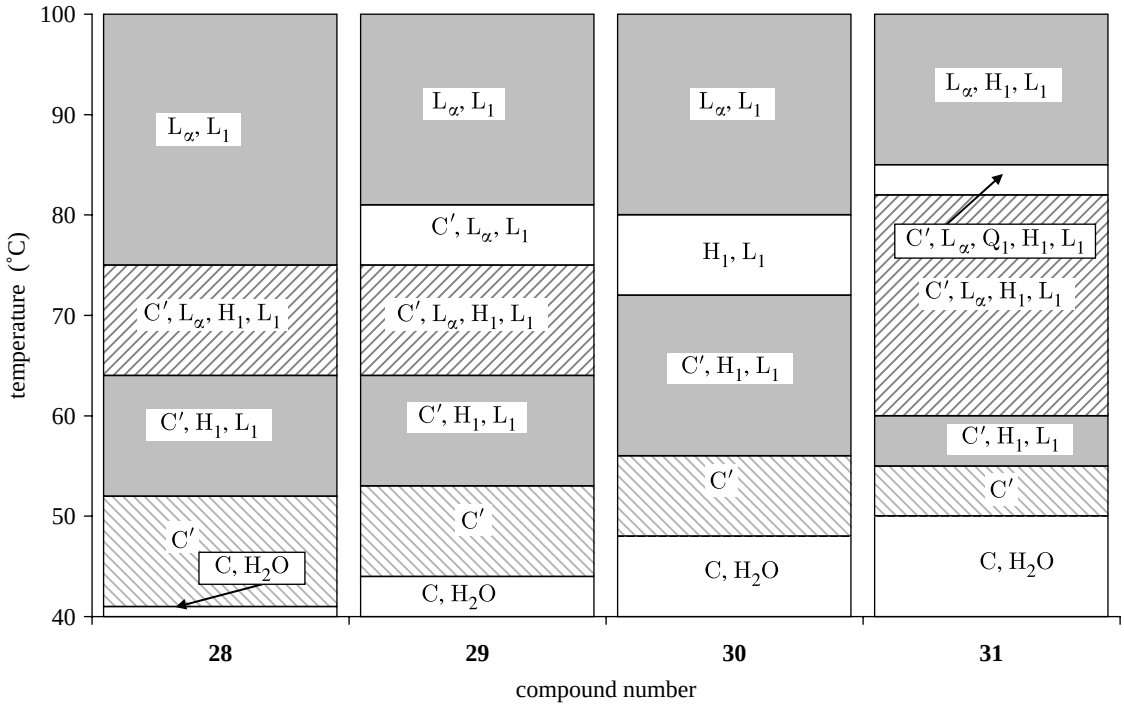


Figure 3. Lyotropic liquid crystal contact preparations of ALP analogues with heterocyclic headgroups. Definitions of terms are the same as given legend of figure 2.

predict, fewer than six carbon units certainly contribute to the headgroup region and thus compound **21** is type I as confirmed by phase studies. Compound **22** has a second alkyl chain of six units and it is consequently harder to assign as type I. Critically, the second alkyl chain increases potency; compound **23**, which lacks the hexyl chain, is much less active than compound **22**, but whether or not the increase in activity is due to increased type I properties or increased partitioning is unclear. Compound **20** illustrates that hydrophobicity is not key to determining activity since compounds **20** and **23** are likely to have very similar hydrophobicity but have very different activities.

3.1.2. Cyclic phosphoramides. Compounds **24**–**27** are all type 0 amphiphiles. The lack of a hydrated crystal phase in compound **24** ($R_3 = CH_3$) compared with compound **26** ($R_3 = H$) suggests that hydrogen bonding plays a role in the aggregation behaviour of compound **26**, a factor that should be taken into account when considering the ED_{50} of these compounds.

When the cytotoxicity of these compounds is examined (table 2), the least active compound is the hexyl analogue **25**. This is unsurprising, as is the case for compound **14**; the short alkyl chain reduces the need for partitioning into the membrane and thus the delivery of the active compound into the cell is hindered.

Compound **26** is a type 0 lipid, confirmed by phase studies and owing to its structural similarity compound **27** will also be type 0. Both these compounds show considerable activity, which is intriguing when compared with the less active compound **24**. Clearly this change in activity is due to the inclusion of a methyl group at R_3 and consequently the role of hydrogen bonding and hydration when present in a membrane becomes important. Using the lateral pressure profile, it is quite clear why this is the case: if the net stress in area γ is lowered, then the desire for type II behaviour increases. Hydrogen bonding increases the hydration in this region and thus increases the effective headgroup volume making the compounds **26** and **27** more type I than compound **24**, agreeing with the prediction that more type I lipids will be more potent.

3.2. Evaluation of ALP analogues with heterocyclic headgroups

The lysophospholipids with heterocyclic headgroups (table 3) were problematic to assess by optical microscopy due to miscibility problems. An assessment of lipid typology is shown in figure 3. Compounds **28**–**31** were evaluated as sodium salts and were all shown to be type I lipids on the basis of the presence of a stable hexagonal phase in all the preparations. Compounds **32**–**38** were more difficult to assign due to low solubility, but because they are lysophosphatidylcholine analogues it is not unreasonable to expect them to be type I amphiphiles.

Compounds **28**–**31**, the 2-hydroxy-1,3 dioxa-2-phosphacyclohexane-2-oxide analogues (table 3), show a clear relationship of increasing activity with chain length, with the octadecyl analogue (**31**) exhibiting the highest activity. This increase in activity with chain length is consistent with the qualitative trends predicted from equation (1.2), reflecting an increase in the partitioning of more hydrophobic, longer chain length compounds into the membranes. The series of homologues **32**, **33** and **34** all have the same length of alkyl chain but differ in the nature of the heteroatoms in their headgroups. On the basis of the criteria outlined earlier, it is predicted that **33** should be the most active of this set, owing to the overall larger size of the headgroup, while **32**, with the smallest headgroup, should be the

least active. These predictions are in reasonable agreement with the experimental observations, which show that **33** and **34** have broadly similar activities, both of which are higher than **32**).

The series of compounds **35**, **36** and **37** are predicted to exhibit an increase in activity as the chain length increases from 14 to 18; however, it is not possible to differentiate between the activities of these homologues within the resolution of the experiment. The only observation for this group of amphiphiles that does not support the predictions stemming from our hypothesis is the comparison of the activities of compounds **37** and **38**, for which it would be expected that the oleoyl homologue should be the less active of the two. Experimentally, it is observed that in fact homologue **38** has the higher activity.

3.3. Evaluation of classic type I amphiphiles

Two series of classic type I amphiphiles, namely the trimethylammonium bromide amphiphiles (**39**–**41**) and the octaethylene glycol amphiphiles (**44**–**48**; table 4), were also studied to test our hypothesis. The phase behaviour of the octaethylene glycol amphiphiles is well documented (Mitchell *et al.* 1983), as is the phase behaviour of the alkyltrimethyl ammonium bromide series (Hertel & Hoffmann 1988). It is noted that apart from their amphiphilic characteristics, these compounds bear no obvious chemical relationships to the ALP and APC compounds, but still exhibit the same patterns of cytotoxicity. Firstly, there is the increase in cytotoxicity with increasing chain length, as seen in compounds **39**, **40** and **41**, and more dramatically in the series **42**–**44**, where the homologue with the octadecyl chain (**46**) is the most active.

3.4. Evaluation of the glycolipid analogues

Table 5 shows a series of glycolipid compounds that provide a challenging test to our hypothesis. Clearly in chemical terms, these materials differ substantially from the lipid analogues described earlier. However, investigation of their phase behaviour suggests that they are largely type 0 lipids, as confirmed by phase studies (figure 4). We note that compounds **50** and **51** are type II amphiphiles because their phase behaviour is dominated by inverse topology phases. Applying the qualitative rules we outlined earlier to homologues **48** and **49** would lead to the prediction that **49** should be the more active of the two in view of its longer hydrocarbon chain. This is consistent with the experimental data. Similarly, for the pair **47** and **50** it would be expected that **47** would be the more active, because the shorter chains would create a packing void when mixed with the longer chain lipids found in membranes. As a consequence **47** would act to reduce the stored elastic energy when compared with **50**. The experimental data are consistent with this prediction.

Comparison of compounds **50** and **52** illustrates the effect of an additional sugar moiety in the headgroup. We would expect that **52** should be the more active of the two on the basis of its larger headgroup. This is the opposite to what is observed experimentally, which may be due to the increased solubility of **52** compared with **51** counteracting its effect on the stored elastic energy. This is supported by a comparison of **52** and **53**. Both these homologues have two carbohydrate moieties in their headgroup region; so they would be expected to have comparable solubility. However **53** would be expected to be the less active on account of the linear nature of the headgroup, and consequently smaller headgroup cross section than **52**. The cytotoxicity data are in agreement with this prediction.

Finally for this complex series, we draw comparisons between the activities of **47** and **55**; **52** and **54**; **53** and **54**; and **48** and **49**. We would predict that **47** should be the more active of the two, since the primary amine group would be expected to become protonated under physiological conditions, thereby resulting in strong type I behaviour due to electrostatic repulsions between headgroups. The ED_{50} of **55** is 8 μM, compared with 20 μM for **47**, consistent with our predictions. Compound **52** should be more active than **54** because the skewed headgroup has a wider cross-sectional area than the stacked system in **54**, but the overall solubility is unchanged. The ED_{50} of **52** is 49 μM compared with 58 μM for **54**; within experimental error there is no difference. Comparing compounds **53** and **54**, we would predict very little change in activity because the structural change from an equatorial (**53**) to axial (**54**) hydroxyl group occurs at a distance from the interface. This may have a minor effect on headgroup packing, but hydration is unlikely to change significantly. In the cases of compounds **48** and **49**, **49** should be the more active because it has a longer hydrocarbon chain than **48** and hence it partitions more effectively into the membrane. This accords with our observations.

Figure 5 shows cytotoxicity data for all the compounds evaluated above, compared with their liquid crystal typology. It is clear from figure 5 that the most active compounds are all type I lipids, which include HDPC. In addition some of the compounds evaluated in this study are of greater potency than HDPC and potentially represent new classes of chemotherapeutic compounds.

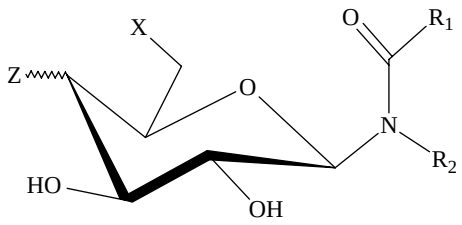
4. DISCUSSION

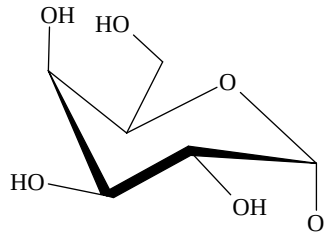
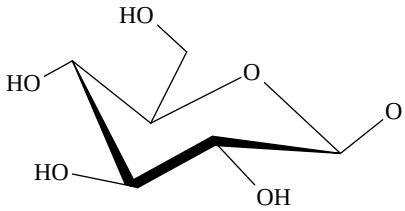
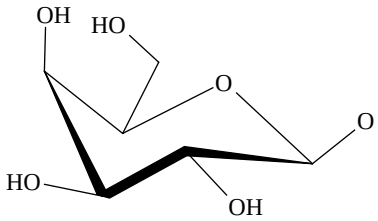
A major problem in interpreting the structure activity data of ALP and APC analogues is the role of the lysis in the mechanism of action. Generally, with respect to the ALP and APC analogues, it is accepted that lysis is a potential mechanism of action but only at higher concentration and this is supported by some experimental evidence (Dive *et al.* 1991). Recently Busto *et al.* (2007) concluded that in the case of ET-18-OMe lysis is not a probable mechanism of cytotoxicity due to the poor ability of ET-18-OMe to breakdown vesicles *in vitro*.

The viability of lysis as a mechanism is dependent on the concentration of the detergent molecules. Below the CMC, amphiphile monomers do not aggregate in solution, instead individual monomers are soluble in the aqueous environment. In the presence of a cellular membrane and below the CMC, the hydrophobic effect will force the amphiphiles into the membrane, creating

Table 5. Structure, cytotoxicity and lipid typology of glycolipid compounds. (Definitions of terms a and b are the same as given in table 2.)

glycolipid general structure



compound number	X	Z	R_1	R_2	ED_{50}/ μM[a]	lipid typology[b]
47	OH	$OH_{(eq)}$	C_7H_{15}	C_8H_{17}	20	type 0
48	OH	$OH_{(eq)}$	C_9H_{19}	C_4H_9	70	type 0
49	OH	$OH_{(eq)}$	$C_{15}H_{31}$	C_4H_9	22	type 0
50	OH	$OH_{(eq)}$	$C_{17}H_{35}$	C_8H_{17}	25	type II
51	OH	$OH_{(ax)}$	$C_{17}H_{35}$	C_8H_{17}	68	type II
52		$OH_{(eq)}$	$C_{17}H_{35}$	C_8H_{17}	49	type 0
53	OH		$C_{17}H_{35}$	C_8H_{17}	55	type 0
54	OH		$C_{17}H_{35}$	C_8H_{17}	58	type 0
55	NH_2	$OH_{(eq)}$	$C_{17}H_{35}$	C_8H_{17}	8	type 0

equilibrium between the membrane and monomers in solution. At concentrations above or around the CMC, the same equilibrium exists, but this time it is between the monomers in the membrane and aggregates in the aqueous solution; thus there is a mechanism for solubilizing cellular components in the extracellular environment. If the ED_{50} is significantly below the CMC, then we believe that lysis is not the dominant mechanism of action. In practice it is easier to consider the ratio of the ED_{50} to the CMC as a guide. At values greater than 1, there are likely to be aggregates of the active material present in the extracellular environment. Such aggregates could cause cell death by both lysis and curvature elastic stress modulation.

Experimental values of the CMC are given alongside the structures, where insufficient material was available to perform surface tension measurements; literature values have been employed to provide estimates. From the literature we have found glycolipid analogues with CMCs in the range of 1×10^{-4} M to 1×10^{-6} M (Takeoka *et al.* 1998; Augusto *et al.* 2002). Since the chain lengths in this study are relatively long, we use an intermediate value of 1×10^{-5} M for compounds **47**–**55**. The phosphoramide analogues (**14**–**27**) are analogues of HDPC (CMC$=8\times10^{-6}$ M; Kotting *et al.* 1992) and are likely to have CMCs of a similar order of magnitude; however, we conservatively use 1×10^{-5} M for all these compounds. Figure 6 shows the corresponding plot of

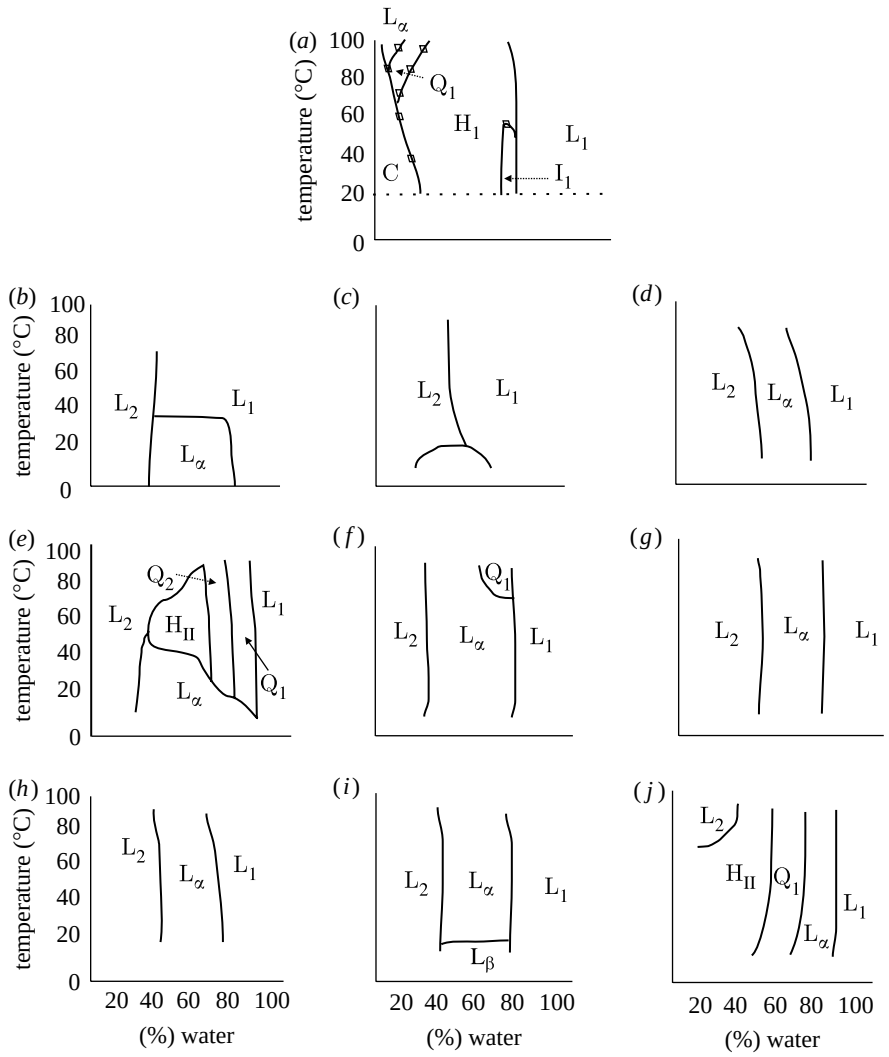


Figure 4. Lyotropic liquid crystal phase diagrams of glycolipid analogues. Definitions of terms are the same as given in figure legend 2. (*a*) HDPC (**2**), (*b*) compound **47**, (*c*) compound **48**, (*d*) compound **49**, (*e*) compound **50**, (*f*) compound **51**, (*g*) compound **52**, (*h*) compound **53**, (*i*) compound **54**, (*j*) compound **55**.

compound versus the ratio of ED_{50}:CMC. It is immediately of note that all the compounds active below their CMC are type I lipids, the most potent modulators of the stored elastic stress. It seems very unlikely that these compounds could be active exclusively through lysis, a view that is supported by our observations in the laboratory, where cells treated with low concentrations of active compound remained intact; at higher concentrations cells were homogenized by lysis.

In our studies the ED_{50} of HDPC ranges from 2.5 to 6.5×10^{-6} M (see figure 5). The CMC of HDPC from the literature is $2.5\text{–}3\times10^{-6}$ M (Rakotomanga *et al.* 2004) and 8×10^{-6} M (Kotting *et al.* 1992), thus for HDPC the ratio ED_{50}:CMC falls in the range of 1–3 and a lytic mechanism cannot be completely ignored (see figure 6). Our most active compounds (**39**, **49**, **41**) are more potent than HDPC and active well below their CMC; potentially, these compounds offer more effective cancer treatments.

Most of the cytotoxic analogues of ALP and APC compounds in the literature follow structure activity trends that agree with the predictions of our simple hypothesis. This is further complemented by the data that we have presented of our own studies on systems. There are a few notable exceptions in the literature which are cytotoxic but are not easily explained by our hypothesis, using the current limited information on their aggregation properties. These exceptions are the plasmanyl-(*N*-acyl)-ethanolamines (Kara *et al.* 1986), a series of naturally occurring compounds with *in vitro* and *in vivo* antitumour activities. Structurally these compounds are dialkylglycerophosphoethanolamines with one of the ethanolamine hydrogens replaced by the moiety $COC_{15}H_{31}$. Effectively, this gives a compound with three alkyl chains, which is likely to exhibit type II lipid behaviour due to the large hydrocarbon region; however, proper lyotropic liquid crystal phase studies will need to be carried out before any firm conclusion is made. The other apparent exception to the hypothesis proposed by us is the compound erucylphosphocholine (EPC). EPC has been studied extensively (Kotting *et al.* 1992; Jendrossek *et al.* 2002, 2003; Jendrossek & Handrick 2003) and shows greater (Zeisig *et al.* 1993) or comparable potency (Kotting *et al.* 1992) to HDPC. The erucyl chain is the *cis*-13-docosenol derivative; it is probably a type 0 lipid, since it forms lamellar structures rather than micelles (Kotting *et al.* 1992). Currently, there are

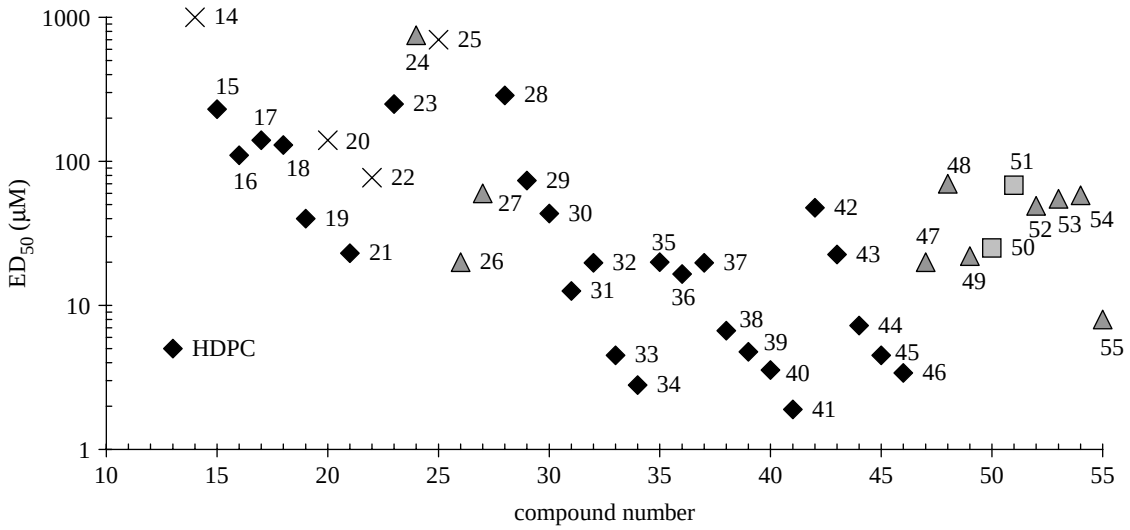


Figure 5. A comparison of ED_{50} against lipid typology for phosphoramide compounds (**14**–**27**), lysophospholipids with heterocyclic headgroups (**28**–**38**), classic type I amphiphiles (**39**–**46**) and glycolipid analogues (**47**–**55**). Diamonds, type I; triangles, type 0; crosses, undetermined; squares, type II.

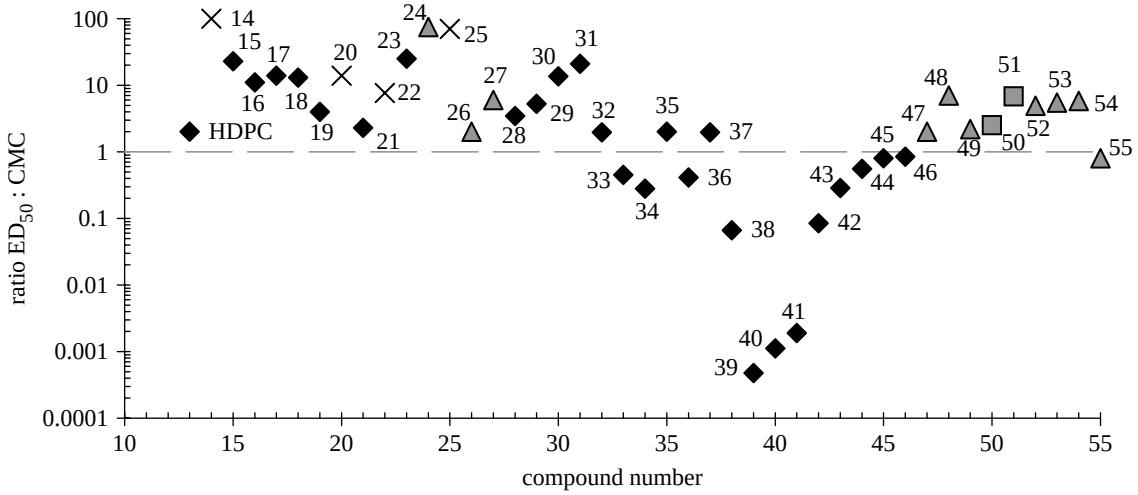


Figure 6. Plot of the ratio of the ED_{50}:CMC for phosphoramide compounds (**14**–**27**), lysophospholipids with heterocyclic headgroups (**28**–**38**), classic type I amphiphiles (**39**–**46**) and glycolipid analogues (**47**–**55**). Compounds with a ratio of greater than 1 probably have a lytic effect on cellular membranes. Diamonds, type I; triangles, type 0; crosses, undetermined; squares, type II.

no detailed phase studies to confirm this; regardless though, it is curious that this single long-chain derivative is active when many studies have concluded that the chain length of 16–18 carbon units gives the highest activity. Of note are the observations that EPC is more active than HDPC in Jurkat, Raji and Ramos cell lines but less active in HL-60 cells (Konstantinov *et al.* 1998*b*). Also EPC is consistently more active than HDPC in a series of brain cell lines (Jendrossek *et al.* 2002) as are a series of other long chain monounsaturated analogues. In our theory the bilayer thickness will change the optimum chain length for highest activity as modelled by the local molecular environment portion of equation (1.3). This may explain why EPC is more active than HDPC in some cell lines and vice versa. If our theory is correct we would predict the saturated analogue of EPC (docosonyl PC) to be more active than EPC in cell lines where EPC is more active than HDPC; currently, there are no data for this analogue in the literature.

The case of EPC illustrates the complexity of the relationship between structure and cytotoxicity, with many studies reporting different orders of potency for EPC and HDPC. Many of these problems could be eliminated by adopting a standard experimental protocol, as we have done after experiencing conflicting data in the literature and problems with our own early work. We suggest using the MTT assay to determine the ED_{50}, but in addition the ED_{50} of every set of compounds should be compared with the ED_{50} of HDPC, measured in the same experiments as a reference compound, shown in equation (4.1).

$$ED_{50}(\text{normalized}) = [ED_{50}\text{cmp}]/n[ED_{50}\text{ref}], \quad (4.1)$$

where n is the number of seed cells; ED_{50}cmp is ED_{50} of the active compound and ED_{50}ref is the ED_{50} of HDPC measured at the same time as the active compound.

The value ED_{50} (normalized) allows the ED_{50} from different determinations of cytotoxicity to be compared with each other. Furthermore, since partitioning

into the cell membrane is a critical part of the mechanism of action for these compounds, normalizing with the number of cells is a critical necessity for quantitative treatments.

We have proposed that the enzyme CCT is the probable cellular target of ALP and APC analogues. Historically, the mechanism of CCT regulation by the composition of lipid membranes has been a matter of debate. An initial model proposed negative membrane surface charge to be the activator of CCT (Cornell 1991*a*) with the physical properties of biomembranes playing a potential role (Cornell 1991*b*); however, these early studies failed to explain the activation or deactivation of CCT by uncharged or electrostatically neutral, zwitterionic lipids. Attard *et al.* (2000) proposed that membrane-free energy, in particular stored curvature elastic stress, regulated CCT translocation and hence activity. This was demonstrated with the zwitterionic lipids dioleoyl phosphoethanolamine (DOPE), a CCT activator, and lyso-myristoyl phosphocholine (MPC), a CCT deactivator. Davies *et al.* (2001) confirmed that CCT deactivation by lyso-oleoyl phosphocholine (OPC) and lyso-oleoyl phosphoethanolamine (OPE) and CCT activation by DOPE correlate with membrane stored elastic energy. OPC, MPC and OPE are type I lipids and ALP compounds, which all deactivate CCT *in vitro*. Other studies have shown that CCT activity is decreased by the type I lipids hexadecyltrimethylammonium bromide (**41**) (Sohal & Cornell 1990) and octaethylene glycol monohexadecyl ether (**45**) (Attard *et al.* 2000), providing further evidence to support our hypothesis that it is the type I lipid properties of ALP and APC compounds which confer their cytotoxicity.

5. CONCLUSIONS

Our analysis of the cytotoxicity data of amphiphilic ALP and APC analogues has shown that the trends in activity in relation to the various moieties of these amphiphiles are consistent with the hypothesis that their primary effect is a decrease in the stored curvature elastic energy of cellular membranes. This suggests that the primary targets of amphiphilic antineoplastic agents are most likely to be proteins whose activity is modulated by the composition of the membranes with which they associate. The most probable candidate appears to be CCT, which from *in vitro* studies has been shown to be strongly modulated by type I amphiphiles. Our results and analysis provide a new rationale for the design of antineoplastic drugs that do not interact with DNA.

This work was supported by the Leverhulme Trust, the Royal Society and the Medical Research Council, UK. The authors would like to thank R.H. Templer, Imperial College London, for many discussions on membrane curvature elastic stress and protein regulation, W.P. Blackaby, J.W. Wang, A. Mackenzie and W. S. Smith for the synthesis and evaluation of antineoplastic compounds, and C McGuigan, University of Wales Cardiff, for collaboration on the ALP analogues and glycolipid analogues.

REFERENCES

Andreesen, R., Modolell, M., Weltzien, H. U., Eibl, H., Common, H. H., Loehr, G. W. & Munder, P. G. 1978 Selective destruction of human leukemic cells by alkyl-lysophospholipids. *Cancer Res.* **38**, 3894–3899.

Attard, G. S., Templer, R. H., Smith, W. S., Hunt, A. N. & Jackowski, S. 2000 Modulation of CTP: phosphocholine cytidylyltransferase by membrane curvature elastic stress. *Proc. Natl Acad. Sci. USA* **97**, 9032–9036. (doi:10.1073/pnas.160260697)

Augusto, L. A., Li, J., Synguelakis, M., Johansson, J. & Chaby, R. 2002 Structural basis for interactions between lung surfactant protein C and bacterial lipopolysaccharide. *J. Biol. Chem.* **277**, 23 484–23 492. (doi:10.1074/jbc.M111925200)

Berkovic, D. 1998 Cytotoxic etherphospholipid analogues. *Gen. Pharmacol.* **31**, 511–517. (doi:10.1016/S0306-3623(98)00081-0)

Blackaby, W. P. 1997 *The synthesis, biological and physical evaluation of glycolipid derivatives*. Southampton, UK: University of Wales.

Boggs, K. P., Rock, C. O. & Jackowski, S. 1995 Lysophosphatidylcholine and 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine inhibit the CDP-choline pathway of phosphatidylcholine synthesis at the CTP: phosphocholine cytidylyltransferase step. *J. Biol. Chem.* **270**, 7757–7764. (doi:10.1074/jbc.270.13.7757)

Boggs, K., Rock, C. O. & Jackowski, S. 1998 The anti-proliferative effect of hexadecylphosphocholine toward HL60 cells is prevented by exogenous lysophosphatidyl-choline. *Biochim. Biophys. Acta* **1389**, 1–12. (doi:10.1016/S0005-2760(97)00145-8)

Brachwitz, H. & Vollgraf, C. 1995 Analogs of alkyllysophos-pholipids: chemistry, effects on the molecular level and their consequences for normal and malignant cells. *Pharmacol. Ther.* **66**, 39–82. (doi:10.1016/0163-7258(95)00001-W)

Brachwitz, H., Langen, P., Otto, A. & Schildt, J. 1979 Halolipids. III. Alkyl glyceryl ether analogs. *J. Prakt. Chem.* **321**, 775–786. (doi:10.1002/prac.19793210509)

Brachwitz, H., Langen, P., Hintsche, R. & Schildt, J. 1982 Halo lipids. V. Synthesis, nuclear magnetic resonance spectra and cytostatic properties of halo analogs of alkyllysophospholipids. *Chem. Phys. Lipids* **31**, 33–52. (doi:10.1016/0009-3084(82)90017-2)

Brachwitz, H., Langen, P. & Schildt, J. 1984 Halo lipids. 7. Synthesis of rac 1-chloro-1-deoxy-2-*O*-hexadecylglycero-3-phosphocholine. *Chem. Phys. Lipids* **34**, 355–362. (doi:10.1016/0009-3084(84)90009-4)

Brachwitz, H., Langen, P., Arndt, D. & Fichtner, I. 1987 Cytostatic activity of synthetic *O*-alkylglycerolipids. *Lipids* **22**, 897–903. (doi:10.1007/BF02535551)

Busto, J. V., Sot, J., Goni, F. M., Mollinedo, F. & Alonso, A. 2007 Surface-active properties of the antitumour ether lipid 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phospho-choline (edelfosine). *Biochim. Biophys. Acta* **1768**, 1855–1860. (doi:10.1016/j.bbamem.2007.04.025)

Cornell, R. B. 1991*a* Regulation of CTP: phosphocholine cytidylyltransferase by lipids. 1. Negative surface charge dependence for activation. *Biochemistry* **30**, 5873–5880. (doi:10.1021/bi00238a010)

Cornell, R. B. 1991*b* Regulation of CTP: phosphocholine cytidylyltransferase by lipids. 2. Surface curvature, acyl chain length, and lipid-phase dependence for activation. *Biochemistry* **30**, 5881–5888. (doi:10.1021/bi00238a011)

Danhauser, S., Berdel, W. E., Schick, H. D., Fromm, M., Reichert, A., Fink, U., Busch, R., Eibl, H. & Rastetter, J.

1987 Structure-cytotoxicity studies on alkyl lysophospholipids and some analogs in leukemic blasts of human origin *in vitro*. *Lipids* **22**, 911–915. (doi:10.1007/BF02535553)

Davies, S. M., Epand, R. M., Kraayenhof, R. & Cornell, R. B. 2001 Regulation of CTP: phosphocholine cytidylyltransferase activity by the physical properties of lipid membranes: an important role for stored curvature strain energy. *Biochemistry* **40**, 10 522–10 531. (doi:10.1021/bi010904c)

Dick, D. L. & Lawrence, D. S. 1992 Physicochemical behavior of cytotoxic ether lipids. *Biochemistry* **31**, 8252–8257. (doi:10.1021/bi00150a019)

Dive, C., Watson, J. V. & Workman, P. 1991 Multiparametric flow cytometry of the modulation of tumor cell membrane permeability by developmental antitumor ether lipid SRI 62-834 in EMT6 mouse mammary tumor and HL-60 human promyelocytic leukemia cells. *Cancer Res.* **51**, 799–806.

Fromm, M., Berdel, W. E., Schick, H. D., Fink, U., Pahlke, W., Bicker, U., Reichert, A. & Rastetter, J. 1987 Antineoplastic activity of the thioether lysophospholipid derivative BM 41.440 *in vitro*. *Lipids* **22**, 916–918. (doi:10.1007/BF02535554)

Geilen, C. C., Haase, A., Wieder, T., Arndt, D., Zeisig, R. & Reutter, W. 1994 Phospholipid analogs: side chain- and polar head group-dependent effects on phosphatidylcholine biosynthesis. *J. Lipid Res.* **35**, 625–632.

Geilen, C. C., Wieder, T. & Orfanos, C. E. 1996 Phosphatidylcholine biosynthesis as a target for phospholipid analogs. *Adv. Exp. Med. Biol.* **416**, 333–336.

Hanauske, A. R., Degen, D., Marshall, M. H., Hilsenbeck, S. G., McPhillips, J. J. & Von Hoff, D. D. 1992 Preclinical activity of ilmofosine against human tumor colony forming units *in vitro*. *Anti-Cancer Drugs* **3**, 43–46.

Helfrich, W. 1973 Elastic properties of lipid bilayers: theory and possible experiments. *Z. Naturforsch.* [*C*] **28**, 693–703.

Hertel, G. & Hoffmann, H. 1988 Lyotropic nematic phases of double-chain surfactants. *Prog. Colloid Polym. Sci.* **76**, 123–131. (doi:10.1007/BFb0114182)

Hertel, G. & Hoffmann, H. 1989 New lyotropic nematics of double chain surfactants. *Liq. Crystals* **5**, 1883–1898. (doi:10.1080/02678298908045696)

Hoffman, D. R., Stanley, J. D., Berchtold, R. & Snyder, F. 1984 Cytotoxicity of ether-linked phytanyl phospholipid analogs and related derivatives in human HL-60 leukemia cells and polymorphonuclear neutrophils. *Res. Commun. Chem. Pathol. Pharmacol.* **44**, 293–306.

Honma, Y., Kasukabe, T., Okabe-Kado, J., Hozumi, M., Tsushima, S. & Nomura, H. 1983 Antileukemic effect of alkyl phospholipids. I. Inhibition of proliferation and induction of differentiation of cultured myeloid leukemia cells by alkyl ethyleneglycophospholipids. *Cancer Chemother. Pharmacol.* **11**, 73–76.

Honma, Y., Kasukabe, T., Hozumi, M., Akimoto, H. & Nomura, H. 1991 Induction of differentiation of human myeloid leukemia HL-60 cells by novel nonphosphorus alkyl ether lipids. *Lipids* **26**, 1445–1449. (doi:10.1007/BF02536583)

Houlihan, W. J., Lee, M. L., Munder, P. G., Nemecek, G. M., Handley, D. A., Winslow, C. M., Happy, J. & Jaeggi, C. 1987 Antitumor activity of SRI 62-834, a cyclic ether analog of ET-18-OCH3. *Lipids* **22**, 884–890. (doi:10.1007/BF02535549)

Hyde, A. J., Langbridge, D. M. & Lawrence, A. S. C. 1954 Soap, water and amphiphile systems. *Discuss. Faraday Soc.* **18**, 239–257. (doi:10.1039/df9541800239)

Hyde, S. T. 2002 Identification of lyotropic liquid crystalline mesophases. In *Handbook of applied surface and colloid chemistry* (ed. K. Holmberg), pp. 299–332. Chichester, UK: John Wiley & Sons.

Ishaq, K. S., Capobianco, M., Piantadosi, C., Noseda, A., Daniel, L. W. & Modest, E. J. 1989 Synthesis and biological evaluation of ether-linked derivatives of phosphatidylinositol. *Pharm. Res.* **6**, 216–224. (doi:10.1023/A:1015961416370)

Jackowski, S. 1994 Coordination of membrane phospholipid synthesis with the cell cycle. *J. Biol. Chem.* **269**, 3858–3867.

Jackowski, S. 1996 Cell cycle regulation of membrane phospholipid metabolism. *J. Biol. Chem.* **271**, 20 219–20 222. (doi:10.1074/jbc.271.33.20219)

Jackowski, S. & Baburina, I. 2002 Lipid interaction with cytidylyltransferase regulates membrane synthesis. In *Biophysical chemistry membranes and proteins*. Special publication no. 283. Cambridge, UK: Royal Society of Chemistry.

Jackowski, S. & Boggs, K. 1998 Antineoplastic phospholipids inhibit phosphatidyl biosynthesis. In *Proceedings of the 7th International Congress on Phospholipids, Brussels, Belgium, September 1996*, pp. 30–44. Champaign, IL: ACOS Press.

Jamil, H., Hatch, G. M. & Vance, D. E. 1993 Evidence that binding of CTP: phosphocholine cytidylyltransferase to membranes in rat hepatocytes is modulated by the ratio of bilayer- to non-bilayer-forming lipids. *Biochem. J.* **291**, 419–427.

Jendrossek, V. & Handrick, R. 2003 Membrane targeted anticancer drugs: potent inducers of apoptosis and putative radiosensitisers. *Curr. Med. Chem. Anti-Cancer Agents* **3**, 343–353. (doi:10.2174/1568011033482341)

Jendrossek, V., Hammersen, K., Erdlenbruch, B., Kugler, W., Kruegener, R., Eibl, H. & Lakomek, M. 2002 Structure–activity relationships of alkylphosphocholine derivatives: antineoplastic action on brain tumor cell lines *in vitro*. *Cancer Chemother. Pharmacol.* **50**, 71–79. (doi:10.1007/s00280-002-0440-8)

Jendrossek, V., Mueller, I., Eibl, H. & Belka, C. 2003 Intracellular mediators of erucylphosphocholine-induced apoptosis. *Oncogene* **22**, 2621–2631. (doi:10.1038/sj.onc.1206355)

Jimenez-Lopez, J. M., Carrasco, M. P., Segovia, J. L. & Marco, C. 2002 Hexadecylphosphocholine inhibits phosphatidylcholine biosynthesis and the proliferation of HepG2 cells. *Eur. J. Biochem.* **269**, 4649–4655. (doi:10.1046/j.1432-1033.2002.03169.x)

Kara, J., Borovicka, M., Liebl, V., Smolikova, J. & Ubik, K. 1986 A novel nontoxic alkyl-phospholipid with selective antitumor activity, plasmanyl-(*N*-acyl)-ethanolamine (PNAE), isolated from degenerating chick embryonal tissues and from an anticancer biopreparation cACPL. *Neoplasma* **33**, 187–205.

Kent, C. 1997 CTP: phosphocholine cytidylyltransferase. *Biochim. Biophys. Acta* **1348**, 79–90.

Konstantinov, S. M., Eibl, H. & Berger, M. R. 1998*a* Alkylphosphocholines induce apoptosis in HL-60 and U-937 leukemic cells. *Cancer Chemother. Pharmacol.* **41**, 210–216. (doi:10.1007/s002800050730)

Konstantinov, S. M., Topashka-Ancheva, M., Benner, A. & Berger, M. R. 1998*b* Alkylphosphocholines: effects on human leukemic cell lines and normal bone marrow cells. *Int. J. Cancer* **77**, 778–786. (doi:10.1002/(SICI)1097-0215(19980831)77:5<778::AID-IJC18>3.0.CO;2-Y)

Kotting, J., Berger, M. R., Unger, C. & Eibl, H. 1992 Alkylphosphocholines: influence of structural variation on

biodistribution at antineoplastically active concentrations. *Cancer Chemother. Pharmacol.* **30**, 105–112. (doi:10.1007/BF00686401)

Kudo, I., Nojima, S., Chang, H. W., Yanoshita, R., Hayashi, H., Kondo, E., Nomura, H. & Inoue, K. 1987 Antitumor activity of synthetic alkylphospholipids with or without PAF activity. *Lipids* **22**, 862–867. (doi:10.1007/BF02535545)

Langen, P., Brachwitz, H. & Schildt, J. 1979 Inhibition of proliferation of Ehrlich ascites carcinoma cells *in vitro* and *in vivo* by halogeno analogues of long chain acyl- and alkylglycerols. *Acta Biol. Med. Ger.* **38**, 965–974.

Langen, P., Maurer, H. R., Brachwitz, H., Eckert, K., Veit, A. & Vollgraf, C. 1992 Cytostatic effects of various alkyl phospholipid analogs on different cells *in vitro*. *Anticancer Res.* **12**, 2109–2112.

Mackenzie, A. N. 1995 *The synthesis of novel phospholipids*. Southampton, UK: University of Southampton.

Marks, D. C., Belov, L., Davey, M. W., Davey, R. A. & Kidman, A. D. 1992 The MTT cell viability assay for cytotoxicity testing in multidrug-resistant human leukemic cells. *Leuk. Res.* **16**, 1165–1173. (doi:10.1016/0145-2126(92)90114-M)

Marsh, D. 2006 Elastic curvature constants of lipid monolayers and bilayers. *Chem. Phys. Lipids* **144**, 146–159. (doi:10.1016/j.chemphyslip.2006.08.004)

McGuigan, C., Wang, Y. & Riley, P. A. 1994 Synthesis and biological evaluation of phosphate triester alkyl lysophospholipids (ALPs) as novel potential anti-neoplastic agents. *Anti-Cancer Drug Des.* **9**, 539–548.

Mitchell, D. J., Tiddy, G. J. T., Waring, L., Bostock, T. & McDonald, M. P. 1983 Phase behavior of polyoxyethylene surfactants with water. Mesophase structures and partial miscibility (cloud points). *J. Chem. Soc. Faraday Trans. 1* **79**, 975–1000. (doi:10.1039/f19837900975)

Morris-Natschke, S., Surles, J. R., Daniel, L. W., Berens, M. E., Modest, E. J. & Piantadosi, C. 1986 Synthesis of sulfur analogs of alkyl lysophospholipid and neoplastic cell growth inhibitory properties. *J. Med. Chem.* **29**, 2114–2117. (doi:10.1021/jm00160a055)

Munder, P. G. 1982 Antitumor activity of alkyllysophospholipids. *Human Cancer Immunol.* **3**, 17–29.

Noseda, A., Berens, M. E., Piantadosi, C. & Modest, E. J. 1987 Neoplastic cell inhibition with new ether lipid analogs. *Lipids* **22**, 878–883. (doi:10.1007/BF02535548)

Principe, P. & Braquet, P. 1995 Advances in either phospholipids treatment of cancer. *Crit. Rev. Oncol.-Hematol.* **18**, 155–178. (doi:10.1016/1040-8428(94)00118-D)

Rakotomanga, M., Loiseau, P. M. & Saint-Pierre-Chazalet, M. 2004 Hexadecylphosphocholine interaction with lipid monolayers. *Biochim. Biophys. Acta* **1661**, 212. (doi:10.1016/j.bbamem.2004.01.010)

Schick, H. D. *et al.* 1987 Cytotoxic effects of ether lipids and derivatives in human nonneoplastic bone marrow cells and leukemic cells *in vitro*. *Lipids* **22**, 904–910. (doi:10.1007/BF02535552)

Seddon, J. M. 1990 Structure of the inverted hexagonal (HII) phase, and non-lamellar phase transitions of lipids. *Biochim. Biophys. Acta* **1031**, 1–69.

Sharma, K. S., Rodgers, C., Palepu, R. M. & Rakshit, A. K. 2003 Studies of mixed surfactant solutions of cationic dimeric (gemini) surfactant with nonionic surfactant C12E6 in aqueous medium. *J. Colloid Interface Sci.* **268**, 482–488. (doi:10.1016/j.jcis.2003.07.038)

Sohal, P. S. & Cornell, R. B. 1990 Sphingosine inhibits the activity of rat liver CTP: phosphocholine cytidylyltransferase. *J. Biol. Chem.* **265**, 11 746–11 750.

Takeoka, S., Sou, K., Boettcher, C., Fuhrhop, J.-H. & Tsuchida, E. 1998 Physical properties and packing states of molecular assemblies of synthetic glycolipids in aqueous dispersions. *J. Chem. Soc. Faraday Trans.* **94**, 2151–2158. (doi:10.1039/a800289d)

Tarnowski, G. S., Mountain, I. M., Stock, C. C., Munder, P. G., Weltzien, H. U. & Westphal, O. 1978 Effect of lysolecithin and analogs on mouse ascites tumors. *Cancer Res.* **38**, 339–344.

Torrecillas, A., Aroca-Aguilar, J. D., Aranda, F. J., Gajate, C., Mollinedo, F., Corbalan-Garcia, S., de Godos, A. & Gomez-Fernandez, J. C. 2006 Effects of the anti-neoplastic agent ET-18-OCH3 and some analogs on the biophysical properties of model membranes. *Int. J. Pharm.* **318**, 28–40. (doi:10.1016/j.ijpharm.2006.03.008)

Unger, C., Fleer, E. A., Kotting, J., Neumuller, W. & Eibl, H. 1992 Antitumoral activity of alkylphosphocholines and analogues in human leukemia cell lines. *Prog. Exp. Tumor Res.* **34**, 25–32.

Vogler, W. R., Olson, A. C., Hajdu, J., Shoji, M., Raynor, R. & Kuo, J. F. 1993 Structure–function relationships of alkyl-lysophospholipid analogs in selective antitumor activity. *Lipids* **28**, 511–516. (doi:10.1007/BF02536082)

Vogler, W. R., Shoji, M., Hayzer, D. J., Xie, Y. P. & Renshaw, M. 1996 The effect of edelfosine on CTP: cholinephosphate cytidylyltransferase activity in leukemic cell lines. *Leuk. Res.* **20**, 947–951. (doi:10.1016/S0145-2126(96)00070-7)

Wan, W. J. 1997 *Novel ether lipids as antineoplastic agents*. Southampton, UK: University of Southampton.

Weber, N. & Benning, H. 1988 Metabolism of ether glycolipids with potentially antineoplastic activity by Ehrlich ascites tumor cells. *Biochim. Biophys. Acta* **959**, 91–94.

Wieder, T., Orfanos, C. E. & Geilen, C. C. 1998 Induction of ceramide-mediated apoptosis by the anticancer phospholipid analog, hexadecylphosphocholine. *J. Biol. Chem.* **273**, 11 025–11 031. (doi:10.1074/jbc.273.18.11025)

Wieder, T., Reutter, W., Orfanos, C. E. & Geilen, C. C. 1999 Mechanisms of action of phospholipid analogs as anticancer compounds. *Prog. Lipid Res.* **38**, 249–259. (doi:10.1016/S0163-7827(99)00004-1)

Zeisig, R., Jungmann, S., Arndt, D., Schuett, A. & Nissen, E. 1993 Antineoplastic activity *in vitro* of free and liposomal alkylphosphocholines. *Anti-Cancer Drugs* **4**, 57–64.