Growth arrest vs direct cytotoxicity and the importance of molecular structure for the in vitro anti-tumour activity of ether lipids

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Summary A panel of 25 different lipid agents was evaluated for in vitro activity against HT29 human colon carcinoma and HL60 promyelocytic leukaemia cells by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The structure–activity relationships seen with this series, including those for four sets of positional or stereoisomers, indicate that specific receptor proteins are unlikely as targets for anti-tumour lipid (ATL) action. Additional data confirm the lack of involvement of the platelet-activating factor receptor in particular and suggest that metabolic stability is a most important determinant of ATL activity. More detailed studies, with 1-O-octadecyl-2-O-methyl-β-d-glucopyranosyl-β-d-glucopyranosyl-sn-glycerol (ET18-OCH3) and (Z)-2-(Hydroxytetradecyl)-2-deoxyoctadecyl-3-methylbutanoyl-phosphocholine-(N,N,N-trimethylammonium) hydroxide (SRI 62-834), suggest three different modes of activity, depending on drug concentration and exposure time. Low doses of up to 5 μM in standard serum-containing medium cause population growth arrest after prolonged exposure. Growth arrest was associated with a leaky G2/M block as determined by flow cytometry. These effects are reversible. Intermediate concentrations (5–40 μM) were cytotoxic, causing a net reduction in cell numbers after 2–3 days. At even higher concentrations, all lipids caused rapid, direct membrane lysis. When the clonogenic assay was used to assess the effects of ATLs, most agents reduced colony formation at concentrations above 5 μM. However, some compounds proved stimulatory at nanomolar concentrations, suggesting that they might possess mitogenic properties. These results, particularly those concerning the concentration and time dependence, may be relevant to current clinical trials with ether lipids.

Keywords: alkyl lyso phosphatidyl; ether lipid; ET18-OCH3; SRI 62-834; hexadecylphosphocholine; cancer cells

Synthetic anti-tumour lipids (ATLs), including the novel alkylphosphocholine derivatives, have emerged as effective agents in model systems and are currently undergoing clinical trials (Berdel et al., 1980; Berdel, 1991; Houlihan et al., 1995). Current preclinical and clinical experience with these agents has recently been comprehensively reviewed (Lohmeyer and Bittman, 1994). They represent a new class of structurally distinct non-DNAinteractive anti-tumour agents whose main site of action appears to be at the plasma membrane (Berdel and Munder, 1987; Diomedee et al., 1990; Grunicke, 1991). Combination chemotherapy studies have indicated that the mechanisms of action of ATLs, DNAinteractive agents and radiation are independent (Andreessen et al., 1982; Nosea et al., 1989c; Hofmann et al., 1989; Neumann et al., 1991). In addition to their direct effects on tumour cells, some ATLs also activate the host immune system (Talmadge et al., 1987; Hilgard et al., 1991; Pignol et al., 1992; Houlihan et al., 1995).

The encouraging results obtained in various model systems have highlighted the therapeutic potential of ATLs. Several compounds, including ET18-OCH3, SRI 62-834, the thioether lipid BM 41.440 and hexadecylphosphocholine (HePC) (see Table I), are scheduled for, or currently undergoing, phase I/II clinical evaluation (Lohmeyer and Bittman, 1994). Considerable success has already been achieved with bone marrow purging (Berdel, 1991; Vogler, 1994). Topical application of HePC in breast cancer has also produced encouraging results (Dummer et al., 1992; Unger et al., 1992). Some responses were seen following systemic administration, and studies are ongoing, but the overall results so far obtained by oral and intravenous dosage have been disappointing (Berdel, 1990; Lohmeyer and Bittman, 1994).

Despite extensive laboratory and clinical studies, the major molecular mechanism of action of ATLs remains unclear (see Berdel, 1991; Lohmeyer and Bittman, 1994; Houlihan et al., 1995). Several plasma membrane proteins have been suggested as targets for ATLs, including the Na+/K+ ATPase membrane pump, Ca2+ channels, protein kinase C (PKC), phospholipase C (PLC) and PI-3-kinase (Shoji et al., 1988; Berdel, 1991; Überall et al., 1991; Powis et al., 1992; Berggren et al., 1993). ATLs also interfere with cellular phospholipid metabolism (Modolell et al., 1979; Berkovic et al., 1992). In addition, they alter membrane fluidity, effect cell shape changes and permeabilise cells by membrane pore formation (Nosea et al., 1989a; Dive et al., 1991). However, direct permeabilisation and lysis may not be the key cytotoxic lesion at lower, pharmacological doses of ATLs (Lohmeyer and Workman, 1992). Sensitivity to ATLs has been correlated with the rate of endocytotic activity, plasma membrane cholesterol content and endogenous alkyl lysosphospholipid concentration (Modolell et al., 1979; Mangold and Weber, 1987; Bazil and Duster, 1990; Diomedee et al., 1991, 1992). ATLs also interfere with the production of infectious HIV-1 virus particles in vitro and inhibit the fusion of intracytoplasmic vesicles with the plasma membrane (Meyer et al., 1991; Kucera et al., 1990, 1993). However, a clear concept of the molecular target of ATL activity has yet to emerge from these interesting, but diverse findings. Indeed, it is possible that there is no single mode of action, but a series of critical events which can act in concert to inhibit tumour cell growth.

We have investigated structure–activity relationships for the in vitro activity of 25 different phospholipids related to platelet-activating factor (PAF). Proliferation assays with differing end points were performed to distinguish between the cytotoxic and/or cytostatic effects of ATLs. Cells were studied using population growth curves, MTT dye reduction and clonogenic assays. Flow cytometric cell cycle analysis provided a further insight into the cytostatic and cytotoxic mechanisms of action of ATLs. We also investigated the dose–response relationships for direct plasma membrane damage using a large selection of agents. Moreover, the potential interaction of ATLs with specific cellular receptors.

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was examined. Experiments were performed on two ATL-sensitive cell lines: HT29 human colon carcinoma and HL60 human promyelocytic leukaemia. Selected agents were also evaluated against the EMT6/VJ mouse mammary tumour.

Materials and methods

Cells

Human promyelocytic HL60 leukaemia cells were cultured in antibiotic-free RPMI-1640 (Gibco Biocult, Paisley, UK) containing 10% fetal calf serum (FCS) (Seralab, Crawley Down, UK) and 1 mM glutamine. HT29 human colon carcinoma cells and EMT6 mouse mammary carcinoma cells were grown in Eagle's MEM (Gibco) with 10% FCS (Seralab), glutamine and antibiotics (penicillin and streptomycin) at 100 IU ml⁻¹ and 100 μg ml⁻¹ respectively. Cells were mycoplasma free and maintained at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide.

Lipid agents and other reagents

The compounds used are illustrated in Table I. ET18-OCH₃, HePC and 3-[4-(chlorophenyl)-9-methyl-6H-thienol[3,2-j][1,2,4]triazolo-[4,3-a][1,4]-diazepin-2-yl]-4-(morpholino)-1-propanone (WEB 2086) were kind gifts from Professor Wolfgang E Berdel (Universitätsklinikum Steglitz, Berlin, Germany). Dr Peter Hilgard (Asta Pharma, Bielefeld, Germany) and Drs Hubert Heuer and Karl-Heinz Weber (Boehringer Ingelheim, Ingelheim am Rhein, Germany) respectively. Dr Bill Houlihan (Sandoz Research Institute, East Hanover, NJ, USA) kindly provided us with SRI 62-537, its pure isomers (SDZ 266-336 and 266-337) and five other analogues (SAH 63-537, 62-817, 62-990, 63-871 and 63-875). PAF₁₆, lyso-PAF, arachidonoyl-PAF, methylcarbamyl-PAF and ET18-OCH₃, were purchased from Peninsula Laboratories (St Helens, Merseyside, UK), while phosphatidic acids and the PAF₁₆ stereoisomers were obtained from Sigma (Poole, UK). The positional isomers BN 52205, BN 52207 and BN 52211 were synthesised by Drs C Broquet and B Vandamme (Institut Henri Beaufour, Les Ulis, France) and kindly made available to us by Dr H Hendriks (EORTC New Drugs Development Office, Amsterdam, The Netherlands). Agents were dissolved in 95% ethanol (phosphatidic acids and arachidonoyl-PAF) or Dulbecco's phosphate buffered saline (PBS) (all others) and stored in glass vials at −20°C in the dark.

MTT was purchased from Sigma. ³²Cr-labelled sodium chromate was obtained from Amersham (Aylesbury, UK) at 10−35 mCi ml⁻¹.

Cytotoxic potency

The antiproliferative potency of ATLs and related lipid agents was assessed using the MTT dye reduction assay, as described previously (Lohmeyer and Workman, 1992).

Membrane damage

The ³⁵Cr radionuclide release assay was performed as described previously (Lohmeyer and Workman, 1993). For the trypan blue dye exclusion assay, HT29 cells were seeded into flat-bottomed 24-well plates and allowed to attach overnight. For analysis, the medium was aspirated and replaced with 200 μl of a 9:1 mixture of medium and trypan blue (2.5 mg ml⁻¹ in PBS). An area with about 250 cells in the field of view was photographed before, and at regular intervals after, addition of the test lipids.

Clonogenic assay

Cells were seeded into 25 cm² flasks and incubated for 2 days. Lipid agents were added on day 3 and after 24 h exposure cells were trypsinised, diluted and seeded into 60 mm tissue culture plates (Nunclon). After 10 days of incubation, the plates were washed twice with PBS, fixed in 100% methanol for 10 min, dried and stained with 0.1% crystal violet. Colonies were counted using a model 980 Artel Colony Counter. Results from four replicate plates were expressed both as a percentage of vehicle control and as the relative plating efficiency. The former expression is more usual, but fails to account for variations in plating efficiency between experiments, which will distort the resulting percentages. The latter was calculated by the formula 100 × (EXP − CTRL)/1000, where EXP is the colony number on treated plates, CTRL is the colony number on control plates and 1000 represents the number of cells plated per dish.

Population growth curve analysis

HT29 or HL60 cells were seeded into 24-well plates (Nunclon). HT29 cells were left to attach to the plastic for 4 h before addition of the test compounds. Two wells were trypsinised and counted for each time point and concentration. In reversibility experiments, drug-containing medium was replaced by drug-free medium at the indicated time. HL60 cells were treated similarly, but trypsinisation was not required and medium was replaced by centrifugation and resuspension.

Table I Structures of lipid agents

| Compound | 1°-substitution | 2°-substitution | 3°-substitution | Stereochemistry |
|----------|----------------|----------------|----------------|----------------|
| PAF₁₆    | OC₃H₇          | OCOCH₃         | O-PC           | R and S        |
| PAF₁₆    | OC₃H₇          | OCOCH₃         | O-PC           | R              |
| Lyso-PAF₁₆| OC₃H₇          | OH             | O-PC           | R              |
| Lyso-PAF₁₆| OC₃H₇          | OH             | O-PC           | R              |
| Arachidonoyl-PAF | OC₃H₇ | OOC,₃H₇ | O-PC           | R              |
| Methylcarbamyl-PAF | OC₃H₇ | OCONHCH₃ | O-PC           | R              |
| ET18-OCH₃ | OC₃H₇          | OCH₃           | O-PC           | R              |
| HePC     | CH₃            | H              | O-PC           | NA             |
| BN 52205 | OCH₃           | NCH₃CH₃H₇     | O-PC           | rac            |
| BN 52207 | NCH₃CH₃H₇     | O-PC           | CH₃            | rac            |
| BN 52211 | NCH₃CH₃H₇     | OCH₃           | O-PC           | rac            |
| Dilauroyl-PA | OOC,₃H₇ | OOC,₃H₇ | OPO₂- OH       | R              |
| Dimyristoyl-PA | OOC,₃H₇ | OOC,₃H₇ | OPO₂- OH       | R              |
| Dipalmitoyl-PA | OOC,₃H₇ | OOC,₃H₇ | OPO₂- OH       | R              |
| Lyso-PA   | OCO-alkyl      | OH             | R              | SAH 62-537     |
| Lyso-PA   | OCO-alkyl      | OH             | R              | SAH 62-817     |

The majority of compounds investigated in this study are based on a glycerol backbone with different substitutions at 1°, 2° and 3° carbons. The six structures differing significantly from this basic scheme are illustrated individually. Note that the R and S stereoisomers of SRI 62-834 are denoted SDZ 266-336 and SDZ 266-337 respectively. SRI 62-834 is present as the racemate. PC, phosphocholine; PA, phosphatidic acid; rac, racemic; NA, not applicable.
Cell cycle studies

Flow cytometry was used to investigate the effect of ATLs on the cell cycle of logarithmically growing cells. Cells were grown and exposed to ATLs as described for the clonogenic assay. Cells were trypsinised (where necessary), resuspended in 150 μl of PBS and fixed by the dropwise addition of 600 μl of ice-cold 70% ethanol with vortexing. For analysis, cells were washed twice with PBS, exposed to 0.1 mg ml⁻¹ RNase A (Sigma) for 15 min at 37°C and then diluted to approximately 10⁶ cells ml⁻¹. Propidium iodide (1 μg ml⁻¹) was added before analysis on a Beckton Dickinson FacStar. Triplicate samples of 10,000 events each were analysed using the flow cytometer software.

Results

Structure–cytotoxicity relationships by MTT assay

Figure 1 shows typical cytotoxicity data in HT29 human colon carcinoma cells, using the MTT assay. Note that there is only a comparatively narrow range over which these agents develop their full effect. Average IC₅₀ values for the complete panel of agents, tested against HT29 and HL60 cells, are given in Table II. Despite differences in cell type (carcinoma vs leukaemia) and growth characteristics (adherent monolayer vs detached single-cell suspension), both cell lines were generally equally sensitive to any given agent. Selected compounds were also evaluated in EMT6 mouse mammary tumour cells. The IC₅₀ values for ET18-OCH₃ and SRI 62-834 in EMT6 cells were 95 ± 10 and 100 ± 20 μM respectively. PAF₁₆ and lyso-PAF₁₆ failed to reach the IC₅₀ at concentrations up to 150 μM. Thus EMT6 cells were at least 3-fold more resistant to PAF and lyso-PAF and about 30-fold more sensitive to the ATLs.

Table I illustrates the chemical structures for all lipid agents tested. Comparison of individual drug potency against HT29 cells revealed that ATLs, as exemplified by ET18-OCH₃ and SRI 62-834, all had IC₅₀ values around 2–5 μM. In contrast, the naturally occurring parent compounds of the PAF and lyso-PAF family were much less potent with IC₅₀ values in the 40–50 μM range. Interestingly, the synthetic S isomer of PAF₁₆ was as potent as some of the ATLs. SAH 63-817 was the only compound which proved more toxic in one particular cell line (HT60) than the other (HT29). Intermediate potencies were seen with SAH 62-537 and the

Table II Cytotoxicity of lipid agents against HT29 and HL60 cells in the presence and absence of WEB 2086

|         | HT29 | HL60 | HT29 + 49 μM WEB 2086 | HL60 + 49 μM WEB 2086 |
|---------|------|------|----------------------|----------------------|
| PAF₁₆   | 45.6 ± 2.4 | 43.8 ± 8.5 | 43.2 ± 4.8           | 50.3 ± 4.8           |
| PAF₁₆ (R)| 53.6 ± 1.9 | ND      | 57.5 ± 4.5           | ND                   |
| PAF₁₆ (S)| 8.5/4.0 | ND      | ND                   | ND                   |
| Lyso-PAF₁₆| 41.4 ± 1.7 | 40.0 ± 4.0 | 45.5 ± 2.2           | 34.6 ± 8.2           |
| Lyso-PAF₁₈| 54.8 ± 3.5 | ND      | 56.6 ± 6.9           | ND                   |
| Arachidonoyl-PAF| 44.3 ± 4.3 | ND      | 39.2 ± 5.7           | ND                   |
| HePC    | 15.5 ± 1.4 | 18.1 ± 1.9 | 17.7 ± 0.3           | 16.4 ± 3.4           |
| Methylcarbamyl-PAF| 3.0 ± 0.2 | 3.3 ± 0.1 | 3.2 ± 0.2            | 2.8 ± 0.5            |
| ET18-OCH₃| 2.5 ± 0.3 | 2.6 ± 0.1 | 2.5 ± 0.3            | 2.2 ± 0.3            |
| ET16-OCH₃| 3.6 ± 1.1 | ND       | 4.1 ± 1.2            | ND                   |
| SRI 62-834| 3.3 ± 0.4 | 2.7 ± 0.1 | 3.1 ± 0.2            | 2.5 ± 0.6            |
| SDZ 266-336 (S)| 2.5 ± 0.2 | 3.0 ± 0.3 | 3.0 ± 0.4            | ND                   |
| SDZ 266-337 (R)| 2.1 ± 0.2 | 2.6 ± 0.2 | 2.6 ± 0.4            | ND                   |
| SAH 62-817| 35.2 ± 4.9 | 21.3 ± 2.9 | 43.3 ± 4.8           | ND                   |
| SAH 62-537| 7.5 ± 1.1 | 9.1 ± 1.1 | 7.5 ± 0.4            | ND                   |
| SAH 62-990| 3.1 ± 0.3 | 4.9 ± 0.9 | 4.0 ± 0.2            | ND                   |
| SAH 63-871| 2.2 ± 0.3 | 2.2 ± 0.2 | 2.7 ± 0.5            | ND                   |
| SAH 63-875| 2.8 ± 0.5 | 2.9/4.2  | 3.6 ± 0.9            | ND                   |
| BN 52205| 2.1 ± 0.2 | 3.0 ± 0.2 | ND                   | ND                   |
| BN 52207| 1.4 ± 0.3 | 2.2 ± 0.1 | ND                   | ND                   |
| BN 52211| 2.3 ± 0.6 | 3.7 ± 0.3 | ND                   | ND                   |
| Dimyristoyl-PA| > 500 | ND     | ND                   | ND                   |
| Dipalmitoyl-PA| > 500 | ND     | ND                   | ND                   |
| Dilauroyl-PA| > 200 | ND     | ND                   | ND                   |
| Lyso-PA| > 100   | ND     | > 100                | ND                   |

IC₅₀ values (μM) are the mean ± standard error of three or more experiments. Where only two experiments were performed, both repeats are given. Suffixes R and S denote isomerically different compounds. ND, not determined; PA, phosphatidic acid.
alkylphosphocholine HePC. The four different phosphatidic acids all proved comparatively inactive with IC_{50} values in excess of 100 μM.

Also evident from Table II is that none of the complementary ATL stereoisomers (i.e. SAH 63-871 and SAH 63-875 or SDZ 266-336 and SDZ 266-337) showed any marked differences in cytotoxic potency. Differences between the positional isomers (i.e. BN 52205, BN 52207 and BN 52211) were also not statistically significant (t-test). Thus molecular stereochemistry clearly does not correlate with anti-tumour activity.

Compounds with differing alkyl chain length (i.e. C_{16} vs C_{18}) were available for PAF, lyso-PAF and ET18-OCH₃. The C_{18} compounds, particularly of PAF and lyso-PAF, were more cytotoxic than their C_{16} counterparts (see Table II). However, the differential was not statistically significant for the more potent anti-tumour agents ET18-OCH₃ and ET16-OCH₃.

Lack of toxicity modulation by WEB 2086

The PAF receptor antagonist WEB 2086 was non-toxic at concentrations exceeding 250 μM. At 49 μM, WEB 2086 had no effect on the cytotoxicities of any of the lipid agents tested in HT29 and HL60 cells (see Table II). These results argue against the involvement of a WEB-sensitive PAF receptor in the cytotoxicity of PAF or its ATL analogues.

Population growth curve analysis

Figure 2 illustrates the effect of continuous SRI 62-834 exposure on HL60 cells. Cytostasis occurs at 3–5 μM and population growth arrest persists for ≥7 days. Similar results were seen in HT29 cells, in agreement with their coordinate MTT data. Again, the dose–response curve is extremely steep for both cell lines with population growth hardly affected at 1 μM, but with almost complete stasis occurring at 2–3 μM.

Figure 3 summarises the comparative effects of ET18-OCH₃, SRI 62-834, HePC and PAFₖ₆ on HL60 cell numbers after 7 days of continuous exposure. Both ET18-OCH₃ and SRI 62-834 are equally active with IC_{50} values between 1 and

2 μM. HePC was less active with an IC_{50} of 10–15 μM. PAFₖ₆ was least potent, effecting half-maximal growth arrest at 40–50 μM. These IC_{50} values agree well with the MTT data (Table II). Complete population growth arrest, i.e. no increase in cell number over the original seeding density, was achieved using ET18-OCH₃, SRI 62-834, HePC and PAFₖ₆ at 3.9, 4.5, 31.6 and ≈125 μM (extrapolated) respectively. Note that these concentrations (IC_{100} values) are only around 2-fold higher than those causing half-maximal growth arrest (Figure 3).

Interestingly, HL60 promyelocytes did not differentiate in response to treatment with ATLs at these doses. Cellular differentiation was not evident under the conditions used, as judged by morphology, adhesion and flow cytometric analysis of cell shape and granularity.

We have previously shown that serum concentration affects the lytic potency of ET18-OCH₃ and SRI 62-834 (Lohmeyer and Workman, 1993). Here we show that serum concentration also affects the non-lytic antiproliferative activity of ATLs. Reducing serum concentrations from 10% to 5.5% and 1% results in markedly slower growth of untreated HT29 cells. Control cell numbers after 7 days were reduced to 55.0% and 9.5% respectively. After continuous exposure to various ATLs, a steep antiproliferative dose–response relationship was evident at all serum levels. The IC_{50} values in 10%, 5.5% and 1% FCS were 1.8, 0.8 and 0.3 μM respectively, confirming the potentiating effects of serum levels on the non-lytic antiproliferative activity of ATLs.

Cell cycle effects of ether lipids

Population growth curves (e.g. Figure 2) show that cell numbers increase at almost the normal rate for the first 24 h after addition ≤5 μM ATL. Only after that period does a significant slowing of cell proliferation (or an increase in cell death) become apparent. This suggests that the majority of cells are able to proceed through one cell division – or complete the one they are currently in – before arresting. At ≥10 μM ATL, however, an immediate reduction in cell growth is apparent after 24 h (Figure 2).

Figure 4 shows DNA histograms of HT29 cells treated for 28 h with 2 and 5 μM ET18-OCH₃. Progressive reduction of

Figure 2: Representative population growth curve for HL60 cells exposed to SRI 62-834. Similar results were obtained with other ATLs in HL60 and HT29 cells. SRI 62-834 was added when indicated (arrow) and cell numbers from two replicate wells, counted in triplicate, were averaged for each data point. Error bars have been omitted for clarity. The data illustrated are representative of three independent experiments. ■, control; ●, 1 μM SRI 62-834; ▲, 2 μM SRI 62-834; ○, 3 μM SRI 62-834; ◆, 5 μM SRI 62-834; ○, 10 μM SRI 62-834.

Figure 3: Comparison of the population growth retarding effects of ET18-OCH₃ (■), SRI 62-834 (▲), HePC (○) and PAFₖ₆ (●) in HL60 cells. HL60 cell numbers after 7 days of continuous exposure (see Materials and methods) are expressed as multiples of the initial cell number seeded (10⁴ cells per well). Concentrations resulted in half-maximal growth arrest (IC₅₀) and no net growth above the initial seeding cell number after 7 days are indicated by dotted lines. Similar results were obtained with HT29 cells. Cell numbers from two replicate wells, counted in triplicate, were averaged for each data point. Error bars have been omitted for clarity. The data illustrated are representative of at least two independent experiments.
cells in G₀ and G₁ phases occurs with a concomitant increase of cells arrested in the G₂ or M-phase of the cell cycle (Figure 4a–c). This shows that cells in the G₀ or M-phase were unable to proceed through mitosis to the next G₁ phase, accumulating instead in the G₂/M compartment. The proportion of cells in S-phase was comparatively unchanged by ET18-OCH₃ treatment. A large amount of DNA-containing debris was also seen routinely at 5 μM doses (Figure 4c). The amount of fluorescent debris increased from just over 2% in control cells to almost 35% of all 'events' collected at 5 μM ET18-OCH₃ (Figure 4c). Thus, ATLs at ≥ 5 μM can induce significant cellular fragmentation within 28 h. Interestingly, the debris was non-random in size and scatter characteristics (debris 'peaks' in Figure 4c). This may suggest a controlled process of cell destruction.

Incubating cells for up to 46 h did not markedly effect further shifts in the cell cycle distribution at any given dose of ATL. The major difference observed was a significant increase in the amount of cell debris at ≥ 5 μM ATL. Here, up to 48.6% of 'events' constituted DNA-containing debris and cell fragments. At 2 μM ATL, the amount of debris collected was low and similar to that after 28 h. Incubating HT29 cells for 6 h with up to 5 μM ET18-OCH₃ did not change normal cell cycle distributions or induce debris formation (not shown). Very similar cell cycle changes were also evident in ATL-treated HL60 cells (Figure 5).

**Reversibility of ATL-induced growth arrest**

The finding that ATLs can induce growth arrest and cellular fragmentation within 28 h led us to investigate the reversibility of ATL-induced damage. After 24 h exposure, HT29 and HL60 cells were hardly affected by ≤ 3 μM ET18-OCH₃ and regrew as soon as the agent was removed. At 5 μM, the population took longer to recover its normal growth rate, while recovery from 10 μM took between 70 and 90 h. The effects of 48 h exposure are illustrated in Figure 6. HL60 cells treated with 2 μM ET18-OCH₃ were able to recover their full growth potential within 24 h – as fast as their 24 h counterparts. However, 3 μM ATL required a recovery time of about 30 h. Between 70 and 90 h were required for 5 μM ET18-OCH₃. Progressive population reduction, indicating large-scale cell death, was evident at 5–10 μM and corroborates our earlier flow cytometry results. Thus reversibility was seen, but recovery time was related to both drug concentration and duration of exposure. Similar data were obtained in HT29 cells (not shown).

As mentioned previously, we did not observe any differentiation of HL60 cells as a result of ATL treatment at these moderate doses. Therefore, we are confident that the
observed 'regrowth' did not result from the proliferation of a small non-differentiated population.

**Effects on clonal growth**

Figure 7 shows typical effects of ET18-OCH₃, lyso-PAF and methylcarbamyl-PAF (MCP) on HT29 cell cloning. Lyso-PAF markedly increased relative plating efficiency by up to 21% (2.3-fold control) and over a wide range of concentrations from 5 nM up to 5 μM (Figure 7). ET18-OCH₃ also gave significant stimulation (up to 2.5-fold control) of clonal growth, while MCP and SRI 62-834 (not shown) gave only comparatively moderate stimulation (1.2 to 1.5-fold control values) at ∼0.1 μM (Figure 7). Neither racemic PAF nor its R or S isomers affected clonal growth up to 5 μM (not shown).

At high lipid concentrations (generally ≥2 μM) a dose-dependent reduction in colony counts was noted. Colony size did not appear to be affected as a result of lipid exposure.

**Membrane-damaging effects**

Exposing HT29 cells to 23 μM (≈10 × MTt IC₅₀) ET18-OCH₃ or SRI 62-834 for up to 60 min had no effect on trypan blue exclusion. In 10% FCS, >98% of cells remained 'viable'. PAF₁₈ and lyso-PAF₁₈ had no immediately lytic effect in HT29 cells up to their MTt IC₅₀ dose. After 60 min at 48 μM, only 5.4% of cells scored trypan blue positive, had lysed or become detached. At 91 μM, however, 50.2% of cells had lost membrane integrity within 7 min, increasing to 72.5% after 60 min. Higher concentrations caused immediate lysis of ≥96% of cells within the first 7 min.

Direct membrane damage was further quantified by ⁵¹Cr release. The R₅₀ values for HL60 and HT29 cell lines are given in Table III. These data confirm that ATLs are not membrane lytic on short-term exposure to concentrations which cause growth inhibition with longer exposures. Moreover, the membrane-damaging potencies of the ATLs were similar to PAF or lyso-PAF. In HT29, the last two compounds both feature R₅₀ values <110 μM, while R₅₀ values for most ATLs are between 139 and 145 μM. Only HePC is significantly less lytic. In HL60 cells the R₅₀ values for PAF and lyso-PAF, as well as for ET18-OCH₃ and SRI 62-834, were between 105 and 120 μM. MCP and HePC retained the same degree of potency seen in HT29 cells.

Note that the trypsinisation process necessary with HT29 cells had no detrimental effect on the membrane integrity or the amount of spontaneous ⁵¹Cr release when applied to HL60 cells (not shown).

The PAF receptor antagonist WEB 2086 caused no increased ⁵¹Cr release at concentrations up to 200 μM and also failed to exert a clear modulating effect on the membrane-permeabilising potencies of ATLs (Table III). This shows that the PAF receptor is not involved in the mechanism of action leading to cell lysis.

**Discussion**

One of the main questions addressed in this study concerns the importance of molecular structure for ATL anti-tumour activity. The great majority of previous structure-activity studies have examined racemic compounds only, ignoring the potential importance of stereochemistry for activity (Berdel et al., 1987; Fromm et al., 1987; Herrmann and Neumann, 1987). Here, we present data for three sets of matched stereo-isomers, chosen to reflect different groups of compounds (PAF, the anti-tumour lipid SRI 62-834 and novel compounds SAH 63-871 and 63-875). Further, we examine the importance of molecular configuration using a set of three positional isomers. This work is also the first to systematically separate and recognise three different antiproliferative activities of ATLs, using a combination of cytotoxicity, proliferation and flow cytometric assays. Another novel aspect of this work is our finding that some ATLs can promote mitogenesis at nanomolar doses. Extending previous studies by ourselves and others, we investigate the serum dependence of the non-lytic anti-tumour activity of ATLs and the importance of interactions with cellular PAF receptors for cytotoxicity and cell lysis (Andreasen et al., 1982; Fleer et al., 1992; Lohmeyer and Workman, 1992).

The extensive structure-activity data reported here show that the human HT29 colon carcinoma and the HL60 pro-myelocytic leukaemia cell line were equally sensitive to any given lipid in our panel. This similarity was seen despite the different biological origins (colon epithelial vs haematopoietic) and modes of growth (attached monolayer vs single-cell suspension). The murine EMT6 mammary carcinoma cell line proved much more resistant to both ATLs and PAF analogues than the human lines. With ATLs, the difference was about 30-fold. With PAF and lyso-PAF, little or no cytostatic effect was seen in EMT6 up to 150 μM – a concentration 3-fold higher than the IC₅₀ in HT29 and HL60 cells. Clearly, EMT6 cells either metabolise and detoxify ATLs more rapidly or lack the specific target(s) responsible for potency in the human lines. Decreased endocytotic activity and/or increased cholesterol levels have also been advanced as a determinant of ATL resistance (Bazill and Dexter, 1990; Diomede et al., 1991; reviewed in Workman, 1991). The effect is unlikely to involve species differences, since other murine cell lines such as WEHI-3B myelomonocytic leu-
kaemia and Meth A fibrosarcoma cells are sensitive to ATLs (Houlihan et al., 1987; Bazill and Dexter, 1990).

To date, three basic key molecular features are recognised for ATL activity: (1) an ether or thioether linked alkyl moiety at the sn-1 position of the glycerol backbone; (2) a small substituent at the sn-2 position; and (3) a phosphocholine head group at the position of sn-3-glycerol (Fleer et al., 1990; Fleer and Vecht, 1990; Kasukabe et al., 1993a). However, these apparent ‘rules’ are being rapidly eroded as medicinal chemists prepare ever more exotic lipid compounds which nevertheless retain anti-tumour activity (Ishaq et al., 1989; Kasukabe et al., 1990; Marasco et al., 1990; Houlihan et al., 1995).

The most important conclusion from our structure–activity data is that molecular conformation at the sn-2 position of the glycerol backbone is not an important determinant of activity. Both isomers SRI 62-834 (i.e. SDZ 226-336 and SDZ 226-337), and SAH 63-871 and SAH 63-875 were equally cytotoxic to spite of their very different conformations. Similarly, the three positional isomers (BN 52205, 52207 and 52211) were found to possess very similar IC₅₀ values. The large panel of agents tested also shows that activity is not related to any particular structural feature at the sn-2 position. Many very different substituents of greatly varying size proved equally active. Thus, a particular biophysical property of these lipids most likely holds the key to their activity.

Interestingly, the S and R isomers of PAF₄ do show a significant difference in cytotoxic potency. The ‘synthetic’ S-PAF was as potent as some of the ATLs, but features the same functional groups as the less toxic R-PAF, just in a different conformation. So why do R-PAF and its natural analogues not possess potent activity, when molecular structure was so clearly not important for activity of the other isomer pairs? The most likely explanation is the rapid metabolism of R-PAF, which may be a general property of nature of ATLs. PAF, lyso-PAF and arachidonoyl-PAF are rapidly metabolised by cellular acetylhydrolases (Shen et al., 1987; Nakagawa and Waku, 1989). Although metabolised to some extent (Bishop et al., 1992; Fleer et al., 1992), synthetic lipids such as MCP, ET18-OCH₃ and HePC are known to be metabolically stable compounds with long half-lives (Arnold et al., 1978; Hoffman et al., 1986; Breiser et al., 1987). At the present time, we believe the most parsimonious hypothesis that these agents are more potent than natural lipids because of their greater metabolic stability.

The precise molecular mode of action of ATLs remains to be elucidated. The potential involvement of a PAF receptor (Honda et al., 1991) was suggested by the close structural similarity of some ATLs to PAF. ET18-OCH₃ and MCP in particular are known to antagonise PAF binding, MCP exhibiting a potent agonist activity (Shen et al., 1987). The triazolodiazepine derivative WEB 2086 potently antagonises the various known PAF effects in vitro and in vivo (Casals-Senzel et al., 1987) and has been described as the ‘reference PAF antagonist’ (Page and Abbott, 1989). Testing a larger panel of agents, we have confirmed our earlier finding (Lohmeyer and Workman, 1992) that WEB 2086 does not affect the cytotoxicity of ATLs or related agents. This conclusion is borne out by other studies showing that the presence of PAF-specific binding sites on neoplastic cells also failed to correlate with their sensitivity to the PAF antagonists (Danhauser-Riedl et al., 1991). Overall, it seems clear that a WEB-sensitive PAF receptor is not involved in the antiproliferative mechanism of ATLs.

Whether ATLs interact in a highly specific way with other cellular proteins is still uncertain (Munder and Westphal, 1990; Berdel, 1991), but this hypothesis becomes increasingly tenuous in the light of our structure–activity data. Given the great structural diversity among active ATLs, it appears unlikely that all of those compounds should be equally active against one or more particular target proteins. Moreover, one might question the significance of various reports of ‘specific’ interactions of ATLs with a plethora of important cellular proteins such as PKC, phospholipases C and A₂, PI-3-kinase, the Na⁺/K⁺-ATPase, Ca²⁺ channels and the epidermal growth receptor (Oishi et al., 1988; Kosano and Takatani, 1989; Powis et al., 1992; Berggren et al., 1993).

All of the proteins known to be affected by ATL exposure are membrane bound or at least membrane associated. Many membrane enzymes and receptors are exquisitely sensitive to their surrounding lipid microenvironment (Epand et al., 1991) and some, e.g. PKC, PLC, phospholipase A₂ (PLA₂) and PI-3-kinase, require lipid co-factors and/or substrates for optimal activity. All of these proteins are thus potentially susceptible to modulation of their boundary lipids and the general phospholipid environment. Postulating such a comparatively indirect interference as a potential mechanism of action accommodates the multitude of membrane proteins affected and may account for the effectiveness of structurally diverse ATLs. However, other more direct mechanisms of action may well be operating in addition to subtle membrane perturbation.

PAF and the ATLs are known to be membrane-active detergents owing to their amphiphilic nature (Noseda et al., 1989b; Sawyer and Andersen, 1989; Kantar et al., 1991). However, an important point to stress is that our experiments show no correlation between the lytic potency of these lipids and their cytostatic potency in long-term proliferation assays. In spite of their very different cytostatic potency, ATLs and PAF were equally lytic. Our results show that ATLs, PAF do not exert direct, gross membrane damage until concentrations greatly exceed those required to produce antiproliferative effects on prolonged exposure. Thus, the much speculated upon detergent effect of ATLs (Noseda et al., 1988) is not the cytotoxic lesion at low concentrations of these agents. However, the IC₅₀ values for the less potent natural lipids are roughly at the point where membrane damage does become significant (≤40 μM). This suggests that the cytotoxic effect seen with PAF and lyso-

Theor, the MTT assay may be more sensitive, but may not be overly sensitive, to effects leading to apoptosis. This finding suggests that the cytotoxicity of ATLs is due to a more subtle, indirect effect on cell membranes.

Taken together, our various studies suggest that ATLs induce cell growth of HL10 cells, HT29 and HL60 cells in a fashion which involves three distinct phases, depending on ATL concentration. (1) At low doses of 1–5 μM in serum-containing medium, ATLs affect a gradual cessation of population growth (cytostasis). We show that this growth arrest is predominantly centred on a G₂/M block and a general slowing of cell cycle progression. However, even at 46 h, a sizeable proportion of cells were still found in the G₁ and S-phases, indicating that the block in G₂ or M is ‘leaky’. Using extremely high concentrations of up to 200 μM over short periods of time, Principe et al. (1992) have reported a similar G₂/M block and some earlier studies have shown that the block in G₂/M is also observed when cells are treated with lower cytostatic concentrations of ATLs.

The ATLs induced cytostasis in HL60 cells without concomitant cellular differentiation. This is unusual in HL60 cells, which will readily differentiate in response to a large variety of agents including retinoic acid, dimethylsulphoxide and other cytotoxic agents (Gallagher et al., 1979; Shoji et al., 1988; Vallari et al., 1988). Some authors have reported differentiation of HL60 cells in response to ATL exposure (Honma et al., 1981, 1991; Vallari et al., 1988; Maurer and Hilgard, 1992), but inhibition of differentiation has also been described in the same model system (Shoji et al., 1988; Kuo et al., 1990; Raynor et al., 1991).

(2) At intermediate concentrations between 5 and 40 μM, a net reduction of viable cell number is observed (cytotoxicity). The precise mechanism of cell death is not yet known, but our flow cytometry data show dramatically increased debris formation which may be indicative of apoptotic cell death.
Apoptosis has been observed in some leukemic cell lines, including HL60, in response to challenge with ATLs (Diomede et al., 1993, 1994), but this response is not universal (Morimoto et al., 1991). (3) As concentrations exceed 40 μM, the detergent properties of the ATLs begin to induce direct lytic membrane damage. At these high concentrations, the toxicity differential between ATLs and naturally occurring ether lipids is progressively eroded, with all types of lipid killing by rapid membrane lysis.

Our results show that the recovery of cell population growth after ATL treatment is both dose and exposure time related. ATLs are widely believed to integrate into the plasma membrane and possibly other cellular membranes (Hoffman et al., 1986). It was perhaps surprising, therefore, that growth inhibition could be removed by simply replacing the drug-containing medium with fresh, drug-free medium. This suggests a rapid equilibrium between the serum-bound and cell-associated lipid. Cells presumably recover their full growth potential by rapid 'back-exchange' onto serum proteins. This putative recovery mechanism has important implications. To be efficiently exchanged with serum proteins, the majority of ATLs must be associated with the outer leaflet of the plasma membrane. The precise location of ATLs within cell membranes has yet to be established and only little is known to date about related phosphocholine lipids (Sleight and Abanto, 1989; Andreason et al., 1982; Bazill and Dexter, 1989).

Interestingly, in addition to the antiproliferative effects above, we noted that some ATLs promoted clonogenic colony formation and cell proliferation in MTT assays (not shown). Submicromolar concentrations were seen to stimulate significant increases in colony counts, while concentrations above about 2 μM reduced colony counts dramatically. This potentially mitogenic activity has been mentioned 'in passing' (Hoffman et al., 1984; Mende et al., 1989; Sobottka et al., 1993), but there have been no data to support the hypothesis that some ATLs can stimulate growth at nanomolar doses.

Other evidence for a potentially mitogenic role of ATLs comes from cell signalling experiments. ATLs can elicit lipid-specific calcium elevations in tumour cells (Lazenby et al., 1990; Seewald et al., 1990; Lohmeyer and Workman, 1993). Under certain circumstances, such as at low ATL concentrations, these calcium changes may conceivably serve as mitogenic signals, thus stimulating cell proliferation. On the other hand, it should be noted that certain conventional anti-tumour drugs, such as doxorubicin, can also induce cell proliferation at very low concentrations, probably via a membrane effect (Vichi and Tritton, 1989). It is not clear whether these results are relevant to the clinical use of ATLs, but one possibility is that they may relate especially to the observed immunostimulatory effects.

A factor of definitive clinical relevance is the schedule dependence of ATLs. Our studies highlight the importance of exposure time for the in vitro anti-tumour effectiveness of ATLs. We found that with pharmacologically relevant concentrations of ATLs, the cytostatic/cytotoxic effect generally developed after the first 24 h of exposure. This 'induction period' was also noted for other ATL activities, such as the suppression of growth factor signalling (unpublished observation; Berens et al., 1988; Seewald et al., 1990). Moreover, recovery of cell growth after 24 h of exposure was comparatively rapid. Longer exposures (≥ 48 h) resulted in more pronounced cytostasis/cytotoxicity, and a much delayed recovery. Under conditions of continuous exposure, 2–3 μM ET18-OCH₃ or SRI 62-834 was cytostatic, maintaining growth arrest for over 7 days. The dependence on prolonged exposure for maximal activity has also been commented upon by others (Seewald et al., 1990; Prinipe et al., 1992). It is possible that the failure of some recent phase I/II clinical trials (Rodriguez et al., 1992; Verweij et al., 1992, 1993) to show activity may lie with the dose schedules used, rather than the efficacy of the drugs per se. Clearly, most of the clinical trials to date have failed to match the promise of the in vitro and in vivo preclinical studies (Lohmeyer and Bittman, 1994; Houlihan et al., 1995). However, success has been reported for local topical administration of HePC in breast cancer (ten Bokkel Huinink et al., 1992; Khayat et al., 1993) and for bone marrow narrowing, where higher drug levels and longer exposures can be effected (Vogler et al., 1993b; Vogler, 1994). Pharmacokinetic monitoring is urgently required to determine whether active concentrations of ATLs can be achieved and maintained clinically. Failure to do this could lead us to discard these mechanistically very interesting agents prematurely.

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Abbreviations: ATL(s), anti-tumour lipid(s); HePC, hexacyclophosphocholine; SRI 62-834, (±)-2-(hydroxytetrahydro-2-(octadeциloxy)methylfuran-2-yl) methoxy phospholipid; N,N,N-trimethyllethanolamin hydroxide; ET18-OCH₃, 1-O-hexadecyl-2-O-methyl-rac-glycerol-3-phosphocholine; ET18-OCH₃, 1-O-octadecyl-2-O-methyl-rac-glycerol-3-phosphocholine; PAF, 1-O-alkyl-2-O-acetyl-sn-glycerol-3-phosphocholine; PAF₃, 1-O-hexadecyl-2-O-acetyl-sn-glycerol-3-phosphocholine; PAF₅, 1-O-octadecyl-2-O-acetyl-sn-glycerol-3-phosphocholine; PAF₆, 1-O-octadecyl-2-O-acetyl-sn-glycerol-3-phosphocholine; lso-PAF, 1-O-alkyl-2-O-acetyl-sn-glycerol-3-phosphocholine; MCP, methylykarbamylo-PF₃; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-3-(4-chlorophenyl)-2H- tetrazolium bromide; WE, 3-(3-[4-(chlorophenyl]-2H-[1,4]-diazepin-2-yl]-1-(4-morpholinyl)-1-propanone; PBS, Dulbecco’s phosphate-buffered saline; FCS, fetal calf serum; IC₅₀, concentration giving half-maximal inhibitory effect; R₅₀, concentration resulting in half-maximal radiosilub release.

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