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Cross-resistance pattern of cell lines selected for resistance towards different cytotoxic drugs to membrane-toxic phospholipids in vitro*

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Summary. The synthetic ether lipids ET-18-OCH_3 and BM41.440 and a derivative, hexadecylphosphocholine, were tested for inhibition of [^{3}H]-thymidine uptake into a Chinese hamster ovarian cell line (AUXB1) and its multidrug-resistant subline selected for colchicine resistance (CH^RC5). The activity of all three compounds against the multidrug-resistant subline was equal to or higher than that against the parent line. The same result was found for their activity against a human leukemic lymphoblastic cell line (CEM/O) and its methotrexate-resistant subline (CEM/MTX). In contrast, two multidrug-resistant cell lines selected for resistance to Adriamycin, the mouse leukemia cell line P388/ADR and the murine sarcoma cell line S180/ADR, expressed modest cross-resistance to the lipids as measured by thymidine uptake. Experiments performed using the trypan-blue dye-exclusion assay yielded comparable results, although this system revealed a slightly different sensitivity in showing the cytotoxicity of the drugs. By this assay, modest cross-resistance for ET-18-OCH_3 and BM41.440 to Adriamycin was found only after 24 h incubation and decreased after 48 h incubation, with almost equal sensitivity to both drugs being shown by the parental (P388/W) and resistant lines (P388/ADR). Furthermore, findings from a human tumor-cloning assay were in accordance with these data, although they did not indicate cross-resistance for the P388/ADR cell line. These results suggest that certain ether lipids and derivatives might represent valuable anticancer drugs warranting further study in the setting of resistant disease.

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Abbreviations used: *ALP*, alkyl-lysophospholipid derivative(s); *TLP*, thioether-lysophospholipid derivative(s); *EL*, ether lipid(s); *ET-18-OCH_3*, 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine; *BM41.440*, 1-hexadecylmercapto-2-methoxymethyl-rac-glycero-3-phosphocholine; *MDR*, multiple drug resistance; *MTX*, methotrexate; *DHFR*, dihydrofolate reductase; *ADR*, Adriamycin; *FCS*, fetal calf serum; *HTCA*, human tumor-cloning assay; *IC_{50}*, drug concentration inhibiting the 48 h [^{3}H]-thymidine uptake of cells by 50% or resulting in a 50% decrease in trypan-blue dye exclusion or colony formation in the HTCA at the times indicated; *RDR*, relative drug resistance; *HPC*, hexadecylphosphocholine (D18 506); *PK-C*, protein kinase C; *TPA*, 12-O-tetradecanoylphorbol-13-acetate

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Introduction

Synthetic ether lipids (EL) have demonstrated broad antineoplastic activity both in vivo and in vitro (for review see [6, 9]). For example, alkyl-lysophospholipid derivatives (ALP) represent a group of antineoplastic compounds [33] that inhibit the growth [34] and metastasis [7] of syngeneic murine tumors and the growth of some rat tumors [12]. A number of thioether-phospholipid derivatives (TLP), the thioether analogs of ALP, and hexadecylphosphocholine (HPC), a derivative of EL, have been shown to possess similar antineoplastic properties [8, 16, 23, 25].

Apart from enhancement of the cytotoxic properties of macrophages [7], the direct cytotoxic destruction of leukemic [2, 44] and tumor [6] cells in vitro has been shown to account for this therapeutic activity of EL. These results have prompted the initiation of clinical studies of two EL and the phospholipid derivative HPC [9, 11, 24, 48]. Although the molecular mechanism of action is not fully understood, there is evidence that EL interact with the plasma membrane to exert their cytotoxic effect [6, 9, 36]. Furthermore, the ALP ET-18-OCH_3 has been reported to interfere with normal phospholipid metabolism [31] and to inhibit binding of epidermal growth factor to human breast-cancer cell lines [28]. ET-18-OCH_3 and the TLP BM41.440 have been shown to inhibit protein kinase C activity [22, 41].

The development of resistance to cytotoxic drugs has posed a major obstacle to the success of many chemotherapeutic regimens. Of considerable importance for the po-

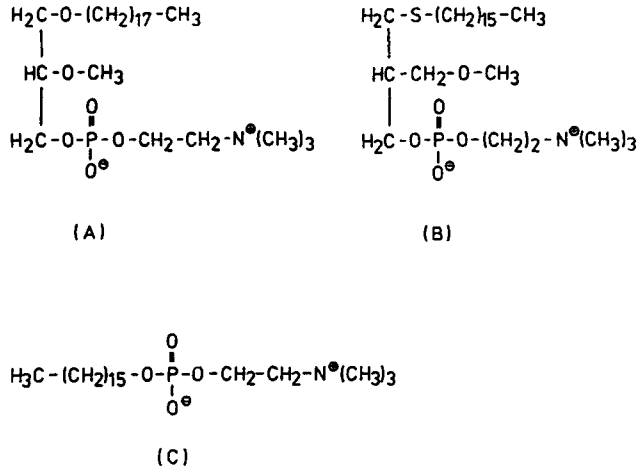

Fig. 1 A–C. Chemical structures of A ET-18-OCH_3, B BM41.440 and C hexadecylphosphocholine

tential clinical use of EL is the question as to whether there is cross-resistance to drugs whose efficacy has been limited by the development of resistance. Tissue-culture systems using sensitive cells as well as sublines selected for resistance by continuous exposure to a variety of cytotoxic drugs have demonstrated different patterns of resistance that are considered to be of clinical significance [37, 38].

The multidrug-resistance (MDR) phenotype (for review see [20, 32]) has been established in hamster ovary cells [29], mouse leukemia cells [26], murine sarcoma cells [42], and different cells of human origin [5, 17]. MDR is due to reduced intracellular drug accumulation [5, 17, 26, 29, 42], associated with increased production of a membrane glycoprotein of 170,000 Da (P-glycoprotein) [15, 27]. This glycoprotein is encoded by an *mdr*-gene, which has been cloned and shows striking homology to a well-characterized bacterial transport system [13]. Transfer of cloned *mdr*-genes confers the MDR phenotype on sensitive cells [47]. A number of substances have been found to reverse MDR, presumably by interfering with the P-glycoprotein transport function [1, 46].

Whereas P-glycoprotein-related detoxifying mechanisms protect MDR cells, resistance to methotrexate (MTX), which has been established in hamster ovary cells [4] and human leukemic cells [39], involves mechanisms that circumvent the attack of MTX on its target enzyme dihydrofolate reductase (DHFR). As a result of gene amplification [40], increased levels of DHFR, altered affinity of DHFR for MTX resulting from mutation of the gene [21] and reduced permeability to the drug by virtue of defects in carrier-mediated transport [4, 39] have been demonstrated as underlying mechanisms.

The present study was conducted to determine whether cross-resistance to the phospholipids exists in various cell lines displaying different patterns of resistance to a broad spectrum of cytotoxic agents.

Materials and methods

Drugs. The ALP ET-18-OCH_3 was obtained from Medmark Chemicals (Gruenwald bei Muenchen, FRG); the TLP BM41.440, from Boehringer Mannheim (Mannheim, FRG); and HPC was synthesized by ASTA (Bielefeld, FRG). 2-LPC, an ester-linked lysophospholipid, was purchased from F. Roth (Karlsruhe, FRG). Figure 1 shows the chemical structures of the substances tested. They were dissolved in RPMI 1640 (Gibco 240, Glascow, Scotland, UK) supplemented with 10% fetal calf serum (FCS) (Gibco I 76) containing 50 IU penicillin and 50 µg streptomycin/ml. The solutions were sterilized by micropore filtration (0.22 µm, Millex; Millipore, Molsheim, France) and stored at –20° C.

Adriamycin was obtained from Farmitalia (Freiburg, FRG); colchicine, from F. Roth (Karlsruhe, FRG); and MTX from Lederle (Wolfratshausen bei München, FRG). The substances were prepared as aqueous solutions that were filtered and diluted in complete culture medium (as defined below). Each solution was prepared immediately before use.

Cell lines and culture conditions. Three MDR cell lines and one MTX-resistant cell line were used in the experiments. The Chinese hamster ovary (CHO) cell line selected for colchicine resistance (CH^RC5) and its sensitive parental line AUXB1 [29] were kindly provided by Dr. V. Ling, Ontario Cancer Institute (Toronto, Canada). The Adriamycin-resistant mouse leukemia line P388/ADR and its sensitive parental line P388/W [26] were originally established by R. K. Johnson of the National Cancer Institute and were kindly provided by Dr. D. Ross, University of Maryland. The Adriamycin-resistant murine sarcoma line S180/ADR and its sensitive parental line S180/W were kindly provided by Dr. Sauer, Deutsches Krebsforschungszentrum (Heidelberg, FRG). The human leukemic lymphoblastic cell line selected for MTX resistance (CEM/MTX) and its parental line CEM/O [39] were obtained from Dr. D. J. Fernandes, Wake Forest University (Winston Salem, N. C., USA).

Stocks of these lines were stored in liquid nitrogen. The cultures used were re-initiated from the frozen stocks 2–3 weeks before the experiments were performed. Cells were maintained as monolayer or suspension cultures in plastic culture flasks (Falcon Plastics, Oxnard, Calif.) at 37° C in an atmosphere containing 5% CO_2 under high humidity, in a medium with supplements as previously specified [26, 29, 39]. In addition, the S180 line was grown in BME medium (Fa. Seromed, F-0215, Berlin, FRG) with Earle's salts (concentration of $NaHCO_3$, 2.2 g/l) containing 10% FCS and supplemented with 50 IU penicillin and 50 µg streptomycin/ml. The CEM/MTX line was grown in the continuous presence of MTX in the medium (final concentration, 1 µmol/l), and Adriamycin was added to the culture medium for S180/ADR cells at a concentration of 1 µmol/l at an interval of 2–3 weeks. Cell cultures were propagated as previously described [8].

Assays for determining drug resistance. The [^{3}H]-thymidine uptake assay and the trypan-blue dye-exclusion assay were performed as recently described [10]. In brief, in both assays the cells were co-incubated with the test compounds at various concentrations in culture medium containing 10% FCS for 24, 48, and 72 h. Cytostatic activity was measured as an inhibition of [^{3}H]-thymidine uptake into the cells, given as a percentage of control values. Usually assays were done six times and standard deviations were <20% of the means. Cytotoxic activity of the substances was assayed as a decrease in trypan-blue dye exclusion of the cells as evaluated under a light microscope. Comparison of simultaneous evaluations by different individuals revealed that the method was reproducible within a range of $\leq 10\%$. Both tests show some correlation with each other and with cloning assays when a variety of neoplastic cells are incubated with membrane-toxic lipids and are thus considered to be reliable for measuring the cytotoxicity of these drugs in vitro [6, 8–10, 35].

The human tumor-cloning assay (HTCA) in glass capillaries ($HTCA_{cap}$) was performed using CHO cells as previously described [30], with the following modification: glass capillaries (1.38 × 126 mm) were sonicated for 30 min in double-distilled water, dried, and sterilized at 180° C. The "capillary incubation mixture" consisted of 300 µl cell suspension (5×10^5 cells/ml in double-enriched Connaught Medical Research Laboratories 1066 medium; ingredients as described by Berdel et al. [10] except that the concentration of mercaptoethanol was 5×10^{-4} M), 705 µl RPMI 1640 medium (Gibco 240, Glasgow, Scotland), 225 µl heat-inactivated horse serum (Gibco 220-6350), and 270 µl 3% agar (Agar Noble 0142-01, Difco Laboratories, Detroit, Mich., USA) diluted (1:3) in RPMI 1640 medium at 37° C. The cells were continuously exposed to the indicated final concentrations of the drugs during the complete assay period after their addition to the capillary incubation

Table 1. Antiproliferative activity (based on [^{3}H]-thymidine uptake) and level of resistance of 3 membrane-toxic lipids and the selective cytotoxic drug to different drug-sensitive and resistant cell lines

Compound	IC_{50}[a] sensitive line (μM)	IC_{50} resistant line (μM)	Relative drug resistance[b]	P[c]
	AUXB1:	CH^RC5:		
Colchicine	0.36[d]	24.66	68.5	0.0061
ET-18-OCH_3	15.50	4.48	0.29	0.0038
BM41.440	14.62	12.7	0.87	0.1930
HPC	> 40	23.9	< 0.6	0.0020
	P388/W:	P388/ADR:		
Adriamycin	< 0.03	4.79	>159.6	0.0038
ET-18-OCH_3	0.09	0.64	7.12	0.0037
BM41.440	0.21	0.81	3.86	0.0039
HPC	4.32	11.57	2.86	0.0037
	S180/W:	S180/ADR		
Adriamycin	0.19	4.38	23.1	0.0038
ET-18-OCH_3	19.3	21.7	1.12	0.0033
BM41.440	19.9	33.8	1.70	0.0036
HPC	105.5	100	0.95	0.32
	CEM/O:	CEM/MTX:		
MTX	0.12	> 40	>333.3	0.0021
ET-18-OCH_3	0.94	0.54	0.57	0.0038
BM41.440	3.13	0.48	0.15	0.0039
HPC	25.7	6.33	0.25	0.0038

[a] IC_{50} was obtained from plots of [^{3}H]-thymidine uptake versus increasing drug concentration after an incubation period of 48 hours. Details see Materials and Methods.

[b] Relative drug resistance as defined in Materials and methods

[c] P values of <0.05 indicate statistical significance

[d] Mean of 6-fold assay; SD, less than ±20%

mixture. In all, 75 μl of the capillary incubation mixture was injected into each capillary by micropipette. This system was evaluated with an inverted microscope (Zeiss, Oberkochen, FRG) before and after an incubation period of 10 days at pH 7.2, 37° C, 5% CO_2, and high humidity as previously described [10]. Numbers of colonies and clusters were obtained with standard deviations of less than ±20% for triplicate cultures, and IC_{50} values were obtained from plots of clonal growth vs increasing drug concentrations.

The HTCA in methylcellulose ($HTCA_{MC}$) was performed using P388 cells as follows. After resuspension and dilution, cells were cultured in alpha-MEM (Gibco 072-1900, Eggenstein, FRG) containing final concentrations of 0.88% methylcellulose (Sigma M-0512, Deisenhofen, FRG), fetal bovine serum (FBS; Hyclone Lab. A-1111-D, Logau, Utah, USA) at concentrations of 5%–30%, 0.2% bovine serum albumin (Sigma A-4503), 9×10^{-4}M mercaptoethanol (Serva 28625, Heidelberg, FRG), 6×10^{-9}M sodium selenite (Sigma S-1382), 0.6% PSN-antibiotic solution (Gibco 043-0564H), and 7.5×10^{-4}M glutamine (Gibco 503). Then, 1 μl aliquots containing 1×10^3 viable (trypan-blue dye exclusion) cells were cultured in triplicate with the respective drug concentration in 35-mm culture dishes (Miles Scientific LUX 5217, Naperville, Ill., USA) at 37° C in 5% CO_2 at high humidity. The system was evaluated before and after the incubation period, and cell aggregates consisting of >40 cells were scored as colonies on day 10 of incubation or as otherwise indicated with an inverted microscope. Standard deviations among triplicates were less than ±20%. IC_{50} values were obtained from plots of clonal growth vs increasing drug concentrations.

The ratio of the drug concentration resulting in 50% inhibition of [^{3}H]-thymidine uptake or a 50% decrease in trypan-blue dye exclusion or colony formation in the HTCA (IC_{50}) in the resistant cell line vs that in the sensitive cell line after an incubation time of 48 h (or as indicated otherwise) was termed relative drug resistance (RDR). For compounds other than the selective drug, this term was considered to be a measure of cross-resistance, which is present when the value of this ratio is >1.

Statistical analysis. Calculation of the significance of differences in the IC_{50} values for the drug-sensitive and resistant cell lines was performed using the Mann-Whitney (two-tailed) test. P-values of <0.05 were considered to indicate significant differences.

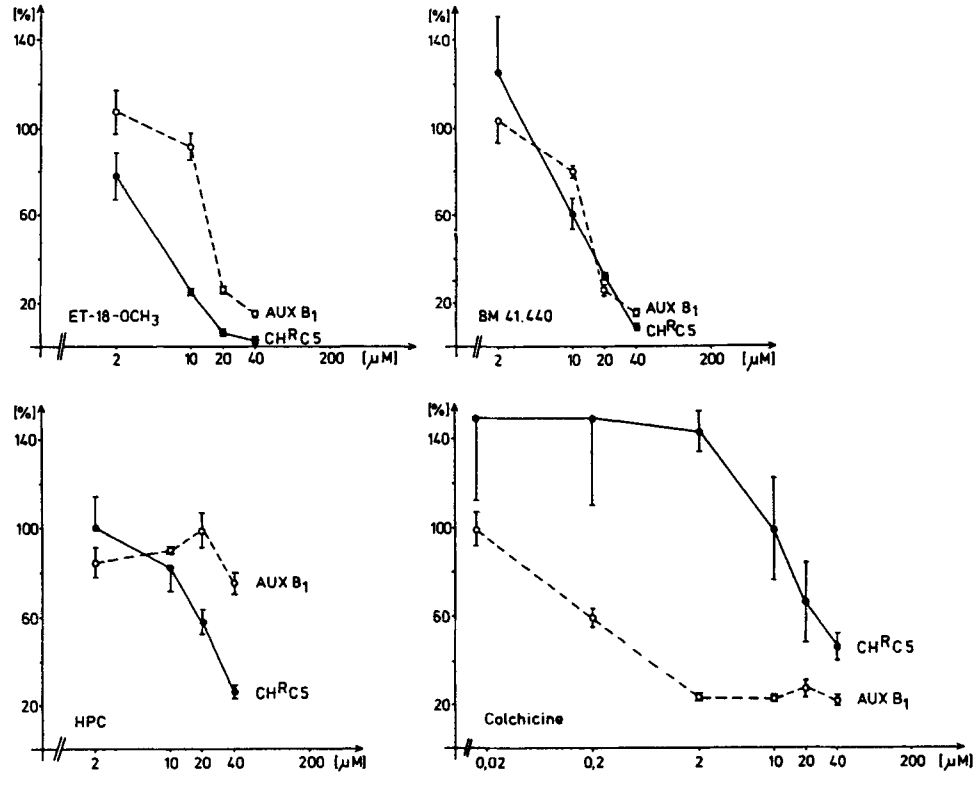

Fig. 2. [^{3}H]-Thymidine uptake expressed as a percentage of control values for CH^RC5 (●——●) and AUXB1 (○——○) with increasing concentrations of ET-18-OCH_3, BM41.440, HPC, and colchicine. Cells were incubated as specified in Materials and methods. Points represent the means of 6 assays; bars, standard deviations

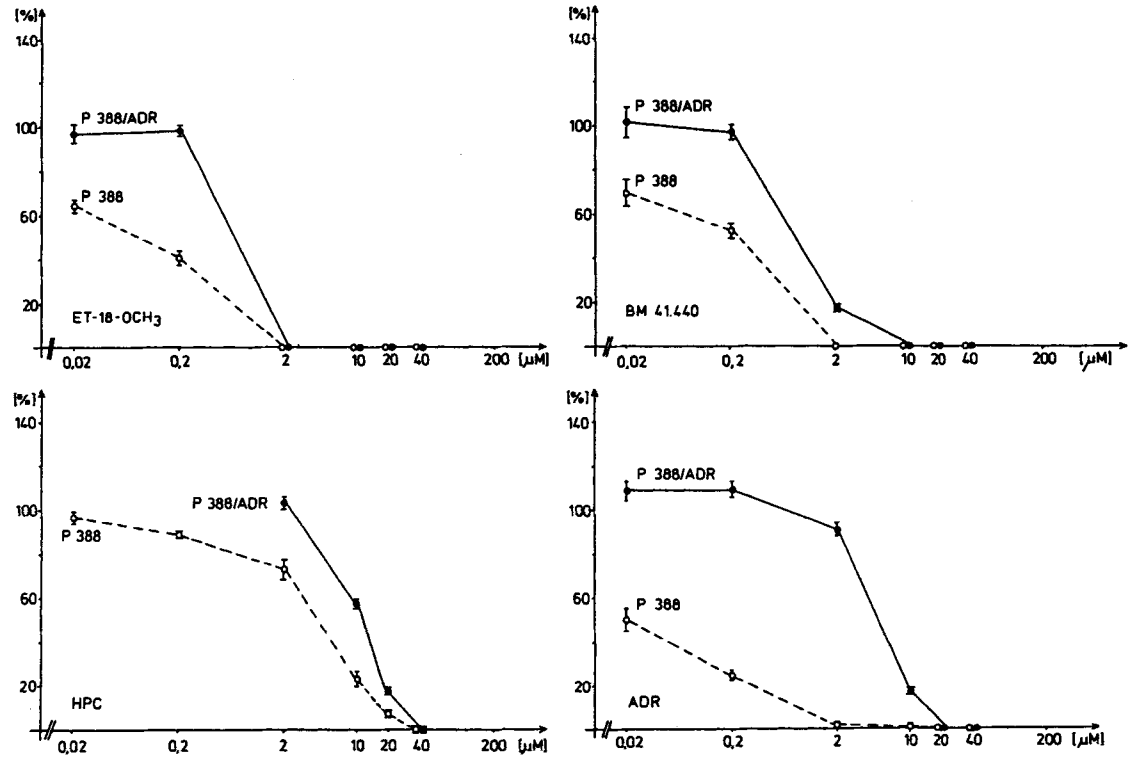

Fig. 3. [^{3}H]-Thymidine uptake expressed as a percentage of control values for P388/ADR (●——●) and P388/W (○——○) with increasing concentrations of ET-18-OCH$_3$, BM41.440, HPC, and Adriamycin. Points represent the means of 6 assays; bars, standard deviations

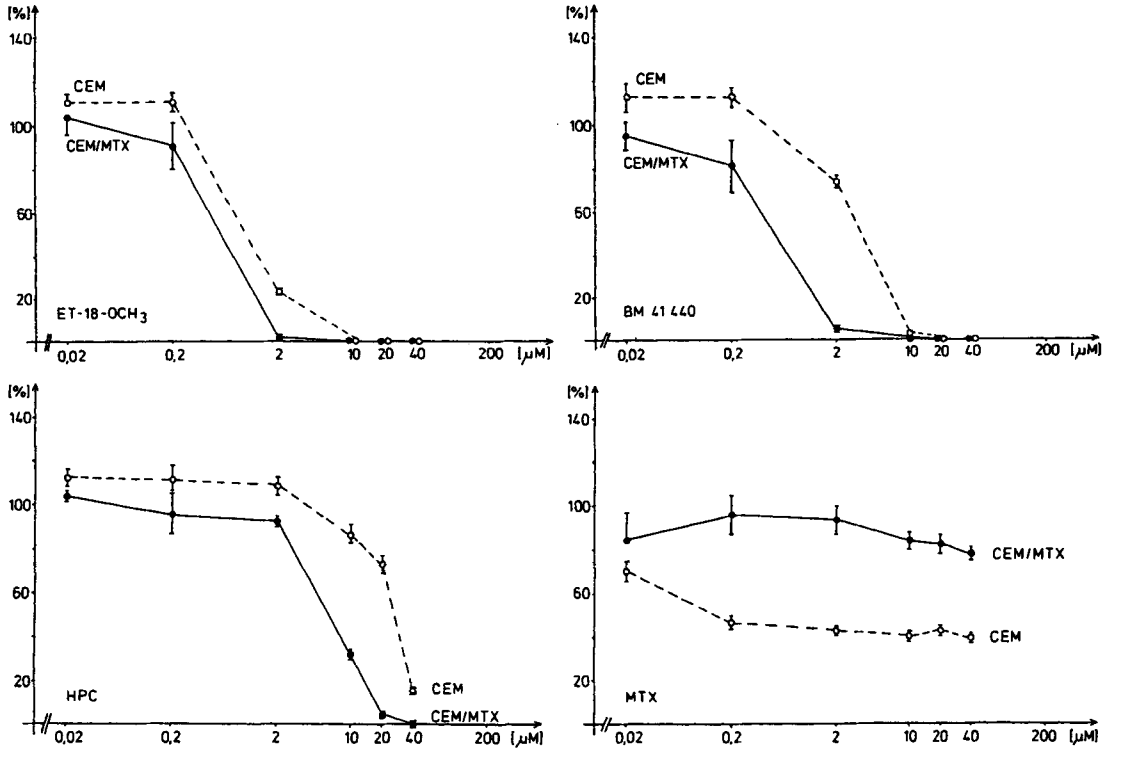

Fig. 4. [^{3}H]-Thymidine uptake expressed as a percentage of control values for CEM/MTX (●——●) and CEM/O (○——○) with increasing concentrations of ET-18-OCH$_3$, BM41.440, HPC, and methotrexate. Points represent the means of 6 assays; bars, standard deviations

Table 2. Cytotoxic activity (based on trypan-blue dye exclusion) and level of resistance of 3 membrane-toxic lipids and the selective cytotoxic drug to 2 drug-sensitive and -resistant cell lines

Compound	IC_{50}[a] sensitive line (μM)	IC_{50} resistant line (μM)	Relative drug resistance[b]
	AUXBl:	CH^RC5:	
Colchicine	1.7	15	8.8
ET-18-OCH_3	33.5	7.5	0.22
BM41.440	24.5	17	0.69
HPC	NT	NT	–
	P388/W[c]:	P388/ADR[c]:	
Adriamycin	4.8	>40	> 8.33
ET-18-OCH_3	1.5	9.5	6.33
BM41.440	3.75	9.0	2.4
HPC	12.5	>40	> 3.2
	P388/W[d]:	P388/ADR[d]:	
Adriamycin	1.3	40	30.77
ET-18-OCH_3	1	1.1	1.1
BM41.440	1.2	1.4	1.17
HPC	7	15.5	2.21

[a] IC_{50} was obtained from plots of cell viability vs increasing drug concentrations
[b] Relative drug resistance as defined in Materials and methods
[c] Indicates results after an incubation period of 24 hours,
[d] Results after a 48-h incubation (explanation, see Results). NT, not tested

Table 3. Cytotoxic activity (based on the human tumor-cloning assay) and level of resistance of 3 membrane-toxic lipids and the selective cytotoxic drug to 2 drug-sensitive and -resistant cell lines

Compound	IC_{50}[a] sensitive line (μM)	IC_{50}[a] resistant line (μM)	Relative drug resistance[b]
	AUXBl:	CH^RC5:	
Colchicine	1.5	24	16
ET-18-OCH_3	50.5	52.5	1.04
BM41.440	51.5	60	1.17
HPC	61	61.5	1.0
	P388/W:	P388/ADR:	
Adriamycin	0.01	0.75	75
ET-18-OCH_3	9.0	7.5	0.83
BM41.440	6.5	6.5	1
HPC	51.5	31.5	0.61

[a] IC_{50} was obtained from plots of clonal growth vs increasing drug concentrations
[b] Relative drug resistance as defined in Materials and methods

Results

The viability of cells based on trypan-blue dye exclusion before testing was >85%. Reliable results in [^{3}H]-thymidine uptake assay were found for all cell lines. All tested lipids reduced the proliferation of both the drug-sensitive and the drug-resistant lines in a time- and dose-dependent way. Incubation for >24 h was necessary to reveal full activity (data not shown).

To determine the levels of resistance and cross-resistance, the drug concentrations required for suppressing [^{3}H]-thymidine uptake to 50% of the control values (IC_{50}) as derived from dose-response plots after an incubation period of 48 h were compared for the drug-sensitive and resistant cell lines. A summary of these results is given in Table 1 and representative plots are shown in Figs. 2–4.

The drug-resistant sublines showed a significant degree of resistance to the selective drug that ranged from 24-fold (S180/ADR) to >300-fold (CEM/MTX) that of control values. No cross-resistance to any of the lipids tested was found in the CH^RC5 line (Table 1, Fig. 2), and the RDR was <0.9 for all compounds. The superior activity of ET-18-OCH_3 and HPC in the resistant subline proved to be of statistical significance. Likewise, there was no cross-resistance in the CEM/MTX cell line to any of the phospholipids tested (Table 1, Fig. 4), and the RDR was <0.6 for all test compounds. Again, this superior activity in the resistant line proved to be statistically significant.

Both cell lines selected for resistance to ADR expressed modest cross-resistance to the phospholipids (Table 1, Fig. 3). The P388/ADR line, which showed a higher degree of resistance to ADR (159-fold) than that of the S180/ADR line (24-fold), also expressed a higher degree of cross-resistance to the lipids. For example, the RDR of the P388/ADR line to ET-18-OCH_3 was 7.12 and that of S180/ADR to ET-18-OCH_3 was 1.12 Statistical significance was indicated for this cross-resistance.

Trypan-blue dye exclusion assay, which was performed to ensure that EL treatment actually killed the neoplastic cells, confirmed the results obtained from the inhibition of [^{3}H]-thymidine uptake. Examples are summarized in Table 2. Experiments were done twice with reproducible results. However, since experiments were not performed as 6-fold assays, no statistical analysis was applied to the results. Also, in this assay P388/ADR expressed modest cross-resistance to the lipids, whereas the latter showed superior activity in the colchicine-resistant CH^RC5 line as compared with the parent line. The trypan-blue dye exclusion assay appeared to have higher sensitivity in showing cytotoxicity in the P388 cell line. Modest cross-resistance of this line to ET-18-OCH_3 and BM41.440 was only found in this assay after 24 h incubation. Cross-resistance subsequently decreased, resulting in an almost equal sensitivity for the parent line and the resistant subline to both drugs after 48 h incubation (see Table 2). Resistance to ADR and cross-resistance to HPC was preserved after an incubation period of 48 h.

Furthermore, experiments using the HTCA were in agreement with the observations obtained in the two other test systems. Examples are summarized in Table 3. Interestingly, in this assay, in which cells were exposed to the drugs throughout the assay period of 10 days, no cross-resistance to ADR and the lipids was detectable in the P388/ADR cell line.

Discussion

We tested the cytostatic and cytotoxic effects of several membrane-active phospholipids on three MDR cell lines, two of which were selected for resistance to ADR and one, for colchicine-resistance, and on an MTX-resistant cell line. The drug-sensitive parental cell lines were tested simultaneously. All resistant lines revealed a >10-fold resistance to the selective drug as compared with the parental line and were phenotypically stable. Thus, they can be used for cross-resistance studies, since it has been estimated that 10-fold resistance in vitro is sufficient to simulate the clinical state of refractoriness [18, 19]. Our results indicate that no in vitro cross-resistance to EL or the derivative HPC exists in the MTX-resistant cell line (see Table 1, Fig. 4). The membrane changes (particularly those in drug transport) partially underlying the resistance to this drug obviously have no effect on the activity of the lipids.

Our experiments using the MDR cell lines yielded somewhat more complex results. There was no cross-resistance to the lipids in the CHO line selected for colchicine resistance (see Tables 1–3; Fig. 2), whereas in the P388 and S180 cell lines resistant to ADR we found modest but statistically significant cross-resistance (see Tables 1, 2; Fig. 3). This result also suggests that MDR is heterogeneous in nature and that mechanisms other than the overproduction of P-glycoprotein may be operative. Several observations are important in explaining this result. First, other evidence has been provided that resistance to ADR is multifactorial. In the ADR-resistant P388 leukemia line, it has been shown that besides enhanced transcription and translation of P-glycoprotein, reduced levels of DNA single- and double-strand breaks contribute to resistance [15]. A possible interpretation of this finding would be that DNA damage is more prolonged in sensitive cells due to altered drug distribution and accumulation. Second, it has been shown that anthracyclines are membrane-active substances that can be cytotoxic without entering the cell [45] and inhibit inositol lipid metabolism in human erythrocytes [43].

Thus, ADR resistance could involve membrane changes other than those described thus far in MDR, which could interfere with lipid toxicity. In view of the observation that protein kinase C (PK-C) inhibition may contribute to the antineoplastic properties of EL, alterations in PK-C regulation and processing in ADR-resistant cells are of particular interest. A recent study has suggested that in HL-60 leukemia cells resistant to ADR, an increased level of PK-C occurs in a calcium/phospholipid-independent form that can phosphorylate vinculin [3]. In drug-sensitive HL-60 cells, this activity could only be shown after treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA). Since EL have been reported to inhibit activation by TPA and diacylglycerol of calcium/phospholipid-dependent PK-C [14, 22], an increased level of its independent form in ADR-resistant cells might cause cross-resistance to the lipids. Further studies concerning alterations of PK-C regulation in drug-resistant cells and the inhibitory effects of EL on PK-C activity are needed.

In conclusion, we found no cross-resistance to membrane-toxic EL or HPC in either an MDR line selected for resistance to colchicine or an MTX-resistant line. However, modest cross-resistance was observed in two MDR cell lines selected for ADR-resistance to the lipids. Further evaluation of the activity of these lipids against drug-resistant cells is recommended, as well as further studies carried out on a wider scale on the effects of membrane-toxic lipids in the setting of drug resistance.

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