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Flow cytometric monitoring of anthracycline accumulation after anti-neoplastic ether phospholipid treatment

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Ether phospholipids are new anti-neoplastic drugs that have been found active against a variety of tumor cell lines, including drug-resistant sublines. We have characterized the antiproliferative activity of three ether phospholipids, i.e. ET-18-OCH_3 (Edelfosine), BM 41.440 (Ilmofosine) and a new aza-derivative (BN 52205), on three leukemic cell lines, i.e. K562 (chronic myeloid leukemia, blast crisis), HL60 (promyelocytic acute leukemia) and CEM (T cell leukemia), and their respective drug-resistant sublines, i.e. K562-ADR (adryamicin resistant), HL60-DNR [daunorubicin (DNR) resistant] and CEM-VLB (vinblastin resistant). These resistant sublines have been found to express the multidrug-resistant phenotype, revealed by the presence of the P-glycoprotein (PgP) using different monoclonal antibodies. Increased cellular accumulation of the fluorescent anthracycline has been found in both sensitive and resistant cell lines after different ether phospholipid treatment times. In resistant cells, the ether phospholipid effect on DNR accumulation has also been found after blocking the PgP function by verapamil and cyclosporin A. These results confirm that the ether phospholipid action is closely linked with the membrane biochemical composition and that these new anti-tumor drugs are able to change the dynamic structural organization of the tumor cell membrane.

Key words: Antineoplastic ether phospholipids, cell membrane, flow cytometry, sensitive and resistant leukemic cells.

Introduction

Ether phospholipids are a chemical species characterized by the presence of an ether bond at position 1 of the glycerol backbone and a metabolically stable substituent at position 2. The chemotherapeutic interest in these new drugs is based on the increasing experimental evidence that their tumoricidal activity is exerted through a direct and indirect effect on tumor cells. Their antineoplastic potential has been evaluated on various *in vitro/in vivo* studies.[1] They can modulate the complex system of host defenses,[2–4] induce tumor cell differentiation[5] and are anti-invasive.[6] In search of more selective and effective anticancer drugs, several ether phospholipids have been synthesized in which different chemical substituents have been included in the glycerol backbone.[1] The analysis of these analogs has led to the conclusion that a long fatty acid chain, the presence of the phosphocholine group, the ether linkage in position 1 and a metabolically stable substituent in position 2 are important requisites for the expression of a direct antiproliferative activity. New aza derivatives have also been synthesized which possess a differential degree of tumor cell growth inhibition.[7,8] The cytotoxic interaction between ether phospholipids and several chemotherapeutic drugs has been studied *in vitro* and *in vivo*. A synergistic interaction has been found between ether phospholipids, spindle poisons and DNA-interactive drugs.[1,9] These findings underline the originality and the diversity of the cytotoxic action of ether phospholipids in comparison with the majority of known anticancer drugs. The cell membrane composition has been found to be important for the selective cytotoxic action of ether phospholipids. We have therefore investigated the effect of ether phospholipid treatment on the accumulation of the anthracycline daunorubicin (DNR) in sensitive and multidrug-resistant leukemic cell lines. Anthracyclines are essential drugs in the treatment of acute leukemia and because of their fluorescence their intracellular accumulation can easily be studied by flow cytometry.[10,11] Many cell lines resistant to chemotherapeutic drugs are characterized by the over-expression of P-glycoprotein (PgP) which is encoded by the *mdr1* gene and functions as a transmembrane drug efflux pump.[12–14] We have characterized the presence of the PgP on the resistant leukemic cell lines using different monoclonal antibodies. Its functional modulation has been evaluated using verapamil and cyclosporin A.[15–18] The results show that ether

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phospholipid treatment induces an increase in DNR accumulation in sensitive and resistant leukemic cell. In these latter cell lines, the ether phospholipid effect on DNR accumulation has also been found after blocking the PgP function by verapamil (VRP) and cyclosporin A (CsA), suggesting that ether phospholipids do not directly interact with PgP function.

Materials and methods

Chemical compounds

1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3) was kindly provided by Dr R Nordstrom (Medmark Pharma, Germany). 1-Hexadecyl-mercapto-2-methoxymethyl-*rac*-glycero-3-phosphocholine (BM 41.440) was gratefully received from Dr D J Herrmann (Boehringer Mannheim, Mannheim, Germany). Methoxy-3-*N*, *N*-methyloctadecylamino-2-propyloxyphosphorylcholine (BN 52205) is a new synthetic aza-substituted ether phospholipid previously described.[7] DNR was from R Bellon Labs (Rhône Poulenc, Antony, France), VRP from Knoll (Levallois-Perret, France) and CsA from Sandoz (Paris, France).

Cell culture

The following human leukemic cell lines were analyzed: the HL60 promyelocytic acute leukemia and its 10^{-6} M DNR-resistant HL60 subline (HL60/DNR); the K562 chronic myeloid leukemia, blast crisis and its 10^{-6} M adriamycin-resistant subline (K562/ADR); and the CEM T cell leukemia and its 10^{-6} M VLB-resistant subline (CEM/VLB). The cell lines K562, K562/ADR, CEM and CEM/VLB were a gift of Dr K Ross (Stanford University, Stanford, CA). The cell lines HL60 and HL60/DNR were kindly received from Dr F Lacombe (Bordeaux, France). These latter cell lines required 20% fetal bovine serum (Seromed Biochrom KG, Société Polylabo, Paris, France) in their growth medium while 10% was required for the other cell lines. All leukemic cells were cultured in RPMI medium (Gibco, Cercy Pontoise, France) supplemented with 2 mM L-glutamine and 50 µg/ml gentamycin. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 in 95% air.

Flow cytometric detection of PgP

The FITC-conjugated C219 monoclonal antibody was obtained from Centocor Diagnostic (Société CIS, Bio-International, Glf sur Yvette, France) while the JSB-1 monoclonal antibody was from Sanbio (Société TEBU, Le Perray-en-Yvellnes, France). Both antibodies react with a conserved cytoplasmic epitope of the PgP, a 170 kDa membrane energy-dependent efflux pump, which confers resistance to numerous chemotherapeutic agents. The third monoclonal antibody MRK16 was a kind gift from Dr Tsuruo (University of Tokyo, Tokyo, Japan). This antibody reacts with an external epitope of the PgP.

For flow cytometric determination of PgP positivity, cell suspensions were divided in three aliquots: the first two were fixed in 100% methanol at −20°C for 15 min, washed with cold TBPBS (0.05% Tween 20, 0.5% BSA in phosphate buffer saline), resuspended in monoclonal antibody solution (C219 and negative antibody, 2.5 µg/ml final concentration; JSB-1 and negative antibody, 1:100 dilution) and incubated for 1 h at 4°C. The third aliquot was used for MRK16 staining which was carried out on non-fixed cells. This antibody was added directly to the cell suspension at 5 µg/ml final concentration. Incubation lasted 30 min at 4°C in the dark. After incubation, MRK16 and JSB-1-stained cells were washed with ice cold TBPBS, resuspended in FITC-labeled second antibody, and incubated for 45 min at 4°C. The C219 monoclonal antibody was directly coupled to FITC. Flow cytometric analyses were carried out on a FACStar plus flow cytometer (Becton-Dickinson, Grenoble, France) equipped with a 2 W argon-ion laser operating at 250 mW and emitting at 488 nm wavelength. Data were collected in list mode. Single FITC histograms were collected for 10 000 events. Electronic gates were set on the isotype controls. Cells with fluorescence higher than the gated population were considered positive.

Evaluation of single and combined ether phospholipids treatment with DNR and PgP inhibitors (VRP and CsA)

The microculture tetrazolium assay (MTT) has been used to evaluate the drugs' cytotoxic effect at the experimental conditions set up for flow cytometric monitoring of DNR uptake. The experimental procedure has been described in detail elsewhere.[9] Briefly, exponentially growing tumor cells were harvested, counted and inoculated at appropriate concentrations (80 µl volume) into 96-well microtiter plates. After 24 h, 20 µl of 6-strength ether phospholipid solution was added to the assigned well. Ether phospholipid treatment (50 µg/ml) was

carried out for 15, 30 and 60 min. In parallel, pretreatment with PgP inhibitors, 10 μM VRP or 1 μM CsA, lasted 30 min before addition of 1 μM DNR for 30 min. Incubations were performed at 37°C in 5% CO_2–95% air. Growth medium (100 μl) was used as a blank and controls without drug were included in the microtiter plate. Each test was set in triplicate. At the end of the treatment time, 50 μl of 1 mg/ml MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide; Sigma) was added to each well and the plates re-incubated at 37°C. The formazan crystals formed inside the living cells were dissolved with dimethyl sulfoxide. The formazan absorbance of each well was read on a microplate reader Multiscan MCC/340 (Titertek, Labsystem, Les Ulls, France) at 570 nm wavelength. Automatic reading and blank subtraction were operated using the Deltasoft. MacIntosh software. The drugs' cytotoxic effect is referred to in terms of cell surviving fraction expressed as percentage of treated versus untreated cells.

Flow cytometric monitoring of DNR uptake

Ether phospholipid treated (50 μg/ml for 15, 30, 45 and 60 min) and untreated sensitive and resistant leukemic cells were incubated for 30 min with 1 μM DNR to measure DNR uptake. To inhibit PgP function, the resistant cells were treated for 30 min with 10 μM VRP or 1 μM CsA prior to DNR incorporation. Flow cytometric analyses were carried out on a FACStar plus flow cytometer equipped with a 2 W argon-ion laser operating at 250 mW and emitting at 488 nm wavelength. Fluorescence emission (FL2; above 530 nm) and forward angle light scatter (FSC) were collected, log amplified and scaled to generate dot plot cytograms (10 000 events were analysed for each sample).

Results

The data obtained from the flow cytometric detection of PgP are presented in Figure 1. No positive staining was present in sensitive cells. A different degree of staining was measured in resistant cells where each monoclonal antibody detected 80–100% positive staining. Although the three monoclonal antibodies are useful for determining PgP presence in resistant tumor cells, in our experience a better distinction between negative and positive cells has been obtained using the monoclonal antibodies C219 and MRK16.

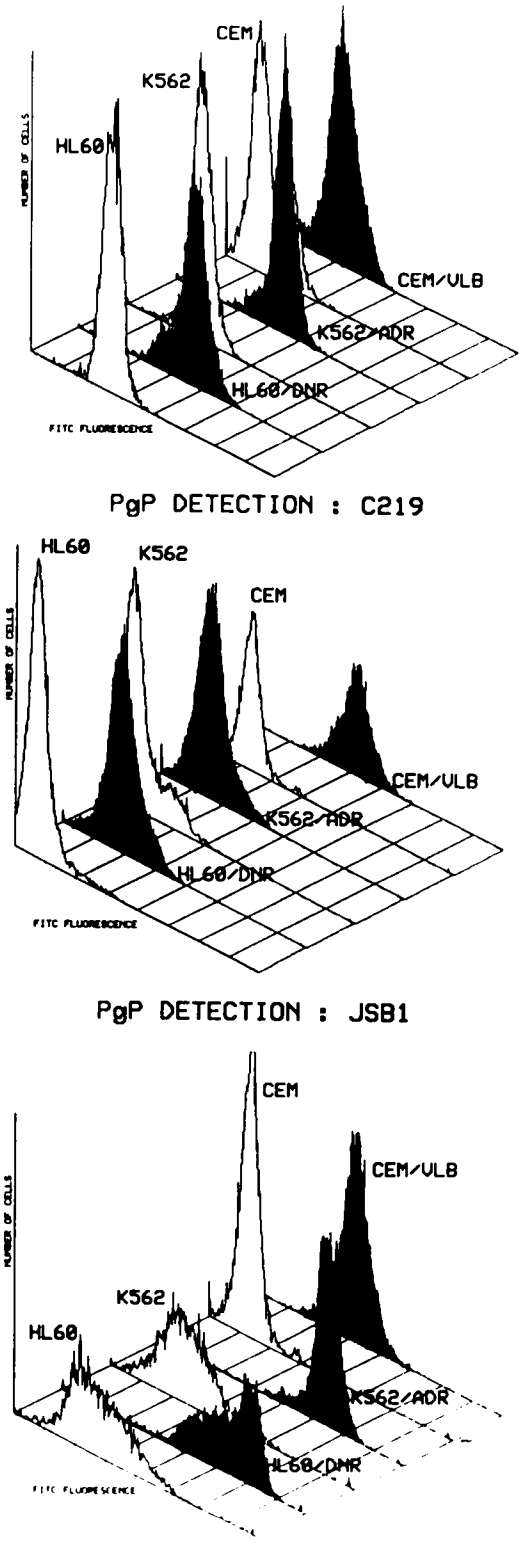

Figure 1. Flow cytometric detection of PgP using the C219, JSB1 and MRK16 monoclonal antibodies in sensitive (open histograms) and MDR resistant (solid histograms) leukemic cell lines.

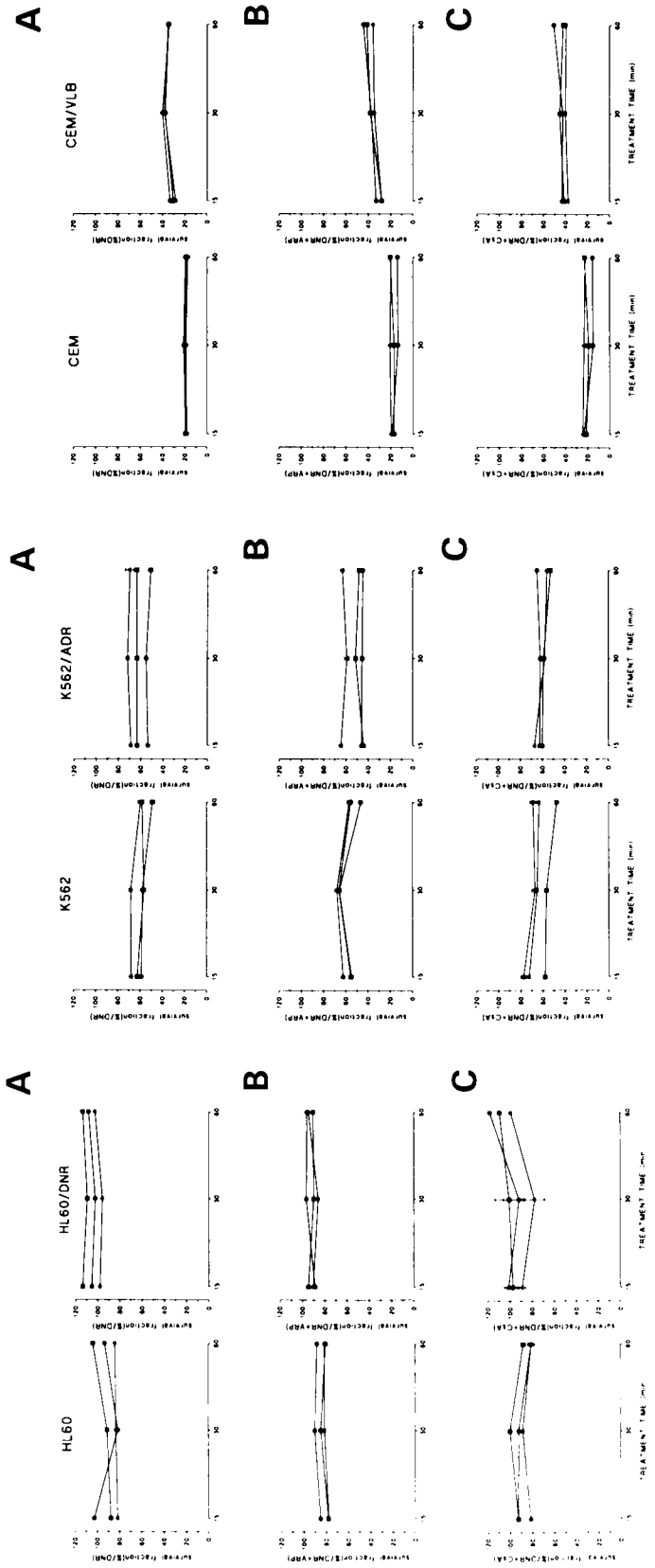

Figure 2. Evaluation of the cytotoxic effect from single and combined ether phospholipid treatment with DNR and PgP modulators, VRP and CsA. For each cell line, the survival fraction (ordinate) is expressed as a function of treatment time (abscissa). The plots in panel (A) are relative to the cytotoxic effect of ether phospholipid followed by DNR treatment. Values are expressed as percentage of those obtained after DNR treatment to extrapolate the effect due to ether phospholipid treatment. The plots in panel (B) are relative to the cytotoxc effect of ether phospholipid followed by VRP and DNR treatment. The survival fraction is expressed as percentage of (VRP + DNR) treatment. In the plots in panel (C), VRP has been replaced by CsA treatment.

Figure 2 shows the results from the evaluation of single and combined ether phospholipid treatments with DNR and PgP modulators, VRP and CsA. For each cell line, panel A shows the plots relative to the cytotoxic effect of ether phospholipids followed by DNR treatment. The survival fraction is expressed as a percentage of the DNR uptake to extrapolate the cytotoxic effect due to ether phospholipids. Panel B shows the plots relative to the cytotoxic effect of ether phospholipids followed by VRP and DNR treatment. The survival fraction is expressed as a percentage of the (VRP + DNR) treatment to extrapolate the cytotoxic effect due to ether phospholipids. In Panel C, VRP is replaced by CsA treatment.

No cytotoxic effect was detected after treatment with DNR, VRP and CsA on all cell lines. As regards the cytotoxic effect due to ether phospholipids, the CEM and CEM/VLB cell lines were the most sensitive. With the exception of HL60/DNR, the resistant cell lines were equally sensitive to ether phospholipid cytotoxicity.

Figure 3 shows the graphs relative to the flow cytometric monitoring of DNR uptake in resistant tumor cells pre-treated with ether phospholipids following the same protocol as that described for the study of the cytotoxicity in single and combined treatment. The DNR incorporation values are indicated in the ordinate (mean FL2) versus treatment times (abscissa). In two of the three cell lines tested, ether phospholipid treatment resulted in increased DNR uptake. In K562/ADR cells, the DNR increment was maximal at 45 min for the three ether phospholipids tested. In VRP and CsA pre-treated K562/ADR cells, DNR incorporation appeared higher than in K562/ADR cells treated with ether phospholipids alone. However, this difference was found not to be significative. No significant difference has been found between the three ether phospholipids tested for each of the three resistant cell lines. The BN 52205 gave the best results in CEM/VLB compared with the other compounds.

Discussion

Flow cytometric analysis of anthracycline accumulation has been demonstrated to be a useful and effective approach to assess the functional capacity of the PgP.[10,11,19] Although several major mechanisms have been proposed to be involved in a lower accumulation of several drugs observed in multidrug-resistant (MDR) as compared with sensitive cells, as higher drug efflux, decreased drug influx or intracellular drug binding, the most consistent biochemical change associated with the MDR phenotype is the over-expression of the ATP-dependent PgP. This protein is believed to act as a pump that actively removes drugs from the cells.[12–14] Kartner *et al.*[20] produced several PgP-specific monoclonal antibodies whose binding to plasma membrane of MDR cells correlated with the degree of resistance. These monoclonal antibodies were directed against three distinct epitopes. One among these antibodies is now commercially available, i.e. C219. Scheper *et al.*[21] produced another monoclonal antibody, JSB1, which is also commercially available. A third monoclonal antibody, MRK16, directed against an extracellular epitope of PgP was described by Hamada and Tsuruo.[22] The C219, JSB1 and MRK16 antibodies have been extensively used for detecting PgP expression in MDR cells of different origin. In our experience C219 and MRK16 gave uniformly and reproductive detection of PgP with the cell lines examined. Similar conclusions were reached in other reports aimed at comparing the efficacy of the different monoclonal antibodies available for PgP detection.[19–23]

The *in vitro/in vivo* ability of CsA and VRP to reverse the MDR phenotype in tumor cells has been well documented.[15–18,24] Although it has been suggested that VRP can correct drug transport defects by a mechanism independent of binding to PgP,[24] both CsA and VRP have been reported to interfere with PgP function.[15–18] Based on these findings, we have used both PgP modulators to alter the pump function in order to examine if ether phospholipid treatment may directly affect PgP function. We have obtained a similar ether phospholipid cytotoxic effect in both sensitive and resistant cell lines. In PgP 'positive' cells, similar profiles were found with both PgP modulators. These data suggest that ether phospholipid treatment is not only effective against resistant leukemic cells but also that these new anticancer drugs do not directly interfere with PgP function. To verify this latter interpretation, we have then monitored, using flow cytometry, the DNR uptake in resistant cells pre-treated with ether phospholipids and PgP modulators. Increased DNR accumulation was found in both CEM/VLB and K562/ADR cell lines after ether phospholipid treatment, and similar profiles were obtained with CsA and VRP in each cell line. Both VRP and CsA failed to correct the defect in DNR accumulation in HL60/DNR cells which were also much less susceptible to ether phospholipid cytotoxicity. Thus, a direct relationship seems to exist betwen the ability of ether phospholipid to change the biophysical and bio-

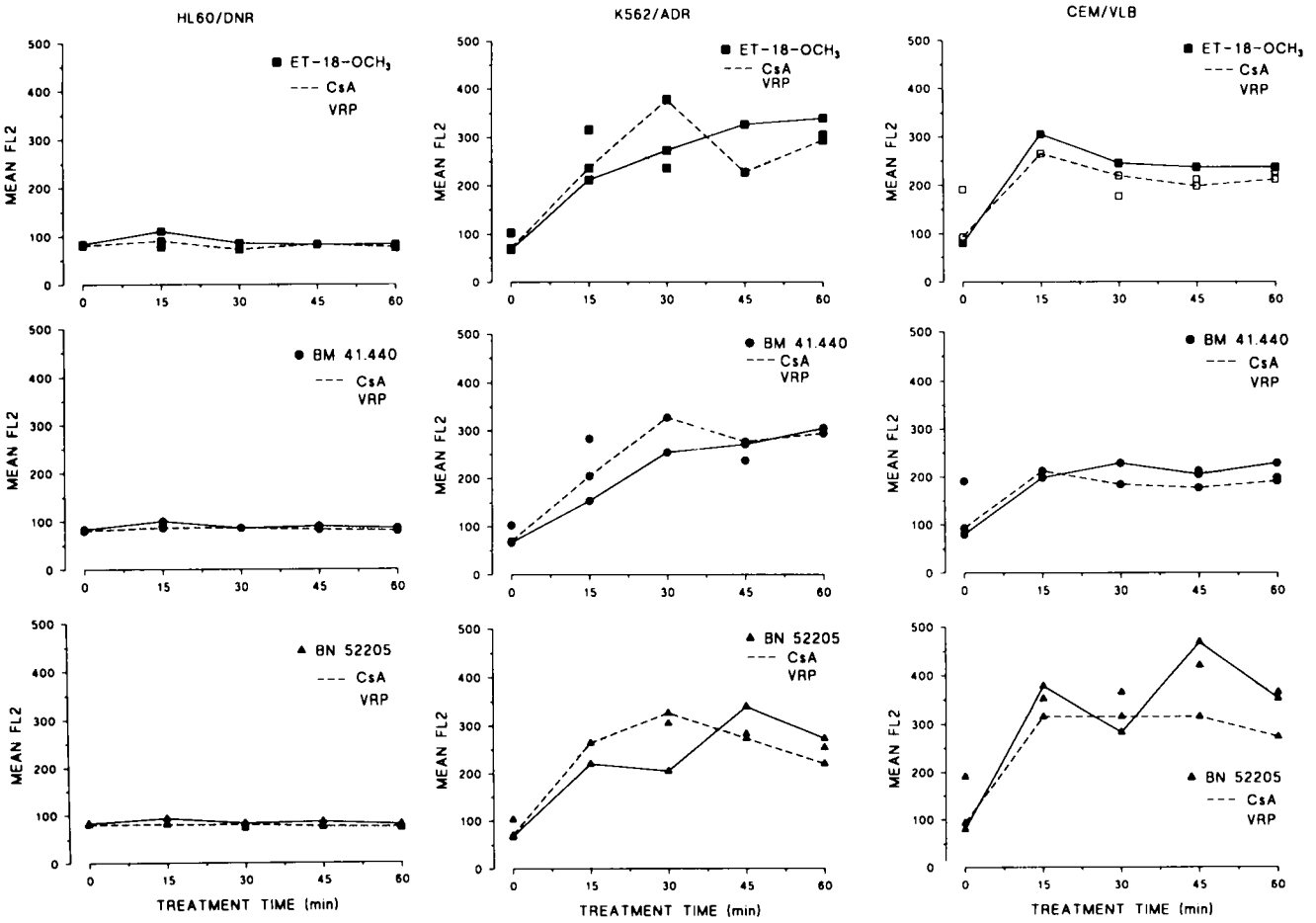

Figure 3. The graphs relative to the flow cytometric monitoring of DNR uptake in MDR leukemic cells pre-treated with ether phospholipids following the protocol described in Materials and methods. The values relative to DNR uptake are reported on the ordinate (mean FL2) versus treatment times (abscissa).

chemical composition of the tumor cell membrane[1] and the observed increase in DNR accumulation. The lack of responsiveness of HL60 sensitive and resistant cells appears to be linked to the fact that HL60 cells possess a higher membrane cholesterol content than other leukemia- and carcinoma-derived cell lines.[25,26] Membrane cholesterol content modulates ether phospholipid cytotoxicity[25–27] and permeation of vincristine through the plasma membrane, thus accounting for lower vincristine accumulation in resistant cells.[28] Experimental evidence has been provided on the passive diffusion of ether phospholipids into the plasma membrane[29] and a great number of reports in the literature support the hypothesis that ether phospholipids, by insertion into the membrane bilayer, induce a cascade of events that ultimately causes tumor cell death.[1] *In vitro* and *in vivo* combination of ether phospholipids and conventionally used chemotherapeutic agents resulted in a synergistic cytotoxic effect in different tumor models[1,9] and interference with tumor cell kinetics did not involve DNA damage.[30]

There is a great need to improve the efficacy of cancer chemotherapy. The heterogeneity of tumor cell behavior calls for innovative treatment strategies. Ether phospholipids belong to a novel generation of anticancer agents whose original mode of action appears to complement that of clinically established drugs and this is likely to result in a greater therapeutic advantage.

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