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Evaluation of combinations of antineoplastic ether phospholipids and chemotherapeutic drugs

Paola Principe,[CA] Hélène Coulomb, Colette Broquet and Pierre Braquet

The authors are at the Institut Henri Beaufour, 1, Avenue des Tropiques, 91952 Les Ulis Cedex, France. Tel: 33 1 69 07 91 15. Fax: 33 1 69 82 92 30.

Combinations of drugs are used clinically for the therapeutic advantages they may provide over single agents. We have studied the cytotoxic interaction between four ether phospholipids ET-18-OCH_3, BM 41.440, BN 52205 and BN 52211, and several chemotherapeutic drugs (ADM, CDDP, VLB, VP-16, MMC, BLM and MTX) on two human tumor cell lines, A427 (lung) and HT29 (colon). We have used the MTT colorimetric assay to evaluate growth inhibition and performed isobologram analysis on the IC_{50} data. For both cell lines a synergistic effect has been found between each of the four ether phospholipids in association with CDDP and ADM. In both cell lines only BM 41.440 and BN 52211 act synergistically with VLB while, in A427 cells, only BN 52205 behaves similarly with MMC. These results show that a positive interaction exists between ether phospholipids, spindle poisons and DNA-interactive drugs.

Key words: Additive (supra-, sub-) response, anti-cancer drugs, cytotoxicity, ether phospholipids, human tumor cells, synergy.

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. Their antineoplastic potential has been evaluated on various *in vitro*/*in vivo* studies.[1–9] They can modulate the complex system of host defenses,[10,11] induce tumor cell differentiation[12,13] and are anti-invasive.[14] Some ether phospholipids are currently being evaluated in clinical trials.[9] The mechanisms on which their cytotoxic and cytostatic activity are based are under active investigation. Unlike most antitumor agents, ether phospholipids do not induce DNA damage. Interaction with cell cycle traverse results in a progressive arrest in the exit from the G_1 and G_2 phases. Tumor cells in late G_1 at the time of treatment can progress through S before being blocked in G_2. In a similar fashion, tumor cells in late G_2 at the time of treatment can go through M but are then halted in G_1.[15,16] There is increasing evidence that the biochemical and biophysical properties of the tumor cell membrane play a crucial role in mediating the tumoricidal activity of ether phospholipids. Changes in cellular lipid synthesis have been reported during ether phospholipid-induced cytolysis.[17,18] Cellular uptake and accumulation as well as the ether phospholipid endocytosis rate are important events in determining tumor cell sensitivity.[19] Inhibition of protein kinase C and Na^+–K^+-ATPase activities[20,21] has been reported together with inhibition of phosphatidylinositol phospholipase C.[22] Perturbation of cellular acylation processes has also been reported and no correlation with inhibition of tumor cell proliferation has been found.[23] A strict correlation does not exist between the quantity of ether phospholipid accumulated and the sensitivity of the tumor cells to the cytotoxic effect.[24] However, the cell membrane composition has been found to be important for the selective cytotoxic action. Pre-loading of cell membranes with cholesterol or high levels of cholesterol in the incubation medium led to attenuation of the ether phospholipid lytic action.[25] Increased ether phospholipid cytotoxicity was obtained by reducing the membrane cholesterol content of diverse leukemic cells.[26] Critical biophysical alterations such as fluidization[27] and permeabilization[28] are also crucial events in the multi-step cytotoxic process of ether phospholipids.

Overall, these findings underline the originality and the diversity of the cytotoxic action of ether phospholipids in comparison with the majority of known anti-cancer drugs. Consequently, ether phospholipids appear as ideally suited candidates to be used in combination with conventional antitumor agents for a more effective cancer chemotherapy.

[CA] Corresponding Author

Materials and methods

Chemical compounds

1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3; Edelfosine) was from Bachem Feinchemikalien AG (Switzerland). 1-Hexadecyl-mercapto-2-methoxymethyl-*rac*-glycero-3-phosphocholine (BM 41.440; Ilmofosine) was kindly provided by Dr Herrmann, Boehringer Mannheim (Germany). Methoxy-3-*N*,*N*-methyl-octadecyl amino-2-propyloxyphosphorylcholine (BN 52205) and *N*,*N*-methyloctadecyl amino-1-methoxy-2-propyloxyphosphorylcholine (BN 52211) are new synthetic aza derivatives.[16] The four ether phospholipids have been combined with the following anti-cancer drugs: mitomycin C (MMC; Sigma, France), adriamycin (ADM; Farmitalia Carlo Erba, Italy), cisplatyl (CDDP; R Bellon Laboratories, France), bleomycin (BLM; R Bellon Laboratories), methotrexate (MTX; R Bellon Laboratories), vinblastine (VLB; R Bellon Laboratories) and vepesid (VP-16; Sandoz, France).

Cell culture

A427 (lung) and HT29 (colon) carcinoma cells were obtained from the American Type Culture Collection (Rockville, MD). The A427 cells were cultured in EMEM medium (Gibco, UK) supplemented with 1% sodium pyruvate (Gibco), 1% non-essential amino acids (Flow Laboratories, France), 2 mM L-glutamine (Seromed Biochrom KG, Germany) and 50 μg/ml gentamycin (Gibco). The HT29 cells were cultured in McCoy's 5a medium (Gibco) supplemented with 2 mM L-glutamine and 50 μg/ml gentamycin. Both growth media contained 10% fetal bovine serum (Seromed Biochrom KG). The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 in 95% air.

Cytotoxic activity *in vitro*

The MTT-microculture tetrazolium assay has been used to evaluate single and combined drug cytotoxicity.[29] The experimental conditions required for the metabolic conversion of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) were previously set up for both tumor cell lines.[30] Single and combined drug cytotoxicity was assessed according to the following protocol: exponentially growing tumor cells were harvested, counted and seeded at appropriate density into 96-well microtiter plates (80 μl/well). Growth medium (100 μl) was used as a blank and controls (untreated cells) were included in the microtiter plate. Eight wells were set up for each point. Tumor cells were continuously exposed to the drugs for 48 h. At the end of this treatment time, 50 μl of 1 mg/ml MTT (Sigma) was added to each well and the microtiter plates re-incubated at 37°C for 4 h in 5% CO_2–95% air. The formazan crystals were dissolved by adding 100 μl dimethylsulfoxide per well. The formazan absorbance was read on a microplate reader (Multiscan MCC/340; Titertek, France) at 570 nm wavelength. Automatic reading and blank subtraction was operated using the Deltasoft Macintosh software. The surviving cell fraction after drug treatment has been expressed as the percentage of the untreated population and plotted as a function of drug concentration. The relative drug concentration required to cause 50% growth inhibition (IC_{50} values) was calculated using linear or non-linear regression to fit the data.[31]

Isobologram analysis

The *in vitro* cytotoxic effect of combinations of ether phospholipids and chemotherapeutic drugs was evaluated using isobologram analysis[32,33]. For each drug combination, either for (compound A + compound B) or (compound B + compound A), we have constructed isobolograms for the isoeffect 50, defined as the relative concentration of drug A or B required to kill 50% of tumor cells at the same incubation time (48 h). In some cases, the isobolograms for the isoeffect 70, defined as the relative concentration of drug A or B required to kill 70% of tumor cells at the same incubation time (48 h), have also been analyzed. For simplicity, only the isobolograms for the isoeffect 50 are presented. An additive relationship between ether phospholipids and chemotherapeutic drugs would produce a straight line whose extremes are represented by the IC_{50} (or the IC_{70}) values calculated for each drug tested alone. Points to the right of this line indicate a sub-additive or antagonistic relationship, whereas points to the left of the line indicate a supra-additive or synergistic relationship.[32]

Results

The cytotoxic effect of various concentrations of each drug tested has been evaluated on HT29 and A427 tumor cell lines, as shown in Figure 1 (HT29)

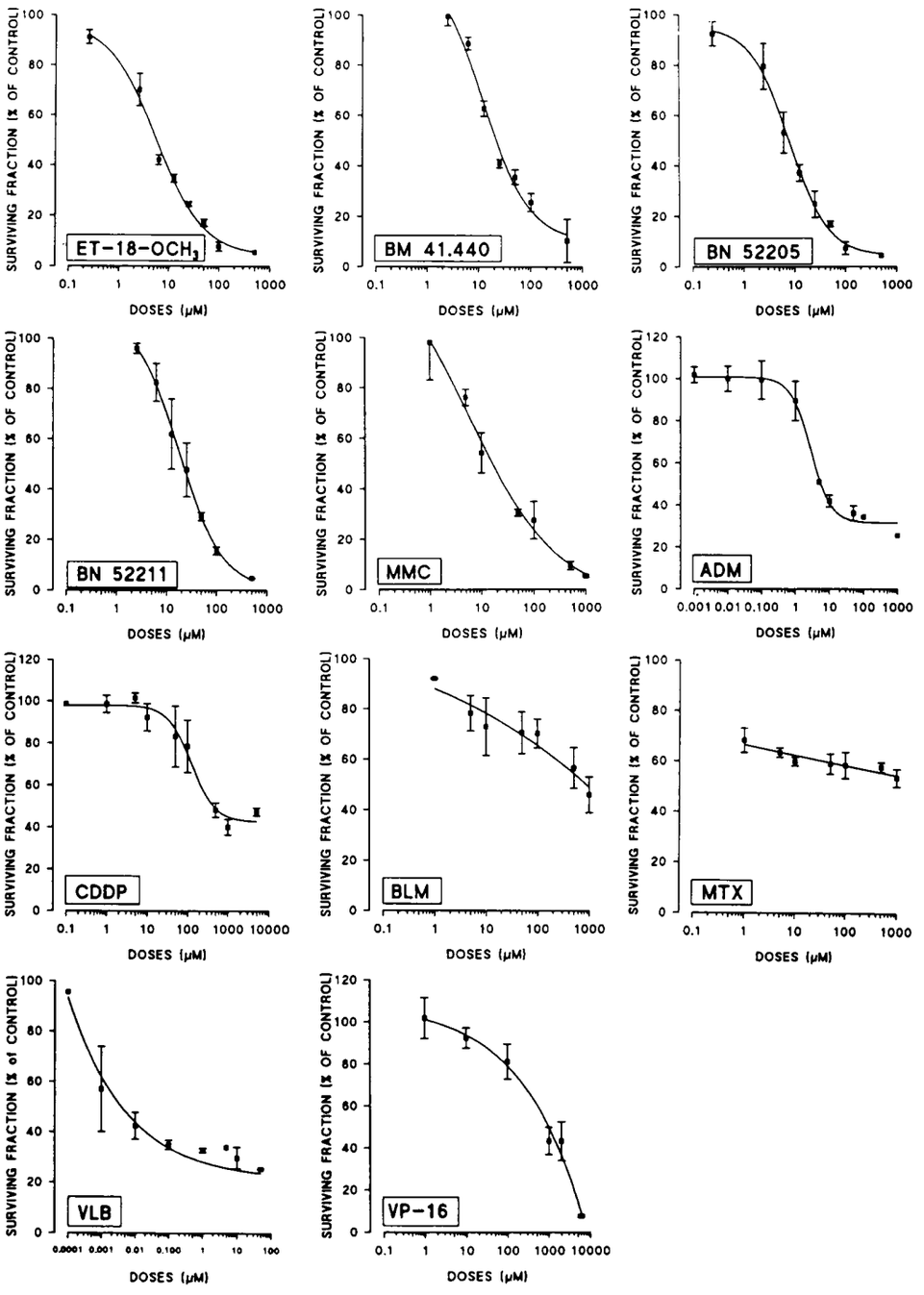

Figure 1. The dose–response plots relative to HT29 human colon adenocarcinoma cells treated for 48 h with ether phospholipids (ET-18-OCH_3, BM 41.440, BN 52205 and BN 52211) and several conventional chemotherapeutic drugs (MMC, ADM, CDDP, BLM, MTX, VLB and VP-16).

and Figure 2 (A427). The chemotherapeutic agents have been chosen because their antiproliferative activity is based on different mechanisms of action to those known for the four ether phospholipids tested. A dose-dependent effect has been observed for each ether phospholipid in both cell lines. A similar dose–response plot has been obtained for the MMC compound in the two cell lines. However, a greater cytotoxic effect was observed in A427 cells with the same ADM concentration in comparison

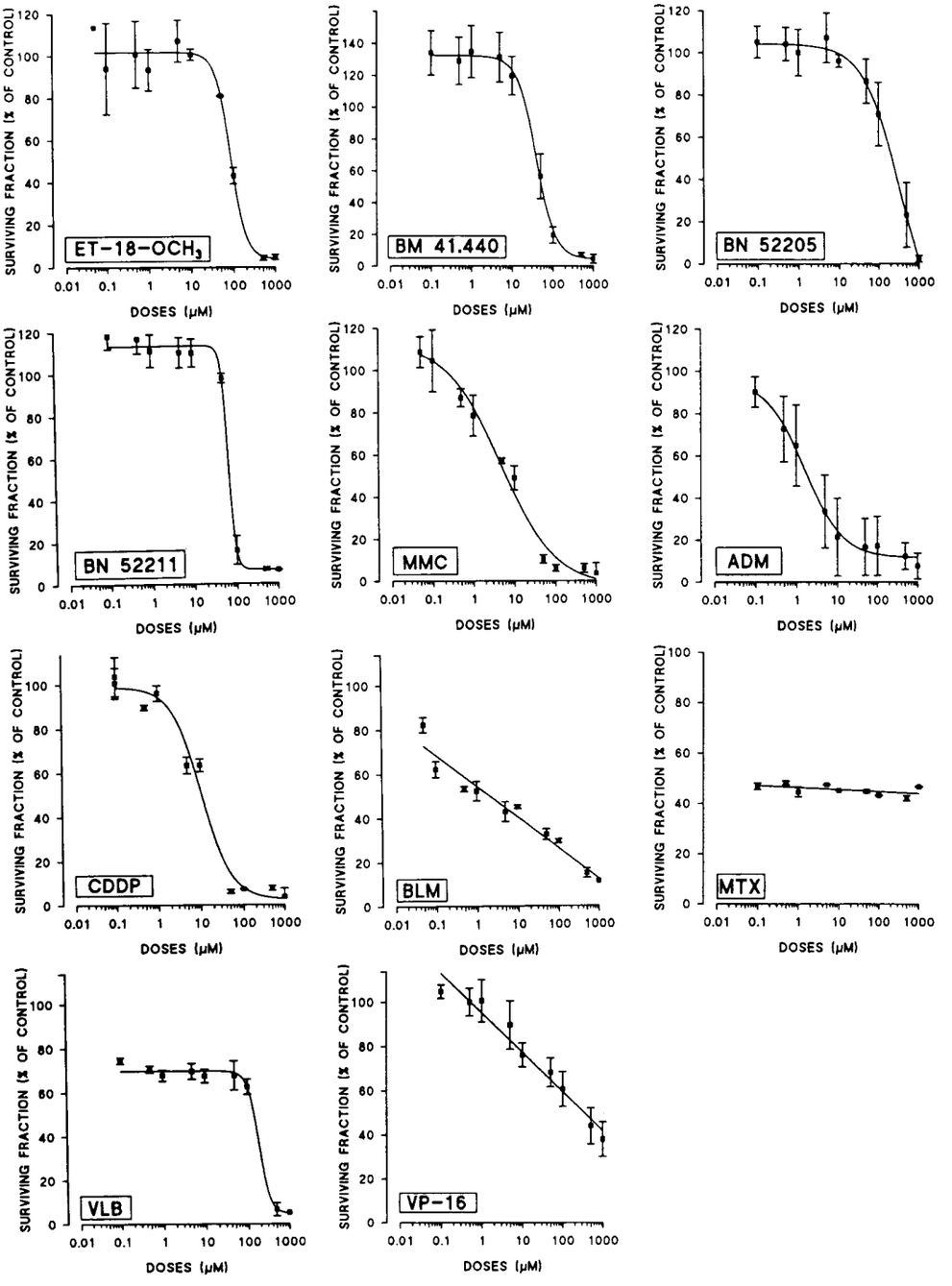

Figure 2. The dose–response plots relative to A427 human lung carcinoma cells treated for 48 h with ether phospholipids (ET-18-OCH_3, BM 41.440, BN 52205 and BN 52211) and several conventional chemotherapeutic drugs (MMC, ADM, CDDP, BLM, MTX, VLB and VP-16).

with HT29 cells, where a plateau was reached at 30% cell survival at the highest concentration tested. Similarly, only 40% cell survival was obtained with the highest CDDP concentration in HT29 cells while nearly 100% mortality was induced in A427 cells. Linear dose–responses were obtained for BLM and VP-16 in A427 cells in comparison with HT29 cells. Both cell lines were moderately responsive to MTX and this prevented the calculation of the IC_{50} and, in consequence, its

association with the ether phospholipids. The shape of the VLB curve was found to be very different for the two cell lines: A427 cells were responsive over a narrow dose range, towards the highest concentrations tested, in contrast with HT29 cells which remained sensitive even at the lowest concentration tested (10^{-10} M). From the dose–response plots reported in Figures 1 and 2, the IC_{50} values were calculated and these values have been classified as shown in Figure 3. For the two cell lines, the different drugs can be clustered according to their cytotoxic efficiency. For HT29 cells, the first group includes VLB which possesses the lowest IC_{50} value equivalent to 5.4×10^{-9} M. Due to its two-order of magnitude difference with all the other IC_{50} values calculated, this value has not been plotted for graphic convenience. In the second group are ADM ($IC_{50} = 4.3 \times 10^{-6}$ M), ET-18-OCH_3 ($IC_{50} = 5.6 \times 10^{-6}$ M) and BN 52205 ($IC_{50} = 7.4 \times 10^{-6}$ M). In the third group are MMC ($IC_{50} = 12.6 \times 10^{-6}$ M), BN 52211 ($IC_{50} = 14.2 \times 10^{-6}$ M) and BM 41.440 ($IC_{50} = 16 \times 10^{-6}$ M). In the fourth group are CDDP ($IC_{50} = 330 \times 10^{-6}$ M), BLM ($IC_{50} = 416 \times 10^{-6}$ M) and VP-16 ($IC_{50} = 595 \times 10^{-6}$ M). For A427 cells, three groups can be distinguished: in the first group are VP-16 ($IC_{50} = 1.4 \times 10^{-6}$ M), ADM ($IC_{50} = 1.4 \times 10^{-6}$ M), BLM ($IC_{50} = 1.5 \times 10^{-6}$ M), MMC ($IC_{50} = 5.4 \times 10^{-6}$ M) and CDDP ($IC_{50} = 6.3 \times 10^{-6}$ M). In the second group are BM 41.440 ($IC_{50} = 39.6 \times 10^{-6}$ M), ET-18-OCH_3 ($IC_{50} = 59 \times 10^{-6}$ M) and BN 52211 ($IC_{50} = 79.1 \times 10^{-6}$ M). In the third group are VLB ($IC_{50} = 103 \times 10^{-6}$ M) and BN 52205 ($IC_{50} = 133 \times 10^{-6}$ M). In conclusion, this classification reflects the differences in sensitivity that the two tumor cell lines have revealed towards each drug tested.

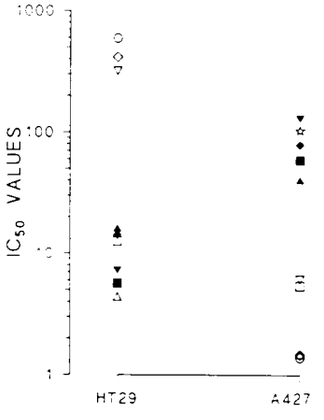

Figure 3. The classification of the IC_{50} values calculated from the dose–response plots relative to each drug tested. ■, ET-18-OCH_3; ▲, BM 41.440; ▼, BN 52205; ◆, BN 52211; □, MMC; △, ADM; ▽, CDDP; ◇, BLM; ☆, VLB; ○, VP-16.

The *in vitro* cytotoxic effect of combinations of ether phospholipids and chemotherapeutic drugs was evaluated using the isobologram analysis. For both cell lines, the data obtained from the association of each ether phospholipid with each chemotherapeutic drug have been grouped and reported in Figure 4 for ET-18-OCH_3, in Figure 5 for BM 41.440, in Figure 6 for BN 52205 and in Figure 7 for BN 52211. An additive relationship between ether phospholipids and chemotherapeutic drugs would produce a straight line, as indicated by the 'dotted line', whose extremes are the IC_{50} values calculated for each drug tested alone. Points to the right of this line indicate a sub-additive (antagonistic) relationship, whereas points to the left of the line indicate a supra-additive (synergistic) relationship.

In HT29 cells, a supra-additive relationship has been obtained in the association between ET-18-OCH_3 and MMC, ADM, CDDP, BLM, VLB and VP-16 (Figure 4). In A427 cells, the same conclusion can be drawn with ADM, CDDP and BLM (Figure 4). However, for these cells, a sub-additive relationship has been found between ET-18-OCH_3, VLB and VP-16 (Figure 4). The association between ET-18-OCH_3 and MMC for the isoeffect 50 produced an undefined relationship. However, a supra-additive relationship was found for the isoeffect 70. In HT29 cells, BM 41.440 acts synergistically with all six chemotherapeutic agents but in A427 cells this relationship has been found only with ADM, CDDP and VLB (Figure 5). In these cells the association of BM 41.440 and MMC, BLM and VP-16 gives rise to a sub-additive relationship (Figure 5). It is apparent from the isobolograms reported in Figure 6 that the interaction between BN 52205, ADM and CDDP is synergistic in both tumor cell lines. However, while in A427 cells MMC interacts synergistically with BN 52205, it has a sub-additive effect in HT29 cells. A different result has also been obtained with VLB which acts synergistically with BN 52205 in A427 cells but has an additive effect in HT29 cells. For both cell lines, the association between BN 52205, BLM and VP-16 results in a contradictory relationship for the isoeffect 50. There is a tendency towards synergy when low concentrations of BN 52205 are used with low concentrations of BLM. However, the isobolograms for the isoeffect 70 revealed no supra-additive activity. Figure 7 shows

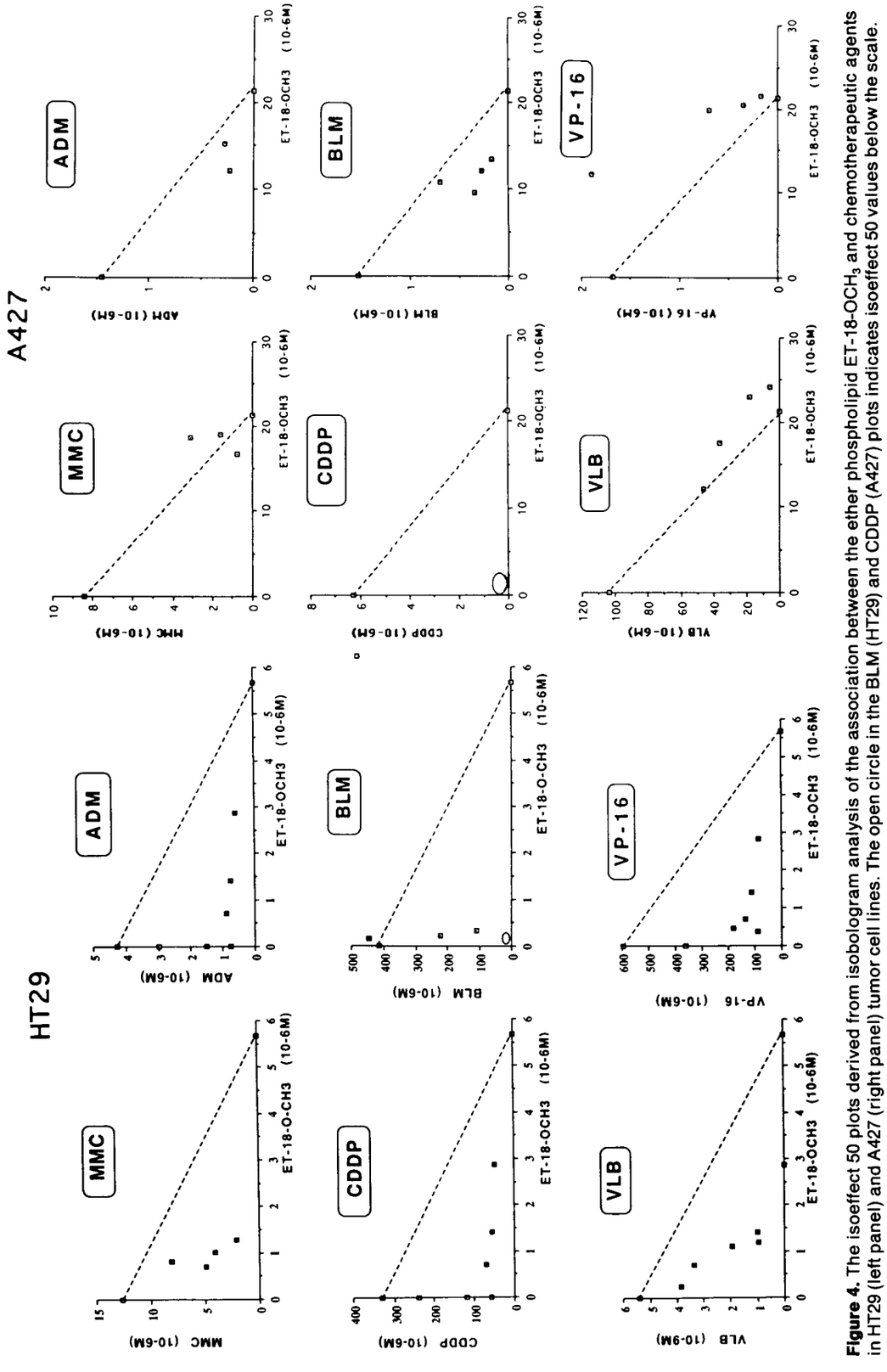

Figure 4. The isoeffect 50 plots derived from isobologram analysis of the association between the ether phospholipid ET-18-OCH_3 and chemotherapeutic agents in HT29 (left panel) and A427 (right panel) tumor cell lines. The open circle in the BLM (HT29) and CDDP (A427) plots indicates isoeffect 50 values below the scale.

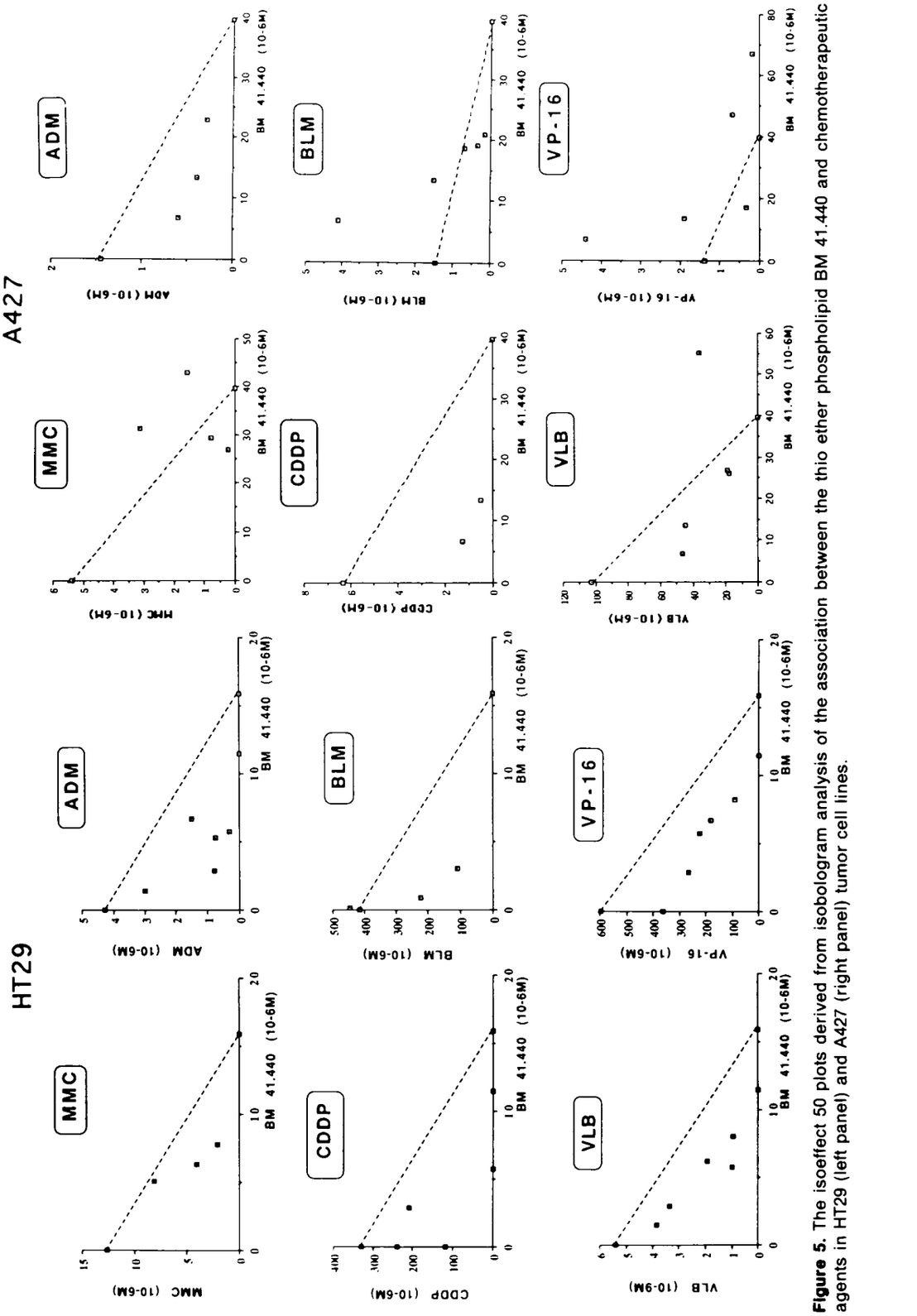

Figure 5. The isoeffect 50 plots derived from isobologram analysis of the association between the thio ether phospholipid BM 41.440 and chemotherapeutic agents in HT29 (left panel) and A427 (right panel) tumor cell lines.

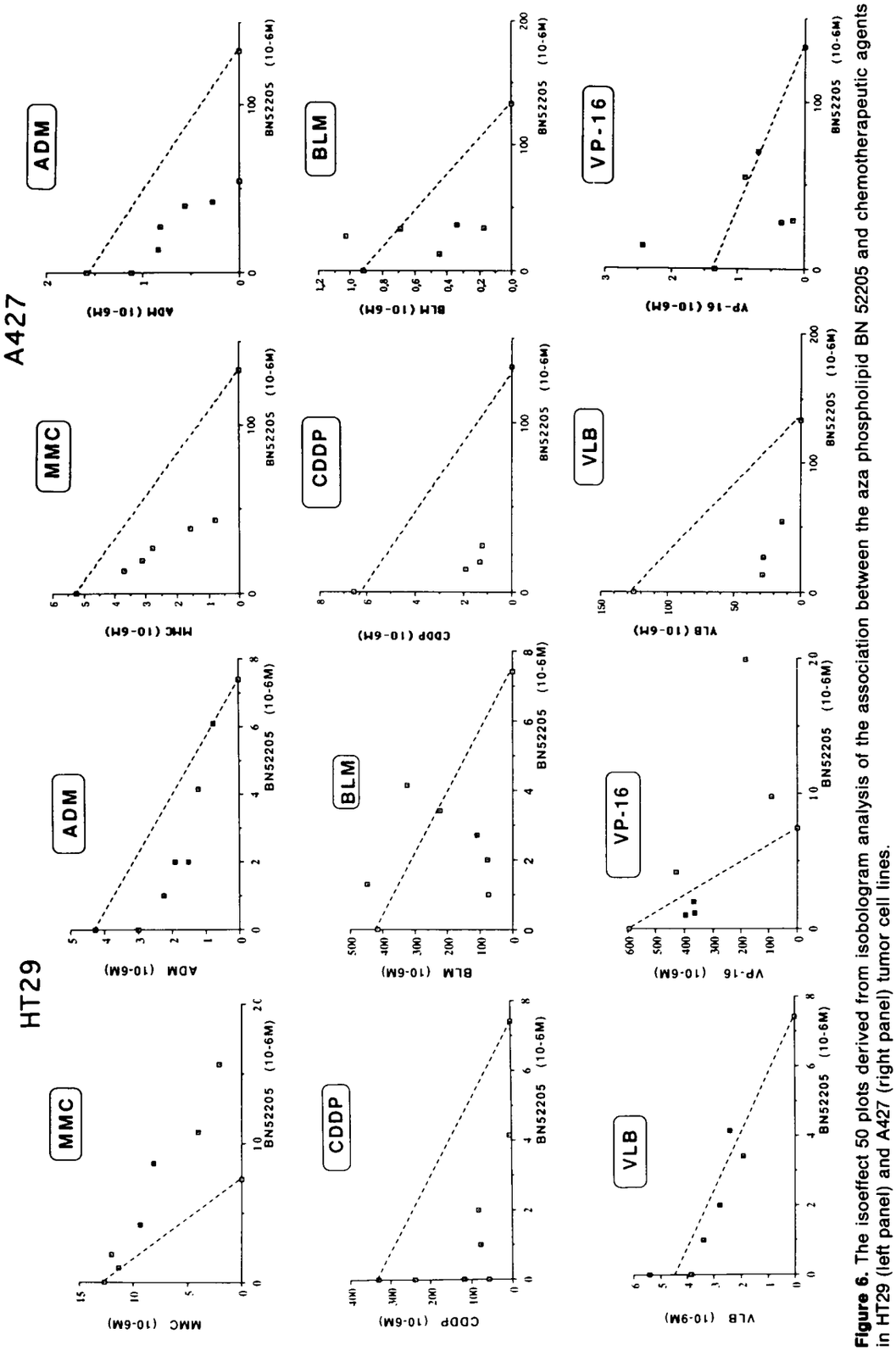

Figure 6. The isoeffect 50 plots derived from isobologram analysis of the association between the aza phospholipid BN 52205 and chemotherapeutic agents in HT29 (left panel) and A427 (right panel) tumor cell lines.

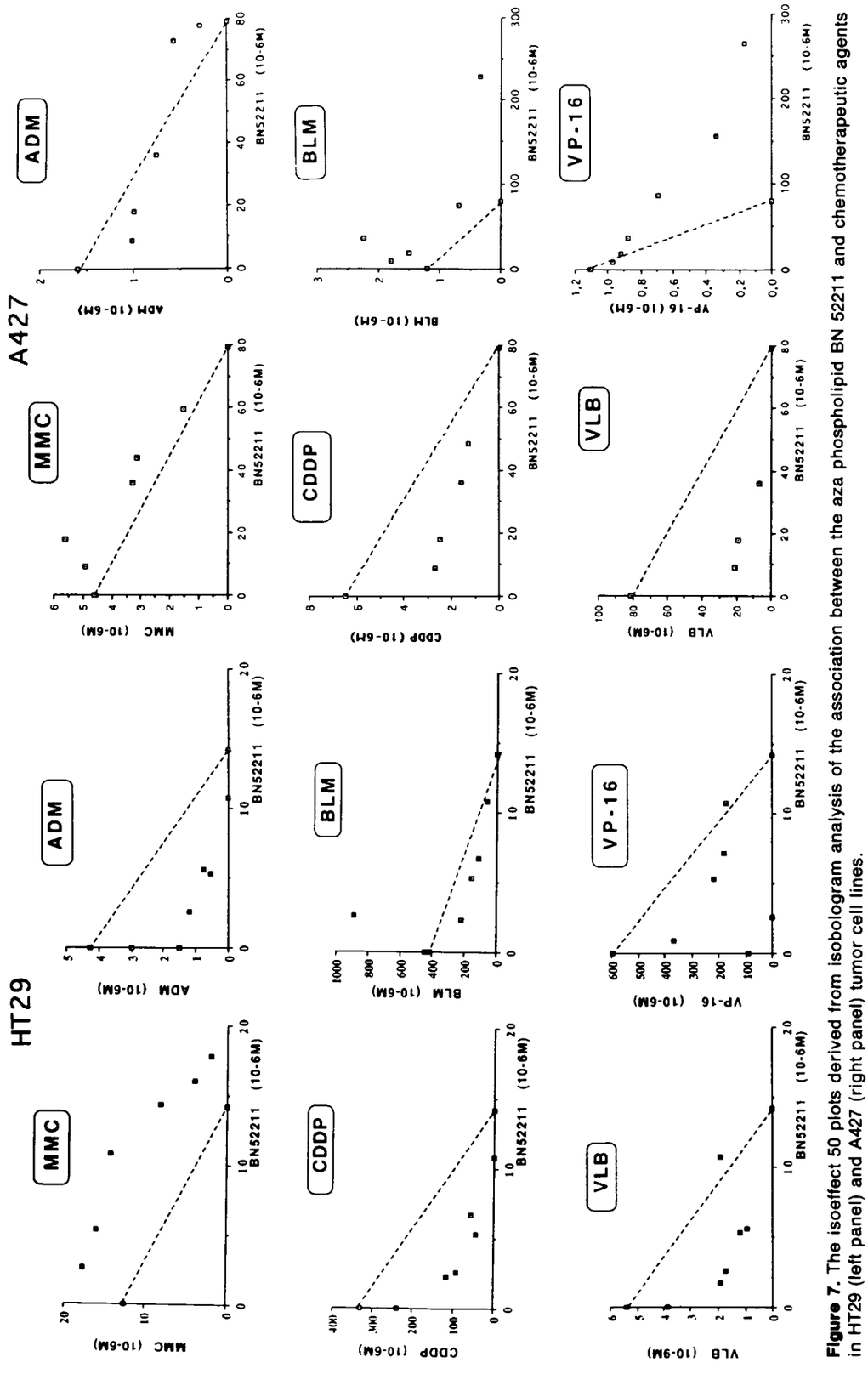

Figure 7. The isoeffect 50 plots derived from isobologram analysis of the association between the aza phospholipid BN 52211 and chemotherapeutic agents in HT29 (left panel) and A427 (right panel) tumor cell lines.

the isobolograms obtained from the association between the aza-alkylphospholipid BN 52211 and the chemotherapeutic agents. In HT29 cells, a supra-additive relationship has been found in the case of ADM, VP-16, CDDP and VLB. In A427 cells, these latter two drugs also behave in synergy with BN 52211. In A427 cells, the association between BN 52211 and ADM results in a synergistic effect for low doses of both drugs but an antagonistic relationship has been found for higher doses. For both cell lines, a sub-additive relationship has been found with MMC. In A427 cells, a sub-additive relationship exists between BN 52211, BLM and VP-16. In HT29 cells, the association between BN 52211 and BLM for the isoeffect 50 indicates a tendency towards an additive effect which has been confirmed by isobologram analysis of the isoeffect 70.

Discussion

Combinations of drugs are used clinically for the therapeutic advantages they may provide over single agents. We have studied the interaction between four ether phospholipids, i.e. edelfosine (ET-18-OCH_3), ilmofosine (BM 41.440), BN 52205 and BN 52211, and several chemotherapeutic drugs chosen because of their different cytotoxic properties. The drugs were ADM, CDDP, VLB, VP-16, MMC, BLM and MTX. The analysis has been carried out on two human carcinoma cell lines, A427 (lung) and HT29 (colon), which have expressed different sensitivity to ether phospholipid cytotoxicity. MTX is the only agent for which the association with ether phospholipids has not been possible because of the difficulty in calculating the IC_{50} and IC_{70} values due to the moderate dose–response obtained in both cell lines. Evaluation of the association of ether phospholipids with chemotherapeutic agents has been performed using isobologram analysis, which represents a precise and widely used method to validate drug interactions. Our data demonstrate that combinations of ether phospholipids and chemotherapeutic agents very often result in potentiation of the single drug cytotoxic effect. A constant synergistic effect has been obtained for each ether phospholipid associated with CDDP and ADM in both cell lines. Interestingly, VLB has a supra-additive relationship with each ether phospholipid but there are two exceptions: in HT29 cells, it has an additive effect in association with BN 52205 and, in A427 cells, it has a sub-additive effect with ET-18-OCH_3. This result emphasizes not only the analytical capability of the isobologram analysis but also the importance of the diversity of the biological target. The latter may explain why, on ovarian adenocarcinoma cells, neither synergism nor antagonism was found from the association between ET-18-OCH_3, ADM and CDDP.[34] However, a synergistic enhancement of the antiproliferative activity of BM 41.440 in association with CDDP was obtained in Walker carcinoma cells.[35] In conclusion, the cytotoxic activity of ether phospholipids is stronger when these drugs are used with conventional chemotherapeutic agents. This observation prompts investigation of this relationship in *in vivo* studies. Although the mechanisms at the base of the synergistic relationship are not yet known, a positive outcome of these studies will not only reinforce the originality of the cytotoxic action of ether phospholipids but will also introduce new alternatives in cancer chemotherapy.

Conclusion

A synergistic relationship has been found between the cytotoxic activity of ether phospholipids tested in association with CDDP and ADM in HT29 and A427 human tumor cell lines. The thio ether phospholipid BM 41.440 acts in synergy with VLB in both cell lines while the aza phospholipid BN 52205 has the same relationship with MMC in A427 cells. In conclusion, the cytotoxic effect of ether phospholipids is enhanced in association with spindle poisons and DNA-interactive drugs.

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