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Antiproliferative effect of complexes of platinum (II) with plasmanyl-(*N*-acyl)-ethanolamine, an inhibitor of protein kinase C

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Antiproliferative activities of combinations of semisynthetic plasmanyl-(*N*-acyl)-ethanolamine [PNAE(s)], an inhibitor of protein kinase C, with two antitumor complexes of platinum (II) [cisplatin and ammine(cyclopentylamine)-*S*-(−)-malatoplatinum (cycloplatam)] were investigated. The exposure of human melanoma BRO cells in culture simultaneously with cisplatin (1–10 μM) and PNAE(s) (100 μM–1 mM) in a molar ratio of 1/100 for 24 h induced a considerable decrease in the ability of these cells to incorporate [^{3}H]thymidine into DNA. A considerable antiproliferative synergism of these agents was observed. The effect of cycloplatam/PNAE(s) combination in similar experiments was significantly different from cisplatin/PNAE(s), i.e. interaction of these agents was complex and synergism was not found.

Key words: Antiproliferative activity, cisplatinum, cycloplatam, drug combination, plasmanyl-(*N*-acyl)-ethanolamine.

Introduction

We have established recently that PNAE(s), which are alkyl-phospholipids shown by Kara *et al.*[1] to exhibit antitumor activity towards a number of mouse and human models in culture, depressed protein kinase C (PKC) isolated from human melanoma BRO cells.[2] Many alkyl-phospholipids are known to be inhibitors of PKC and simultaneously enhance the antiproliferative activity of cisplatin.[3,4] Although the correlation between these events was not obvious, the data suggest that it may be possible to raise the antiproliferative effect of cisplatin by semisynthetic plasmanyl-(*N*-acyl)-ethanolamine [PNAE(s)].

The effect of two coordination compounds of platinum (II), i.e. cisplatin and ammine(cyclopentylamine)-*S*-(−)-malatoplatinum (II) (cycloplatam), was studied on human melanoma BRO cells in culture. The antitumor activity of cisplatin is well known;[5] it is used in clinical practice for both mono- and combination therapy. Therefore, it seemed interesting to test the antiproliferative activity of cisplatin in combination with PNAE(s). In contrast, cycloplatam is a new second-generation complex which is now under phase I clinical trial. It has been synthesized in the Institute of Common and Inorganic Chemistry in Russia.[6] Cycloplatam differs from cisplatin with regard to the spectrum of sensitive experimental tumors. For this reason we decided to study the cycloplatam/PNAE(s) combination.

Materials and methods

Chemicals

[^{3}H]Thymidine (45 Ci/mmol) was purchased from the Radiochemical Centre, Institute of Nuclear Physics (Uzbekistan). All chemicals were purchased from Sigma (USA). Cisplatin was obtained from Lachema (Czechoslovakia). Cycloplatam was obtained from the Institute of Common and Inorganic Chemistry, Russian Academy of Sciences (Russia). The preparation of PNAE(s) was kindly donated by Dr J Kara (Laboratory of Evolutionary Biology, Czechoslovakia). The culture of BRO cells was obtained from the Laboratory of Experimental Models, Cancer Research Centre, Russian Academy of Medical Sciences (Russia).

Cells and culture techniques

The unpigmented BRO cell line was obtained from a patient with malignant melanoma. Cells were

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cultured in RPMI 1640 medium (Flow Laboratories, UK) supplemented with 10% (v/v) heat-inactivated (30 min, 56°C) fetal bovine serum and 50 μg/ml of gentamicin (Pharmachim, Bulgaria) in tissue culture flasks (Costar, UK). Cells were incubated in an atmosphere of 5% CO_2 in air at 37°C.

Cell proliferation

Cell proliferation was determined by measuring the incorporation of [^{3}H]thymidine into DNA. [^{3}H]-Thymidine (10 μCi/sample), 50 μM unlabeled thymidine and 5 μM deoxycytidine were added to 5×10^4 cells in 96-well plates. After 2 h of incubation, the excess of labeled thymidine was removed and cells were suspended in 20 mM EDTA. The cells were lysed with 10% (w/v) trichoroacetic acid and cell lysates were collected on fibroglass filters with a Scatron Cell Harvester. Radioactivity of filters was determined by liquid scintillation counting. The depression of [^{3}H]thymidine incorporation into DNA was examined after exposure of cells to drugs for 24 h. The compounds were added to the cell culture 24 h after passage.

Results

The action of PNAE(s) combined with a complex of platinum (II) on the proliferation of human melanoma BRO cells in culture was studied. Two antitumor coordination compounds of platinum, i.e. cisplatin and cycloplatam, were tested. Our data indicate that BRO cells were sensitive to PNAE(s), cisplatin and cycloplatam with values of IC'_{50} = 750 μM, IC''_{50} = 6.2 μM and IC'''_{50} = 2 μM, respectively (Figure 1). These preliminary values allowed us to choose concentrations for combining agents. Thus, the concentration interval for both complexes of platinum was 1–10 μM and for PNAE(s) was 100 μM–1 mM (molar ratio 1/100).

The data in Figure 2(A) indicates that the cisplatin/PNAE(s) combination significantly depresses thymidine incorporation into DNA of cells. To clarify the character of the antiproliferative interaction of the drugs, the qualitative analysis of the dose–effect relationship discussed by Chou and Talalay was applied.[8] According to this method we then computed the combination index (CI) with respect to the depression of cell proliferation ['fraction affected' (Fa)] occurring under the action both

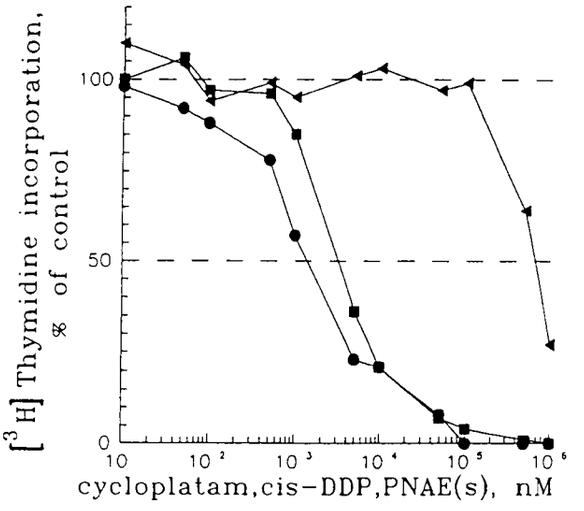

Figure 1. Depression of [^{3}H]thymidine incorporation into DNA of melanoma BRO cells treated with PNAE(s) (▲), cisplatin (■) and cycloplatam (●). The experiment was repeated 6 times. The data represent a typical experiment.

of each distinct agent in its own concentration interval, and their combination in molar ratio used (e.g. Fa = 0.4 equates to 40% depression of cell proliferation). The nature of the agents' relationship is judged by the value of CI. As outlined by these authors[8] CI yields a value of CI = 1 when summation is indicated, CI > 1 points to an antagonistic relationship and CI < 1 indicates synergism. We found CI < 1, which indicates that these drugs act synergistically at the concentrations used.

Figure 3(A) shows the effects of the cycloplatam/PNAE(s) combination on the proliferation of BRO cells, and Figure 3(B) represents the CI function. It is obvious that the nature of the agents' relationship is considerably different from that of cisplatin/PNAE(s). The plot of CI crosses over the value CI = 1 when Fa = 0.4. This means that when Fa < 0.4 (the inhibition of cell proliferation is less than 40%) these agents act synergistically; when Fa = 0.4 a summation of action occurs and when Fa > 0.4 an antagonism is indicated.

Discussion

Evidence indicates that many alkyl-phospholipids modulate the antiproliferative activity of platinum complexes. Some authors, e.g. Noseda *et al.*[4] who investigated the combination of 1-thyohexadecyl-2-

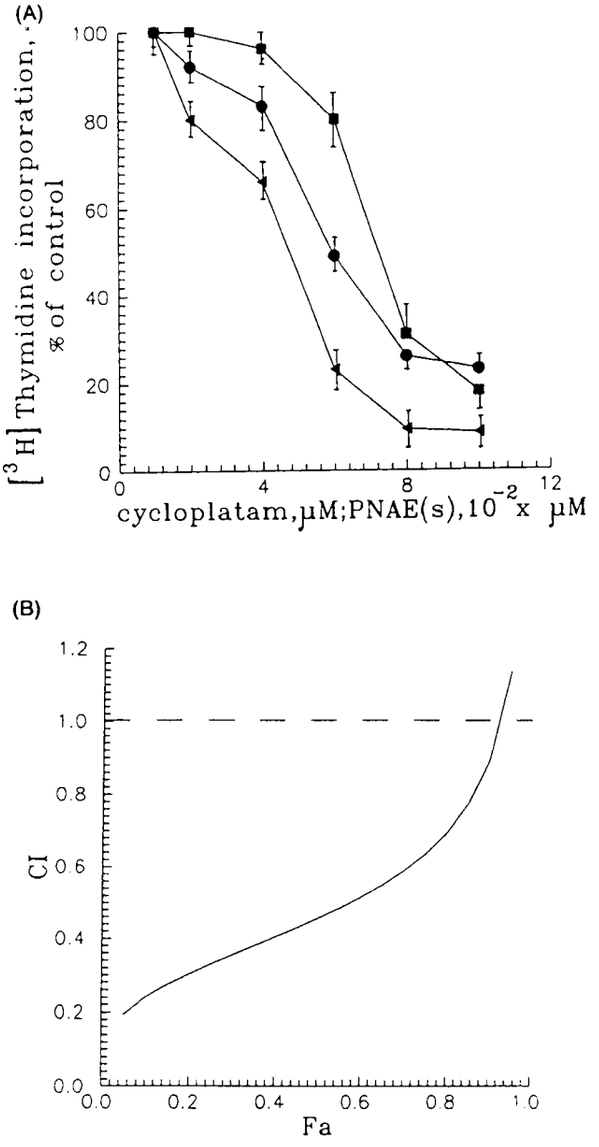

Figure 2. (A) Inhibition of proliferation of melanoma BRO cells by cisplatin (●), PNAE(s) (■) and a 1/100 combination of cisplatin/PNAE(s) (▲). Points are means of six replicate samples taken from a representative experiment; bars, SE. The experiment was repeated 3 times. (B) Computed plot of CI with respect to inhibition of cell proliferation expressed Fa. Computation was performed according to Chou and Talalay[8] employing data from (A).

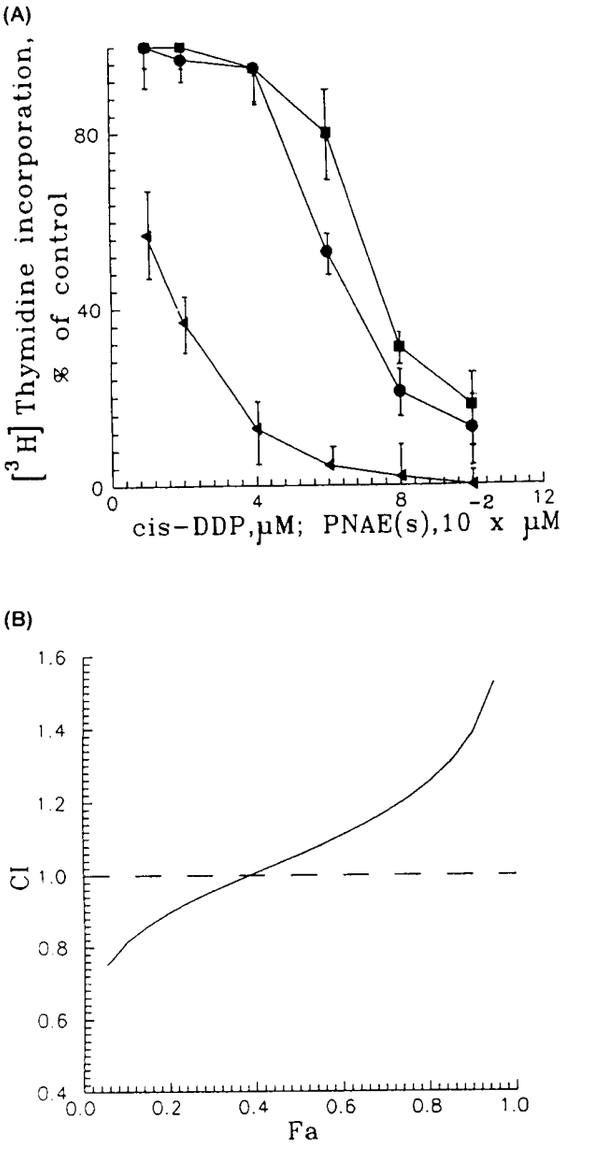

Figure 3. (A) Inhibition of proliferation of melanoma BRO cells by cycloplatam (●), PNAE(s) (■) and a 1/100 combination of cycloplatam/PNAE(s) (▲). Points are means of six replicate samples taken from a representative experiment; bars, SE. The experiment was repeated 3 times. (B) Computed plot of CI with respect to inhibition of cell proliferation expressed as Fa. Computation was performed according to Chou and Talalay[8] employing data from (A).

ethyl-1-rac-glycero-3-phosphocholin with cisplatin, reported an additive effect between these compounds. Other drugs show a synergistic modulation of cisplatin activity. For instance, Hofman *et al.*[3] found that the alkyl-phospholipid analog 3-hexadecyl-mercapto-2-methoxy-methyl-propyl-1-phosphocholine synergistically increased the antiproliferative activity of cisplatin towards Walker carcinoma cells *in vitro*.[3] With regard to cisplatin, we found evidence indicating a synergistical mechanism. Moreover, as discussed by Chou and Talalay,[8] the lower the CI, the higher the degree of

synergism. Interestingly, we showed extremely high ranges of synergism for the cisplatin/PNAE(s) combination. In the case of cycloplatam/PNAE(s) combinations, taking into account deviations of CI values from 1 upwards or downwards, the action generally seems to be simply additive.

The mechanism of enhancement of the antiproliferative activity of complexes of platinum is unknown. In our opinion, this enhancement, most likely, results from an easier influx for these compounds into the cell under the action of alkyl-phospholipids. The inclusion of exogenic amphiphilic molecules, such as PNAE(s), into the plasma membrane is now considered to lead to the formation of local lesions in the regular bilayer structure.[9] The PNAE(s) molecule has a small head group and three hydrophobic hydrocarbon chains, so it can probably either spread apart the host phospholipids or facilitate the formation of inverted structures. This eventually leads to the formation of a large quantity of holes in the membrane that can accelerate the passive influx of platinum compounds into the cell. In fact, a 24–48 h incubation of HEp-2 cells with 50 μM PNAE(s) has been observed by Kara *et al.*[1] to induce membrane holes that can be clearly seen under scanning electron microscopy. Moreover, the modification of membrane phospholipids at the expense of PNAE(s) can promote the active transport of platinum complexes by non-specific systems, e.g. the one that provides the internalization of the cisplatin/transferrin complex.[10]

It seems likely that additional mechanisms, in particular modulation of PKC action, are involved in the enhancement of the antiproliferative activity of platinum complexes by alkyl-phospholipids. We recently established that PNAE(s) can inhibit PKC activity in a cell-free system.[2] Inhibition of PKC may trigger a cascade of reactions that eventually lead to modulation of the action of platinum complexes.

Our data show that the antiproliferative activity of cisplatin/PNAE(s) combinations is different from that of cycloplatam/PNAE(s) combinations. The nature of the interaction of complexes of platinum and alkyl-phospholipids is likely to depend on the properties of the platinum drug. DNA is known to be the main target for platinum complexes. It has been found that damage of DNA by cisplatin is higher than that by cycloplatam.[11] Cisplatin induces two polypeptides with molecular weights of 12 and 45 kDa in the nuclear matrix that are absent in intact cells or in cycloplatam-treated cells.[12] In addition, the dynamics of DNA synthesis in cells treated with cisplatin are not the same as in cells treated with cycloplatam. However, not only the DNA, but also other parts of the cell, may be affected by platinum complexes. The cytotoxic effect of platinum compounds may result from two events: transient action on signal transduction systems and long-term effect on DNA.[13] Recently, we have shown that one of the points affected by platinum drugs is PKC, which could be irreversibly inhibited by both cisplatin and cycloplatam in cell-free systems.[14] Our unpublished data also indicate that cisplatin is a more potent inhibitor of PKC activity and of phosphorylation of PKC substrates that cycloplatam. Thus, the difference between the antiproliferative effects of cisplatin/ PNAE(s) and cycloplatam/PNAE(s) may reflect, firstly, a different action of these platinum drugs on cell targets and, secondly, that different cell systems are affected by these agents.

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