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ORIGINAL ARTICLE

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Potentiation of cisplatin and carboplatin cytotoxicity by amphotericin B in different human ovarian carcinoma and malignant peritoneal mesothelioma cells

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Abstract An in vitro study of the combined cytotoxicity of either cisplatin (CDDP) or carboplatin and amphotericin B (AmB) was undertaken on a set of different ovarian carcinoma (IGROVI, IGROVI-C10, OAW42) and peritoneal malignant mesothelioma (CFB-CARP1) cell lines and ascitic cells freshly obtained from ovarian cancer patients so as to investigate the possibility of overcoming their resistance to platinum compounds. Growth-inhibition curves obtained 6 days after a 2-h period of exposure to the drugs showed that AmB at 5–10 mg/l allowed a 5- to 10-fold decrease in the 50% growth-inhibitory concentrations (IC_{50}) of CDDP and carboplatin on either sensitive or resistant cells. Intracellular platinum assays with IGROVI cells showed that AmB acted by increasing dramatically the platinum uptake at a proportion that accounted for the increase in cytotoxicity. In the subline IGROVI-C10, a 10-fold resistant subline of IGROVI, AmB at 10 mg/l allowed recovery to the level of sensitivity seen in the parental cell line in the absence of AmB but not to the level observed in the presence of AmB. Acquisition of resistance mechanisms that are independent of the regulation of platinum uptake might be involved in this cell line. Thus, AmB might act by increasing the intracellular concentration of platinum without modifying the resistance mechanism involved downstream. However, in our models an increase in the intracellular level of platinum was always sufficient for the recovery of chemosensivity in vitro. We also show that the phosphodiesterase inhibiting methylxanthines act synergistically with AmB. The latter drugs are weakly toxic and could also attenuate the nephrotoxicity of AmB.

Key words Ovarian carcinoma · Malignant mesothelioma · Carboplatin · Cisplatin · Amphotericin B

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Introduction

The platinum derivatives have constituted a major advance in the treatment of solid tumors such as ovarian carcinoma, testicular cancer, esophageal carcinoma, and head and neck cancers. However, many tumors show a natural or acquired resistance to cisplatin (CDDP) that does not involve multidrug resistance and is responsible for most therapy failures. Different mechanisms of resistance have been described, including a decrease in the intracellular accumulation of CDDP [19]; an increase in the intracellular level of glutathione and metallothioneins, which are associated with an important decrease in interstrand linkage [8]; and an increase in DNA-repair activity [6]. Mitochondrial modifications also seem to be involved in a yet unidentified way in acquired resistance to CDDP [1].

This resistance can be overcome by the use of either other chemotherapeutic agents acting via a different cytotoxic mechanism, such as Taxol [12], or platinum derivatives that do not show cross-reactivity in the resistance mechanism, such as the Pt(IV) derivatives [9], or drugs that help to overcome resistance mechanisms. Such is the case, for instance, for cyclosporin, which specifically inhibits the expression of some oncogenes [14], particularly those involved in signal transduction, and for 3-aminobenzidine and methylxanthine, which induce a pharmalogical modification of DNA-repair mechanisms developed by the cells during acquisition of the resistant phenotype [4, 15, 18], or other molecules that induce a decrease in mechanisms of detoxication [8]. However, the extrapolation of these in vitro assays to the pathological situation in vivo is very difficult, and no modulator suitable for clinical use is yet available.

Nevertheless, amphotericin B (AmB), an antifungic molecule used in the clinical setting, seems to offer perspectives for reversal of the resistance phenomenon by increasing the intracellular accumulation of CDDP in several cell lines in vitro [10, 16, 17, 21]. AmB is a polyenic hapten that can form insoluble complexes with membrane sterols, thus inducing alterations in cell permeability. This property, as well as its clinical use as an antifungic, makes it a potential candidate as a modulator of the cytotoxicity of platinum derivatives, although its systemic use is limited by its nephrotoxicity, which is cumulative to that of CDDP. However, its use can be considered either for local treatment of some solid tumors such as ovarian carcinomas or in association with non nephrotoxic platinum derivatives such as carboplatin.

Therefore, we chose to study the possibility of overcoming resistance to CDDP and carboplatin by using them in combination with AmB. Our work concerns various cancers with a peritoneal dissemination, accessible for local treatment, such as ovarian carcinoma and malignant peritoneal mesothelioma. The conventional therapy of these malignancies involves platinum derivatives, but they frequently present intrinsic or acquired resistance to these compounds. The present paper describes an in vitro study of the combined cytotoxicity of either CDDP or carboplatin and AmB on a set of different ovarian carcinoma and peritoneal mesothelioma cell lines and ascitic cells freshly obtained from patients. We also studied the effects of the phosphodiesterase-inhibiting methylxanthines at the same time as those of AmB. These molecules may act by inducing an excess of cyclic adenosine monophosphate (cAMP), which could lead the cells to start a new cell cycle before repairing their DNA lesions, leading to an increase in the cytotoxicity of DNA-damaging agents [15, 18, 22]. The use of various modulators acting at different levels in the cellular pharmacology of platinum compounds appears promising from the viewpoint of reduction of chemoresistance.

Materials and methods

Cell lines

The cell lines IGROV1 and OAW42 were established from human ovarian adenocarcinomas [3, 23]. IGROV1 was provided by Dr. J. Bénard and OAW42 was obtained from the ECACC (Cerdic, Nice, France). An IGROV1 subline (IGROV1-C10) that is 10 times more resistant to CDDP than the parental cell line was established in the laboratory after 3 cycles of 3×2-h treatments with increasing doses of CDDP starting at 5 mg/l. The cell line CFB-CARP1 was obtained as described below from the ascitic fluid of a patient treated at the Centre François Baclesse for peritoneal carcinosis. This is a cell line of differentiated malignant mesothelioma of the epithelial type.

IGROV1 and CFB-CARP1 cells were grown in 25-cm^2 flasks (Falcon) in RPMI 1640 medium (Eurobio) supplemented with 10% fetal calf serum, 2 m*M* glutamine, penicillin 100,000 UI/l, streptomycin at 1 mg/l, 33 m*M* sodium bicarbonate, and 20 m*M* HEPES. The cell line OAW42 was cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, bovine insulin at 20 UI/l (Gibco), 1 mm sodium pyruvate, 2 m*M* glutamine, and antibiotics as described above. The cultures were split twice a week by mild trypsinization and replated at 500,000 cells/flask. Cell viability was assessed by the trypan-blue exclusion test. In these conditions the doubling time was 20 h for IGROV1 and CFB-CARP1 cells and 38 h for OAW42 cells. The absence of mycoplasma was assessed using 4,6-diamino-2-phenylindol dihydrochlorate (Boehringer-Mannheim) fluorescent staining and an immunological test against four strains (Boehringer-Mannheim).

Ascitic cells

Ascitic fluids were collected during the normal treatment of patients. For 15 min following the puncture the ascitic fluid was centrifuged at 1,200 rpm. The supernatant was discarded and the cell pellet was resuspended in RPMI 1640 supplemented with 20% fetal calf serum. The suspension was plated into culture flasks. The mesothelial cells were eliminated by partial trypsinization. When the growth became faster the supplementation with fetal calf serum was progressively reduced to 10%.

Tumor and epithelial markers (cytokeratins, carcinoembryonic antigen, CA125, CA 15–3) were revealed by immunocytochemistry on cells grown on coverslips and fixed in paraformaldehyde according to conventional protocols (Dako). Sensitivity to various anticancer drugs was established as soon as the early passages in short (2 h) or long periods of exposure (4 days). We thus obtained three cultures referred to as AU1, CR1, and FOR1.

The AU1 cells came from a patient with a peritoneal ovarian tumor of mixed mullerian origin before any treatment. At the second passage these cells exhibited moderate sensitivity to CDDP, 5-fluorouracil, and Adriamycin after a 4-day period of exposure and displayed resistance to CDDP after a 2-h exposure period. The results depicted in Fig. 7 were obtained after the second passage.

The CR1 cells were obtained from the ascites of a patient with an ovarian cancer of serous papillary cystadenocarcinoma type prior to any treatment. The results shown in Fig. 7 were obtained after the fifth subculture. These cells were immediately resistant to CDDP, but the mesothelial cells contaminating the culture during the cytotoxicity test could have altered the interpretation.

The FOR1 cells were obtained during the recurrence of an ovarian adenocarcinoma of serous papillary type after four treatments with a combination of Adriamycin, cyclophosphamide, and CDDP at 50 mg/m^2. The results shown in Fig. 7 were obtained at

the second passage. These cells were slightly less sensitive than IGROV1 cells to CDDP.

Cytotoxicity test

The cells were distributed in a 96-well microplate (Falcon) at 10,000 cells/well. After 48 h of incubation at 37 °C the cells were rinced twice with Hanks' balanced solution and then incubated for 2 h in the presence of increasing concentrations of the different drugs diluted in serum-supplemented medium. At the end of the exposure period the cells were rinced twice with Hanks' balanced solution and replaced in complete RPMI 1640 medium for 6 days at 37 °C. The cell viability was assessed according the procedure of Scudiero et al. [20]. In brief, 50 µl of a solution containing XTT at 1 mg/ml (Sigma) and phenazine methosulfate at 7.65 µg/ml (Sigma) in RPMI 1640 was added to each well. After incubation for 4 h at 37 °C the optical density at 450 versus 650 nm was measured by a microplate reader (Molecular Devices). Each value represents the mean of triplicate determinations. Standard errors were always below 10% and were not plotted. One representative experiment of at least three is shown. In the tests involving AmB the cells were grown for 3 days in an antibiotic-free medium and then treated according to the protocol described above. Carboplatin (Paraplatine) and AmB (Fungizone) were graciously provided by Bristol-Myers Squibb.

Measurement of intracellular platinum

The cells were treated in 25-cm^2 flasks and then dissociated by trypsinization, centrifuged, and resuspended in 500 µl ultrapure water (Milli-Q, Millipore). This suspension was then sonicated for 30 min and slowly frozen and thawed three times to lyse the cells. Lysates were diluted in 0.1 *N* HCl prior to the platinum assay by atomic absorption spectrophotometry using a Perkin-Elmer 3030 Zeeman apparatus with a graphite furnace. Samples were dried at 100° and then 140 °C, mineralized at 1,400 °C, and, finally, atomized at 2,700 °C. The protein content was assayed according to the technique of Bradford using Biorad reagents and bovine serum albumin (Merck) as the standard.

Results

Figure 1 shows the cytotoxicity of CDDP alone (Fig. 1A) or in presence of AmB at 10 mg/l (Fig. 1B) as a function of the duration of exposure of the cell line IGROV1. The cytotoxicity of CDDP was weak after 1 h

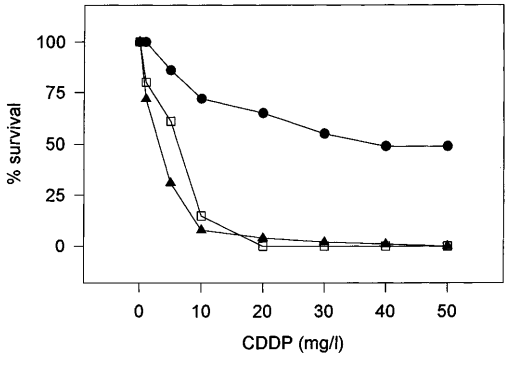

Fig. 1 Cytotoxicity of CDDP alone after various periods of exposure as observed in IGROV1 cells (● 1 h, □ 2 h, ▲ 4 h)

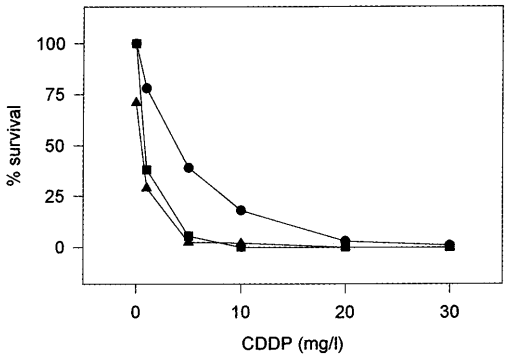

Fig. 2 Cytotoxicity of CDDP after a 2-h period of exposure in the presence of various concentrations of AmB as observed in IGROV1 cells (● CDDP alone, ■ AmB at 5 mg/l, ▲ AmB at 10 mg/l)

and reached a stable level after 2 h. In the presence of AmB at 10 mg/l the cytotoxicity was greatly enhanced, with a maximal effect being observed after 2 h of exposure. We thus chose 2 h of exposure for subsequent experiments. In these experimental conditions, comparable with the clinical exposure, the 50% growth-inhibitory concentration (IC_{50}) of CDDP is around 5 mg/l. In the presence of various doses of AmB (Fig. 2) we observed a dose-dependent potentiation of the cytotoxicity. AmB at 5 mg/l reduced 5-fold the IC_{50} of CDDP ($P < 0.01$). The Fungizone excipient, sodium desoxycholate, had no effect on the cytotoxicity of CDDP at the concentrations used in these experiments (results not shown). AmB alone was cytotoxic only at doses higher than 10 mg/l, and its IC_{50} was around 40 mg/l.

On IGROV1 cells, 2 h of exposure to carboplatin led to weak cytotoxicity; the IC_{50} could not be obtained at 500 mg/l (Fig. 3). This could have been due to a slow incorporation of carboplatin in these cells, since 24 h of exposure elicited a dramatic increase in cytotoxicity, leading to an IC_{50} of around 25 mg/l. A potentiating effect was found with AmB after 2 h of exposure, the IC_{50} was about 100 mg/l for carboplatin together with 10 mg/l for AmB.

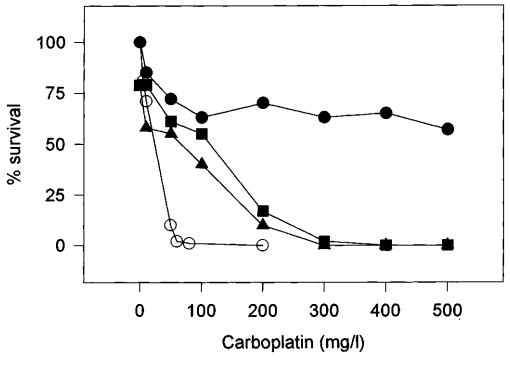

Fig. 3 Cytotoxicity of carboplatin after a 2-h period of exposure in the presence of various concentrations of AmB as observed in IGROV1 cells (● Carboplatin alone, ■ AmB at 10 mg/l, ▲ AmB at 20 mg/l, ○ carboplatin alone after 24 h of exposure)

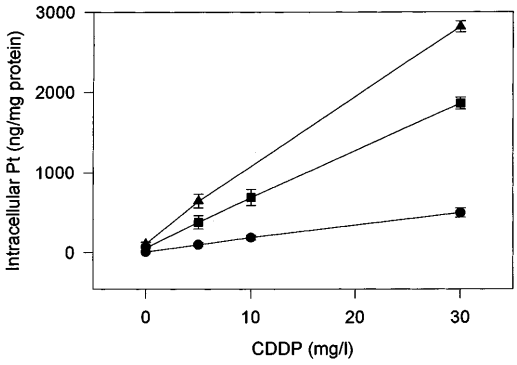

Fig. 4 Intracellular platinum accumulation as observed in IGROV1 cells immediately after a 2-h period of exposure to various concentrations of CDDP in the absence (●) or presence of AmB at 5 mg/l (■) and 10 mg/l (▲)

Measurement of the intracellular platinum content (Fig. 4) showed that the uptake was linearly dependent of the dose and that AmB enhanced this uptake at a proportion that accounted for the increase in cytotoxicity, e.g., AmB at 5 mg/l elicited a 3.6-fold increase in the intracellular platinum level and a 5-fold decrease in the IC_{50}.

The subline IGROV1-C10, which was obtained by successive exposure to CDDP, exhibited 10-fold greater resistance to CDDP as compared with the parental cell line (Fig. 5). This resistance could be overcome in the presence of AmB at 10 mg/l, which allowed recovery to the parental-cells IC_{50} obtained without AmB ($P < 0.005$). At 5 mg/l AmB a 5-fold decrease in the IC_{50} was accompanied by a 2-fold increase in the intracellular level of platinum. We looked for the possibility of reversing more completely the resistance of cell line IGROV1-C10 using methylxanthines at the same time as AmB. The results we obtained showed that pentoxifylline (Fig. 6) and theophyllin (results not shown) acted synergistically with AmB to potentiate the cytotoxicity of CDDP. Similar results were obtained with a subline of CFB-CARP1, which became 2 times more resistant to CDDP after successive in vitro exposure to the drug.

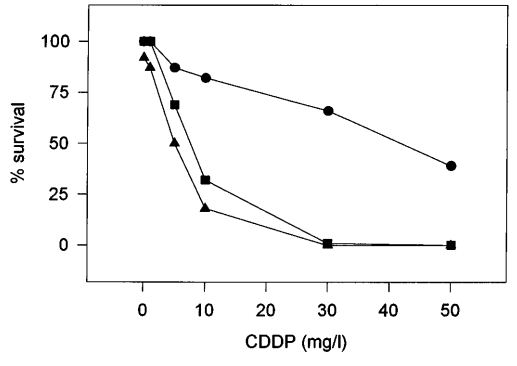

Fig. 5 Cytotoxicity of CDDP after a 2-h period of exposure in the presence of various concentrations of AmB as observed in IGROV1-C10 cells (● CDDP alone, ■ AmB at 5 mg/l, ▲ AmB at 10 mg/l)

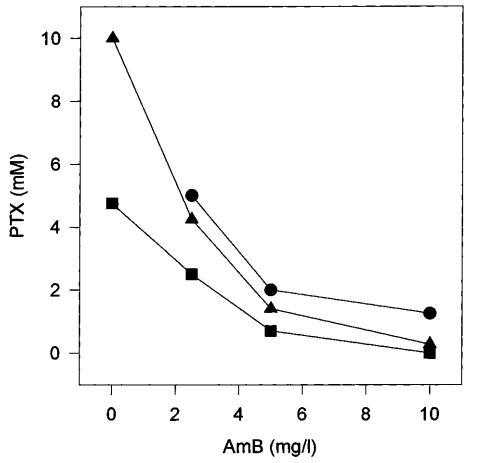

Fig. 6 Isobolograms of the potentiation of CDDP cytotoxicity by pentoxifylline (*PTX*) and AmB as observed in IGROV1-C10 cells. Isobolograms were established using the cytotoxicity curves obtained after a 2-h period of exposure of cells to various concentrations of PTX and AmB in the presence of CDDP at 10 mg/l (● IC_{50}, ▲ IC_{40}, ■ IC_{25})

Figure 7 summarizes the results obtained in the different ovarian carcinoma (IGROV1, OAW42) and malignant mesothelioma (CFB-CARP1) cell lines and in ovarian ascitic cells (FOR1, CR1, AU1) with CDDP and carboplatin. In all cases, AmB at 10 mg/l decreased the IC_{50} by a factor of 5–10, except in OAW42 cells, which

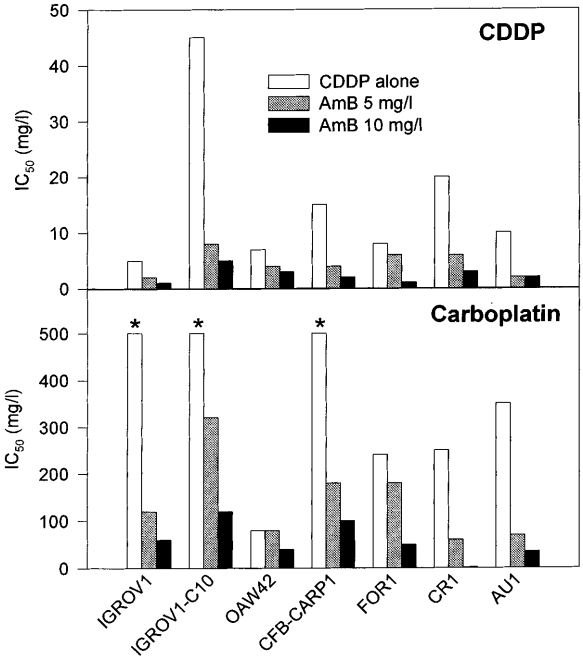

Fig. 7 IC_{50} of CDDP or carboplatin after a 2-h period of exposure in the absence or presence of various concentrations of AmB as observed in various human cell lines (*IGROV1, OAW42* Ovarian adenocarcinoma cell lines; *IGROV1-C10* in-vitro-established CDDP-resistant subline of IGROV1; *CFB-CARP1* peritoneal mesothelioma cell line; *FOR1, CR1, AU1* ascitic cells freshly obtained from ovarian carcinoma-bearing patients; * $IC_{50} > 500$ mg/l)

exhibited a very high degree of sensitivity to carboplatin in the absence of AmB.

Discussion

AmB modulates in a dose-dependent way the cytotoxicity of CDDP in cell line IGROV1. In the presence of each concentration tested the IC_{50} was lower than 1 mg/l. Taking into account the cytotoxicity of AmB itself, 5 mg/l appears to be the optimal concentration since it allows a reduction in the IC_{50} by 5 orders of magnitude. This AmB concentration is slightly above the plasmatic peak obtained 6–8 h after an intravenous injection of 1 mg/kg, which is about 3–4 mg/l [5]. Similar results were obtained in all the cell lines and ex vivo cultures studied, independently of their level of sensitivity to CDDP. This shows the potentiality of AmB as a modulator in vitro. These results confirm those obtained in different tumor cell lines of various origin, including ovarian cell lines. In IGROV1 cells our results show that this potentiation is accompanied by an increase in the intracellular uptake of platinum that is proportional to the increase in cytotoxicity. This result suggests that in this model, in agreement with different authors [10, 11, 16], AmB may act by modifying membrane permeability to CDDP. In the subline IGROV1-C10, 10 mg/l allowed recovery to the level of sensitivity of the parental cell line seen in the absence of AmB but not to the level observed in the presence of AmB. Acquisition of resistance mechanisms independently of the regulation of platinum uptake, such as an increase in the mechanisms of DNA-adduct repair, for instance, might be involved in this cell line. Thus, AmB could act by increasing the intracellular concentration of platinum but may not modify the resistance phenomenon involved downstream. However, in our models an increase in the intracellular level of platinum was always sufficient for the recovery of chemosensivity in vitro.

Our results also show that the potentiating effect of AmB is very efficient in terms of the cytotoxicity of carboplatin. Surprisingly, the IGROV1 and OAW42 cell lines, which show the same level of sensitivity to CDDP, exhibit very differing degrees of sensitivity to carboplatin. This difference is abolished when the cells are exposed for 24 h to carboplatin (IC_{50} of 25 and 30 mg/l for IGROV1 and OAW42, respectively), suggesting a different time course for incorporation of the drug. AmB is very active in potentiating carboplatin cytotoxicity when the incorporation of the platinum derivative is slow (IGROV1), whereas its activity is weak when carboplatin incorporation is rapid (OAW42). This suggests that, as with CDDP, AmB may act by increasing the cellular uptake of carboplatin.

Previous in vivo studies have yielded variable results [11, 21] but show that the efficiency of AmB is lower in vivo than in vitro, perhaps due to an inhibitory effect of serum proteins [2]. Furthermore, the CDDP-AmB association appears too nephrotoxic for clinical use. However, our results show that the AmB-carboplatin association is very efficient against ex vivo cultures of tumor cells from patients, including resistant ones, and against a spontaneously resistant malignant mesothelioma cell line. We also show that other modulators, such as the methylxanthines, act synergistically with AmB. These molecules are particularly interesting since they are weakly toxic and could attenuate the nephrotoxicity of AmB [7, 13]. Further studies in animal models using AmB-carboplatin and AmB-methylxanthine-carboplatin combinations are now necessary.

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