ENHANCEMENT OF PLATINUM-DRUG CYTOTOXICITY IN A HUMAN HEAD AND NECK SQUAMOUS CELL CARCINOMA LINE AND ITS PLATINUM-RESISTANT VARIANT BY LIPOSOMAL AMPHOTERICIN B AND PHOSPHOLIPASE A2-II

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ABSTRACT:

Platinum drugs comprise one of the main classes of chemotherapy drugs that can induce remissions in various solid tumors. Although tumors often regress on treatment with cis-diaminedichloroplatinum II (cisplatin) or cis-diammine-1,1-cyclobutane dicarboxylate platinum II (carboplatin), they usually relapse as a drug-resistant tumor. Most mechanisms of platinum resistance could be overcome by increasing the amount of drug that is accumulated by tumor cells. Amphotericin B (Amph B) is efficient at increasing platinum drug uptake, but because of nephrotoxicity associated with extended usage, and the potential for synergistic nephrotoxicity when used with platinum drugs, Amph B has not been used clinically for this purpose. A liposomal preparation of Amph B (LipoAmph B), which is substantially less nephrotoxic, was studied for its ability to enhance platinum-drug toxicity to a human oral squamous cell carcinoma line, HN-5a, and its carboplatin-resistant variant, 5a/carbono-15a, in which cisplatin accumulation was reduced by approximately 40%. Amph B at 10 μg/ml enhanced cisplatin accumulation by approximately 100% in both cell lines, enhancing cytotoxicity of the drugs by 35 to 60%, and completely reversed resistance to both cisplatin and carboplatin. LipoAmph B in the presence of phospholipase A2-II (PLA2-II) was able to enhance cisplatin and carboplatin cytotoxicity as effectively as free Amph B in both cell lines. At optimal concentrations, LipoAmph B plus PLA2-II enhanced drug uptake sufficiently to abolish resistance in the platinum-resistant line. Because PLA2-II is elevated in some tumor microenvironments and in plasma of ill patients, LipoAmph B has potential clinical usefulness as a modulator of platinum-drug efficacy.

Since the discovery of the anticancer activity of cis-diaminedichloroplatinum II (cisplatin; Rosenberg et al., 1969)1, this drug has become the mainstay of cytotoxic treatment of various neoplasms. Particularly, when used in combination protocols, cisplatin has greatly improved the treatment of several solid tumors (Durant, 1980). cis-Diammine-1,1-cyclobutane dicarboxylate platinum II (carboplatin) is effective against a similar spectrum of tumors, but causes less toxicity (Wagstaff et al., 1989). In spite of a good response to cisplatin and carboplatin, the majority of tumors recur, refractory to additional platinum treatment, resulting in the death of 90% of patients within 2 years (Wagstaff et al., 1989). To improve the success of this treatment, methods must be developed to enhance the antitumor effectiveness of this important class of drugs.

Because clinical chemotherapy is administered at the highest tolerable doses, a tumor need only acquire a 2- to 5-fold level of resistance to escape the maximum effect of a drug (Jekunen et al., 1992). A host of phenotypes has been described in association with platinum drug resistance, the four most common being: 1) reduced intracellular drug accumulation; 2) increased removal of platinum-DNA adducts; 3) increased expression of glutathione and/or glutathione-metabolizing enzymes; and 4) increased expression of metallothionein (Andrews and Howell, 1990; Eastman, 1991; Scanlon et al., 1991; Gately and Howell, 1993; Ferguson, 1995). Because these mechanisms of resistance are time and/or concentration dependent, each could be overcome, at least in part, by increasing the cellular accumulation of drug. Of a variety of agents that have been shown to reverse resistance, amphotericin B (Amph B) is the most universal with respect to the spectrum of cell lines, and most efficient in terms of degree of enhancement, by increasing the cellular accumulation of platinum drugs (Morikage et al., 1991a, 1993; Kikkawa et al., 1993; Sharp et al., 1994; Beketic-Oreskovic and Osmak, 1995). However, the danger of cumulative nephrotoxicity limits the clinically achievable plasma concentrations of Amph B to 7 to 20% of the concentration required to effectively enhance platinum-drug cytotoxicity to tumor cells (Sculier et al., 1988; Barriere, 1990; Morikage et al., 1991a). Therefore, the present study examined a liposomal preparation of Amph B, AmBisome, which may be used more safely in the

1 Abbreviations used are: cisplatin, cis-diaminedichloroplatinum II; carboplatin, cis-diammine-1,1-cyclobutane dicarboxylate platinum II; Amph B, amphotericin B; LipoAmph B, liposomal amphotericin B; HNSCC, head and neck squamous cell carcinoma; PLA2-II, phospholipase A2-II; FAAS, flameless (graphite furnace) atomic absorption spectroscopy; DC, deoxycholate.

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It is important that studies on cellular resistance to anticancer drugs be conducted in a model system that reflects the clinical situation as closely as possible. To this end, a 4-fold carboplatin-resistant variant was selected from a unique head and neck squamous cell carcinoma (HNSCC) cell line in two increments of carboplatin concentration, to reflect the resistance levels that are most likely to be observed clinically. This is the first HNSCC cell line selected for resistance to carboplatin.

Materials and Methods

Drugs. Carboplatin (CBDCA; JM-8; NSC 241240; Paraplatin) and cisplatin (BMY-25936; Platinol-AQ) were a generous donation from Bristol-Myers Squibb (Saint-Laurent, Quebec). Amph B was purchased as Fungizone (Life Technologies, Inc., Burlington, Ontario). Liposomal Amph B (LipoAmph B; as AmBisome) and empty liposomes were generously provided by NeXstar, Inc., (Boulder, CO). Melphanal (phenylalanine mustard) was a gift from Burroughs Wellcome (Research Triangle Park, NC). Ouabain was kindly provided by Dr. M. Karmazyn (Department of Pharmacology and Toxicology, University of Western Ontario).

Other Supplies. Cell culture plasticware was obtained from Life Technologies and Fisher Scientific (Unionville, Ontario). Cell culture medium (Dulbecco’s modified Eagle’s medium) and fetal bovine serum were purchased from Life Technologies. PLAA-II (bee venom, specific activity approx. 2400 U/mg) was purchased from Boehringer Mannheim Canada (Laval, Quebec). (one unit of PLAA-II activity is equivalent to 1 μmol of fatty acid released per minute). All other reagents were obtained through commercial sources.

Cell Culture. HNSCC cell line HN-5a was established at this institution from the gingival tumor of a patient not previously treated with chemotherapy or radiation (Lapointe et al., 1992). HN-5a was cloned from the primary HNSCC cell line by limiting dilution. Cells were maintained in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum and penicillin (50 U/ml)/streptomycin (50 μg/ml) (growth medium). Cultures were incubated in a humidified atmosphere of 5% CO2 at 37°C. The carboplatin-resistant cell lines were selected by culturing HN-5a cells in the presence of carboplatin without any exposure to mutagens. Colonies, which had propagated from single cells, were selected and expanded into separate variant lines. In the first step of selection, cells were exposed continuously for 3 weeks to 6 μM carboplatin (105 cells/60-mm Petri dish) or 10 to 20 μM carboplatin (104 cells/dish), with a weekly change of drug-containing medium. At concentrations less than 6 μM, cells grew to confluence. Distinct colonies were obtained from cells in 6 and 15 to 20 μM carboplatin, but those in 15 to 20 μM were unable to sustain proliferation in this concentration of drug. The cells in 10 μM carboplatin, not having formed distinct colonies at 2 weeks, were harvested collectively by trypsinization, and recultured at 105/dish for an additional 3 weeks in 10 μM carboplatin. This reduced the viable cell number to approximatively 10/dish, allowing propagation of colonies from single cells. Cells expanded from one of these colonies, 5a/carbo-10c, were then reselected in higher concentrations of carboplatin. Colonies were obtained after 3 weeks from 15 and 20 μM carboplatin, including the 5a/carbo-15a line used in these studies. Colonies were not obtained in higher concentrations of carboplatin. The carboplatin-resistant lines expanded from individual colonies were exposed to drug at every alternate passage, and were maintained drug-free for at least 3 days before initiation of an experiment.

For the purpose of cytotoxicity assays, cultured cells were exposed to drug as reported previously (Ferguson and Cheng, 1987). Briefly, replicate flasks of rapidly proliferating cells were exposed to a range of drug concentrations, including modifiers where indicated. Drug exposure was initiated by addition of 0.2 volume of an appropriate concentration of the agent of interest, in growth medium. At initiation of drug exposure and after 4 days, cell numbers were determined using an electronic particle counter (Coulter Electronics, Hialeah, FL). The drug effect was measured by the inhibition of proliferation of the cultured cells as a percentage of the respective controls (absence of platinum drug). Controls for Fungizone included desoxycholate, the solubilizing agent for Amph B. IC50 and IC90 values were obtained by interpolation of plotted data. For experiments involving 4-h exposures to drugs, pretreatments were added to cultures 24 h after establishment of cultures, and after an additional 24-h incubation, cisplatin in growth medium was added directly (without changing medium). After 4 h, drug-containing medium was aspirated and substituted with growth medium, and cells were incubated for another 4 days.

Determination of Cisplatin Uptake. After 3 days of proliferation to approximately 50% confluence, replicate 25-cm2 flasks of cells were exposed to cisplatin by addition of drug as described above. Cells were exposed to 50 μM cisplatin for 4 h at 37°C, using as a background control a 0°C, 1-s exposure (nonspecific, external binding). After the 4-h exposure, samples were prepared according to Mann et al. (1990). Each flask was washed 3 x with ice-cold phosphate-buffered saline (0.15 M NaCl + 0.67 mM KH2PO4, pH 7.4), and then removed by scraping with a plastic scraper in 0.5 ml of Trition-HCl [0.1% (v/v) Triton X-100 in 0.35 N HCl (modified)]. Cells were disrupted by a cycle of freezing and thawing followed by sonication. The protein concentration was determined using Coomassie staining (BioRad reagent) (Bradford, 1976) for normalization of results. Duplicate samples of replicate flasks were analyzed by flameless (graphite furnace) atomic absorption spectroscopy (FAAS) using a Varian Automated Graphite Furnace Atomizer attached to a Varian Spectra 30 Atomic Absorption Spectrometer (Varian Canada, Georgetown, Ontario). Platinum concentrations were determined by the computer from a standard curve generated before each run.

Statistical Analysis. Statistical significance was determined using a 2-tailed Student’s t test, except for drug accumulation studies, which were analyzed by a paired t test.

Results

Establishment of a Carboplatin-Resistant Human Tumor Line That Is Cross-Resistant to Cisplatin. The HN-5a/carbo cell lines were selected under minimal stringency to establish resistant lines that would reflect phenotypes displayed by refractory patient tumors. The two-step carboplatin selection of the resistant variant cell line 5a/
Cells were exposed continuously over 4 days to the conditions presented, as controls for the combined exposure to carboplatin or cisplatin (Fig. 2). The data were obtained from the same experiments presented in Figs. 2 and 3. The concentration of empty liposomes at 200 μg/ml is equivalent to that of the liposomes present in a preparation of LipoAmph B with an Amph B concentration of 20 μg/ml. The combination of Amph B and desoxycholate is constituted by the use of Fungizone. The combination of Amph B and liposomes is constituted by the use of LipoAmph B. Statistical analysis (t test, compared with drug-free medium): *p < .05; **p < .01; ***p < .001.

carro-15a is detailed in Materials and Methods. The carboplatin-selected cell line had the same protein content as the parent line (Table 1). The proliferation rate of 5a/carbo-15a did not differ from the parent in the absence of drug, but was significantly slower in 20 μM carboplatin (a slightly higher concentration of carboplatin than the selection concentration). 5a/carbo-15a cells displayed a similar level of resistance to both carboplatin and cisplatin. This line was more sensitive than the parent HN-5a line to ouabain. 5a/carbo-15a cells were cross-resistant to melphalan, but sensitivity to cadmium was unaffected.

LipoAmph B in the Presence of PLA2-II Enhances Platinum Drug Cytotoxicity. LipoAmph B was compared with Amph B (Fungizone) for its ability to enhance platinum cytotoxicity in the parent HN-5a and carboplatin-resistant 5a/carbo-15a lines. As a control, the effect of the Amph B formulations on proliferation of both cell lines was tested each time, in the absence of carboplatin (Fig. 1). Nearly all of the treatments were somewhat inhibitory of proliferation, in an Amph B concentration-dependent manner, thus limiting the concentration of Amph B that could be used. The resistant line was more sensitive to inhibition. Addition of PLA2-II increased the antiproliferative activity of LipoAmph B. The combination of 25 μg/ml LipoAmph B and 400 U/liter PLA2-II, in the absence of any platinum drug, reduced proliferation of 5a/carbo-15a cells to only 10% of the control (data not shown). LipoAmph B (50 μg/ml) plus 200 U/liter PLA2-II killed all 5a/carbo-15a cells (not shown). Desoxycholate, the solubilizing agent for Fungizone, was used as a control for free Amph B. None of the formulations that were later used along with platinum drugs significantly inhibited proliferation in the absence of Amph B.
experiments) in the resistant subline, approximating the enhancement caused by free Amph B. Similar results were observed at the level of the IC90 for carboplatin (data not shown) as that presented for the IC50.

The combination of LipoAmph B and PLA2-II was subsequently tested for its ability to enhance cisplatin cytotoxicity in both cell lines, and again the enhancement was similar to that achieved with free Amph B (Fig. 3). In both cell lines, there was a significant enhancement of cisplatin cytotoxicity, in the presence of LipoAmph B, by the addition of PLA2-II. The incremental increase in PLA2-II concentration also caused a significant increase in enhancement of cisplatin cytotoxicity under the following conditions: 1) in HN-5a, 400 versus 100 U/liter PLA2-II in 25 μg/ml LipoAmph B (p,.05); 2) in 5a/carbo-15a, 200 versus 50 U/liter in the presence of 20 μg/ml LipoAmph B (p,.01); and 3) 100 versus 50 U/liter in 25 μg/ml LipoAmph B (p,.05).

A 24-h Exposure to LipoAmph B plus PLA2-II Enhances Cytotoxicity of a Subsequent 4-h Exposure to Cisplatin. Cellular platinum uptake, in the absence or presence of modulators, must be measured after 4 h to allow time for measurable amounts of drug to accumulate, but before the cytotoxic action is manifested. Therefore, the ability of Amph B to alter cisplatin cytotoxicity after a 4-h exposure was first assessed. Figure 4 demonstrates equivalent enhancement of cisplatin cytotoxicity in both cell lines by Amph B over a 4-h exposure. Toxicity due to Amph B alone was minimal.

The combination of LipoAmph B and PLA2-II was then tested for its effect on cytotoxicity of a 4-h cisplatin exposure. Figures 5 and 6 indicate the significant enhancement of cisplatin cytotoxicity by the LipoAmph B/PLA2-II combination in both the sensitive and resistant cell lines, respectively, equivalent to the effect observed during the continuous exposure. Because Amph B must be liberated from LipoAmph B by the digestion by PLA2-II, cells were incubated with the combination of LipoAmph B and PLA2-II for 24 h before the 4-h exposure to cisplatin. Because in previous experiments the empty liposomes were shown to have little effect on cisplatin toxicity over an extended period, in these experiments the influence of treatments on cisplatin toxicity were compared with cisplatin in the absence of any other treatment.

In HN-5a cells (Fig. 5), there were no statistically significant differences in the enhancement of cytotoxicity between the different concentrations of PLA2-II, although the trend was clear as the PLA2-II concentration was increased. For the 5a/carbo-15a cells (Fig.
The data presented above describe the ability of a liposomal preparation of Amph B, in the presence of PL2-II, to enhance cytotoxicity of platinum drugs by increasing drug accumulation in cultured tumor cells. Because anticancer drugs are dose limited by patient toxicities, the degree of resistance required for a tumor cell to survive a drug treatment can be conferred by a minor change in its phenotype. Therefore, an increase of only 50% in tumor cell platinum accumulation could be enough to overcome clinical levels of drug resistance.

Most mechanisms of resistance to platinum drugs could be circumvented, to some degree, by enhancing cellular accumulation of the drug, because the mechanisms rely on time dependence of repair or uptake, or a limited store of intracellular drug scavengers (e.g., glutathione). The carboplatin-resistant cell line used in this study appears to express the phenotypes of decreased drug uptake and increased glutathione content, without any change in metallothionein content. These resistance-associated phenotypes are evidenced by the increased sensitivity to ouabain (Ohmori et al., 1993) and decreased cisplatin uptake, cross-resistance to melphalan (Andrews et al., 1986) (putatively glutathione-dependent), and lack of change in sensitivity to cadmium (Singh et al., 1995) (Table 1). Increased glutathione could contribute to enhanced cisplatin efflux (Ishikawa and Ali-Osman, 1993) as well as increased DNA repair (Eastman, 1987; Andrews and Howell, 1990; Meijer et al., 1990). However, there is no evidence at present to indicate whether DNA repair is altered.

Amph B usually enhances accumulation and cytotoxicity of platinum drugs in both platinum-resistant and -sensitive cell lines (Morikage et al., 1993; Sharp et al., 1994; Beketic-Oreskovic and Osmak, 1995), but in some sensitive lines it has no effect (Morikage et al., 1993; Sharp et al., 1994; Beketic-Oreskovic and Osmak, 1995). Although the ionicophore effect of Amph B should not be ruled out completely as contributing to increased platinum uptake, the ability to enhance cytotoxicity in some cell lines and not others suggests a specific mode of action (Sharp et al., 1994).

It has been proposed that Amph B enhances uptake by acting directly on a putative, metal-regulatory, gated pore protein, inducing it to

**Discussion**

Cells were exposed for 24 h to the various treatments indicated, followed by concomitant 4-h exposure to cisplatin, then 4 days drug free. Control conditions in the absence of cisplatin (A) were assessed as a control against which to normalize the data from assays that included cisplatin (B). The IC<sub>50</sub> value for cisplatin for this set of experiments was 5.08 ± 1.20 μM (n = 5). Statistical analysis (t test, compared with cisplatin treatment alone): *p < .05; **p < .01.

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amph B required to reverse cisplatin resistance are in the range of 5 to 30 µg/ml (depending on the cell line), the clinically achievable plasma concentration is limited to 1 to 2 µg/ml (Sculler et al., 1988; Barriere, 1990; Morikage et al., 1991a). Also, the nephrotoxicity caused by free Amph B and by cisplatin could become synergistic if the two are used in combination. Thus, the approach was taken to study liposomally encapsulated Amph B, a preparation that has been shown to substantially avoid the kidney toxicity observed with Amph B alone. LipoAmph B is demonstrated to be safe in patients at efficacious doses, which yield plasma concentrations of Amph B up to 20 µg/ml (Meunier et al., 1991).

It is hypothesized that a liposomal preparation of Amph B prevents nephrotoxicity by maintaining a lower concentration of free Amph B than efficacious doses of aqueous Amph B. The antifungal activity of LipoAmph B depends on yeast cells’ production of a variety of phospholipases (Takahashi et al., 1991) that are thought to destabilize the liposome, allowing free Amph B to be released in the immediate vicinity of the organism. Optimal exploitation of LipoAmph B as a chemomodulator of platinum-drug cytotoxicity may entail a process by which free Amph B can be released in the local tumor milieu. PLA2-II, which preferentially hydrolyzes zwitterionic phospholipids, such as phosphatidylcholine (lecithin), effectively catalyzes the liposome (Dr. G. Jensen, NeXstar Inc., personal communication). Both PLA2-I and -II are found in the circulating plasma, but the latter is generally in much higher amounts (Kortesuo et al., 1992), ranging from 2 U/liter in healthy subjects up to 500 U/liter in patients with severe infections and neoplasms (Kortesuo et al., 1992; Nevalainen et al., 1992; Aufenanger et al., 1993; Rintala and Nevalainen, 1993). The PLA2-II level in peritoneal and pleural effusions from patients with various cancers generally averages between 40 and 60 ng/ml (approximately 100–150 U/liter), and up to 150 to 180 ng/ml (375–450 U/liter) in some patients (Abe et al., 1997), similar to the in vitro concentrations that were effective in this study. Also, PLA2-II is frequently overexpressed in more aggressive colon and breast tumors (Murata et al., 1993; Yamashita et al., 1994).

In this in vitro study, the action of PLA2-II appears to have released Amph B from the liposomes. The antiproliferative action of PLA2-II plus LipoAmph B (up to 45 and 70% against HN-5a and 5a/carbo-15a, respectively; Fig. 1) was much greater than that of PLA2-II plus empty liposomes (approximately 10–15% in HN-5a, 10–20% in 5a/carbo-15a, specific data not shown), thus rejecting the possibility that the release of free lipids caused the antiproliferative activity. It is of interest that the carboplatin-resistant cells are more sensitive to the antiproliferative action of Amph B than the parent line, a finding observed by others (Beke et al., 1995).

It was possible that the liposome would have an adverse effect on the antitumor activity of a platinum drug, because, in several animal studies, liposomally encapsulated platinum drugs had reduced or no activity compared with free drug (Reszka et al., 1987; Steerenberg et al., 1988; Fichtner et al., 1993). However, in the present study, free liposomes alone did not significantly alter carboplatin cytotoxicity in vitro (Fig. 2).

The efficacy of LipoAmph B in combination with platinum drugs has not been well documented. LipoAmph B significantly elevated the activity of cisplatin against cisplatin-resistant PC-14 in nude mice (Morikage et al., 1991b). Otherwise, to our knowledge, there are no other reports on the antitumor activity of the combination of LipoAmph B and cisplatin. The treatment of 5a/carbo-15a cells with free Amph B or some combinations of LipoAmph B plus PLA2-II completely abolished resistance to cisplatin (this report). Also, resistance of 5a/carbo-15a cells to carboplatin was reduced from 2.5- (at the IC90 level) to 1.3-fold, compared with the untreated parent line.

The use of LipoAmph B was proposed because this preparation largely avoids the kidney toxicity of Amph B, but it was not clear whether sufficient active Amph B would be released in the environment of the tumor cells to enhance drug uptake. The data presented here suggest that the phospholipase present in tumor microenvironments, as well as in serum of some tumor-bearing patients, can potentially release sufficient Amph B from LipoAmph B in the vicinity of the tumors to enhance platinum-drug cytotoxicity. LipoAmph B could be particularly effective in enhancing cisplatin toxicity
in peritoneal tumors. The potential efficacy of this combination warrants additional investigation.

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