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ORIGINAL ARTICLE

Lidija Beketic-Oreskovic · Maja Osmak

Modulation of resistance to cisplatin by amphotericin B and aphidicolin in human larynx carcinoma cells

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Abstract The aim of this study was to examine whether resistance to cisplatin [*cis*-diamminedichloroplatinum (II)] (CDDP) could be overcome by amphotericin B, cyclosporin A and aphidicolin in two sublines of human larynx carcinoma HEp2 cells. The sensitivity of parental and cisplatin-resistant CA3 and CK2 cells to amphotericin B, cyclosporin A and aphidicolin, and also the effects of these drugs (given in maximal nontoxic concentrations) on cisplatin sensitivity were determined by clonogenic survival assay. CA3 ad CK2 cells were sensitive to amphotericin B, and resistant to cyclosporin A and aphidicolin, compared with their parental cells. Amphotericin B increased cisplatin toxicity 2-fold in CA3 cells and 2.7-fold in CK2 cells, while it had no effect in parental HEp2 cells. Cyclosporin A did not influence the sensitivity of examined cells to cisplatin. The sensitizing effect of aphidicolin was more obvious in cisplatin-resistant cells. Cisplatin toxicity was increased by aphidicolin: 1.5-fold in HEp2 cells, 2-fold in CA3 cells, and 1.9-fold in CK2 cells. Therefore, the resistance to cisplatin in human larynx carcinoma CA3 and CK2 cells can be partially reversed by amphotericin B and aphidicolin.

Key words Cisplatin · Modulation of drug resistance · Human tumor cells

Introduction

Cisplatin [*cis*-diamminedichloroplatinum (II)] (CDDP) is one of the most effective cancer chemotherapeutic agents and is used especially in the treatment of solid tumors [1]. However, the development of resistance to this agent significantly limits the cure rates. Therefore, understanding the molecular basis of cisplatin resistance and devising strategies to circumvent this resistance are important research and clinical aims.

During the past few years a number of cisplatin-resistant cell lines have been established by exposure to escalating doses to cytostatics (reviewed in [37]). Different mechanisms of cisplatin resistance have been described: decreased accumulation of this drug (reviewed in [1, 12]), increased inactivation by the intracellular detoxification molecules (glutathione with related enzymes, metallothioneins, reviewed in [1, 27, 37, 45], and increased ability of the cells to repair and/or tolerate DNA lesions (reviewed in [4]). According to some reports, cisplatin-resistant cells could have undergone changes in signal transduction pathways or have overexpressed some of the oncogenes (reviewed in [37]). In general, mechanisms of cellular resistance to cisplatin are considered to be multifactorial and no one mechanism predominates in all cell lines tested.

In attempts to overcome cisplatin-resistance, various non-chemotherapeutic agents that could potentiate cisplatin efficiency have been used (reviewed in [37, 41, 42]). Some of them are membrane-active agents (calcium channel blockers, calmodulin inhibitors, or amphotericin B) that enhance cisplatin accumulation in cisplatin-resistant cells. Others (like buthionine sulfoximine, or ethacrinic acid) increase cisplatin toxicity by acting on the cytoplasmic defense system. Agents that could affect signal transduction pathways, like tamoxifen, or cyclosporin A, modulated cisplatin cytotoxicity in some cell lines. Similar effects could be obtained by inhibitors of DNA repair (azydothymidine or aphidicoline). However, the results concerning the circumvention of cisplatin-resistance are, because of multifactorial mechanisms involved in cisplatin resistance, different, often controversial, and dependent on the cell line examined [42].

L. Beketic-Oreskovic (✉)[1] · M. Osmak
Ruder Boskovic Institute, Department of Molecular Medicine, Bijenicka 54, 41000 Zagreb, Croatia
Fax (385-41) 425-497

[1] *Present address* Stanford University School of Medicine, Division of Oncology, Rm. M-211, Stanford, CA 94305-5306, USA
fax (+) 415 725 1420

We reported previously the establishment of cisplatin-resistant sublines of human larynx carcinoma HEp2 cells: CA3 and CK2. Those cell lines were obtained by two different treatment protocols [31, 32]. We identified decreased platinum accumulation as an important mechanism of cisplatin resistance in those cells. We found that buthionine sulfoximine and ethacrinic acid (specific inhibitors of glutathione and glutathione transferase) and verapamil (P-glycoprotein inhibitor) were unable to modify cisplatin resistance in those cells [3]. The aim of this study was to examine whether amphotericin B, cyclosporin A and aphidicolin could reverse resistance to cisplatin in CA3 and CK2 cells.

Materials and methods

Cell lines and culture conditions

Human larynx carcinoma HEp2 cells were used in this study. The cells were maintained as a monolayer culture in Eagle's minimum essential medium (Gibco Life Technologies, U.K.), supplemented with 10% fetal calf serum (Gibco) and antibiotics in a humidified atmosphere containing 5% CO_2.

Drugs

Cisplatin (Sigma, St. Louis, Mo.) was dissolved in water. Aphidicolin (Sigma) was dissolved in dimethylsulfoxide (Sigma). These stock solutions and amphotericin B (Fungisone, Gibco Life Technologies, U.K.) were stored at − 20° C, while cyclosporin A (Sandimmune, Sandoz, Pharmaceuticals, Feltham, Middlese U.K.) was stored at room temperature.

Resistance development

The cell lines resistant to cisplatin were established by intermittent exposure of the HEp2 human larynx carcinoma cell line to increasing concentrations of cisplatin, as described earlier [31, 32]. Briefly, CA3 cells were selected from HEp2 cells, treated for 1 h with cisplatin concentrations of 6–48 μM in serum-free medium. CK2 cells were selected from HEp2 cells, treated for 24 h with cisplatin concentrations ranging from 0.3 to 2.4 μM in medium supplemented with serum. The resistance ratio, which is the quotient when the IC_{50} of cisplatin for the resistant cells is divided by the IC_{50} for the parental cells (IC_{50} is the drug concentration that reduces the number of viable cells by 50%), was 2.3 for CA3 cells and 3.7 for CK2 cells [32].

Survival studies

The sensitivity of the parental and cisplatin-resistant cells to amphotericin B, cyclosporin A and aphidicolin, as well as the effects of these drugs on cisplatin sensitivity, were determined by clonogenic survival assay. The cells were plated at appropriate concentrations, three dishes for each experimental point, and treated after overnight incubation. To determine the maximal nontoxic concentration of the drugs examined, the cells were incubated with various concentrations of amphotericin B (for 3 h), cyclosporin A (for 18 h) and aphidicolin (for 48 h). To examine the effect of amphotericin B on cisplatin sensitivity, the samples were treated with 15 μg/ml of amphotericin B for 2 h, and then treated for 1 h in serum-free medium with various concentrations of cisplatin with or without addition of amphotericin B. To determine the effect of cyclosporin A on cisplatin sensitivity, the samples were treated with 5 μg/ml of cyclosporin A for 18 h. After that, the cells were treated with various concentrations of cisplatin for 1 h serum-free medium. One half of the samples were treated with cisplatin only. To examine the effect of aphidicolin on cisplatin sensitivity, the cells were treated with cisplatin for 1 h in serum-free medium. Thereafter, the samples were exposed to 0.5 μg/ml of aphidicolin for 48 h in medium supplemented with 0.5% of serum. One half of the samples were treated with cisplatin only and kept for a further 48 h in medium supplemented with 0.5% of serum. After that, the cells were grown for 10–15 days, after which the colonies were stained. Survival was calculated as the percentage of colonies in treated vs untreated samples.

Statistical analysis

Each experiment was performed in triplicate and repeated three times. Significance of the differences in the cell sensitivity was assessed by Student's *t*-test. The level of significance was set at 0.05.

Results

Cisplatin-resistant CA3 and CK2 cells were more sensitive to amphotericin B than parental HEp2 cells, as shown in Fig. 1. The maximal concentration of amphotericin B nontoxic for all cell lines examined was 15 μg/ml. As shown in Fig. 2a and b, amphotericin B increased cisplatin cytotoxicity in CA3 and CK2 cells, while it had no effect in HEp2 cells. When compared at the IC_{50} of cisplatin, amphotericin B increased cisplatin cytotoxicity 2-fold in CA3 cells (Fig. 2a) and 2.7-fold in CK2 cells (Fig. 2b).

The sensitivity of parental and cisplatin-resistant cells to cyclosporin A is shown in Fig. 3. CA3 and CK2 cells were more highly resistant to cyclosporin A than were HEp2 cells. The maximal concentration of cyclosporin A

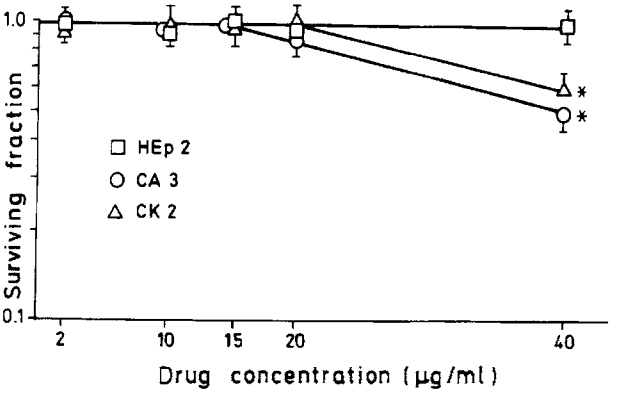
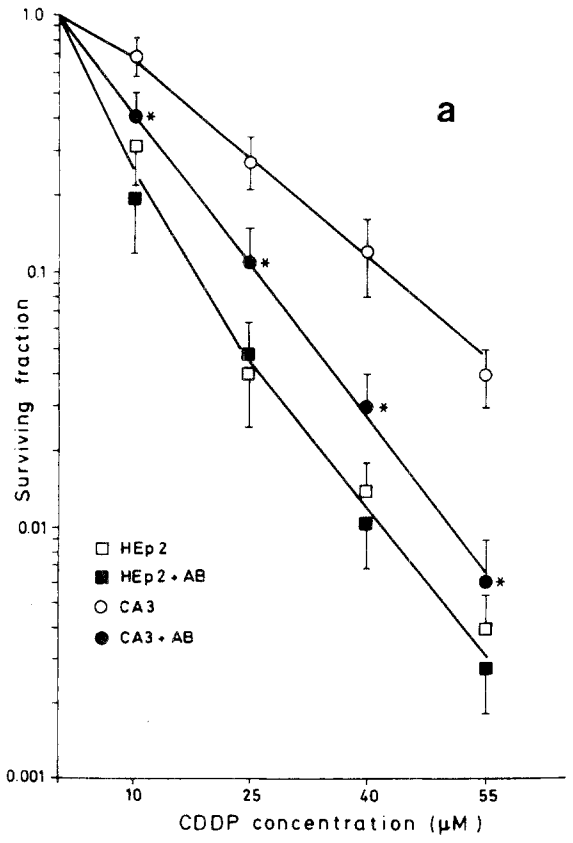
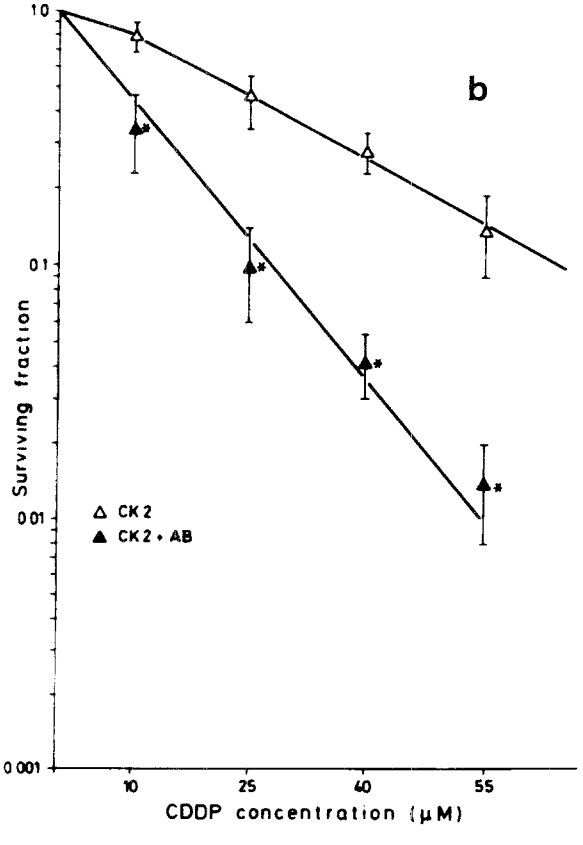


Fig. 1 The sensitivity of HEp2, CA3 and CK2 cells to various concentrations of amphotericin B given over 3 h. Values are the means of three independent experiments done in triplicate ± SD. *Significantly different from the value in parental cells ($P < 0.05$)





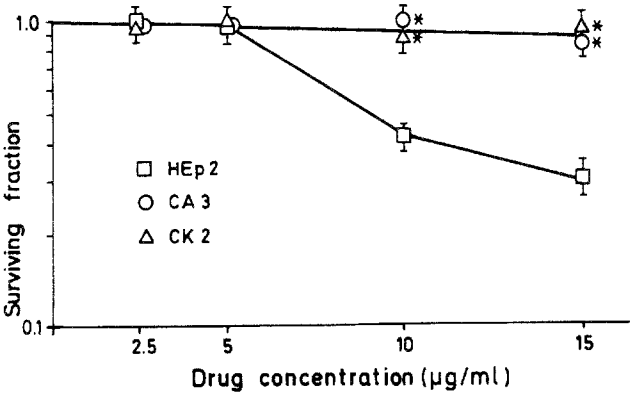


Fig. 3 The sensitivity of HEp2, CA3 and CK2 cells to various concentrations of cyclosporin A, given for 18 h. Values are the means of three independent experiments done in triplicate ± SD. *Significantly different from the value in parental cells ($P < 0.05$)

that exerted no toxic effect on parental or on cisplatin-resistant cells was 5 μg/ml. Cyclosporin A did not influence the sensitivity of resistant or parental cells to cisplatin (Fig. 4).

Cisplatin-resistant CA3 and CK2 cells were more highly resistant to aphidicolin than parental HEp2 cells (Fig. 5). The maximal nontoxic concentration of aphidicolin, for all cell lines examined, was 0.5 μg/ml. Aphidicolin increased cisplatin cytotoxicity in all cell lines examined (Fig. 6a, b). This effect was more obvious in cisplatin-resistant cells. When compared at the IC_{50} of cisplatin, aphidicolin increased cisplatin cytotoxicity 1.5-fold in HEp2 cells, 2-fold in CA3 cells (Fig. 6a) and 1.9-fold in CK2 cells (Fig. 6b).

Discussion

According to the literature data, various agents could be used to modulate cisplatin resistance, depending on the mechanisms involved in the resistance in examined cell lines (reviewed in [37, 42]). Previously, we found that buthionine sulfoximine and ethacrinic acid, specific inhibitors of glutathione and glutathione transferase, had no effect on cisplatin sensitivity in cisplatin-resistant human larynx carcinoma CA3 and CK2 cells. Similarly, verapamil, an inhibitor of P-glycoprotein, did not influenced the sensitivity of the cells examined to cisplatin [3]. In the present study we evaluated the sensitizing effect of amphotericin B, cyclosporin A and aphidicolin on cisplatin cytotoxicity in these cells.

Fig. 2a,b The sensitivity of **a** HEp2 and CA3 cells or **b** CK2 cells to various concentrations of cisplatin with or without addition of 15 μg/ml of amphotericin B (*AB*). The cells were incubated with amphotericin B for 2 h before and during cisplatin treatment. Values are the means of three independent experiments done in triplicate ± SD. *Significantly different (+ AB vs − AB) ($P < 0.05$)

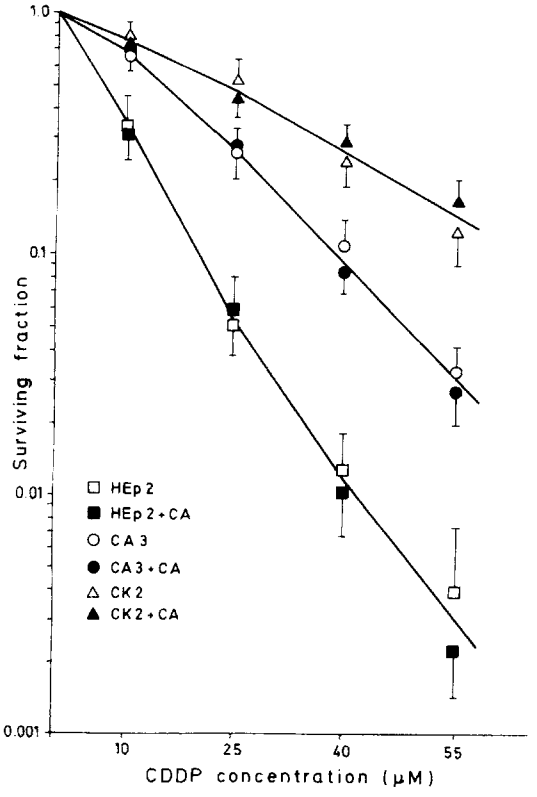


Fig. 4 The sensitivity of HEp2, CA3 and CK2 cells to various concentrations of cisplatin with, or without, 18 h preincubation with 5 μg/ml of cyclosporin A (*CA*). Values are the means of three independent experiments done in triplicate ± SD. *Significantly different (+ CA vs − CA) ($P < 0.05$)

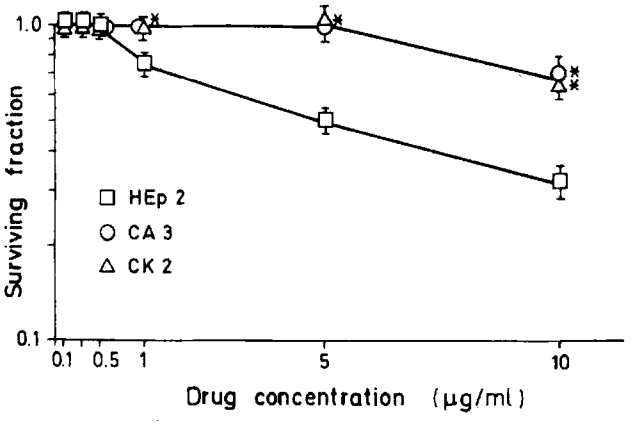
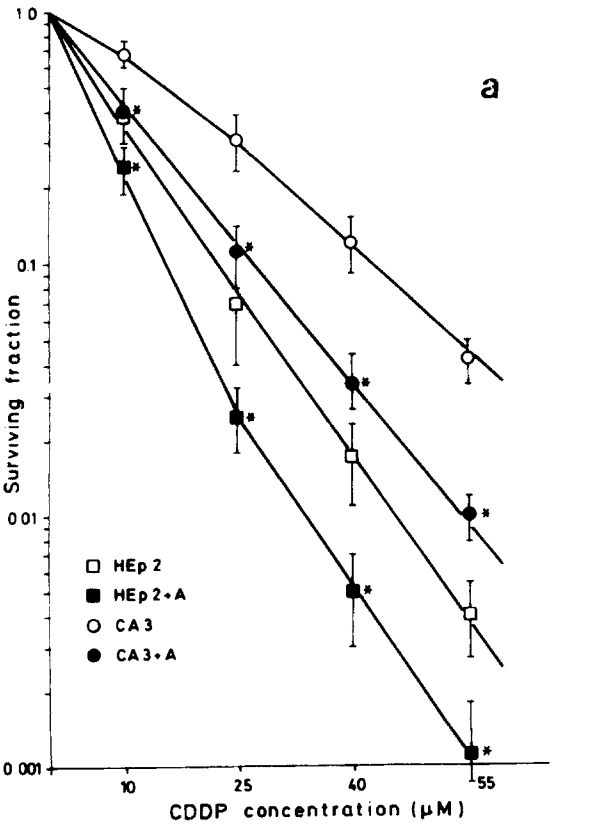


Fig. 5 The sensitivity of HEp2, CA3 and CK2 cells to various concentrations of aphidicolin given for 48 h. Values are the means of three independent experiments done in triplicates ± SD. *Significantly different from the value of parental cells ($P < 0.05$)



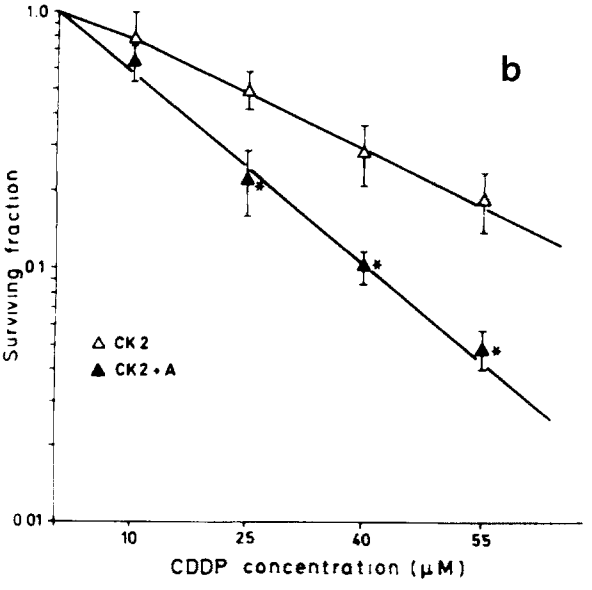


Fig. 6a,b The sensitivity of **a** HEp2 and CA3 cells, and **b** CK2 cells to various concentrations of cisplatin with, or without addition of, 0.5 μg/ml of aphidicolin (A). The cells were incubated with aphidicolin for 48 h after CDDP treatment. Values are the means of three independent experiments done in triplicates ± SD. *Significantly different (+ AB vs − AB) ($P < 0.05$)

Amphotericin B is a macrolide polyene antibiotic, which is used in the therapy of disseminated fungal diseases. Although direct cytostatic activity of amphotericin B was not proved, some *in vitro* and *in vivo* studies indicated that this drug could potentiate the efficiency of different chemotherapeutic agents (reviewed in [6, 4]). It was found that amphotericin B increased the accumulation of nitrogen mustard [33, 34], Adriamycin [19], and cisplatin [24, 28, 29] and its analogues [29]. It is considered that amphotericin B interacts with membrane sterol molecules, resulting in increased permeability of the cell membrane [24]. This drug could also cause leakage of intracellular potassium ions and alter the membrane charge [44].

Cisplatin-resistant CA3 and CK2 cells were more sensitive to amphotericin B than were their parental cells. In contrast to our results, Morikage at al. found that cisplatin-resistant PC-9 human lung carcinoma cells had the same sensitivity to amphotericin B as their parental cells [28]. Masuda et al. observed that A2780 human ovarian cells were more resistant to amphotericin B than their parental cells. They explained this resistance by an increased level of glutathione [24]. Although CA3 and CK2 cells had elevated levels of glutathione, as we reported earlier [3], they were sensitive to amphotericin B. The reason for this enhanced sensitivity is not known. One possible explanation might be in the differences in cholesterol levels and sensitivity to potassium ion leakage between parental and cisplatin-resistant cells, as suggested for 2780 human ovarian cancer cells resistant to cisplatin [24].

The effect of amphotericin B seems to be complex. For example Ozols et al. demonstrated that amphotericin B increased the permeability of human ovarian cancer cello line (COLO 319) for Adriamycin, but not for melphalan [33]. Masuda et al. observed that higher doses of amphotericin B increased cisplatin toxicity in parental and cisplatin-resistant A2780 human ovarian cancer cells, while lower doses (under 2.4 μg/ml) selectively potentiated cisplatin toxicity in cisplatin-resistant cells, decreasing even cisplatin toxicity in sensitive cells [24].

We demonstrated that amphotericin B was able selectively to potentiate cisplatin toxicity in cisplatin-resistant cells, without having any such effect in parental HEp2 cells. Our results are comparable with those of Morikage et al.: they found that amphotericin B reversed cisplatin resistance by increasing the platinum accumulation in PC-9 human non-small-cell lung cancer line resistant to cisplatin, but with no effect on the parental cells [28]. The concentration of amphotericin B used for modulation of cisplatin resistance in CA3 and CK2 cells was 15 μg/ml. This concentration of amphotericin B has been used for reversal of cisplatin resistance *in vitro* [28], but it is also achievable *in vivo*. It has been shown that amphotericin B, given entrapped in sonicated liposomes to cancer patients with fungal infections, can be used in a concentration of 15 μg/ml [38]. In our previous study we identified decreased platinum accumulation as an important mechanism of cisplatin resistance in CA3 in CK2 cells [3]. Therefore, it is most likely that amphotericin B increased drug accumulation, causing the potentiation of cisplatin toxicity. Although amphotericin B significantly increased cisplatin toxicity in CA3 and CK2 cells, the recovery from cisplatin resistance was not complete. It suggests that decreased drug accumulation was not the only mechanism involved in cisplatin resistance.

Cyclosporin A is an immunosuppressive agent that is used in organ transplantations. This cyclic polipeptide inhibits the initial activation of lymphocytes and other processes essential for an immune response [10]. This drug has been also used as a potentiator of chemotherapeutic efficiency. Cyclosporin A modulates multidrug resistance (MDR) in different cell lines [14, 40], and some clinical trials have been also performed, with similar results [21, 33, 46]. This drug increased cisplatin toxicity in cisplatin-resistant human ovarian A2780 carcinoma cells [17, 36], and also in PC-9 human lung carcinoma cells [13].

The precise mechanism of action of cyclosporin A on drug resistance is not completely understood. It has been found that cyclosporin A can increase drug accumulation by competitive inhibition of P-glycoprotein [11] or by alterations to the cell membrane potential [40]. This drug could also act on signal transduction pathways in the cells and modulate the expression of oncogenes [2, 10]. It was found that cyclosporin A suppressed cisplatin-induced expression of c-*fos* and c-H-*ras* oncogenes in cisplatin-resistant human ovarian carcinoma A2780 cells, decreasing the resistance to cisplatin [17, 36].

Cyclosporin A, given in a dose of 5 μg/ml, which is usually required for the modulation of resistance to some cytostatics [21, 41], had no effect on cisplatin resistance in CA3 and CK2 cells. This suggests that cyclosporin A does not necessarily interfere with the mechanisms of the resistance to cisplatin. The mechanism of cross-resistance of CA3 and CK2 cells to cyclosporin A is not known. As we reported recently, CA3 and CK2 cells exhibited a significant decrease in cisplatin accumulation, most probably because of the changes in the cell membrane potential [3]. As cyclosporin A could also affect cell membrane potential [43], the reason for the resistance to this agent could be a decrease in its accumulation in cisplatin-resistant cells.

Aphidicolin is a tetracyclic ditepernoid obtained from *Aphidicolium cephalospora*. It is a specific inhibitor of polymerase α, the enzyme that is necessary for DNA synthesis and repair [15]. Polymerase-α requires DNA lesions of 30 nucleotides or more to initiate DNA synthesis (long-patch excision repair). Aphidicolin adheres to nucleotide-binding sites on DNA polymerase-α, thereby causing an incomplete repair process and accumulation of DNA lesions [26].

It has been shown that aphidicolin can potentiate cisplatin toxicity in some cell lines resistant to cisplatin, by inhibiting the increased DNA repair in those cells [5, 20, 22, 23]. Aphidicolin glycianate, a water-soluble form of aphidicolin, given in combination with cisplatin in the treatment of transplantable murine tumors *in vivo*, significantly increased the toxicity of cisplatin [30]. In contrast to this, Damia et al. found that potentiation of the activity of cisplatin by aphidicolin glycianate was moderate in mice bearing cisplatin-sensitive and cisplatin-resistant M5076 murine reticular cell sarcoma [8]. Katz et al. found that aphidicolin glycianate potentiated cisplatin toxicity in both cisplatin-resistant and parental human ovarian carcinoma cells, with the same efficiency [18]. Demke et al. showed that aphidicolin glycianate had no effect on the removal of platinum-bound DNA adducts in logarithmically growing ovarian tumor cells resistant to cisplatin [9]. Both studies were performed on the proliferating cells. The effect of aphidicolin on the reduction of DNA repair in drug-resistant cells was apparent only in non-proliferating cells with no replicative DNA synthesis [16].

To examine the effect of aphidicolin on DNA repair and to minimize replicative DNA synthesis, we incubated the cells in medium with 0.5% of serum after cisplatin treatment. These conditions were used by Creissen et al. to measure the aphidicolin-inhibitable DNA repair [7]. The more marked effect of aphidicolin on cisplatin toxicity in CA3 and CK2 cells than in the parental cells suggests that the increased DNA repair is one of the mechanisms of cisplatin-resistance in these cells. That is to say, because of the increased repair of cisplatin lesions in the resistant cells, more polymerases were needed and the effect of aphidicolin would be more obvious, as described for A2780 human ovarian carcinoma cells resistant to cisplatin [25]. We found that cisplatin-resistant cells of both the CA3 and CK2 lines were also resistant to UV light (data not shown). As long-patch excision repair is involved in UV-radiation-induced DNA damage [26], it is most likely that CA3 and CK2 cells have increased capacity of DNA repair. Cross resistance of CA3 and CK2 cells to aphidicolin could be explained by increased level of DNA polymerases, as found in colon carcinoma HCT8 cells resistant to cisplatin [35]. Cross resistance to aphidicolin was also found in A2780 human ovarian cancer cells resistant to cisplatin, which was explained by the increased level of polymerase-α, but also by an increased level of glutathione, which might enhance the pool sizes of DNA precursors and protect the cells from aphidicolin [25]. CA3 and CK2 cells had increased levels of gluathione, as we have reported previously [3]. Although it possibly protected the cells from aphidicolin, glutathione was not involved in the mechanisms of resistance to cisplatin as we showed by means of buthionine sulfoximine, a specific inhibitor of glutathione synthesis [3]. The concentration of aphidicolin used in our study for modulation of cisplatin resistance was 2 μg/ml. This concentration was required to potentiate the cisplatin effect in a cisplatin-resistant human ovarian cancer cell line [22], but could be also achieved *in vivo*. At the maximal tolerated dose for aphidicolin glycianate, the plasma concentration of this drug was 3 μg/ml, as determined in a phase I clinical study of aphidicolin glycianate [39].

In conclusion, it was possible to obtain partial reversal of cisplatin resistance in two human larynx carcinoma cell lines with amphotericin B and aphidicolin, while cyclosporin A had no effect. But, as the mechanisms of cisplatin resistance can be different and vary among the cell lines, the relevance of these results to other cisplatin-resistant cell lines and to the clinical setting remains to be determined.

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