Cancer Chemother Pharmacol (1990) 26: 163–167

Cancer Chemotherapy and Pharmacology

The influence of Na^+, K^+-pump blockade on doxorubicin-mediated cytotoxicity and DNA strand breakage in human tumor cells*

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Received 2 January 1990/Accepted 30 January 1990

Summary. We have previously shown that blockade of the Na^+, K^+-pump by the cardiac glycoside ouabain produces doxorubicin resistance and decreases topoisomerase II-mediated DNA strand breakage in hamster cells. To determine if this were a general phenomenon, the effect of pump blockade on doxorubicin resistance was assessed in three human tumor cell lines: A549 lung and HT29 colon adenocarcinomas and U1 melanoma. When cells were exposed to 1 μM ouabain prior to and during incubation with doxorubicin, cytotoxicity was markedly reduced. Ouabain had no effect on either the influx or the efflux of doxorubicin. However, all cell lines showed a ouabain-induced decrease in doxorubicin-induced topoisomerase-mediated DNA strand breakage (SSB). These data suggest that blockade of the Na^+,K^+ pump decreases doxorubicin cytotoxicity in human tumor cells by inhibiting topoisomerase-mediated SSB. Furthermore, they indicate that altered ionic gradients are a potential cause of resistance to drugs that use topoisomerase II as a target.

Introduction

Although the interaction between doxorubicin and human tumor cells is complex, much evidence suggests that doxorubicin-mediated stabilization of DNA-topoisomerase II complexes is a critical step in the chain of events leading to cell death [6]. Topoisomerase II is an enzyme that enables DNA double-strand passage in supercoiled DNA, thereby decreasing the linking number [23]. Although the function of topoisomerase II in higher eukaryotes is not yet clear, recent evidence suggests roles in replication and transcription [5].

Since the intracellular ionic environment influences some of these same cellular functions [7, 16], we hypothesized that it might affect the cytotoxicity of drugs that use topoisomerase II as a target. In fact, blockade of the Na^+,K^+ pump, which is the chief regulator of the intracellular ionic environment, by the cardiac glycoside ouabain was found to protect V79 hamster cells from doxorubicin- [9] and etoposide-mediated [10] cytotoxicity. Protection was demonstrated to correlate with a decrease in the number of drug-induced DNA-topoisomerase II complexes ("cleavable complexes"). These findings showed that the activity of these two agents, which use topoisomerase II as a target, could be modulated by Na^+,K^+-pump blockade. Additional evidence suggested that protection results directly from the change in the intracellular ionic environment caused by ouabain treatment [10].

It was then of interest to determine whether these findings applied to human tumor cells. If ouabain decreased doxorubicin-induced cytotoxicity in several human tumor cell lines through decreasing the formation of cleavable complexes, it would suggest that Na^+,K^+-pump blockade affects a fundamental step leading to doxorubicin cytotoxicity that is general to all cells rather than being an artifact of hamster cells. Furthermore, it would establish that alterations in the Na^+,K^+-pump could represent a potential mechanism for doxorubicin resistance in human tumor cells. Therefore, the effect of ouabain on doxorubicin-mediated cytotoxicity and cleavable complex formation was assessed in three human tumor cell lines.

Materials and methods

Cell culture. Human lung adenocarcinoma cells (A549), melanoma cells (U1), and colon adenocarcinoma cells (HT29) were obtained from American Type Culture Collection (Rockville, Md.). All cell lines were maintained at 37° C in a humidified atmosphere of 7% CO_2 and 93% air. Cells were cultured in RPMI medium 1640 containing 10% fetal bovine serum. Media was supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), and glutamine (10 m*M*). Under these conditions the plating efficiency of A549 and U1 cells was 40%–60%, and both doubling times

* This research was supported in part by a grant from the Rackham Foundation. Presented in part at the meeting of the American Association for Cancer Research in May, 1989

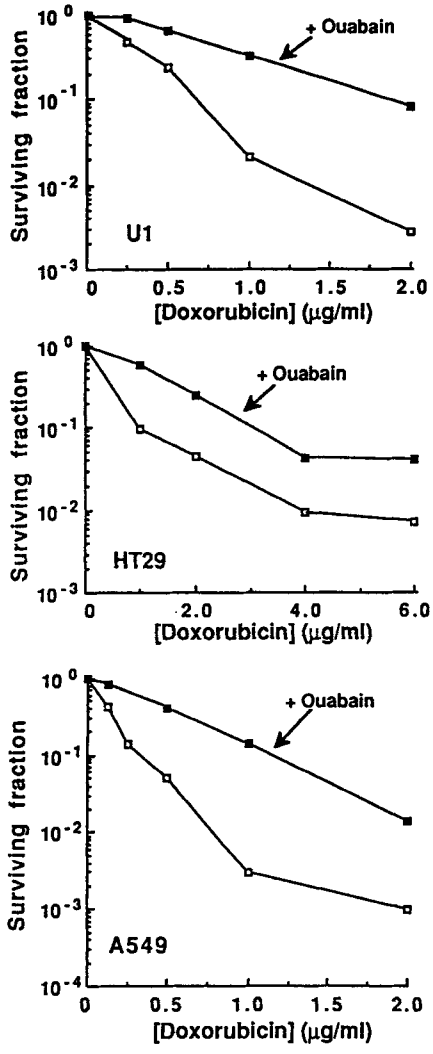

Fig. 1. Ouabain protects A549 lung cancer cells, U1 melanoma cells, and HT29 colon cancer cells from doxorubicin-mediated cytotoxicity. Cells were incubated in medium alone (□) or with 1 μM ouabain (■) for 1 h prior to exposure to various concentrations of doxorubicin. After doxorubicin exposure, cells were rinsed twice with PBS, returned to the preincubation medium for 3 h, and assessed for cell survival

were approximately 24 h. The plating efficiency of HT29 cells was 70%–90%, and the doubling time approximately 21 h.

Drug treatment. Ouabain (Sigma) was made fresh prior to each experiment. The ouabain concentration used in all experiments was 1 μM, which has previously been shown to cause maximal Na^+,K^+-pump blockade in human tumor cells ([9]; unpublished observations). Doxorubicin HCl (obtained as a freeze-dried powder with lactose; Adria Laboratories) was made as a 500-μg/ml stock in phosphate-buffered saline (PBS) (NaCl, 154 m*M*; KH_2PO_4, 1.06 m*M*; Na_2HPO_4, 5.6 m*M*), aliquotted, frozen at –70° C, and diluted on the day of the experiment. All doxorubicin incubations were carried out for 1 h at 37° C.

Cell survival assay. For all cell survival experiments, cells were incubated in media alone or in 1 μM ouabain for 1 h prior to exposure to various concentrations of doxorubicin. Cells were then rinsed twice with PBS and returned to the preincubation medium for 3 h. Cells were washed with PBS, removed from the dishes with PBS containing 0.03% trypsin and 0.27 m*M* EDTA, and diluted into culture dishes to yield between 20 and 200 colonies per plate. Dilutions were carried out in triplicate. After 10–12 days, the plates were fixed with methanol-acetic acid, stained

Table 1. Ouabain protects three human tumor cell lines from doxorubicin cytotoxicity

Cell line	[Doxorubicin] (μg/ml)[a]:	
	Doxorubicin alone	Doxorubicin + Ouabain
A549	0.6±0.1	1.1±0.2
HT29	1.5±0.3	3.0±0.4
U1	0.6±0.1	1.5±0.2

[a] Mean doxorubicin concentration that reduced the surviving fraction to 10%

with crystal violet, and scored for colonies containing >50 cells. Doxorubicin cell-survival curves from experiments using preincubations with ouabain were corrected for the plating efficiency of cells treated with the preincubation medium alone.

Determination of intracellular doxorubicin. After drug treatment, cells were removed from the plates with glucose-free trypsin that contained 10 m*M* sodium azide to inhibit doxorubicin efflux during cell preparation [19], and the intracellular doxorubicin content was measured according to the method of Bachur et al. [2], with minor modifications as previously described [9]. Assays were conducted in duplicate. Data were expressed as a percentage of the maximal doxorubicin concentration measured after a 60-min incubation with the drug.

Alkaline elution. Alkaline elution was performed according to the method of Kohn et al. [8], with minor modifications as previously described [9]. Elutions were carried out at a rate of approximately 0.04 ml/min using an internal standard irradiated with 1.5 Gy. Assays were conducted in duplicate. Multiple elutions were compared by the expression of single strand breaks (SSBs) in terms of cGy (1 rad = 1 cGy) equivalents.

Statistical analysis. For cell-survival assays, the standard error was typically <15% of the mean and is contained within the size of the symbol unless otherwise indicated. For intracellular doxorubicin assays and alkaline elutions, the duplicate determinations were within 10% of the mean. Data represent the mean ± SEM. All experiments were carried out at least three times.

Results

Ouabain had previously been shown to protect rodent cells (V79) and human lung adenocarcinoma cells (A549) from the effects of doxorubicin cytotoxicity [9]. The effect of ouabain on doxorubicin-mediated cytotoxicity was then assessed using two other human cell lines (U1 melanoma cells and HT29 colon adenocarcinoma cells) that show differing sensitivities to doxorubicin. Ouabain protected all three cell types from doxorubicin cytotoxicity (Fig. 1). Table 1 indicates the concentration of doxorubicin that reduced the surviving fraction to 10% of control levels and shows that both A549 and U1 cells were substantially more sensitive to the drug than were HT29 cells. However, in each cell line the presence of ouabain resulted in approximately a doubling of the concentration of doxorubicin required to reduce the surviving fraction to 10%.

To examine the possibility that ouabain exerted its effect by decreasing intracellular doxorubicin levels, the influx and efflux of doxorubicin were measured under conditions identical to those used in cell-survival experiments (Fig. 2). The data are expressed as the percentage of

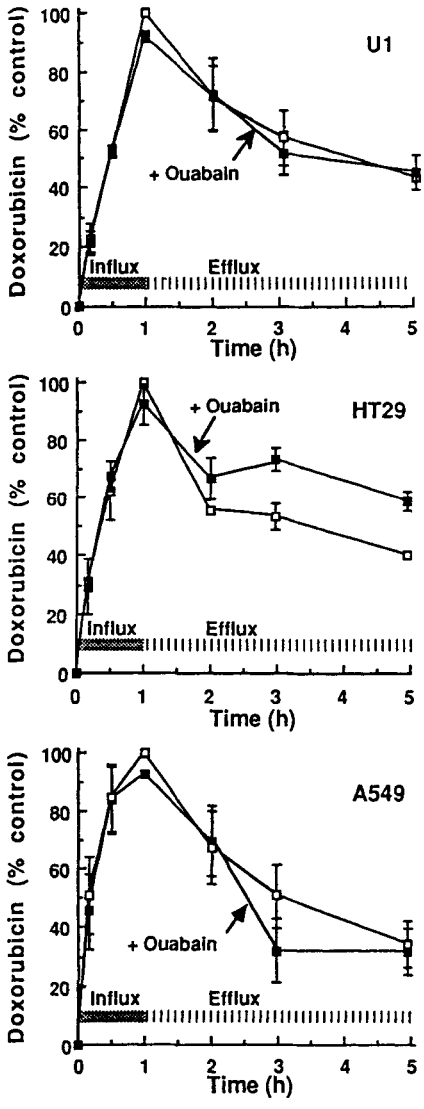

Fig. 2. Ouabain affects neither the influx nor the efflux of doxorubicin. Cells were incubated in medium alone (□) or with 1 μM ouabain (■) as described in Fig. 1. For assessment of influx, cells were exposed to doxorubicin (4 μg/ml for HT29 cells; 2 μg/ml for U1 and A549 cells) for increasing periods of up to 1 h. For determination of efflux, cells were incubated for 1 h in the same concentration of doxorubicin that was used to test influx, rinsed twice with PBS, and returned to the preincubation medium for the indicated times. Cells were then assessed for doxorubicin content. Data for each experiment are expressed as a percentage of the doxorubicin content attained in the absence of ouabain at the end of the influx period (1 h). The average of 4 experiments is shown

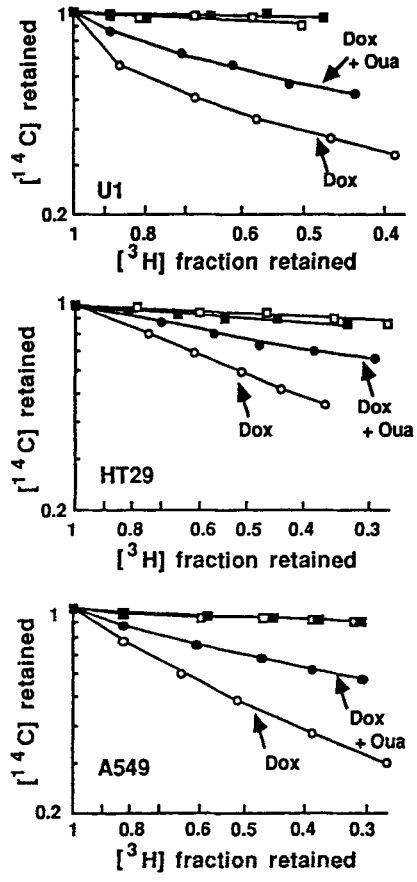

Fig. 3. Ouabain decreases doxorubicin-induced DNA SSBs. Cells were incubated as described in Fig. 1 with medium alone (□), 1 μ*M* ouabain alone (■), doxorubicin alone (*Dox*, ○) (2 μg/ml for HT29 cells; 1 μg/ml for U1 and A549 cells), or doxorubicin plus ouabain (*Dox + Oua*, ●) and assessed for DNA SSBs

the doxorubicin content achieved in the absence of ouabain at the end of the 1-h influx period. This level was 194 ± 65 ng/10^6 A549 cells, 341 ± 112 ng/10^6 HT29 cells, and 298 ± 90 ng/10^6 U1 cells. Although the maximal doxorubicin content attained was different for each cell line, as were the rates of influx and efflux, ouabain had no effect on any of these parameters in any cell line.

Since ouabain did not reduce intracellular doxorubicin content, it was possible that, as was shown to occur in V79 cells [9], ouabain conferred protection by inhibiting the formation of DNA-topoisomerase II complexes. This was assessed by use of the alkaline elution technique under the same conditions as were used for cell survival assays. Preincubation with ouabain reduced the number of cleavable complexes induced by doxorubicin in each of the cell lines studied (Fig. 3).

The results of the alkaline elution experiments were compared with the assays of cell survival in two manners. First, the SSBs (expressed as cGy equivalents) were determined for the concentration of doxorubicin that reduced the surviving fraction to 10% of control values, as shown in Table 1. For this level of cytotoxicity (10% surviving fraction), the number of SSBs induced was approximately the same in the absence and presence of ouabain (Table 2). This suggests that pump blockade does not alter the relationship between cleavable complex formation and cytotoxicity but simply decreases the number of complexes formed. Second, the effect of ouabain was assessed by comparing cytotoxicity ratios (for the cell-survival experiments) and SSB ratios (for the alkaline elutions). A cytotoxicity ratio was defined as the ratio of the concentrations of doxorubicin that produced a 10% surviving fraction in the presence and absence of ouabain. The SSB ratio was similarly defined as the ratio of SSBs produced by the concentrations of doxorubicin that resulted in an approximately 10% surviving fraction in the absence and presence of ouabain. Cytotoxicity and SSB ratios were approximately the same for each of the cell lines (Table 3).

Table 2. Ouabain does not affect the relationship between SSBs and cytotoxicity

Cell line	Doxorubicin alone (cGy equivalent)[a]	Doxorubicin + ouabain (cGy equivalent)[a]
A549	63 ± 11	58 ± 5
HT29	61 ± 20	47 ± 5
U1	93 ± 49	87 ± 49

[a] SSB frequency for the conditions that produced the 10% surviving fraction shown in Table 1

Table 3. Ouabain-mediated protection correlates with a decrease in SSBs

Cell line	Cytotoxicity ratio	SSB ratio
A549	1.9 ± 0.4	2.3 ± 0.4
HT29	2.1 ± 0.4	2.5 ± 0.3
U1	2.4 ± 0.2	2.4 ± 0.3

Discussion

These data show that blockade of the Na^+,K^+ pump by ouabain protects three human tumor cell lines (A549 lung adenocarcinoma, U1 melanoma, and HT29 colon adenocarcinoma cells) from the toxicity of doxorubicin. The degree of protection was similar despite the differing sensitivities of these cell lines to doxorubicin. Protection did not appear to be mediated by an effect on the intracellular concentration of doxorubicin, since ouabain affected neither the influx nor the efflux of doxorubicin. In contrast, pump blockade markedly decreased the formation of doxorubicin-induced cleavable complexes as measured by alkaline elution, and this decrease was correlated with the decrease in cytotoxicity. These findings suggest that a change in the intracellular ionic environment produced by blockade of the Na^+,K^+-pump affects a critical step in the pathway by which doxorubicin causes cell death in human tumor cells, that of cleavable complex formation. Therefore, although doxorubicin has multiple potential mechanisms of action, including cell membrane damage [20] and free radical generation [12], the current study suggests that complex formation is an important step leading to cell death in these human tumor cell lines, as has also been demonstrated in several other systems [14, 17, 22, 24]. It is likely that the decrease in doxorubicin-mediated cleavable complex formation reported in the present study results from a decrease in topoisomerase II activity caused by the ouabain-induced change in the intracellular ionic environment, as isolated topoisomerase II has been shown to be approximately 30% less active in an ionic environment made to simulate that produced by Na^+,K^+-pump blockade, as opposed to the normal intracellular ionic milieu [10].

The results of this study suggest that alterations in the Na^+,K^+-pump and the resulting effects on the intracellular ionic environment represent a potential mechanism of resistance to drugs that use topoisomerase II as a target. Such drug resistance would be quite distinct from that associated with the multiple drug resistance (MDR-1) phenotype [13, 15], since it does not depend on changes in intracellular drug accumulation. For instance, some tumor cells secrete a factor with ouabain-like activity, which could make them resistant to drugs that use topoisomerase II as a target [11]. It is also of interest that cultured human and murine cancers that have been made resistant to daunorubicin have a lower membrane potential than the parental, sensitive cells [21]. Since the Na^+,K^+-pump is the chief determinant of the transmembrane potential [18], this lower membrane potential could be the result of an alteration in Na^+,K^+-pump activity. Likewise, low concentrations of the potassium ionophore valinomycin confer sensitivity to drug-resistant Chinese hamster ovary cells, possibly by increasing the membrane potential [4]. This could occur only if Na^+,K^+-pump activity increased adequately to maintain the elevated transmembrane potential. Based on these observations, one would hypothesize that stimulation of the Na^+,K^+-pump might represent a possible mechanism for reversing drug resistance in selected settings. Since the Na^+,K^+-pump can be stimulated both in vitro [3] and in patients [1] by β-agonists, it would enable the possibility of reversing drug resistance in patients whose tumors are resistant secondary to an altered intracellular ionic environment.

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