Reduction of Doxorubicin Cytotoxicity by Ouabain: Correlation with Topoisomerase-induced DNA Strand Breakage in Human and Hamster Cells

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ABSTRACT

The cardiac glycoside ouabain, which is a specific inhibitor of the Na\(^+\), K\(^-\)-pump, confers dramatic protection from the cytotoxic effects of doxorubicin (Adriamycin). This effect was documented in cultured A549 cells (human lung adenocarcinoma). CCL210 cells (human fibroblasts), and V79 cells (hamster fibroblasts). Maximum protection from doxorubicin cytotoxicity was achieved with 1 \(\mu \text{M}\) ouabain for A549 and CCL210 cells and 300 \(\mu \text{M}\) ouabain for V79 cells. These concentrations correlated well with the concentrations of ouabain required to induce Na\(^+\), K\(^-\)-pump blockade, which was assessed using the K\(^+\) analogue \(^{86}\)Rb\(^+\). This suggests that protection is mediated by pump blockade. Addition of ouabain at the same time as doxorubicin was just as protective as preincubation with ouabain for an hour, demonstrating that the ouabain acts rapidly. Ouabain treatment affected neither influx nor efflux of doxorubicin. Ouabain also had no effect on verapamil-induced inhibition of doxorubicin efflux. However, ouabain partially blocked the verapamil-induced potentiation of the cytotoxic effects of doxorubicin. Therefore, ouabain does not protect by affecting intracellular doxorubicin levels. Fluorescence microscopy showed that the ability of doxorubicin to reach the nucleus was not influenced by ouabain. Alkaline elution studies demonstrated that ouabain greatly decreased doxorubicin-induced DNA strand breakage. Protection from cytotoxicity correlated closely with this decrease in strand breakage. These studies suggest that the stabilization of DNA-topoisomerase II complexes is closely linked to the mechanism of doxorubicin cytotoxicity and that this stabilization is influenced by the intracellular ionic milieu.

INTRODUCTION

Although doxorubicin (Adriamycin) is one of the most active chemotherapeutic agents, its mechanism of action remains unknown (1). Much recent investigation has focused on the effect of doxorubicin on DNA-topoisomerase II complexes. Initially, it was observed that doxorubicin caused DNA-protein cross-links that were revealed as single strand DNA breaks after treatment with proteinase K (2). It now appears that DNA-protein cross-links represent complexes of DNA and topoisomerase II that have been stabilized by doxorubicin (3, 4). Additional support for this model comes from the demonstration that the combination of isolated DNA and purified topoisomerase II exhibits this same behavior when exposed to doxorubicin and other related drugs (5, 6). Although the exact role of DNA-topoisomerase II has not yet been established in eukaryotic cells, it is likely that it plays an important role in DNA replication and transcription (7).

The intracellular ionic environment is known to play a critical role in replication and transcription (8, 9), and it is possible that this role is at least partially mediated by an effect on topoisomerases. If this were the case, blockade of the Na\(^+\), K\(^-\)-pump, which is an important regulator of the intracellular ionic composition, would affect topoisomerase activity and, potentially, doxorubicin cytotoxicity. Therefore, experiments were conducted to investigate if ouabain, a specific inhibitor of the Na\(^+\), K\(^-\)-pump, affected doxorubicin cytotoxicity. It was discovered that ouabain dramatically protects cells from doxorubicin. Studies were then conducted to characterize the dose dependence and time course of protection. Since many drugs that affect doxorubicin cytotoxicity do so through alteration of intracellular drug levels (10-14), a detailed study of the effect of ouabain on doxorubicin levels was undertaken. When it was determined that ouabain had no measurable effect on intracellular doxorubicin levels, the influence of ouabain on doxorubicin-induced stabilization of DNA-topoisomerase II complexes was assessed.

MATERIALS AND METHODS

Cell Culture. Human adenocarcinoma cells (A549) and human lung fibroblasts (CCL210) were obtained from American Type Culture Collection, Rockville, MD. All cell lines were maintained at 37°C in a humidified atmosphere of 5% CO\(_2\) and 95% air. A549 and V79 cells were cultured in RPMI medium and F-12 medium, respectively, each supplemented with 10% fetal bovine serum that was heat inactivated. All media were supplemented with penicillin and streptomycin. Under these conditions, the plating efficiencies of V79 cells, A549 cells, and CCL210 cells were 80-90%, 50-75%, and 10-20%, and the doubling times were approximately 9 h, 24 h, and 24 h, respectively.

Drug Treatment. Ouabain and verapamil hydrochloride (Sigma) were made fresh prior to each experiment. Doxorubicin hydrochloride (obtained as a freeze-dried powder with lactose; Adria Laboratories) was made as a 500-\(\mu\)g/ml stock in PBS (154 mM NaCl: 1.06 mM KH\(_2\)PO\(_4\); 5.6 mM Na\(_2\)HPO\(_4\)), aliquoted, frozen at -70°C, and diluted on the day of the experiment. Stocks were remade every 3 mo. All doxorubicin incubations were for 1 h at 37°C.

Cell Survival Assay. After drug treatment, cells were washed twice with PBS, removed from the dishes with PBS containing 0.03% trypsin and 0.27 mM EDTA, and diluted into culture dishes in numbers to yield between 20 and 200 colonies per plate. Dilutions were performed in triplicate. After 6-7 days (for V79 cells) or 12-14 days (for A549 and CCL210 cells), the plates were fixed with methanol-acetic acid, stained with crystal violet, and scored for colonies containing more than 50 cells. Doxorubicin cell survival curves from experiments using preincubations with ouabain and/or verapamil were corrected for the plating efficiency of cells that were treated with the preincubation medium alone.

\(^{86}\)Rb\(^+\) Content. Cells were incubated overnight with 0.01-0.1 \(\mu\)Ci/ml of \(^{86}\)Rb\(^+\) (1 \(\mu\)Ci/ml, 240 \(\mu\)Ci/ml; Amersham). The following day, ouabain was added in a small volume of medium that was heat inactivated at 56°C for 1 h. CCL210 cells were maintained in F-12 medium with 15% fetal bovine serum that was not heat inactivated. All media were supplemented with penicillin and streptomycin. Under these conditions, the plating efficiencies of V79 cells, A549 cells, and CCL210 cells were 80-90%, 50-75%, and 10-20%, and the doubling times were approximately 9 h, 24 h, and 24 h, respectively.

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The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.1 Present address: University of Michigan Medical Center, Department of Radiation Oncology, UH-B2C490/0010, 1500 E. Medical Center Drive, Ann Arbor, MI 48109-0010.

1 The abbreviations used are: PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; AMSA, 4'-(9-acridinylamino)methanesulfonlyl-m-anisidide; CHO, Chinese hamster ovary.

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Determination of Intracellular Doxorubicin. After drug treatment, V79 cells were removed from the plates with glucose-free trypsin-EDTA which contained 10 mM sodium azide to inhibit doxorubicin efflux during cell preparation (15). Cells were washed twice more with ice-cold azide-PBS, and the cell pellet was extracted with 2 ml of a 0.3 N HCl:50% ethanol solution, as described by Bachur (16). Fluorescence was determined with a Perkin-Elmer spectrophotometer (excitation, 470 nm; fluorescence, 585 nm) and compared to standards prepared in the same solvent. The fluorescence from samples extracted from untreated cells was subtracted from experimental samples. Assays were performed in duplicate. Data are expressed as ng of doxorubicin per 10⁶ cells.

Fluorescence Microscopy. V79 cells were grown on No. 1 coverslips in 60-mm culture dishes. Cells were then exposed to drugs as described above, mounted in PBS, and viewed immediately using a Nikon Optiphot microscope equipped with fluorescence optics. Photographs were taken with Tri-X film and developed with Accufine developer to yield an approximate ASA of 1200. For each experiment all films were exposed, developed, and printed identically to allow comparison between experimental conditions.

Alkaline Elution. V79 cells were incubated overnight in either 0.05 µCi/ml of [2-¹C]thymidine (59.3 mCi/mmol) or 0.2 µCi/ml of [methyl-³H]thymidine (20 Ci/mmol) (New England Nuclear). After a 3-h chase with medium without label, [¹⁴C]thymidine-labeled cells were exposed to drugs as indicated. [³H]Thymidine-labeled cells were irradiated on ice in PBS with 1.5 Gy and then used as an internal standard (17). Alkaline elution was then performed according to the method of Kohl (18) with minor modifications. Briefly, 5 x 10⁶ [¹⁴C]thymidine-labeled cells and 2 x 10⁶ [³H]thymidine-labeled cells were filtered onto 0.8-µm polycarbonate filters in cold buffered PBS and lysed at 25°C with two 4-ml aliquots of a solution containing 2% SDS, 20 mM EDTA, and 0.1 M glycine (pH 9.6) which flowed through by gravity. Filters were then exposed to this same solution containing proteinase K (0.5 mg/ml) to unmask single-strand breaks. Alkaline elution was then carried out at a rate of approximately 2 ml/h with a solution of 20 mM EDTA (acid form), tetrapropylammonium hydroxide to a pH of 12.2, and 0.1% SDS. Eluted fractions were collected and counted for radioactivity according to published methods (19). Assays were performed in duplicate. Data from individual elutions are expressed as the fraction of [¹⁴C]thymidine retained on the filter with increasing time. Since the elution rate varies as a first-order function of the number of strand breaks, these profiles can be used to estimate the relative ratios of breaks that occur under different conditions during the same experiment. Multiple elutions were compared by normalizing to a fixed amount of [³H]standard retained on the filter (17).

Statistical Analysis. For cell survival assays, the standard error was typically less than 15% of the mean and is contained within the size of the symbol unless otherwise indicated. For assays of intracellular ⁸⁶Rb⁺, DNA alkaline elution, and doxorubicin, the duplicate determinations were within 10% of the mean. Results from individual experiments are shown; all experiments were repeated at least once.

RESULTS

Doxorubicin cytotoxicity was assessed in cell lines chosen to allow comparison between human cells (A549 and CCL210) and rodent cells (V79), since the latter are far more resistant to ouabain (20). A 1-h incubation with doxorubicin caused a dose-dependent decrease in the survival of clonogenic cells of all 3 lines (Fig. 1). This cytotoxicity was greatly inhibited when the cells were incubated with ouabain for 5 h beginning 1 h prior to doxorubicin exposure. In the cases of A549 and V79 cells exposed to 1.5–2 µg/ml of doxorubicin, ouabain caused an approximately 100-fold increase in surviving cells. For CCL210 cells, the presence of ouabain led to a 10-fold increase in cell survival.

This protective effect was dependent on the concentration of ouabain (Fig. 1). For A549 and CCL210 cells, ouabain was effective at concentrations in the submicromolar range, with a maximum effect at 1 µM. For V79 cells, the maximum protective effect of ouabain was seen at a concentration of 300 µM.

The time course of ouabain protection was examined in detail in V79 cells (Fig. 2). In experiments in which cells were preincubated with ouabain for 1 h prior to doxorubicin addition, the protective effect was seen at a concentration of 300 µM.
rubricin exposure. Protection reached a maximum when the incubation was continued for 4 h after doxorubicin exposure.

In order to further evaluate the time course of ouabain's protective effect, the timing of the ouabain exposure was varied (Fig. 3). The same protective effect was seen when ouabain incubation was begun either 1 h prior to doxorubicin exposure, or concurrent with the doxorubicin exposure. This demonstrates that ouabain's protective effect had a rapid onset. Oua-

bain addition could be delayed until the end of the doxorubicin incubation and still confer some protection. If ouabain were added 1 h after doxorubicin exposure, there was no protection from cytoxicity. Therefore, ouabain did not appear to be able to reverse doxorubicin cytoxicity after a short critical period had elapsed.

Since ouabain is a potent inhibitor of the Na+, K+-pump, it seemed likely that ouabain’s protective effect was mediated by changes in the intracellular ionic composition. If this were the case, the dose-response and time-course characteristics of these changes in ionic composition should parallel those described for ouabain-mediated protection from doxorubicin toxicity. Intracellular ionic composition was estimated by measuring 86Rb+ leakage from preloaded cells after exposure to ouabain. It has been shown in many systems that cells process 86Rb+ (half life, 18.6-days) and 42K+ (half life, 12 h) similarly (21, 22). When these three cell types were exposed to ouabain, they rapidly lost 86Rb+ (Fig. 4). Submicromolar concentrations of ouabain were effective in causing 86Rb+ loss from A549 and CCL210 cells, while much higher concentrations were required to induce 86Rb+ loss than V79 cells. The concentrations of ouabain that caused 86Rb+ leakage for each cell type were the same as those that protected against doxorubicin cytoxicity.

In order to rule out the possibility that ouabain exerted its protective effect by decreasing the intracellular doxorubicin level, doxorubicin influx and efflux were assessed in V79 cells under conditions and concentrations identical to those used for cell survival studies (Fig. 5). After a 1-h incubation, intracellular doxorubicin levels were a linear function of the extracellular doxorubicin concentration. These levels were completely unaffected by the presence of 1 mM ouabain, demonstrating that ouabain had no effect on doxorubicin influx.

It was particularly important to determine if ouabain affected doxorubicin efflux, since increased efflux has been reported in many instances of doxorubicin resistance (12-14). As was the case for influx, efflux of doxorubicin was unaffected by 1 mM ouabain (Fig. 5). To investigate this further, studies with the Ca2+ blocker verapamil were conducted. Verapamil blocks dox-

orubicin efflux (Fig. 5) and greatly increased doxorubicin cy-
totoxicity (Fig. 6). Ouabain had no effect on the verapamil-

induced blockade of doxorubicin efflux. However, increased
doxorubicin cytotoxicity caused by verapamil was significantly reversed by ouabain. Therefore, the protection by ouabain from doxorubicin toxicity was not caused by a change in intracellular doxorubicin levels.

The above data were all measurements of total cellular dox-
orubicin levels. A possible effect of ouabain could be to block doxorubicin from reaching the nucleus, which is the presumed site of action of this drug. Studies using fluorescence micros-
copy show that there was no detectable difference in nuclear fluorescence between ouabain-treated and control cells that were exposed to the same concentration of doxorubicin (Fig. 7). In both cases, most of the doxorubicin fluorescence was in the nucleus. Therefore, ouabain did not prevent doxorubicin from reaching its site of action in the cell nucleus.

These data indicated that ouabain, by altering the intracellu-

lar environment, inhibited doxorubicin that was present inside the cell nucleus from becoming cytoxic. This suggested that ouabain acted by interfering with the interactions between dox-
orubicin and DNA. In fact, ouabain greatly inhibited doxorubicin-induced formation of DNA single-strand breaks in V79 cells (Fig. 8). Eighty-three % (±4%, n = 4) of the strand breaks induced by 1.5 μg/ml of doxorubicin was inhibited by preincu-
bation with 1 mM ouabain for an hour. Nearly identical elution
Fig. 5. Ouabain has no effect on intracellular doxorubicin levels. In A, V79 cells were preincubated for 1 h with 1 mM ouabain (B), 10 μM verapamil (A), both ouabain and verapamil (A), or under control conditions (C). Cultures were then rinsed twice with PBS and processed immediately for intracellular doxorubicin content (time = 0) or returned to medium containing the preincubation drugs and assessed at varying times after drug exposure. In B, V79 cells were preincubated for 1 h with 1 mM ouabain or under control conditions prior to the addition of varying concentrations of doxorubicin. After 1 h, cultures were assessed for doxorubicin content.

Fig. 6. Ouabain partially reverses verapamil potentiation of doxorubicin cytotoxicity. V79 cells were preincubated with 1 mM ouabain (B), 10 μM verapamil (A), both verapamil and ouabain (A), or under control conditions (C). Cells were then exposed to varying concentrations of doxorubicin for 1 h, rinsed twice with PBS, and returned to medium containing preincubation drugs. Cultures were assessed for cell survival 4 h later.

 Profiles were obtained from cells treated with 0.25 μg/ml of doxorubicin alone and those exposed to 1.5 μg/ml of doxorubicin in the presence of ouabain (data not shown). Since cell survival was the same under these two conditions (approximately 10%; see Fig. 1), there appeared to be a close correlation between DNA strand breaks and cell survival.

To better explore this possibility, the data from multiple elutions were combined and normalized as described in “Materials and Methods” to allow comparison with cell survival throughout the range of doxorubicin concentrations used (Fig. 9). Ouabain prevented the formation of strand breaks at both low (0.5 μg/ml) and high (1.5 μg/ml) concentrations of doxorubicin. Cell survival data from Fig. 1 were overlaid on the elution data to facilitate direct comparison. A close association between doxorubicin-induced DNA strand breaks and cell lethality was evident.

DISCUSSION

These results demonstrate that ouabain, a specific inhibitor of the Na⁺, K⁺-pump, confers protection from doxorubicin cytotoxicity. This protective effect occurs at the same concentrations of ouabain that result in pump blockade and decrease in intracellular K⁺ (as assessed by the K⁺ analogue ⁸⁶Rb⁺). This correlation between pump inhibition and protection is clearly demonstrated through comparison of a rodent cell line (V79) and human cell lines (A549 and CCL210). The Na⁺, K⁺-pump of rodent cells is approximately 300-fold more resistant to ouabain than other mammalian cells (20), and this difference is quantitatively reflected in the present study by the higher concentrations of ouabain needed to protect V79 cells, compared to A549 or CCL210 cells, from doxorubicin cytotoxicity.

Studies were conducted to elucidate the mechanism by which blockade of the Na⁺, K⁺-pump leads to doxorubicin protection.

Fig. 7. Ouabain does not affect intranuclear doxorubicin. V79 cells were preincubated for 1 h under control conditions (A and C) or with 1 mM ouabain (B and D). They were then exposed to 0.5 μg/ml (A and B) or 1.5 μg/ml (C and D) of doxorubicin for 1 h and photographed using fluorescence optics as described in “Materials and Methods.”

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confirmed these findings and showed that ouabain partially inhibits the potentiation of cytotoxicity without affecting the verapamil-induced increase in intracellular doxorubicin. Therefore, ouabain does not appear to protect cells from doxorubicin cytotoxicity by affecting doxorubicin influx, efflux, or access to DNA.

It, therefore, appeared likely that ouabain interfered with the ability of doxorubicin to interact with DNA. As described in the “Introduction,” doxorubicin stabilizes DNA-topoisomerase II complexes, which are detected as single- and double-strand DNA breaks. Indirect evidence has suggested that these intercalator-induced strand breaks are related to cell cytotoxicity. For instance, m-AMSA, which causes more single-strand breaks than its analogue o-AMSA, is also more cytotoxic than o-AMSA (25). However, other examples exist in which drugs that cause a similar degree of strand breakage have very different effects on cell survival (4). Stronger support for a direct correlation between cell survival and strand breaks comes from studies of CHO mutants which are more sensitive to doxorubicin cytotoxicity than wild-type CHO cells. These mutants also show more single-strand breaks after doxorubicin exposure (26). In the present study, direct evidence is presented that doxorubicin cytotoxicity correlates with the induction of single-strand breaks. In the presence of ouabain, both cytotoxicity and strand breakage were decreased dramatically and remained tightly correlated. The data also demonstrate a critical window beginning at the time of exposure and extending to approximately 1 h after exposure during which doxorubicin damage becomes fixed. Ouabain treatment after this time did not confer any protection from cytotoxicity. It will be of interest to determine if the DNA-topoisomerase II complexes that have been shown to exist many hours after doxorubicin exposure (25) are affected by ouabain at a time when cytotoxicity is not. For instance, if ouabain caused complexes to dissociate under these conditions, it would suggest that the doxorubicin-induced lethal event occurs within the first hour after exposure and that cell death does not depend on the continued presence of the stabilized complex. Such experiments should better elucidate the link between the formation of DNA-topoisomerase II complexes and cytotoxicity.

The mechanism by which ouabain blocks doxorubicin-mediated stabilization of DNA-topoisomerase II complexes is uncertain. The rapid onset of protection correlates best with the rapid decrease in intracellular K+ (as assessed by 86Rb+) that occurs when ouabain inhibits the Na+, K+-pump. This would suggest that doxorubicin-induced stabilization of DNA-topoisomerase II complexes depends directly on the concentrations of intracellular K+ and Na+. Another possibility is that the ouabain-induced increase in intracellular Na+ triggers the Na+, Ca2+ exchange pump that exists in many cells (27). This would cause an increase in intracellular Ca2+ levels. Indeed, ouabain’s ability to reverse the acute negative ionotropic effect of doxorubicin on isolated cardiac muscle has been attributed to this mechanism (28). It is conceivable that an elevation of intracellular Ca2+ could also affect doxorubicin-induced DNA-topoisomerase II stabilization. However, it should be noted that the activity of purified topoisomerase II is critically dependent on the concentration of Mg2+ and is independent of Ca2+ (29). In addition, if Ca2+ were the mediator of ouabain’s protective effect, one would expect an interaction between the effects of ouabain and the Ca2+ blocker verapamil on doxorubicin cytotoxicity. However, the present study reveals no such interaction. Ouabain, through its effect on intracellular K+, does have additional intracellular effects, such as inhibition of protein and DNA synthesis.
DNA synthesis. However, these effects do not occur until hours after exposure (30–32). This slower time course does not appear to be rapid enough to account for the finding that, when ouabain is added simultaneously with doxorubicin, complete protection occurs.

The current study suggests that it may be possible to influence the efficacy of doxorubicin chemotherapy through manipulation of the Na⁺, K⁺-pump. Pump activity has been shown, in many cases, to be dramatically different in tumor or transformed cells compared to their normal counterparts (33, 34). Although in the current study ouabain protected both tumor (A549) and normal (CCL210 and V799) cells from doxorubicin, it remains possible that differences in Na⁺, K⁺-pump activity between tumor and normal cells could be exploited to improve the therapeutic index of doxorubicin chemotherapy. It will clearly be important to determine if ouabain-induced protection from doxorubicin occurs in other normal and tumor cell lines, and in settings other than cell culture.

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