Differential Effect of the Calmodulin Inhibitor Trifluoperazine on Cellular Accumulation, Retention, and Cytotoxicity of Anthracyclines in Doxorubicin (Adriamycin)-resistant P388 Mouse Leukemia Cells

Ram Ganapathi, Dale Grabowski, William Rouse, and Francis Riegler

Research Division, Cleveland Clinic Foundation, Cleveland, Ohio 44106

ABSTRACT

Calmodulin inhibitors enhance cytotoxic effects of doxorubicin (DOX) in DOX-resistant (P388/DOX) P388 mouse leukemia cells by increasing cellular accumulation and retention of drug. In P388/DOX cells treated for 3 hr, cytotoxic effects (based on colony formation in soft agar) of daunorubicin (DAU) in the presence of trifluoperazine (TFP) were DAU concentration-dependent and enhanced 2- to 100-fold. Additionally, in the presence of TFP, on a molar basis, equitoxic doses of DAU were 4-fold lower than DOX for P388/DOX cells. However, in P388/DOX cells treated for 3 hr with other anthracyclines, except for a slight enhancement in the cytotoxic effects of aclacinomycin A (ACM) with TFP, colony formation in soft agar of cells treated with N-trifluoroacetyladriamycin-14-valerate (AD32) and N-trifluoroacetyladriamycin were similar in the absence and presence of TFP. In DOX-sensitive (P388/S) P388 mouse leukemia cells treated for 3 hr, some enhancement in the cytotoxic effects due to TFP were observed with DAU and DOX but not with ACM, AD32, or N-trifluoroacetyladriamycin. Although accumulation of ACM and AD32 in P388/S and P388/DOX cells was similar and unaffected by TFP, the retention of ACM but not AD32 was enhanced 1.5-fold only in TFP-treated P388/DOX cells. In contrast, DAU accumulation in P388/S cells was 4-fold higher than in similarly treated P388/DOX cells, and the 2- and 4-fold increase due to TFP in the accumulation and retention, respectively, of DAU in P388/DOX cells was not observed in P388/S cells. Results from this study indicate that in P388/DOX cells, the calmodulin inhibitor TFP is more effective with DAU than DOX, significantly less effective with ACM, and ineffective with AD32 and N-trifluoroacetyladriamycin.

INTRODUCTION

The anthracycline antibiotics DAU and DOX play a prominent role in the chemotherapeutic management of human cancers (5, 28). An inevitable reality limiting the success of chemotherapy is the presence of intrinsically drug-resistant cells and/or the emergence of resistant clones following repeated courses of chemotherapy (21, 29). In order to understand the mechanisms involved with cellular resistance to anthracyclines, tumor model systems have been developed, and a common characteristic of these resistant sublines is the reduced accumulation and/or retention of cytotoxic concentrations of the drug (9, 14, 17, 24, 27). Recent studies by us (12, 13) and Tsuruo et al. (30, 31) indicate that calmodulin inhibitors and calcium antagonists are capable of partially reversing resistance in DOX-resistant sublines of mouse leukemia P388, primarily by increasing cellular retention of drug.

In the search for anthracyclines with broader spectrum of antitumor activity and less cardiotoxicity than DAU and DOX, a number of naturally occurring and semisynthetic anthracyclines with significantly different cellular pharmacokinetics, antitumor activity, and toxicity characteristics have been evaluated (8, 33). Although reduced drug accumulation appears to be a primary reason for resistance, anthracyclines with potent antitumor activity, and which are rapidly accumulated in cells due to their high lipophilicity, have been of limited value in circumventing resistance in DOX-resistant sublines (18). In the present study, we have attempted to determine, in DOX-resistant cells, the relationship between cellular drug levels and cytotoxicity of anthracyclines and the potential role for TFP in modulating these effects. Specifically, using the DOX-sensitive (P388/S) and DOX-resistant (P388/DOX) P388 mouse leukemia model system, the effect of the calmodulin inhibitor TFP on the cellular accumulation, retention, and cytotoxicity of the anthracyclines, namely, DAU, AD32, AD41, and ACM, which are distinctly different from DOX, was determined.

MATERIALS AND METHODS

The source of the P388/S and P388/DOX cells and conditions for their maintenance in vitro are similar to those reported previously (12, 14). TFP was a generous gift from Dr. Carl Kaiser, Smith, Kline & French Laboratories, Philadelphia, PA. DAU and ACM were provided by Dr. Ven Narayan, Chief, Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. AD32 and AD41 were a generous gift from Dr. Mervyn Israel, Department of Pharmacology, University of Tennessee Center for Health Sciences, Memphis, TN.

Anthracycline Cytotoxicity in Vitro. Cytotoxicity studies were carried out using a soft-agar colony-forming assay. Stock solutions of DOX, DAU, ACM, and TFP were prepared in sterile-glass distilled water and in the case of AD32 and AD41, stock solutions were prepared in DMSO. Working dilutions were made in RPMI 1640 supplemented with 25 mM N-2-hydroxyethylpiperazine-N-ethanesulfonic acid buffer (M. A. Btoprod-...
and 0.05 to 2.0 μg/ml for P388/DOX cells; ACM, 0.01 to 0.5 μg/ml for P388/S cells and 0.05 to 1.0 μg/ml for P388/DOX cells; AD32 and AD41, 0.1 to 5.0 μg/ml for P388/S and 0.5 to 10.0 μg/ml for P388/DOX cells. The cells following treatment were centrifuged (80 × g), washed twice with drug-free RPMI 1640 supplemented with 10% FBS, and plated in triplicate in 35 × 10-mm Petri dishes. The plating medium used was RPMI 1640 supplemented with 20% FBS, 10 μM 2-mercaptoethanol, conditioned medium from culture supernatants of P388/S or P388/DOX cells, and 0.3% agar. Following incubation for 120 hr in a humidified 5% CO2 plus 95% air atmosphere, colonies (>50 cells) in untreated control and treated plates were counted in an Omnicon Feature Analysis System (Bausch and Lomb, Rochester, NY). Plating efficiency of control untreated P388/S and P388/DOX cells under these conditions was approximately 30%.

### Cellular Anthracycline Accumulation In Vitro. P388/S and P388/DOX cells in RPMI 1640 supplemented with 10% FBS were treated with DAU (0.1 and 1.0 μg/ml), ACM (0.1 and 1.0 μg/ml), and AD32 (0.5 and 5.0 μg/ml) in the absence and presence of 5 μM TFP at 37°C. Duplicate 1-ml aliquots of (1 × 10^6) cells were harvested following a 3-hr incubation were centrifuged (100 × g) and washed twice with 7 ml of ice-cold 0.85% sodium chloride solution. The cells following the final wash were mixed thoroughly in a vortex mixer with 50% ethanol-0.3 n hydrochloric acid and were centrifuged at 700 × g, and anthracycline content in the supernatant was determined fluorimetrically (1,12) in an Aminco Bowman spectrophotofluorometer (American Instrument Co., Silver Spring, MD). Excitation and emission wavelengths of 470 and 585 nm, respectively, were used for DAU, AD32, and AD41; and for ACM, the excitation and emission wavelengths were 450 and 585 nm, respectively. Anthracycline content was computed from standard curves prepared with DAU, AD32, AD41, ACM, and were expressed as ng/10^6 cells.

### Cellular Anthracycline Retention In Vitro. P388/S and P388/DOX cells in RPMI 1640 supplemented with 10% FBS were pretreated for 1 hr at 37°C with DAU (0.5 μg/ml for P388/S cells and 2.0 μg/ml for P388/DOX cells), ACM (0.5 μg/ml), and AD32 (1.0 μg/ml) in the absence and presence of 5 μM TFP. Cells were then centrifuged, resuspended in anthracycline-free medium (RPMI 1640 supplemented with 10% FBS) in the absence and presence of 5 μM TFP, and incubated at 37°C. Duplicate aliquots of (10^6) cells retrieved at the end of 1-hr pretreatment drug accumulation phase and at the end of 15, 30, 60, and 90 min during retention phase, were centrifuged (100 × g) and washed twice with 7 ml of ice-cold 0.85% sodium chloride solution. Cellular levels of DAU, ACM, AD32, and AD41 following the final wash were then quantitated fluorimetrically as outlined earlier under accumulation experiments.

### RESULTS

**Effect of TFP on the Cytotoxicity of Anthracyclines In Vitro.** Survival of P388/S and P388/DOX cells treated with DOX and DAU in the absence and presence of TFP is presented in Table 1. In P388/S cells, cytotoxic effects following treatment with DOX for 3 hr was markedly dose-dependent and ~1.5-fold and ~3.5-fold enhancement in cytotoxic effects in the presence of TFP were observed at DOX concentrations of 0.02 and 0.04 μg/ml, respectively. Cytotoxic effects of DOX in P388/DOX cells were relatively independent of dose at drug concentrations of 0.05 to 2.0 μg/ml, and a marked DOX dose-dependent enhancement in cytotoxicity was apparent only in the presence of TFP. These results are in agreement with our earlier data for 1-hr exposure to DOX in the absence and presence of TFP (12) and further demonstrate as reported (25), increased cell kill with longer drug treatment. At equimolar doses, DAU was 2-fold more cytotoxic than DOX in P388/S cells, and a 1.5- and 8-fold enhancement in the cytotoxic effects of DAU concentrations of 0.005 and 0.01 μg/ml, respectively, in the presence of TFP was observed in cells treated for 3 hr. In P388/DOX cells treated for 3 hr, the cytotoxic effects of DAU were not dose-dependent at drug concentrations of 0.05 to 1.0 μg/ml, and significant reductions in survival with increasing drug concentrations were apparent only in the presence of TFP. Furthermore, with a 3-hr drug treatment in the presence of TFP, on a molar basis, equitoxic doses of DAU were 4-fold lower than DOX for P388/DOX cells.

The cytotoxic effects of AD32, AD41, and ACM in the absence and presence of TFP in P388/S and P388/DOX cells is shown in Table 2. In P388/S cells, cytotoxic effects of AD32 and AD41 were drug concentration dependent, and TFP had no effect in enhancing the cytotoxic activity of either drug with a 3-hr treatment. In P388/DOX cells, although AD32 was relatively noncytotoxic at concentrations of 0.5 to 10 μg/ml, AD41 was signifi-
Table 2

<table>
<thead>
<tr>
<th>Drug concentration</th>
<th>AD32</th>
<th>AD41</th>
<th>ACM</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 μM TFP</td>
<td>100 ± 0</td>
<td>100 ± 0</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>0.05 μg/ml</td>
<td>97 ± 2</td>
<td>99 ± 2</td>
<td>97 ± 2</td>
</tr>
<tr>
<td>0.05 μg/ml + TFP</td>
<td>74 ± 4</td>
<td>98 ± 2</td>
<td>74 ± 3</td>
</tr>
<tr>
<td>0.1 μg/ml</td>
<td>42 ± 6</td>
<td>80 ± 2</td>
<td>42 ± 6</td>
</tr>
<tr>
<td>0.5 μg/ml + TFP</td>
<td>67 ± 2</td>
<td>97 ± 3</td>
<td>97 ± 3</td>
</tr>
<tr>
<td>0.5 μg/ml</td>
<td>0.3 ± 0.3</td>
<td>15 ± 3</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>5.0 μg/ml + TFP</td>
<td>9.3 ± 2</td>
<td>93 ± 2</td>
<td>9.3 ± 2</td>
</tr>
<tr>
<td>5.0 μg/ml</td>
<td>0.2 ± 0.2</td>
<td>6 ± 2</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>10.0 μg/ml + TFP</td>
<td>0.1 ± 0.1</td>
<td>17 ± 1</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>10.0 μg/ml</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
</tbody>
</table>

*a* P388/S and P388/DOX cells were treated with various concentrations of AD32, AD41, and ACM in the presence and absence of 5 μM TFP for 3 hr and plated in soft agar.

*b* Mean ± S.E. from triplicate experiments. Colony counts are expressed as a percentage of the untreated control.

*c* Values are from triplicate plates in duplicate experiments.

Significantly cytotoxic at a concentration of 5.0 μg/ml for a 3-hr drug exposure. Furthermore, unlike results with DAU and DOX, TFP had no effect on the cytotoxic activity of AD32 or AD41 in P388/DOX cells. As shown in Table 2, the cytotoxicity of ACM in P388/S and P388/DOX cells treated for 3 hr was drug dose-dependent over the concentration range of 0.01 to 1.0 μg/ml. Although cytotoxic effects of ACM in P388/S cells were similar in the absence and presence of TFP, in P388/DOX cells, an enhancement in cell kill with ACM in the presence of TFP was observed at the lower drug doses of 0.05 and 0.1 μg/ml.

Effect of TFP on Cellular Anthracycline Accumulation in Vitro. The 3-hr cellular accumulation of DAU, AD32, and ACM by P388/S and P388/DOX cells, in the absence and presence of TFP, is shown in Charts 1, 2, and 3, respectively. In accordance with data in an earlier report (17), accumulation of DAU in P388/DOX cells was 4-fold lower than in similarly treated P388/S cells. Cellular levels of DAU (Chart 1) in P388/S and P388/DOX cells were dose dependent (1.0 μg/ml > 0.1 μg/ml), and in contrast to P388/S cells, TFP produced a significant 1.5- to 2.5-fold increase in cellular levels of DAU in P388/DOX cells. As shown in Charts 2 and 3, accumulation of AD32 and ACM by P388/S and P388/DOX cells is dose dependent and, unlike the results with DAU (Chart 1), cellular levels of AD32 and ACM in P388/S and P388/DOX cells were similar and not significantly different in the absence or presence of TFP.

Effect of TFP on Cellular Anthracycline Retention in Vitro. Due to the differential effect of TFP on the cellular accumulation and cytotoxicity of the various anthracyclines, the role of the calmodulin inhibitor TFP on drug retention, an important determinant of resistance, was evaluated. DAU retention in P388/S and P388/DOX cells treated without and with TFP is shown in Chart 4. As observed earlier with DOX (12), a dramatic increase...
Chart 3. Accumulation of ACM in P388/S (left) and P388/DOX (right) cells in the absence and presence of TFP. A, ACM, 0.1 μg/ml; B, ACM, 1.0 μg/ml. Values, means from triplicate experiments; bars, S.E.; ACM alone; ACM plus 5 μM TFP.

Chart 4. Effect of TFP on the cellular retention of DAU in P388/S and P388/DOX cells. Points, mean value from triplicate experiments; bars, S.E.; ACM, 0.5 μg/ml; TFP, 0.5 μg/ml; ACM plus 5 μM TFP; DOX, 2.0 μg/ml; ACM plus 5 μM TFP; DOX-resistant P388.

The retention of DAU in P388/S cells was about 60 to 70% of the drug initially accumulated and not affected by TFP, whereas in P388/DOX cells, retention of DAU in the absence and presence of TFP was <20% and >40%, respectively. The effect of TFP on retention of AD32 and ACM in P388/S and P388/DOX cells is shown in Chart 5. Similar to the accumulation data, TFP had no effect on the retention of AD32 in P388/S or P388/DOX cells, and between 30 and 40% of the drug initially accumulated was retained at the end of the 90-min incubation in both sensitive and DOX-resistant cells. Retention studies with ACM indicated that drug levels in P388/S cells were not affected by TFP, and represented about 75 to 80% of the amount initially accumulated. In contrast, retention of ACM in P388/DOX cells was similar to that of P388/S cells only with TFP treatment, and cellular drug levels were about 1.5-fold higher than in the absence of TFP.

DISCUSSION

Studies with DOX- and DAU-resistant tumor model systems have demonstrated that cellular resistance to drug-induced cytotoxic effects are primarily due to reduced drug accumulation and/or retention (9, 14, 17, 24, 27). The impaired retention of drug in the resistant sublines has been mainly attributed to efflux of intracellular drug by an energy-dependent mechanism (9, 17, 27). Recent studies attempting to possibly circumvent cellular DOX and vincristine resistance suggest that both calmodulin inhibitors and calcium antagonists (12, 13, 31) enhance DOX- and vincristine-induced cytotoxic effects in resistant sublines.
primarily by increasing cellular accumulation and retention of drug. At the present time, the specific mechanism of action of these agents in enhancing sensitivity to the cytotoxic effects of DOX and vincristine sensitivity in resistant sublines is not clear, and the involvement of a calcium-dependent and/or calmodulin-mediated process is circumstantial and based on the following evidence. (a) Enhancement of sensitivity to cytotoxic effects of DOX in resistant P388 cells by phenothiazines correlate with the potency of these agents to inhibit calmodulin-mediated processes (13). Additionally, it was also demonstrated that N-(4-aminobutyryl)-5-chloro-2-naphthalenesulfonamide (W-13) but not N-(4-aminobutyryl)-2-naphthalenesulfonamide (W-12) was effective in enhancing DOX cellular levels and cytotoxic effects in the resistant P388 cells (13). (b) Vincristine-resistant P388 mouse leukemia cells contain significantly greater amounts of calcium (especially in the form of ethyleneglycol bis(4-aminoethyl ether)-N,N,N',N'-tetraacetic acid-removable surface bound calcium) than do parent sensitive cells (32).

The DOX-resistant subline of mouse leukemia P388 used in the present study has been found to be cross-resistant to a variety of anthracyclines, the Vinca alkaloids, and actinomycin-D (18). Although reduced DOX accumulation has been identified as a major mechanism of resistance in this cell line, the lack of cytotoxic effects with highly lipophilic, rapidly transported anthracyclines is enigmatic. Our data from the present study demonstrate that TFP has a differential effect on the cellular levels and cytotoxicity of anthracyclines DAU, AD32, AD41, and ACM which are distinctly different from DOX in their cellular pharmacokinetics and antitumor activity. Although the in vitro cytotoxicity data (Table 1) indicate that P388/DOX cells are >100-fold resistant to DAU and DOX, in the presence of TFP, on a molar basis, equitoxic doses of DAU were 4-fold lower than DOX, and this difference may in part be due to the more rapid cellular accumulation characteristics of DAU. A comparison of our earlier data on the effect of TFP with DOX (12) and the present data with DAU, indicate that overall, TFP has a more pronounced effect on cellular accumulation, retention, and cytotoxicity of DAU in P388/DOX cells. Additionally, although TFP is effective in enhancing the cytotoxic activity of DAU and DOX in P388/DOX cells following a 1- and 3-hr drug exposure, enhanced cell kill in the presence of TFP with DAU and DOX in P388/S cells are observed only with 3-hr but not 1-hr drug treatment.

AD32 is a highly lipophilic analogue of DOX which does not bind to DNA (22), and cellular fluorescence is reported to be localized exclusively in the cytoplasm (20). Data from the present study indicate, that in spite of similar accumulation and retention of AD32 in P388/S and P388/DOX cells, the drug is nontoxic to P388/DOX cells at concentrations as high as 10.0 µg/ml (Table 2). Seebeler et al. (26) in an earlier study, also demonstrated that accumulation of AD32 in DAU-sensitive and -resistant Ehrlich ascites cells was similar, and suggested that the lack of difference may in part have been due to the membrane effects of the Tween 80 used to solubilize the drug. In the present study, however, DMSO was used to dissolve AD32, and since a final concentration of 1% DMSO in the tissue culture medium does not enhance accumulation or cytotoxicity of other anthracyclines, it is possible that resistance to AD32 in P388/DOX cells (in spite of similar drug accumulation and retention characteristics as in P388/S cells) could be due to the reduced ability for cellular breakdown of the drug to a cytotoxic metabolite. AD41 is the “active” metabolite of AD32, and to determine if indeed AD32 was being handled differently in vitro by P388/S and P388/DOX cells, cytotoxicity studies with AD41 in the absence and presence of TFP were carried out (Table 2). From the data in Table 2, it is apparent that AD41 is significantly more cytotoxic, and on a molar basis is >5-fold more potent than AD32. Furthermore, unlike DOX and DAU, P388/DOX cells are only ~10-fold resistant to AD41. AD41 like AD32 (22) does not bind to DNA, and it is certainly interesting that in P388/DOX cells, TFP had no effect in enhancing the cytotoxic activity of these lipophilic non-DNA-binding anthracyclines.

Similar to results with AD32, accumulation studies indicated that cellular levels of the class II anthracycline ACM in P388/S and P388/DOX cells were comparable and unaffected by TFP. This observation is in agreement with a similar finding in DAU-sensitive and -resistant Ehrlich ascites cells (26) and Friend leukemia cells (11), although Egorin et al. (10), using DOX-sensitive and -resistant P388 cells, reported lower ACM levels in the resistant subline, and the basis for this discrepancy is not clear. In contrast to the accumulation data, both ACM retention and cytotoxicity (only at lower ACM concentrations of 0.05 and 0.1 µg/ml) in P388/DOX cells was enhanced in the presence of 5 µM TFP, and data were similar to those of P388/S cells treated with similar concentrations of ACM in the absence of TFP. The differential effect of TFP on accumulation versus retention and cytotoxicity of ACM in P388/DOX cells suggests the possible alteration in intracellular ACM binding (11).

The P388/DOX cells used in the present study are also cross-resistant to the Vinca alkaloids and actinomycin-D (18). In a recent report, Beck et al. (2) suggested that reduced retention of vinblastine in vinblastine-resistant CCRF-CEM human leukemic lymphoblasts is possibly due to energy-dependent reduced drug binding. Since TFP does modulate cellular levels and cytotoxic effects of anthracyclines and Vinca-alkaloids in DOX-resistant and vincristine-resistant sublines of P388 cells, the effects of TFP on altering the ratio of free versus bound drug may be an important mechanism of action responsible for enhanced drug sensitivity in the resistant sublines.

A number of studies with a variety of anthracycline and Vinca alkaloid-resistant model systems have demonstrated the presence in the plasma membrane of a glycoprotein with a molecular weight of approximately 150,000 to 180,000 (3, 19, 23). Although it has been suggested that this glycoprotein present in some of the resistant sublines (3, 19, 23) may be responsible for reduced drug permeation, recent studies suggest that this high-molecular-weight glycoprotein was not found in other DOX-resistant human and murine tumors (7, 16). The relationship between phosphorylation of the high-molecular-weight glycoprotein and resistance has also been suggested (4, 6); although a M, 180,000 glycoprotein has been identified in the P388/DOX cells used in this study (7), it is not clear if the pronounced effect of TFP in enhancing cellular levels of DOX in the DOX-resistant subline of P388 cells is related to superphosphorylation of the glycoprotein, as reported by Center (8) and Garman et al. (15) with DOX-resistant Chinese hamster lung HT-1 cells.

The differential effect of TFP on the cellular levels and cytotoxicity of various anthracyclines in P388/S and P388/DOX cells is summarized in Table 3. From the data in Table 3, it is apparent that the effects of TFP appear to be more specific and striking for the strong DNA-binding anthracyclines DAU and DOX. Fur-
thermore, cellular anthracycline levels alone (especially with highly lipophilic anthracyclines) may not be a reliable indicator to predict drug-induced cytotoxic effects in resistant cells, suggesting that multiple mechanisms may be operational in the cellular expression of anthracycline resistance.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Mervyn Israel for his helpful discussions concerning AD32 and AD41. The authors also gratefully acknowledge Njolle Mazzells and Catherine Reinhard for their excellent secretarial assistance and Joseph Pangrace of the Art-Medical Illustrations and Photography Department for skilful preparation of the artwork.

REFERENCES

Differential Effect of the Calmodulin Inhibitor Trifluoperazine on Cellular Accumulation, Retention, and Cytotoxicity of Anthracyclines in Doxorubicin (Adriamycin)-resistant P388 Mouse Leukemia Cells

Ram Ganapathi, Dale Grabowski, William Rouse, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/44/11/5056

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.