ABSTRACT

Resistance to the cytotoxic effects of daunomycin and Adriamycin (ADR) in sublines of Ehrlich ascites and P388 mouse tumors has been demonstrated to be due to reduced cellular accumulation and retention of drug. In this study, the effect of the calmodulin inhibitor trifluoperazine on the cellular accumulation, retention, and cytotoxic effects of ADR in ADR-sensitive (P388/S) and ADR-resistant (P388/R) P388 mouse leukemia cells was determined. In cells treated in suspension culture for 24 hr or for 1 hr followed by plating in soft agar, a noncytotoxic concentration of 4 μM trifluoperazine enhanced the sensitivity to ADR 2- to 6-fold in P388/R but not in P388/S cells. A marked enhancement in cellular retention rather than accumulation of ADR in only P388/R cells was obtained with trifluoperazine treatment. This study suggests the possible novel use of phenothiazines to improve drug sensitivity of tumors resistant to ADR treatment.

INTRODUCTION

The emergence of drug-resistant tumor clones continues to be a serious challenge in cancer chemotherapy. Repeated courses of chemotherapy administered to eradicate a tumor provide an opportunity for the selection of drug-resistant clones during exposure to suboptimal drug levels. This is further compounded by the heterogeneous nature of tumor cells which contain subpopulations varying in their sensitivity to the cytotoxic effects of a drug (3, 15). Studies on DAU and ADR-resistant Ehrlich ascites (5) and P388 (8, 9) mouse tumors indicate that resistance is secondary to reduced cellular accumulation and retention of drug. The decreased cellular retention of DAU and ADR has been demonstrated to be due to active efflux of intracellular drug by an energy-dependent mechanism (5, 9).

Based on these suggested mechanisms of resistance, the availability of an inhibitor to circumvent this uptake and/or efflux mechanism could potentially lead to increased drug sensitivity in the resistant cells. Danos (5) evaluated the effect of ouabain, an inhibitor of Na⁺/K⁺-ATPase, and found that DAU uptake by resistant cells was not altered by such treatment. Skovsgaard (14) reported that an analogue of daunorubicin, N-acetylodaunorubicin, inhibited active efflux of DAU in both wild type-sensitive and DAU-resistant Ehrlich ascites tumor cells. Further, in vivo treatment with N-acetylodaunorubicin was found to significantly enhance the antitumor activity of DAU only against the DAU-resistant variant of Ehrlich ascites cells. More recently, verapamil (16) and reserpine (7), which control calcium fluxes across membranes, have been shown to increase drug uptake, reduce efflux, and increase sensitivity to drug treatment in vincristine and ADR-resistant P388 mouse leukemia.

Since phenothiazines are "inhibitors" of calmodulin (17) and can stabilize membranes by impairing calcium fluxes (11), an evaluation of their role in reversing ADR resistance was considered a potential alternative. In the present study, we have determined the effect of the phenothiazine, TFP, a potent inhibitor of calmodulin on the cellular accumulation, retention, and cytotoxicity of ADR in ADR-sensitive (P388/S) and in >100-fold ADR-resistant (P388/R) mouse leukemia cells.

MATERIALS AND METHODS

The P388/S (10) and P388/R (10) cells were obtained from Dr. Randall K. Johnson, Arthur D. Little, Inc., Cambridge, Mass., and were maintained in female DBA/2 mice by weekly i.p. transplantation. In vitro cultures of P388/S and P388/R cells were established from mouse ascites and were maintained for no longer than 3 months in RPMI 1640 supplemented with 25 mM N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid buffer (M. A. Bioproducts, Walkersville, Md.), 10% fetal bovine serum (Sterile Systems, Inc., Logan, Utah), 10 μM 2-mercaptopethanol, penicillin, and streptomycin. P388/R cultures were maintained in the absence of ADR, and no change in the pattern of ADR sensitivity was observed. TFP was a generous gift from Dr. Carl Kaiser, Smith Kline and French Laboratories, Philadelphia, Pa.

In Vitro Cytotoxicity. Log-phase suspension cultures of P388/S and P388/R cells were treated with ADR (0.01 to 5.0 μg/ml) in the presence and absence of 4 μM TFP for 24 hr at 37°C. Cell counts in control and treated cultures were then determined by trypan blue dye exclusion in a hemacytometer.

In long-term drug exposure experiments in soft agar, P388/S and P388/R cells in RPMI 1640 supplemented with 20% FBS, 10 μM 2-mercaptopethanol, and 0.3% agar were plated (~2 × 10⁴ cells/plate) with ADR (0.005 P388/S only), 0.01, 0.05, 0.1, and 0.5 μg/ml) or with ADR plus 4 μM TFP in triplicate, in 35-mm × 10-mm Petri dishes. Following incubation for 6 days at 37°C in a humidified 5% CO₂_-95% air atmosphere, colonies (>50 cells) in untreated control and treated plates were determined in an Omnicom Feature Analysis System II (Bausch and Lomb, Rochester, N. Y.). Plating efficiencies of control P388/S and P388/R cells under these conditions were approximately 37 and 29%, respectively.

Cytotoxic effects following short-term drug exposure were determined by treating P388/S and P388/R cells with ADR (0.01 to 0.1 μg/ml and 0.5 to 2.0 μg/ml, respectively), for 1 hr in the presence and absence of 4 μM TFP. The treated cells were centrifuged, washed twice with drug-free RPMI 1640 supplemented with 10% FBS, and plated in soft agar as described above. Colony counts in control and treated plates after a 5-day incubation in a humidified 5% CO₂_-95% air atmosphere were determined as described earlier.

Cellular Accumulation and Retention of ADR. In drug accumulation studies, P388/S and P388/R cells (1 × 10⁶/ml) in RPMI 1640 supplemented with 10% FBS, were treated at 37°C with ADR (1 and 5 μg/ml) in
the presence and absence of 4 \( \mu M \) TFP. Triplicate aliquots of \( 1 \times 10^6 \) cells were removed at various times (1 to 4 hr), centrifuged (100 \( \times \) g), washed 3 times with 7 ml of cold 0.85% sodium chloride solution, extracted with 50% ethanol-0.3 \( \text{n} \) hydrochloric acid, and centrifuged (1). ADR fluorescence in the supernatant was determined at excitation and emission wavelengths of 470 and 585 nm, respectively, in an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silver Spring, Md.). ADR content was computed from standard curves prepared with ADR hydrochloride.

In ADR retention experiments, P388/S and P388/R cells (\( 1 \times 10^6/\text{ml} \)) in RPMI 1640 supplemented with 10% FBS were pretreated with ADR (1 and 5 \( \mu \text{g/ml} \)) in the presence and absence of 4 \( \mu \text{M} \) TFP. Following incubation for 1 hr at 37\( ^\circ \), cells were centrifuged (100 \( \times \) g), washed, and resuspended in drug-free medium (RPMI 1640 supplemented with 10% FBS) with and without 4 \( \mu \text{M} \) TFP. Aliquots (\( 1 \times 10^6 \) cells) of cells retrieved at various times (15 to 90 min) were centrifuged and washed with 7 ml of cold 0.85% sodium chloride solution, and cellular ADR was quantitated fluorimetrically.

RESULTS AND DISCUSSION

The effect of ADR (0.01 to 5.0 \( \mu \text{g/ml} \)) alone or in combination with 4 \( \mu \text{M} \) TFP on the proliferation of P388/S and P388/R cells in suspension culture is shown in Chart 1. In preliminary studies with TFP (1 \( \mu \text{M} \) to 0.1 \( \text{mm} \)), concentrations less than 5 \( \mu \text{M} \) were found to be noncytotoxic and did not inhibit the proliferation of P388/S and P388/R cells. The cytotoxic effects of ADR alone in P388/S and P388/R cells in suspension culture were similar to that previously reported (6). Cell counts in P388/S cells treated with ADR alone were markedly dependent on drug concentration, and the combination of ADR with TFP did not significantly change the pattern of cell count. In P388/R cells, however, the cytotoxic effects of ADR were found to be significantly enhanced and drug concentration dependent only in the presence of TFP. The addition of TFP with ADR enhanced drug-induced cytotoxicity by a factor of approximately 2, 4, and 6 at ADR concentrations of 1.0, 2.5, and 5.0 \( \mu \text{g/ml} \), respectively, in P388/R cells.

Due to the limitations of the dye exclusion method as a predictive measure of drug-induced cell lethality (13), the ability of TFP to enhance the cytotoxic effects of ADR following long-term and short-term treatment was determined based on colony-forming ability of P388/S and P388/R cells in soft agar. The survival of P388/S and P388/R cells in soft agar following long-term treatment with various concentrations of ADR alone or in combination with 4 \( \mu \text{M} \) TFP is shown in Chart 2. Unlike results with suspension culture, following continuous drug exposure for 144 hr in soft agar, the cytotoxic effects of ADR were enhanced by TFP in P388/S cells. Survival of P388/S cells treated with ADR (0.005 \( \mu \text{g/ml} \) and 0.01 \( \mu \text{g/ml} \)) were 63 and 34%, respectively, in the absence of TFP and 3 and <1%, respectively, in the presence of TFP. At ADR concentrations above 0.01 \( \mu \text{g/ml} \), survival of P388/S cells was <1% with or without TFP. In P388/R cells treated with ADR (0.01, 0.05, and 0.1 \( \mu \text{g/ml} \)) alone or in combination with 4 \( \mu \text{M} \) TFP, survival was not markedly different,
and colony counts were 75 to 95% of the untreated control. However, at an ADR concentration of 0.5 µg/ml, survival in the absence and presence of TFP was 70% and <1%, respectively. Survival in soft agar of P388/S and P388/R cells following short-term treatment with ADR and ADR plus TFP is shown in Table 1. It is apparent that with P388/S cells, reductions in survival were ADR dose dependent, and incubation of cells with ADR plus TFP did not result in any marked enhancement of cytotoxic effects. Survival of P388/R cells treated with ADR (0.5, 1.0, and 2.0 µg/ml) was not drug dose dependent, and colony counts were 70 to 84% of the untreated control. However, in P388/R cells treated with ADR plus TFP, reductions in survival were ADR dose dependent with an approximately 2- and 4-fold decrease in survival at ADR concentrations of 1.0 and 2.0 µg/ml, respectively.

Since TFP had a marked effect in enhancing the sensitivity of P388/R cells to the cytotoxic effects of ADR, and reduced drug levels have been shown to be a determinant of ADR resistance in P388/R cells (9), the effect of TFP on cellular accumulation and retention of ADR was determined. Chart 3 shows the effect of TFP on ADR accumulation in P388/S and P388/R cells. Cellular drug levels in both P388/S and P388/R cells treated with ADR (5 µg/ml) were 3-fold higher than following treatment with ADR (1 µg/ml). In P388/R cells, drug levels in cells treated with ADR plus TFP were significantly higher (p < 0.01) than for ADR alone at concentrations of 1 and 5 µg/ml. In similarly treated P388/S cells, TFP had only a marginal effect (3 to 10% increase) in enhancing cellular accumulation of ADR. Overall, TFP treatment appeared to have a more pronounced effect in increasing ADR levels in P388/R cells.

The effect of TFP on retention of ADR in P388/S and P388/R cells in vitro is shown in Chart 4. In P388/S cells, retention of ADR was similar in the presence and absence of 4 µM TFP, and drug levels retained represented about 60 to 70% of the ADR accumulated prior to incubation in drug-free medium. In contrast, TFP treatment markedly increased retention of ADR in P388/R cells, and drug levels retained between 60 and 90 min in the presence of 4 µM TFP were about 2-fold higher than in the absence of TFP.

The results on ADR accumulation and retention in P388/R cells suggest that TFP has a significant effect on drug retention?

![Chart 3. Cellular accumulation of ADR by P388/S (—) and P388/R (—) cells treated in vitro at 37°C. A, O, ADR (1 µg/ml); ◯, ADR (1 µg/ml) plus 4 µM TFP; B, □, ADR (5 µg/ml); ■, ADR (5 µg/ml) plus 4 µM TFP. Cellular ADR content was determined fluorimetrically as outlined in "Materials and Methods." Points, mean value from at least triplicate experiments; S.D. < 10%.

![Chart 4. Effect of TFP on cellular retention of ADR in P388/S (—) and P388/R (—) cells in vitro at 37°C. P388/S and P388/R cells were pretreated with ADR (1 µg/ml) or ADR (1 µg/ml) plus 4 µM TFP, and ADR (5 µg/ml) or ADR (5 µg/ml) plus 4 µM TFP for 1 hr, washed, and resuspended in drug-free medium with (●) and without (□) 4 µM TFP. Cellular ADR content was determined fluorimetrically as outlined in "Materials and Methods." Percentage of ADR retention was obtained from the ratio of the amount of retained drug to that originally accumulated in 1 hr. Although percentage of drug retained was similar at the various time intervals, ADR levels retained in P388/S and P388/R cells pretreated with 5 µg/ml were about 2- to 3-fold higher than 1 µg/ml. Points, means from triplicate experiments; bars, S.E.]

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Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P388/S cells</td>
<td>100 ± 4°F</td>
</tr>
<tr>
<td>TFP (4 µM)</td>
<td>93 ± 6</td>
</tr>
<tr>
<td>ADR (0.01 µg/ml)</td>
<td>93 ± 6</td>
</tr>
<tr>
<td>ADR (0.05 µg/ml)</td>
<td>87 ± 3</td>
</tr>
<tr>
<td>ADR (0.1 µg/ml)</td>
<td>33 ± 4</td>
</tr>
<tr>
<td>ADR (0.1 µg/ml) + TFP (4 µM)</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>ADR (2.0 µg/ml)</td>
<td>69 ± 5</td>
</tr>
</tbody>
</table>

| P388/R cells | 98 ± 1 |
| TFP (4 µM) | 98 ± 1 |
| ADR (0.5 µg/ml) | 84 ± 2 |
| ADR (0.5 µg/ml) + TFP (4 µM) | 84 ± 2 |
| ADR (1.0 µg/ml) | 66 ± 1 |
| ADR (1.0 µg/ml) + TFP (4 µM) | 66 ± 1 |
| ADR (2.0 µg/ml) | 42 ± 3 |
| ADR (2.0 µg/ml) + TFP (4 µM) | 42 ± 3 |

n° P388/S and P388/R cells were treated with the various concentrations of ADR in the presence and absence of 4 µM TFP for 1 hr and plated in soft agar as outlined in "Materials and Methods."

°F Survival is based on colony counts expressed as a percentage of the untreated control.

Mean ± S.E. from triplicate experiments.
and that the higher ADR level during accumulation could be partially due to enhanced retention. In contrast, TFP treatment had little if any effects on accumulation or retention of ADR in P388/S cells. The survival data following short-term drug treatment with ADR or ADR + TFP (Table 1) further support the observations on the effect of TFP in enhancing cellular accumulation and retention of ADR in P388/R cells. The lack of any significant effect of TFP in enhancing the cytotoxic activity of ADR in P388/S cells following short-term drug treatment (Table 1) suggests that the reduced survival using continuous drug exposure with ADR and TFP in P388/S cells (Chart 2) is probably due to an enhancement in the cytotoxic effects of ADR by a mechanism other than an increase in cellular drug levels.

Altering membrane permeability with Tween 80 has been shown to enhance cellular accumulation but not retention of ADR in P388/R cells (8). More recently, the use of tunicamycin to suppress glycoprotein synthesis, which has been implicated with the development of drug resistance, was also not found to alter the cellular uptake, retention, or cytotoxic effects of DAU in P388/R cells (4). Our results with TFP, in P388/R cells, and of verapamil (16) and reserpine (7) in increasing drug accumulation, retention, and cytotoxicity in vincristine- and ADR-resistant P388 leukemia suggest that the mechanism involved in reversal of resistance with these agents could possibly be related to inhibition of a calmodulin-mediated process. Further studies are necessary to determine if the increased sensitivity to ADR and vincristine in the presence of TFP, verapamil, and reserpine is also true for other resistant tumors. In preliminary studies (data not shown) with another phenothiazine, chlorpromazine, increased sensitivity to ADR in P388/R cells was achieved only at higher dose level, which is in agreement with reported differences in potency between TFP and chlorpromazine as inhibitors of calmodulin (11, 12).

In summary, this study demonstrates that TFP treatment significantly enhances the cytotoxic effects of ADR in ADR-resistant P388 cells. The effect of TFP on cellular ADR levels in P388/R cells suggest that enhanced sensitivity to the cytotoxic effect of ADR is largely due to an increase in cellular retention rather than to the accumulation of drug. Since the antipsychotic drugs have a large therapeutic index (2), the use of phenothiazines to improve the drug sensitivity of tumors resistant to ADR treatment could be a novel approach in cancer chemotherapy.

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Enhancement of Sensitivity to Adriamycin in Resistant P388 Leukemia by the Calmodulin Inhibitor Trifluoperazine

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