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DIFFERENTIAL EFFECT OF THE CALMODULIN INHIBITOR TRIFLUOPERAZINE IN MODULATING CELLULAR ACCUMULATION, RETENTION AND CYTOTOXICITY OF DOXORUBICIN IN PROGRESSIVELY DOXORUBICIN-RESISTANT L1210 MOUSE LEUKEMIA CELLS

LACK OF CORRELATION BETWEEN CELLULAR DOXORUBICIN LEVELS AND EXPRESSION OF RESISTANCE*

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Abstract—Calmodulin inhibitors are effective in enhancing cytotoxic effects of doxorubicin (DOX) in DOX-resistant cells, possibly by enhancing cellular levels of drug. In the present study, L1210 mouse leukemia cells adapted to grow *in vitro*, in the presence of 0.025 to 0.25 μg/ml DOX, and identified as L1210/DOX0.025, L1210/DOX0.05, L1210/DOX0.1, and L1210/DOX0.25 were approximately 5-, 10-, 20-, and 40-fold DOX resistant, respectively, compared to parent-sensitive cells (L1210/S). Using a soft agar colony assay and 3-hr drug exposure, the IC_{50} concentration of DOX in the progressively DOX-resistant (5- to 40-fold) L1210 cells ranged from 0.25 to 2.0 μg/ml and from 0.08 to 0.25 μg/ml in the absence and presence of a non-cytotoxic concentration of 5 μM trifluoperazine (TFP) respectively. Further, based on the observed *in vitro* cytotoxic response, the IC_{50} concentration of DOX in the presence of 5 μM TFP was 2.5-, 4-, 6.7- and 8-fold lower than DOX without 5 μM TFP in the L1210/DOX0.025, L1210/DOX0.05, L1210/DOX0.1, and L1210/DOX0.25 resistant sublines respectively. In contrast, the IC_{50} of DOX in L1210/S cells was approximately 0.05 μg/ml with or without 5 μM TFP. Cellular accumulation of DOX was 15–50% lower in the progressively resistant L1210 sublines compared to similarly treated L1210/S cells. However, in the presence of 5 μM TFP, cellular accumulation of DOX in the L1210/DOX0.05 and L1210/DOX0.1 but not L1210/DOX0.25 was comparable to the L1210/S cells. Cellular retention of DOX in the absence or presence of 5 μM TFP was comparable in similarly treated L1210/S, L1210/DOX0.05 and L1210/DOX0.1 cells, and a 2-fold reduction in the retention of DOX in the absence versus the presence of 5 μM TFP was apparent only in L1210/DOX0.25 cells. At the IC_{50} of DOX in the presence of 5 μM TFP, although cellular accumulation of DOX was concentration dependent over the range of 1–20 μM TFP, enhancement in cytotoxicity of DOX was dose dependent at 1–5 μM TFP but not 5–20 μM TFP. In cells treated for 3 hr at the IC_{50} concentration of DOX alone or DOX plus 5 μM TFP, cellular accumulation of DOX was 7- to 14-fold and 2.5- to 3.5-fold higher, respectively, in resistant than in sensitive cells. Additionally, following treatment for 3 hr at the IC_{50} dose of DOX in the absence or presence of 5 μM TFP, drug retention at 3 hr was 4- to 6-fold and 1.5-fold higher, respectively, in the resistant versus sensitive cells. Results from this study demonstrate that: (1) the effect of TFP on DOX cytotoxicity was dependent on the level of acquired resistance; (2) in contrast to sensitive cells, the resistant sublines required much higher cellular levels of DOX in the absence versus the presence of TFP to achieve equivalent cytotoxicity; and (3) differences in cellular levels of DOX during accumulation or retention in sensitive versus resistant sublines did not appear to correlate with the magnitude in expression of resistance.

The antitumor antibiotic doxorubicin (Adriamycin) is widely recognized for its potent antitumor effects in a spectrum of animal and human tumors [1, 2]. Although earlier studies suggested that the major locus of action and cytotoxicity of doxorubicin (DOX‡) was related to the binding to nucleic acids, it has become apparent that the antitumor effects are multifactorial and include free radical formation, membrane binding and metal ion chelation as other biochemical events contributing towards a cytotoxic response [3].

In spite of the potent antitumor activity of DOX, failure of DOX chemotherapy is encountered, due to the emergence of tumor cells with intrinsic or acquired drug resistance. The inability to identify the factors governing the mechanism of action of DOX has also complicated an understanding of the mechanisms involved in the expression of resistance. Studies on acquired DOX resistance have been carried out over the past several years using murine and human tumor model systems [4–7], and it is widely accepted that reduced drug accumulation and/or

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‡ Abbreviations: DOX, doxorubicin; TFP, trifluoperazine dihydrochloride; and FBS, fetal bovine serum.

retention are primary mechanisms of resistance to DOX [4, 5]. The mechanism responsible for reduced cellular drug levels in resistant sublines has been attributed to the presence of a plasma membrane glycoprotein (M_r 150,000–180,000) widely referred to as the P-glycoprotein [7]. The functional role of the P-glycoprotein in resistance has been suggested to involve either reduced influx and/or enhanced efflux of cellular drug [7]. Membrane permeability as a determinant of resistance has also been substantiated by studies demonstrating that non-specific perturbation by non-ionic detergents sensitizes resistant cells to the cytotoxic effects of DOX and daunorubicin [8, 9]. Reduced drug accumulation and/or retention of drug as factors governing resistance is also an attractive hypothesis based on the cross-resistance of DOX-resistant cells to a number of other antitumor agents that are different structurally and in their mechanism of action [10–12].

Recent studies from our laboratory and that of Tsuruo *et al.* demonstrated that acquired resistance to DOX and vinca-alkaloids can be reduced markedly in the presence of calmodulin inhibitors [13–15] and calcium antagonists [15, 16], and the use of these calcium modifying agents in modulating resistance *in vitro* and/or *in vivo* using a variety of multidrug resistant tumor model systems has been documented [16–20]. Studies demonstrating that modulation of cytotoxicity by calcium modifiers in multidrug resistant cells is related to enhanced drug accumulation and/or retention appear to be true for vinca-alkaloid resistance [12, 15]. However, in the case of DOX-resistant cells, the magnitude of alterations in cellular drug levels is not convincing to account for the expression of resistance or the marked potentiation of cytotoxicity with calcium antagonists or calmodulin inhibitors [21–23]. Most studies on DOX resistance have been carried out in model systems that are at least 50-fold resistant, and little is known about the cellular pharmacokinetics and cytotoxicity of DOX at low levels of progressive resistance which are more likely to be encountered clinically. Additionally, the modulation of DOX cytotoxicity by calmodulin inhibitors at low levels of resistance could provide valuable information in the design of therapeutic modalities. In this study we describe the characterization of L1210 mouse leukemia cells during the development of progressive acquired resistance to DOX with reference to alterations in cellular pharmacokinetics (accumulation and retention) and cytotoxicity, as well as the potential for modulation of these parameters by the calmodulin inhibitor trifluoperazine (TFP).

MATERIALS AND METHODS

L1210 lymphoid leukemia in female DBA/2n mice was obtained from Dr. Joseph G. Mayo, Chief, Animal Genetics and Production Branch, DCT Tumor Repository, National Cancer Institute, Frederick Cancer Research Facility, Frederick, MD. The L1210 tumor cells were transplanted every 7 days into naive female DBA/2n mice by implanting 1×10^5 cells intraperitoneally. Five to seven days following tumor growth as ascites in the mice, *in vitro* cultures of L1210 cells from ascites were established 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, UT) and 10 μM 2-mercaptoethanol. The *in vitro* cell line of L1210 mouse leukemia has a doubling time of approximately 11 hr and, following injection i.p. into DBA/2n mice, demonstrated growth characteristics, tumorigenicity and mortality similar to cells maintained in mice by weekly transplantation. Trifluoperazine (TFP) was a gift from Dr. Carl Kaiser, Smith Kline & French Laboratories, Philadelphia, PA.

Isolation of progressively DOX-resistant L1210 mouse leukemia cells. The parent sensitive L1210 cells (L1210/S) cultured *in vitro* in RPMI 1640 supplemented with 25 mM *N*-2- hydroxyethylpiperazine-*N*-ethanesulfonic acid buffer (buffered RPMI 1640), 10% fetal bovine serum (FBS) and 10 μM 2-mercaptoethanol were treated with increasing extracellular concentrations of 0.025 to 0.25 μg/ml DOX. L1210 cells adapted to grow in the presence of 0.025, 0.05, 0.1, and 0.25 μg/ml DOX were maintained in DOX-free medium for at least 2 weeks prior to preparation of stock frozen cultures and were subsequently never maintained in the presence of DOX for use in experiments. The extracellular concentration of DOX was increased to obtain cells at a higher level of resistance when the doubling time and growth characteristics *in vitro* were comparable to the L1210/S cells. In general, progressively DOX-resistant L1210 cells were obtained by culturing in the presence of the aforementioned concentrations of DOX for 8–10 weeks. The L1210 mouse leukemia parent line and sublines adapted to grow in the presence of 0.025, 0.05, 0.1 and 0.25 μg/ml DOX are identified as L1210/S, L1210/DOX0.025, L1210/DOX0.05, L1210/DOX0.1 and L1210/DOX0.25 respectively.

Doxorubicin cytotoxicity in vitro. Cytotoxicity studies were carried out using a soft-agar colony forming assay. Log-phase cultures of parent sensitive and progressively DOX-resistant sublines of L1210 mouse leukemia cells at a density of 1×10^6 cells/ml in buffered RPMI 1640 supplemented with 10% FBS were treated with 0.01 to 2.0 μg/ml of DOX in the absence or presence of 5 μM TFP for 3 hr at 37° in a humidified 5% CO_2 plus 95% air atmosphere. Treated cells were centrifuged at 80 *g* and washed twice in drug-free buffered RPMI 1640 supplemented with 10% FBS. Cells were then plated in triplicate at a density of 5×10^3 cells per 35×10 mm Petri-dish using RPMI 1640 supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N*-ethanesulfonic acid buffer, 20% FBS, and 10 μM 2-mercaptoethanol. Following incubation for 96 hr in a humidified atmosphere of 5% CO_2 plus 95% air at 37°, colonies with >30–50 cells [24] in untreated control and treated plates were counted in an Omnicon Feature Analysis System II (Bausch & Lomb, Rochester, NY). Under these conditions, the colony count (mean ± SE) in untreated controls was 1385 ± 50, corresponding to a plating efficency of approximately 28%.

Using the IC_{50} of DOX in the presence of 5 μM TFP (concentration required to inhibit colony formation by 50%), cytotoxicity studies with DOX in

the absence or presence of 1, 2.5, 5, 10, and 20 μM TFP were also carried out in the L1210/S and DOX-resistant L1210 sublines. The length of treatment was 3 hr, and untreated controls and treated cells were processed for the soft-agar colony assay as described earlier.

Cellular accumulation of doxorubicin in vitro. Log-phase cultures of parent-sensitive (L1210/S) and progressively DOX-resistant (L1210/DOX0.05, L1210/DOX0.1 and L1210/DOX0.25) L1210 mouse leukemia cells at a density of 1×10^6 cells/ml in buffered RPMI 1640 supplemented with 10% FBS were treated with 0.1, 0.5, and 2.0 μg/ml DOX in the absence or presence of 5 μM TFP at 37° in a humidified 5% CO_2 plus 95% air atmosphere. Duplicate aliquots (1×10^6 cells/sample) removed at the end of 1 and 3 hr of treatment were centrifuged (100 *g*) and washed twice with 7 ml of ice-cold 0.85% sodium chloride solution. The cell pellet following the final wash was resuspended in 50% ethanol/0.3 N hydrochloric acid, mixed thoroughly in a vortex mixer and centrifuged at 700 *g*; DOX content in the supernatant fraction was determined fluorimetrically [25, 26] in an Aminco–Bowman spectrophotofluorometer (American Instrument Co., Silver Spring, MD) at excitation and emission wavelengths of 470 and 585 nm respectively. Standard curves of DOX prepared in 50% ethanol/0.3 N hydrochloric acid were used for computation of DOX content which was expressed as ng/10^6 cells. To determine possible metabolism of DOX *in vitro*, cells following treatment with DOX in the absence or presence of 5 μM TFP were washed and centrifuged as described earlier, sonicated, and extracted with 2×5 ml of ethyl acetate–*n*-propanol (9:1, v/v). Extracts were combined, evaporated to dryness under a stream of nitrogen and reconstituted in methanol, and thin-layer chromatography was carried out in silica gel HF plates (Analtech, Inc., Newark, DE) using chloroform–methanol–acetic acid–water (80:20:14:6, by vol.). The thin-layer chromatographic system which is capable of resolving doxorubicin, doxorubicinol, their respective aglycones, and their respective 7-deoxy-aglycones demonstrated no metabolism of doxorubicin in samples of the cell extracts, suggesting that fluorometric analysis represents unchanged cellular DOX levels.

Additional studies to evaluate the effect of TFP concentration on DOX accumulation were also carried out in the L1210/S, L1210/DOX0.05, L1210/DOX0.1 and L1210/DOX0.25 cells. Briefly, at the IC_{50} concentration of DOX in the presence of 5 μM TFP, DOX accumulation at 1 and 3 hr was determined using 0, 1, 2.5, 5, 10 and 20 μM TFP. The experimental protocol for quantifying cellular DOX levels was similar to that described earlier. The mean cellular DOX levels were compared using the Tukey test to determine statistical significance of differences between treatments and cell lines.

Cellular retention of doxorubicin in vitro. L1210/S, L1210/DOX0.05, L1210/DOX0.1, and L1210/DOX0.25 cells in buffered RPMI 1640 supplemented with 10% FBS were pretreated for 1 hr at 37° in a humidified 5% CO_2 plus 95% air atmosphere with 1.0 μg/ml DOX alone (2.0 μg/ml DOX alone for L1210/DOX0.25) or 0.5 μg/ml DOX in the presence of 5 μM TFP. These different concentrations of DOX were utilized in order to possibly compensate for differences in cellular accumulation of DOX between the sensitive and resistant sublines treated with or without TFP. Cells treated with DOX in the absence or presence of TFP were centrifuged, resuspended in DOX-free medium (buffered RPMI 1640 supplemented with 10% FBS) with or without 5 μM TFP, and incubated at 37°. Duplicate aliquots of cells (10^6 cells/sample) retrieved at the end of the 1-hr pretreatment drug accumulation phase and subsequently at 15, 30, 60, 90 and 120 min during the retention phase, were centrifuged (100 *g*) and washed twice with 7 ml of ice-cold 0.85% sodium chloride solution. Cellular levels of DOX in the pellet were then quantitated fluorimetrically as described earlier under accumulation experiments.

In a separate series of experiments, L1210/S, L1210/DOX0.05, L1210/DOX0.1 and L1210/DOX0.25 cells were pretreated with the IC_{50} concentration of DOX in the absence or presence of 5 μM TFP for 3 hr at 37° in a humidified 5% CO_2 plus 95% air atmosphere. Cells were then centrifuged, resuspended in drug-free medium (buffered RPMI 1640 supplemented with 10% FBS), and incubated at 37°. Duplicate aliquots of cells (1×10^6 cells/sample) were retrieved at the end of 3-hr accumulation phase, and subsequently at 30, 60, 120 and 180 min during the retention phase. All other experimental details on processing of samples and quantitation of cellular DOX levels were similar to those described earlier. The mean cellular DOX levels were compared using the Tukey test to determine statistical significance of differences between treatments and cell lines.

RESULTS

Characteristics of DOX resistant cells. The sensitive and DOX-resistant sublines of L1210 mouse leukemia proliferated *in vitro* as single cell suspension cultures with a doubling time (mean ± SE) of approximately 10.7 ± 0.1 hr. Flow cytometric analysis of propidium iodide stained nuclei revealed that the G_1 DNA content of the DOX-resistant sublines was similar to the diploid parent sensitive L1210/S cells, using thymocytes as a reference standard. Tumorigenicity in female DBA/2n mice of the progressively DOX-resistant sublines was similar to the sensitive L1210 cells, and the mean survival time of mice implanted i.p. with 1×10^5 cells was 8.5 to 11 days. The DOX-resistant sublines were stably resistant in the absence of DOX for at least 3 months (about 200 doublings) during *in vitro* culture.

Effect of TFP on doxorubicin cytotoxicity in vitro. Survival of parent-sensitive and progressively DOX-resistant sublines of L1210 mouse leukemia treated with various concentrations of DOX in the absence or presence of 5 μM TFP is outlined in Table 1. An extracellular concentration of 5 μM TFP was non-cytotoxic in the sensitive and progressively DOX-resistant sublines, and no collateral sensitivity to 5 μM TFP was observed in the L1210 cells with increasing DOX resistance. Doxorubicin dose-dependent cytotoxicity was observed in the L1210/S and DOX-resistant sublines over a wide range of

Table 1. Effect of TFP on cytotoxicity of DOX in sensitive and progressively DOX-resistant L1210 mouse leukemia cells

Drug concentration*	Survival†‡ (% of control)				
	L1210/S	L1210/ DOX0.025	L1210/ DOX0.05	L1210/ DOX0.1	L1210/ DOX0.25
5 μM TFP	100 ± 0	95 ± 2	94 ± 2	92 ± 3	100 ± 3
0.01 μg/ml DOX	98 ± 2	100 ± 0			
0.01 μg/ml DOX + TFP	97 ± 3	97 ± 0			
0.05 μg/ml DOX	60 ± 5	85 ± 4	96 ± 2		
0.05 μg/ml DOX + TFP	52 ± 5	75 ± 3	80 ± 5		
0.1 μg/ml DOX	13 ± 3	59 ± 5	92 ± 4	90 ± 7	95 ± 7
0.1 μg/ml DOX + TFP	12 ± 4	39 ± 3	58 ± 2	65 ± 8	78 ± 2
0.5 μg/ml DOX	0.1 ± 0.1	0.4 ± 0.2	56 ± 3	79 ± 3	90 ± 4
0.5 μg/ml DOX + TFP	0.2 ± 0.1	0.2 ± 0.1	1.6 ± 0.4	5 ± 1	16 ± 5
1.0 μg/ml DOX			27 ± 4	52 ± 5	76 ± 1
1.0 μg/ml DOX + TFP			0 ± 0	0.3 ± 0.2	1.8 ± 0.7
2.0 μg/ml DOX				14 ± 3	51 ± 4
2.0 μg/ml DOX + TFP				0.1 ± 0.1	0.1 ± 0.1

* Cells were treated with various concentrations of DOX in the absence and presence of 5 μM TFP for 3 hr, washed, and plated in soft-agar.

† Values are expressed as mean ± SE of triplicate experiments. Survival is based on colony counts.

‡ Cells were plated at a density of 5×10^3 cells per 35 × 10 mm Petri dish, and the colony count (mean ± SE) in the untreated control was 1385 ± 50, corresponding to a plating efficiency of approximately 28%.

concentrations, and the observed IC_{50} for DOX alone in the L1210/S, L1210/DOX0.025, L1210/DOX0.05, L1210/DOX0.1 and L1210/DOX0.25 cells was approximately 0.05, 0.25, 0.5, 1.0, and 2.0 μg/ml DOX respectively. In accordance with our studies in other tumor cell lines [13], no remarkable decrease in the IC_{50} of DOX in the presence of 5 μM TFP was observed in L1210/S cells. In contrast, with the progressively DOX-resistant sublines, marked reductions in the IC_{50} concentration of DOX were apparent in the presence of 5 μM TFP, and the effect of TFP in enhancing cytotoxicity of DOX was dependent on the level of DOX resistance. A plot of the estimated IC_{50} of DOX with or without 5 μM TFP (determined by regression analysis of data from Table 1) in the parent-sensitive and progressively

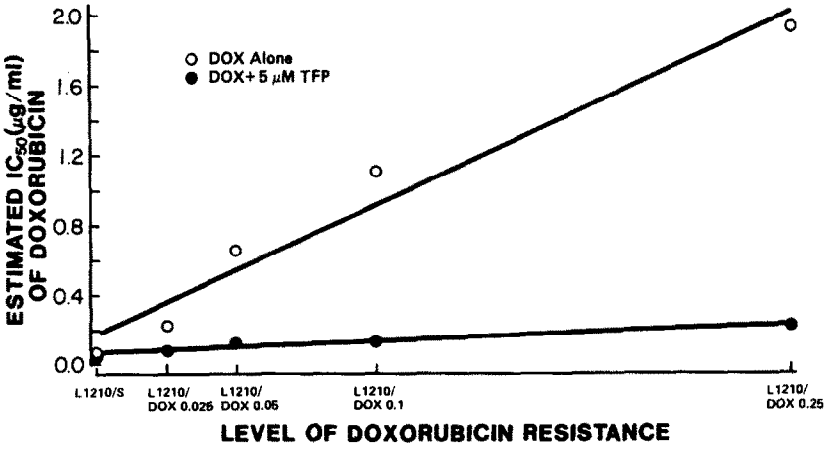

Fig. 1. Relationship between IC_{50} concentration of DOX with or without 5 μM TFP in sensitive and progressively DOX-resistant sublines of L1210 mouse leukemia. The estimated IC_{50} concentration of DOX was determined by regression analysis of data from Table 1. The 95% confidence intervals (C.I.) for the estimated IC_{50} of DOX (μg/ml) in the absence of 5 μM TFP were: L1210/S: C.I. = (0.016, 0.193); L1210/DOX0.025: C.I. = (0.079, 0.374); L1210/DOX0.05: C.I. = (0.435, 0.886); L1210/DOX0.1: C.I. = (0.652, 1.577); and L1210/DOX0.25: C.I. = (1.505, 2.387). In the presence of 5 μM TFP, 95% confidence intervals (C.I.) for the estimated IC_{50} of DOX (μg/ml) were: L1210/S: C.I. = (0.014, 0.187); L1210/DOX0.025: C.I. = (0.033, 0.188); L1210/DOX0.05: C.I. = (0.066, 0.260); L1210/DOX0.1: C.I. = (0.032, 0.521); and L1210/DOX0.25: C.I. = (0.112, 0.466).

DOX-resistant sublines is shown in Fig. 1. It is apparent from these results that, although the IC_{50} of DOX in the absence or presence of 5 μM TFP was comparable in L1210/S cells, the IC_{50} of DOX versus the IC_{50} of DOX plus 5 μM TFP was approximately 2.5-, 4-, 6.7-, and 8.0-fold higher in the L1210/DOX0.025, L1210/DOX0.05, L1210/DOX0.1, and L1210/DOX0.25 sublines respectively.

Given the observations that DOX-resistance is mediated by membrane alterations [7] and that an agent such as TFP possibly alters the resistance phenotype by non-specific perturbations of the plasma membrane [18], modulation in cytotoxicity of the IC_{50} of DOX with 5 μM TFP was evaluated at concentrations of 1, 2.5, 5, 10, and 20 μM TFP. Results from these studies are presented in Fig. 2. Although concentrations <5 μM TFP alone were relatively non-cytotoxic, a 10–25% reduction in survival at 10 μM TFP and 20 μM TFP alone was apparent in the sensitive and progressively DOX-resistant sublines. In the presence of DOX, reductions in cell survival were TFP concentration dependent over the range of 1 to 5 μM TFP and, at these concentrations of TFP, the effects on cell survival were more pronounced with increasing DOX resistance. Concentrations of 10 and 20 μM TFP were not remarkably different from 5 μM TFP in potentiating cytotoxicity of DOX in the sensitive and progressively resistant DOX sublines, since TFP alone at these concentrations was cytotoxic.

Effect of TFP on cellular doxorubicin accumulation in vitro. Accumulation of DOX at 1 and 3 hr in L1210/S, L1210/DOX0.05, L1210/DOX0.1 and L1210/DOX0.25 cells treated with 0.1, 0.5, and 2.0 μg/ml DOX in the absence or presence of 5 μM TFP is presented in Fig. 3. In all cell lines examined, accumulation of DOX was both concentration and time dependent. As observed in earlier studies with other DOX-sensitive tumors [13], <15% increase in cellular accumulation of DOX in the presence of 5 μM TFP was apparent in L1210/S cells. Cellular levels of DOX in the resistant sublines decreased with increasing DOX resistance compared to similarity treated L1210/S cells and, overall, DOX levels in the absence of 5 μM TFP were 15–50% lower in the L1210/DOX0.05, L1210/DOX0.1 and L1210/DOX0.25 sublines. In contrast, cellular accumulation of DOX in the presence of 5 μM TFP was

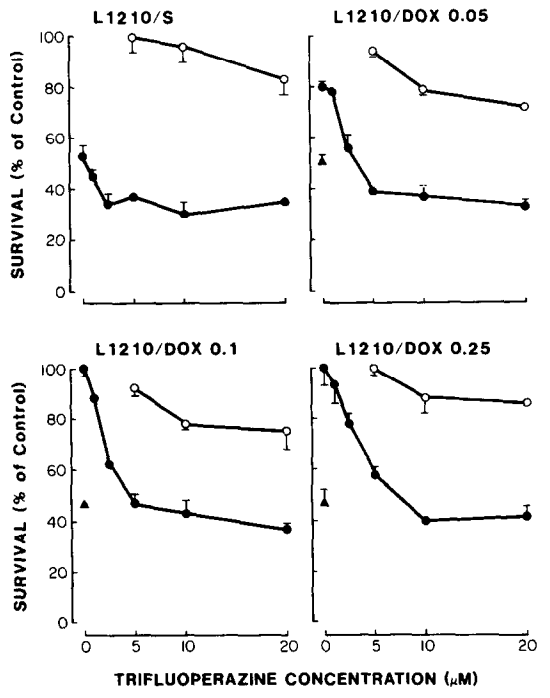

Fig. 2. Effect of TFP concentration on the survival in soft-agar of sensitive and progressively DOX-resistant L1210 cells treated with the IC_{50} of DOX in the presence of 5 μM TFP. Key: L1210/S, (○) TFP alone and (●) 0.05 μg/ml DOX plus TFP; L1210/DOX0.05, (▲) 0.5 μg/ml DOX, (○) TFP alone, and (●) 0.125 μg/ml DOX plus TFP; L1210/DOX0.1, (▲) 1.0 μg/ml DOX, (○) TFP alone, and (●) 0.15 μg/ml DOX plus TFP; L1210/DOX0.25, (▲) 2.0 μg/ml DOX, (○) TFP alone, and (●) 0.25 μg/ml DOX plus TFP. Cells were plated at a density of 5×10^3 cells per 35×10 mm Petri dish, and the colony count (mean ± SE) in the untreated control was 1385 ± 50, corresponding to a plating efficiency of approximately 28%. Each point is the mean value from triplicate experiments; bars, SE.

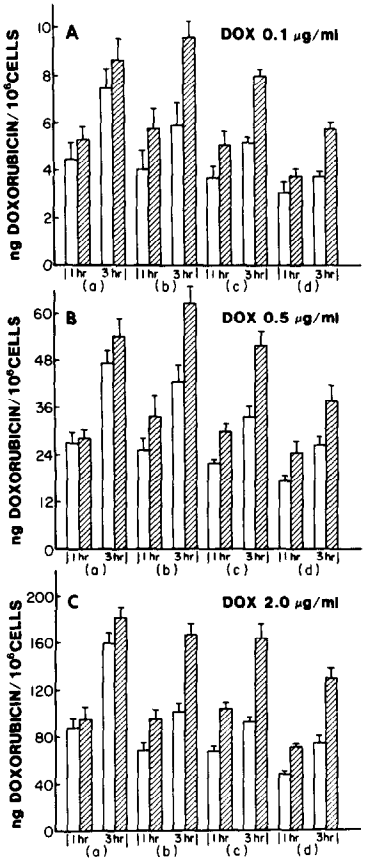

Fig. 3. Cellular accumulation of DOX without TFP (□) or with 5 μM TFP (▨) at 1 and 3 hr in sensitive and progressively DOX-resistant L1210 cells treated with 0.1 μg/ml DOX (A), 0.5 μg/ml DOX (B), and 2.0 μg/ml DOX (C). Key: L1210/S (a), L1210/DOX0.05 (b), L1210/DOX0.1 (c) and L1210/DOX0.25 (d). Values are means of duplicate determinations from triplicate experiments; bars, S.E.

enhanced approximately 1.5-fold in the DOX-resistant sublines, and this increase in DOX accumulation in the presence of 5 μM TFP was not dependent on the level of resistance.

Effect of TFP on cellular doxorubicin retention in vitro. Since reduced cellular retention of DOX has been suggested to be an important mode of resistance [4, 5] and TFP markedly alters the cytotoxic response, retention of DOX in the absence or presence of 5 μM TFP was determined in the L1210/S and progressively DOX-resistant sublines; the results are shown in Fig. 4. In L1210/S cells, retention of DOX between 90 and 120 min was 45–50% of the drug initially accumulated at 1 hr, and the presence or absence of 5 μM TFP during the accumulation and/or retention phase did not alter markedly the pattern of DOX retention. The retention of DOX in the L1210/DOX0.05 and L1210/DOX0.1 sublines was 35–40% at the end of 90–120 min and, similar to results with L1210/S cells, no remarkable enhancement in DOX retention was apparent due to the presence of 5 μM TFP during the accumulation and/or retention phase. In contrast to these results, with the L1210/DOX0.25 cells a nearly 2-fold reduction in DOX retention compared to L1210/S cells was apparent without TFP treatment. Further, DOX retention was >1.5-fold higher ($P < 0.05$) in the presence versus the absence of 5 μM TFP during the retention phase in the L1210/DOX0.25 cells.

Cytotoxicity studies involved exposure to DOX in the absence or presence of 5 μM TFP for 3 hr and subsequent evaluation of survival in the absence of 5 μM TFP. Retention of DOX in the L1210/S and progressively resistant sublines treated with the IC_{50} of DOX in the absence or presence of 5 μM TFP for 3 hr and subsequently incubated for 180 min in DOX- and TFP-free medium was determined; the results are presented in Fig. 5. It is apparent from the results that with all the cell types the free and/or loosely bound DOX was rapidly lost during a 30-min incubation in drug-free medium, and no further appreci-

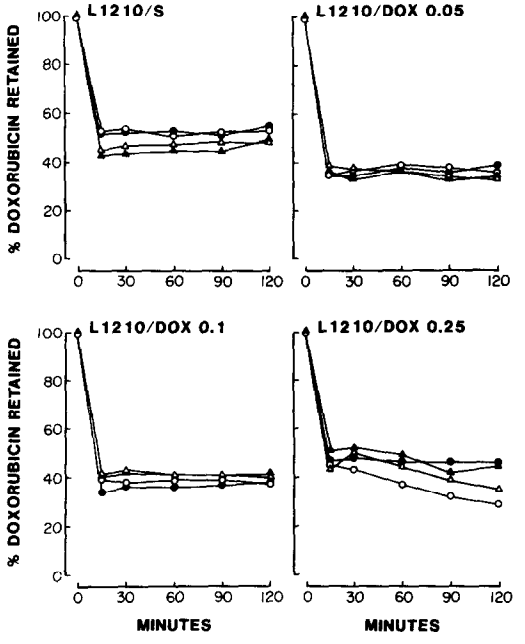

Fig. 4. Cellular retention of DOX in the absence or presence of TFP in sensitive and progressively DOX-resistant L1210 cells. Each point is the mean value of replicate determinations from at least duplicate experiments. Key: (○) accumulation and retention in the absence of 5 μM TFP; (●) accumulation in the absence of 5 μM TFP and retention in the presence of 5 μM TFP; (△) accumulation in the presence of 5 μM TFP and retention in the absence of 5 μM TFP; and (▲) accumulation and retention in the presence of 5 μM TFP. The accumulation at 1 hr of DOX (ng/10^6 cells) in L1210/S, L1210/DOX0.05 and L1210/DOX0.1 cells treated with 1.0 μg/ml DOX was 71.0, 57.4, and 46.0, respectively, and 52.5 in L1210/DOX0.25 cells treated with 2.0 μg/ml DOX. Accumulation of DOX (ng/10^6 cells) at 1 hr in L1210/S, L1210/DOX0.05, L1210/DOX0.1 and L1210/DOX0.25 cells treated with 0.5 μg/ml DOX plus 5 μM TFP was 43.0, 41.1, 34.8, and 24.3 respectively. The retention of DOX in the presence versus the absence of 5 μM TFP was significantly higher ($P < 0.05$) in L1210/DOX0.25 but not in L1210/S, L1210/DOX0.05, and L1210/DOX0.1 cells.

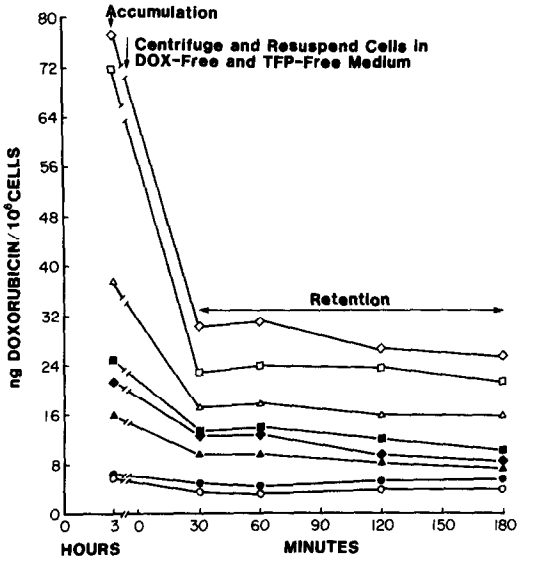

Fig. 5. Cellular accumulation and retention of DOX in sensitive and progressively resistant L1210 cells treated with the IC_{50} of DOX in the absence or the presence of 5 μM TFP. Each point is the mean value of replicate determinations from at least duplicate experiments. Key: (○) L1210/S plus 0.05 μg/ml DOX; (●) L1210/S plus 0.05 μg/ml DOX plus 5 μM TFP; (△) L1210/DOX0.05 plus 0.5 μg/ml DOX; (▲) L1210/DOX0.05 plus 0.125 μg/ml DOX plus 5 μM TFP; (□) L1210/DOX0.1 plus 1.0 μg/ml DOX; (■) L1210/DOX0.1 plus 0.15 μg/ml DOX plus 5 μM TFP; (◇) L1210/DOX0.25 plus 2.0 μg/ml DOX; and (◆) L1210/DOX0.25 plus 0.25 μg/ml DOX plus 5 μM TFP. Doxorubicin levels during accumulation (3 hr) and retention (180 min) following treatment with the IC_{50} of DOX in the absence of 5 μM TFP were significantly higher ($P < 0.05$) in L1210/DOX0.05, L1210/DOX0.1, and L1210/DOX0.25 sublines versus L1210/S cells. Cellular doxorubicin levels following treatment with the IC_{50} of DOX in the presence of 5 μM TFP were significantly higher ($P < 0.05$) during accumulation (3 hr) in L1210/DOX0.05, L1210/DOX0.1, and L1210/DOX0.25 sublines than L1210/S cells and during retention in L1210/DOX0.1 and L1210/DOX0.25 versus L1210/S cells.

able decreases (<15%) in DOX retention were apparent between 120 and 180 min. At the end of the 3-hr accumulation phase, cellular DOX levels in the resistant sublines treated with the IC_{50} for DOX alone were 7- to 14-fold higher ($P < 0.05$) than L1210/S cells, and the retention of DOX at 180 min in these resistant sublines was also 4- to 6-fold higher ($P < 0.05$) than L1210/S cells. However, compared to L1210/S cells, in the resistant sublines treated with the IC_{50} of DOX in the presence of 5 μM TFP, DOX levels were still 2.5- to 3.5-fold higher ($P < 0.05$) at the end of the 3-hr accumulation phase and approximately 1.5-fold higher ($P < 0.05$) at 180 min in drug-free medium during the retention phase.

DISCUSSION

Tumor cell resistance to DOX has been studied in a variety of model systems and the general consensus on mechanisms suggests that reduced drug accumulation and/or retention are the primary determinants [4, 5]. Although the precise mechanism contributing to reduced cellular drug levels in resistant sublines has not been identified, the presence of a high molecular weight glycoprotein (P-glycoprotein) in the plasma membrane has been suggested to modulate both influx as well as the active efflux of drug [7]. The demonstration that calmodulin inhibitors [13, 15] and calcium antagonists [15, 16] modulate the multidrug resistant phenotype of DOX-resistant cells by enhancing cellular drug retention suggests that the membrane-perturbing effects of these calcium modifiers may be responsible. A majority of studies analyzing DOX-resistant cells and modulation of cytotoxicity by calcium modifiers have utilized model systems with high levels of resistance (>50-fold), wherein marked reductions in cellular drug accumulation and/or retention compared to parent-sensitive cells are readily apparent. However, it is not clear in tumors with low levels of acquired resistance to DOX, which are more likely to be encountered clinically, whether drug accumulation and/or retention are important determinants. The role of calmodulin inhibitors or calcium antagonists in modulating DOX resistance has also been complicated by the fact that no specific calcium regulated processes which govern modulation of a cytotoxic response have been identified. Circumstantial evidence for the possible role of calmodulin regulated processes in the expression of DOX resistance are based on studies demonstrating that: (a) modulation of DOX-resistance by calmodulin inhibitors is based on the potency of these agents [14]; and (b) DOX-resistant cells have higher cellular calcium levels compared to the parental population [27].

Based on the data presented in this study, it is apparent that, in progressively DOX-resistant cells, the calmodulin inhibitor trifluoperazine has a differential effect on cellular accumulation, retention and cytotoxicity of DOX, and that there is little correlation between cellular DOX levels and the expression of resistance. A striking observation from the *in vitro* cytotoxicity studies is the relationship between the level of DOX resistance and the magnitude of modulation in DOX cytotoxicity by a non-cytotoxic concentration of 5 μM TFP (Fig. 1). It is also quite apparent that the combination of DOX and TFP is synergistically cytotoxic, since in all the resistant sublines concentrations of DOX or 5 μM TFP that were non-cytotoxic by themselves reduced cell survival by 50% in combination (Fig. 2). Although this synergistic cytotoxicity was nonetheless accompanied by a 1.5-fold increase in DOX accumulation (data not shown), the mere increase in cellular drug levels does not appear to be responsible, since 4- to 8-fold higher concentrations of DOX alone, which also enhanced accumulation by a similar magnitude, were required to achieve equivalent cytotoxicity in the absence of TFP. Although the effects of TFP on DOX cytotoxicity were concentration dependent over the range of 1–5 μM TFP but not at 10 and 20 μM TFP (Fig. 2), cellular accumulation of DOX was TFP dose dependent over the entire range of 1–20 μM (data not shown), suggesting that a mechanism(s) other than mere increases in cellular drug levels is responsible.

Although TFP is a well recognized potent antagonist of calmodulin, its specificity is limited due to numerous other effects [28–30] including inhibitory effects on other important calcium regulated enzymes, e.g. protein kinase C [31]. In preliminary studies we have evaluated 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) a "specific" inhibitor of protein kinase C [32] in L1210/DOX0.25 cells at concentrations of 5–50 μM, and were unable to demonstrate any potentiation of DOX cytotoxicity. Although these experiments do not specifically rule out the role of protein kinase C in the expression of DOX resistance, it appears that effects of TFP in DOX-resistant cells may not involve inhibition of protein kinase C.

It is becoming increasingly apparent in multidrug resistant cells that reduced accumulation and/or retention of vinca-alkaloids but not DOX appears to correlate with the degree of resistance [21–23]. The comparative studies between L1210/S and progressively DOX-resistant cells on characteristics of DOX accumulation and retention when treated under similar conditions further support the hypothesis that cellular DOX levels alone do not relate to the expression of resistance. Although TFP did increase cellular accumulation of DOX in the resistant sublines, the magnitude of reduction in DOX accumulation alone without TFP was insufficient to explain the level of resistance found in cytotoxicity studies. Furthermore, in experiments determining the net accumulation of DOX at 3 hr when treated with the IC_{50} of DOX, cellular DOX levels in L1210/DOX0.05, L1210/DOX0.1 and L1210/DOX0.25 sublines compared to L1210/S cells were 7- to 14-fold higher ($P < 0.05$) and 2.5- to 3.5-fold higher ($P < 0.05$) in the absence and presence of 5 μM TFP respectively (Fig. 5).

Although reduced drug accumulation has been suggested to be an important mode of resistance, reduced drug retention is currently accepted as the more important mechanism. The results from this study evaluating patterns of DOX retention in the L1210/S and progressively DOX-resistant sublines treated similarly further demonstrate that, similar to the accumulation data, the magnitude of differences

in DOX retention do not correlate with the expression of resistance in cells with <20-fold DOX resistance (Fig. 4) since DOX retention was markedly different from L1210/S cells only in the L1210/DOX0.25 cells. Kessel and Wilberding [21] have demonstrated that calcium modifiers enhance drug retention by inhibiting the outward transport process, and this phenomenon appears to be a mechanism only in cells with high levels of resistance, since the retention data presented clearly indicate enhanced DOX retention in the presence of TFP only in L1210/DOX0.25 cells. The results presented in Fig. 5 also suggest that, to achieve equivalent cytotoxicity, both net accumulation and retention of DOX in the resistant sublines compared to L1210/S cells are markedly higher ($P < 0.05$) in the absence versus the presence of TFP.

Center [33] has demonstrated that TFP-induced superphosphorylation of the P-glycoprotein is accompanied by enhanced DOX accumulation and consequently cytotoxicity. Although these results suggest a mechanistic basis for the effects of the calmodulin inhibitor, it is not clear why significantly lower cellular levels of DOX in the presence versus the absence of TFP are required to achieve equivalent cytotoxicity in the resistant sublines. More recently, Hamada and Tsuruo [34] have identified monoclonal antibodies to P-glycoprotein and demonstrated that one of the antibodies is capable of modulating accumulation and cytotoxicity of vincristine and actinomycin D but not DOX in multidrug-resistant cells. Based on results of the present study and those of Center [33] and Hamada and Tsuruo [34], it is therefore possible that the functional role of the P-glycoprotein may be important in mediating resistance to vinca-alkaloids but not DOX.

The precise mechanism of DOX cytotoxicity remains to be established, and the lack of a defined pathway(s) contributing to DOX cytotoxicity has also hampered our understanding of the mechanisms of DOX resistance. We have demonstrated recently that caffeine can protect cells from the cytotoxic effects of DOX, and that TFP can reverse this phenomenon. Ongoing studies to identify the mechanism of caffeine-induced protection indicate that, in both L1210/S and L1210/DOX0.05 cells, prior exposure to TFP reverses the protective effect of caffeine on DOX cytotoxicity (results not shown). It is therefore possible that the resistant sublines are protected from DOX cytotoxicity in a manner similar to that induced by caffeine and that this phenomenon can be modulated by TFP.

In summary, results from this study demonstrate that, in a series of progressively DOX-resistant sublines of L1210 mouse leukemia, the accumulation and retention of DOX did not correlate with the expression of resistance. The effect of TFP in modulating DOX cytotoxicity was dependent on the level of DOX resistance, and the potentiating effect of TFP on DOX cytotoxicity in the resistant sublines was not solely related to restoration of cellular drug levels. Further, in the DOX-resistant sublines, significantly higher cellular drug levels in the absence versus the presence of TFP were required to achieve equivalent cytotoxicity, and TFP did not restore DOX sensitivity comparable to that of L1210/S cells. The mechanism(s) of DOX resistance appears to be multifactorial, and our ongoing studies evaluating the effects of TFP on subcellular distribution of DOX and modulation of DNA damage and/or repair should provide additional information in delineating the role of TFP in modulating DOX resistance.

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