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# Correlation between Potency of Calmodulin Inhibitors and Effects on Cellular Levels and Cytotoxic Activity of Doxorubicin (Adriamycin) in Resistant P388 Mouse Leukemia Cells*

RAM GANAPATHI,† DALE GRABOWSKI, REBECCA TURINIC and RAFAEL VALENZUELA
*Research Division, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44106, U.S.A.*

**Abstract**—*The relationship between potency of phenothiazine and naphthalenesulfonamide calmodulin inhibitors and their effects on cellular levels and cytotoxic activity of doxorubicin was evaluated using the doxorubicin-sensitive and >100-fold doxorubicin-resistant P388 mouse leukemia model system. In cytotoxicity studies using cell counts based on proliferation following a 24-hr drug exposure and in survival based on colony formation in soft-agar after a 2-hr drug exposure, the calmodulin inhibitors significantly enhanced the cytotoxic effects of doxorubicin in the resistant but not parent-sensitive P388 cells. However, survival in soft-agar (based on colony formation) following long-term drug exposure (~120 hr) revealed that the cytotoxic effects of doxorubicin were significantly increased by the calmodulin inhibitors in both sensitive and resistant P388 cells. Laser flow cytometry studies on single-cell doxorubicin levels indicated that treatment with doxorubicin in the presence of trifluoperazine had no effect on drug levels in sensitive cells but significantly enhanced cellular accumulation and retention of doxorubicin in resistant cells. Furthermore, unlike treatment with doxorubicin alone, in the presence of trifluoperazine, heterogeneity in cellular drug levels in the resistant P388 cells was not observed. Among the various calmodulin inhibitors effective in enhancing cellular levels and cytotoxic effects of doxorubicin in the resistant P388 cells, chlorpromazine was approximately two-fold less potent than trifluoperazine or prochlorperazine and only N-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide but not N-(4-aminobutyl)-2-naphthalenesulfonamide was active.*

## INTRODUCTION

RESISTANCE to various antineoplastic agents has been demonstrated to be due to specific pharmacokinetic, biochemical and/or cytokinetic alterations at the cellular level of tumors [1–3]. Doxorubicin (DOX) and daunorubicin (DAU) represent two clinically important cancer chemotherapeutic agents [4, 5] and in studies with various model systems, specific mechanisms(s) for the expression of resistance to these drugs have been demonstrated [6–8]. In the well-characterized DOX- and DAU-resistant tumors, which include Chinese hamster lung DC-3F cells [6], Ehrlich ascites carcinoma [7, 9] and the P388 mouse leukemia [8], the primary mechanisms for resistance have been shown to be reduced cellular accumulation and/or retention of drug [7, 9, 10].

Recent studies [11–13] suggest that calmodulin inhibitors and calcium antagonists enhance the cytotoxic effects of DOX and VCR in resistant P388 mouse leukemia cells primarily by increasing cellular retention of drug. To further evaluate this potential approach to modulate the activity of DOX in resistant cells, we have systematically studied calmodulin inhibitors of differing potency for their effects on the cellular levels (accumulation and retention) and cytotoxic activity of doxorubicin in the DOX-sensitive

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†To whom correspondence and requests for reprints should be addressed.

(P388/S) and DOX-resistant (P388/DOX) P388 mouse leukemia model system.

The calmodulin inhibitors evaluated in the present study were the phenothiazines, *viz.* trifluoperazine (TFP), prochlorperazine (PCR), chlorpromazine (CPZ) and the naphthalenesulfonamides, *N*-(4-aminobutyl)-2-naphthalenesulfonamide (W-12) and *N*-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide (W-13). The rationale for evaluating the less hydrophobic naphthalenesulfonamide calmodulin inhibitors W-12 and W-13 as well was to determine whether the effects observed with the phenothiazines could be primarily related to their non-specific interactions with cell membranes. The choice of W-12 and W-13 was also based on the widely differing potencies (W-13, four-fold more potent than W-12 in inhibiting calmodulin-mediated activation of cyclic nucleotide phosphodiesterase) of these drugs as calmodulin inhibitors, in spite of similarities in chemical structure and hydrophobicity indices [14, 15].

Since the heterogeneous nature of tumor cells could significantly affect drug response [16] and our earlier studies demonstrated heterogeneity in the cellular accumulation of doxorubicin in P388/DOX cells [17], in the present study the effect of calmodulin inhibitors in altering patterns of cellular accumulation and/or retention of DOX in single cells was determined using laser flow cytometry (FCM). The significance of using FCM, in contrast to analysis of drug levels in cell homogenates, is in the ability to rapidly determine the presence of subpopulations which demonstrate heterogeneous characteristics in the cellular levels of doxorubicin.

## MATERIALS AND METHODS

*Cell lines and drugs*

The source of P388/S and P388/DOX cell line and conditions for their maintenance *in vitro* and *in vivo* was similar to that described previously [11, 17]. The phenothiazines, TFP, PCR and CPZ, were a generous gift from Dr Carl Kaiser, Smith Kline and French Laboratories, Philadelphia, PA. The naphthalenesulfonamides, W-12 and W-13, were obtained from Caabco, Inc., Houston, TX.

*Cytotoxicity studies in suspension culture*

Log phase cultures of P388/S and P388/DOX cells in RPMI-1640 supplemented with 25 mM HEPES buffer, 10% fetal bovine serum (FBS) and 10 $\mu$M 2-mercaptoethanol were treated with 0.01–5.0 $\mu$g/ml of DOX in the presence or absence of different concentrations of the calmodulin based on determining cell counts in control and inhibitors for 24 hr at 37°C. Proliferation was treated cultures in a hemacytometer and the number of trypan blue dye-excluding cells expressed as a percentage of the untreated control.

*Cytotoxicity studies in soft-agar*

For cytotoxicity experiments in soft-agar with continuous drug exposure, P388/S and P388/DOX cells were plated ($\sim 2 \times 10^4$ cells/plate) with various concentrations of DOX alone or in combination with the calmodulin inhibitors, in triplicate in 35 × 10-mm Petri dishes. Plating medium for the soft-agar assay was RPMI-1640 supplemented with 25 mM HEPES buffer, 20% FBS, 10 $\mu$M 2-mercaptoethanol and 0.3% agar. Following incubation for 120 hr at 37°C in a humidified 5% $CO_2$ + 95% air atmosphere, colonies (>50 cells) in control and treated plates were determined as described previously [11]. For cytotoxicity experiments following short-term drug treatment, P388/S and P388/DOX cells were treated for 2 hr at 37°C with various concentrations of DOX alone or in combination with the calmodulin inhibitors. Cells were then centrifuged at 80 *g*, resuspended in drug-free medium supplemented with and without the appropriate calmodulin inhibitor and incubated for an additional 1 hr at 37°C. Cells were then washed with drug-free medium and plated in triplicate in 35 × 10-mm Petri dishes using RPMI-1640 supplemented with 25 mM HEPES buffer, 20% FBS, 10 $\mu$M mercaptoethanol and 0.3% agar. Following incubation for 120 hr at 37°C in a humidified 5% $CO_2$ + 95% air atmosphere, colony counts were determined as described earlier.

*FCM analysis of cellular DOX fluorescence*

A Becton–Dickinson FACS II cell sorter equipped with a 2-W argon-ion laser was used for FCM analysis of cellular DOX levels. The instrument was aligned using glutaraldehyde-fixed chicken erythrocytes and conditions for analysis was an excitation wavelength of 488 nm using a laser power of 0.3 W and photomultiplier voltage of 750 V. Cells were analyzed at a flow rate of approximately 200–300 cells/sec and the percentage of fluorescent cells quantified by calculating the number of cells detected by light scatter (LS) vs the number detected based on cellular fluorescence (FL). For cellular accumulation and retention studies by flow cytometry, the incubation conditions for the cells with DOX in the presence and absence of the calmodulin inhibitors were similar to that described earlier for the short-term cytotoxicity experiments.

## RESULTS

*Effect of calmodulin inhibitors on the cytotoxic effects of DOX in suspension culture*

Cell counts in P388/S cells treated with 0.01, 0.05 and 0.1 μg/ml of DOX alone were 74, 41 and 32% respectively of the untreated control, as shown in Fig. 1A. It is further apparent that with the addition of TFP, PCR and CPZ to these concentrations of DOX no significant reduction in cell counts due to the presence of these calmodulin inhibitors was observed. In P388/DOX cells (Fig. 1B) treated with 1.0, 2.5 and 5.0 μg/ml of DOX, cell counts were 74–100% of the untreated control and, in contrast to results with the P388/S cells, significant DOX dose-dependent reductions in cell counts were observed only in the presence of TFP, PCR and CPZ. Results from similar suspension culture studies with the naphthalenesulfonamide calmodulin inhibitors, W-12 and W-13, are shown in Fig. 2. Similar to results with the phenothiazines, DOX-induced inhibition in proliferation of the P388/S cells was not altered by either W-12 or W-13 (Fig. 2A). In contrast, marked reductions in cell number of P388/DOX cells were obtained only with the combination of DOX plus W-13 compared to DOX alone or a very slight effect with DOX plus W-12 (Fig. 2B). It is important to note that the enhancement in the cytotoxic effects of DOX in the P388/DOX cells with the calmodulin inhibitors was obtained at concentrations of the phenothiazines and naphthalenesulfonamides which were not significantly cytotoxic by themselves.

*Effect of calmodulin inhibitors on the cytotoxic effects of DOX in soft-agar*

Due to possible limitations of dye-exclusion assays [18] in further studies evaluating the effect of calmodulin inhibitors in modulating the cytotoxic effects of DOX in P388/S and P388/DOX cells, a soft-agar colony assay was used. In contrast to results in suspension culture with P388/S cells, TFP at concentrations of 1 and 4 μM (Fig. 3A) enhanced the cytotoxic effects of DOX in the soft-agar assay in a dose-dependent manner (4 μM > 1 μM). In the P388/DOX cells using the soft-agar colony assay, reductions in survival with TFP were evident at a much lower concentration of DOX than used in suspension culture (Fig. 1B vs Fig. 3B). Although the survival at a lower DOX concentration of 0.05 μg/ml was similar with or without TFP, at 0.5 μg/ml DOX in the presence of 4 μM TFP the survival of P388/DOX cells was reduced >60-fold compared to DOX alone. However, a lower TFP concentration of 1 μM did not significantly enhance the cytotoxic effects of doxorubicin in P388/DOX cells. In similar soft-agar colony assay studies with equimolar doses of another piperazine side-chain phenothiazine, *viz.* prochlorperazine (data not shown), the survival of P388/S and P388/DOX cells treated with DOX alone or with PCR was comparable to that observed with TFP. However, as shown in Fig. 4 (A and B), in cytotoxicity experiments with the phenothiazine chlorpromazine, which has a tertiary amine side chain group at position 10, two-fold higher concentrations on a molar basis were required to

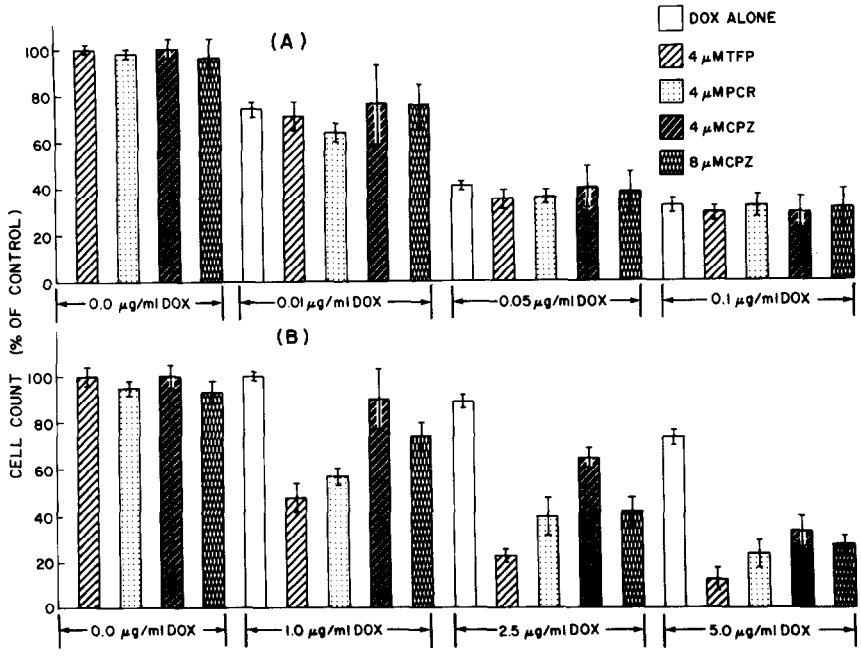


*Fig. 1. Effect of DOX or DOX plus phenothiazines on the proliferation of P388/S (A) and P388/DOX (B) cells in suspension culture. Values are means from at least triplicate experiments; bars, S.E.*

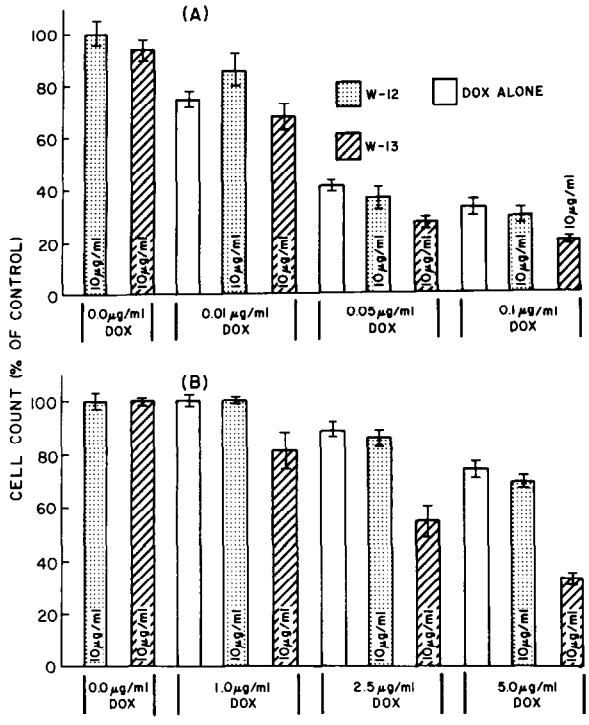


*Fig. 2. Effect of DOX or DOX plus naphthalenesulfonamides (W-12 and W-13) on the proliferation of P388/S (A) and P388/DOX (B) cells in suspension culture. Values are means from at least triplicate experiments; bars, S.E.*

achieve reductions in survival similar to those obtained with TFP and PCR. Results with the naphthalenesulfonamides, W-12 and W-13, on the cytotoxic effects of DOX are shown in Fig. 5. In accordance with results using the phenothiazine calmodulin inhibitors, the cytotoxic effects of DOX were enhanced in P388/DOX cells only with the ~4-fold more potent calmodulin inhibitor W-13 but not with the less active analog W-12.

*FCM studies on cellular DOX levels and correlation with cytotoxic effects in soft-agar*

Since resistance to the cytotoxic effects of DOX is primarily related to reduced cellular accumulation and/or retention of drug [10, 17], alterations in single-cell levels of DOX with the calmodulin inhibitors was determined using FCM. At 1 μg/ml DOX, drug accumulation in P388/DOX cells indicated a minor peak of fluorescence (Fig. 6A), with only 15% of cells detectable based on drug fluorescence, whereas in retention studies no fluorescent peak accompanied by only a slight reduction in the number of detectable cells was observed (Fig. 6B). In contrast, treatment of P388/DOX cells with 1 μg/ml DOX in the presence of TFP for 2 hr (Fig. 6C) resulted in a major fluorecent peak (50% greater) with >99% detectable cells. Analysis of these treated cells for DOX retention (1 hr) indicated a reduction in the fluorescent peak, without any significant decrease in the number of detectable cells (Fig. 6D).

In P388/DOX cells treated with 5 μg/ml of DOX alone for 2 hr (Fig. 7A), a prominent fluorescent peak with >99% detectable cells was obtained. Cellular retention of DOX in these cells after incubation for 1 hr in drug-free medium resulted in nearly a three-fold decrease in fluorescence, with only 30% detectable (Fig. 7B) cells. Following similar treatment of P388/DOX cells with DOX in the presence of TFP, the cells were found to be two-fold more fluorescent than with DOX alone (Fig. 7C) and >99% of cells were detectable based on drug fluorescence. Retention

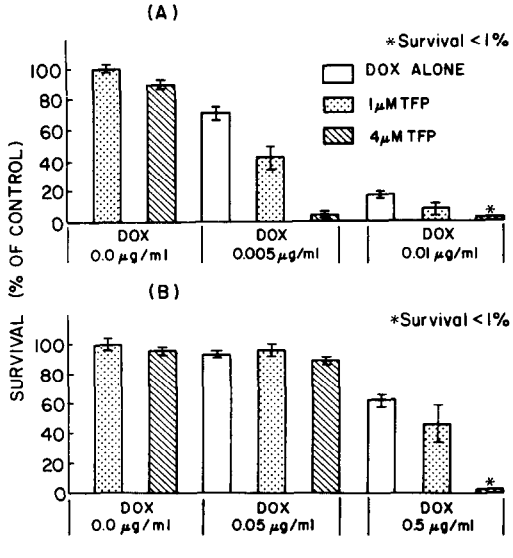


*Fig. 3. Survival in soft-agar of P388/S (A) and P388/DOX (B) cells treated with DOX or DOX + TFP for 120 hr. Values are means from at least triplicate experiments; bars, S.E.*

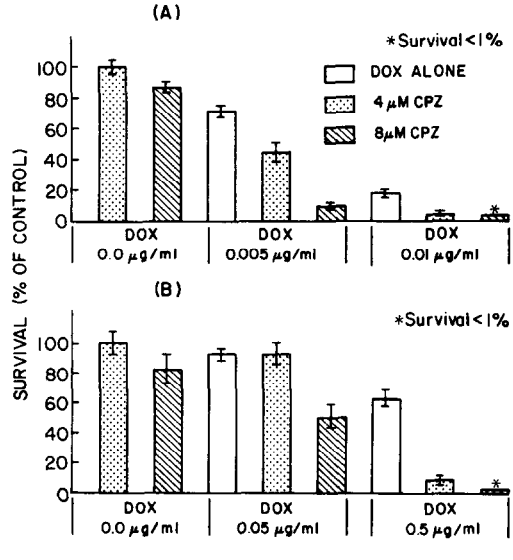


*Fig. 4. Survival in soft-agar of P388/S (A) and P388/DOX (B) cells treated with DOX or DOX + CPZ for 120 hr. Values are means from at least triplicate experiments; bars, S.E.*

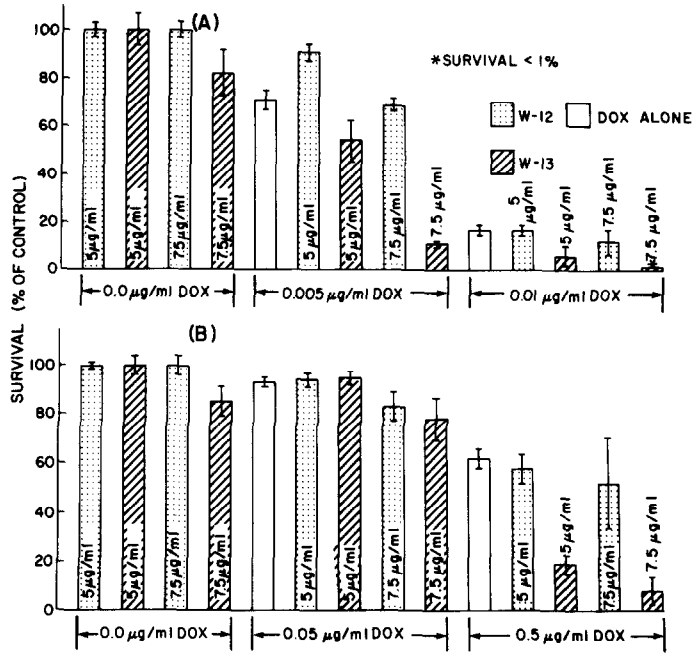


*Fig. 5. Survival in soft-agar of P388/S (A) and P388/DOX (B) cells treated with DOX or DOX + W-12 or DOX + W-13 for* 120 hr. *Values are means from at least triplicate experiments; bars, S.E.*

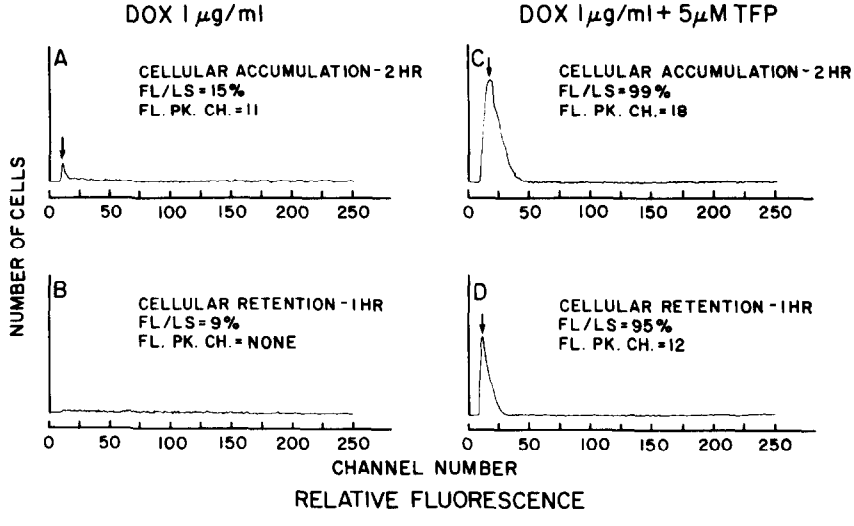


*Fig. 6. Laser flow cytometry profiles of cellular DOX fluorescence during accumulation and retention in P388/DOX cells treated with* 1 μg/ml *DOX (A and B) or* 1 μg/ml *DOX* + 5 μM *TFP (C and D).*

studies in drug-free media of these cells resulted in a reduction of fluorescence (Fig. 7D) but with no change in the number of drug fluorescent cells. Further, in P388/DOX cells treated with TFP, cellular DOX fluorescence was ~3-fold greater than in the absence of TFP. FCM analysis of similarly treated P388/S cells revealed that TFP had a slight effect in enhancing cellular accumulation but not retention of DOX.

In an attempt to correlate cytotoxic effects with cellular accumulation and retention characteristics of DOX in the presence and absence of TFP observed with FCM, survival in soft-agar of P388/S and P388/DOX cells following short-term drug exposure (2 hr) was determined. From the results in Fig. 8A, it is evident that the cytotoxic effects of DOX in P388/S cells were not affected by TFP. However, in P388/DOX cells, DOX dose-dependent reductions in cell survival occurred only with TFP treatment (Fig. 8B).

## DISCUSSION

In the present study we have determined the effect of the phenothiazine and the more specific naphthalenesulfonamide calmodulin inhibitors, on cellular levels and cytotoxic activity of DOX in

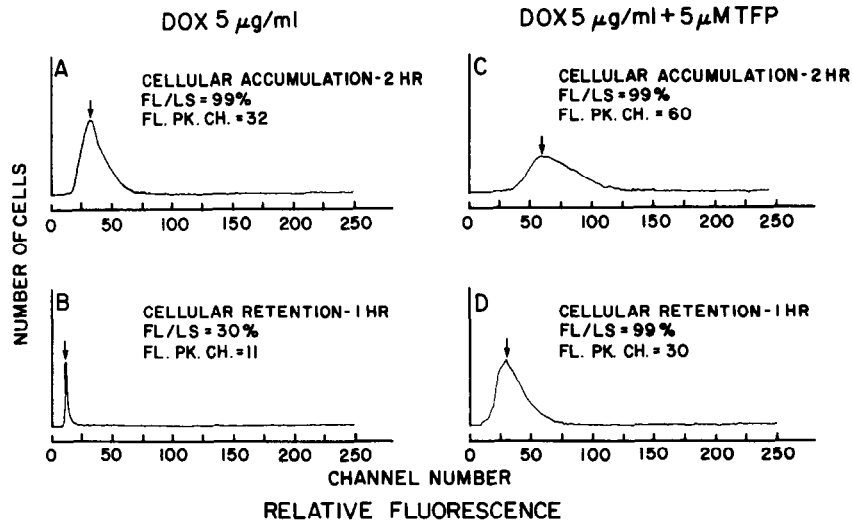


*Fig. 7. Laser flow cytometry profiles of cellular DOX fluorescence during accumulation and retention in P388/DOX cells treated with 5 μg/ml DOX (A and B) or 5 μg/ml DOX + 5 μM TFP (C and D).*

sensitive and >100-fold resistant P388 mouse leukemia cells. Confirming our earlier observation with TFP [11], the results with the various phenothiazines and naphthalenesulfonamides reported in this study further support our hypothesis that enhanced cytotoxic effects with DOX in resistant cells could be due to inhibition of a calmodulin-mediated process. The two-fold higher concentration (on a molar basis) of CPZ compared to TFP (Figs 3 and 4) required to produce comparable cytotoxic effects of DOX in P388/S and P388/DOX cells is in agreement with the reported differences in the $IC_{50}$ concentrations of these phenothiazines for inhibition of calmodulin-mediated processes [19]. The results from the FCM studies on cellular DOX levels in P388/DOX cells (Figs 6 and 7) indicate that in addition to poor cellular retention, some heterogeneity in cellular levels of drug fluorescence is present. It is certainly interesting to note that the FCM studies, in addition to confirming our earlier data by fluorometric analysis on the effect of TFP on DOX levels [11], demonstrate that a major effect of TFP is in significantly reducing the heterogeneity in cellular accumulation and retention of DOX in P388/DOX cells. Furthermore, the increases in drug levels due to TFP is not restricted to a particular subpopulation, since the number of cells detectable based on DOX fluorescence was not significantly different during accumulation or retention. The parallel survival studies in soft-agar with short-term drug treatment (Fig. 8) further confirm that the increases in cytotoxic effects of DOX with TFP treatment in the resistant cells are due to enhanced cellular accumulation and retention of drug. In similar FCM and short-term cytotoxicity studies in P388/DOX cells with the naphthalenesulfonamides (data not shown) and DOX, W-13 increased the number of detectable cells based on drug fluorescence and reduced cell survival compared to W-12 by nearly two-fold. However, W-13 was not as effective as TFP and these results are in accordance with the potency differences between TFP and W-13 observed in both suspension culture and soft-agar studies (Figs 1 and 2 and Figs 3 and 5) and the differences in $IC_{50}$ concentrations of these compounds for inhibiting calmodulin-mediated processes [14, 19]. The specificity of calmodulin inhibitors for enhancing cellular DOX levels and thereby the cytotoxic effects in P388/DOX rather than P388/S cells was more apparent both in FCM and survival

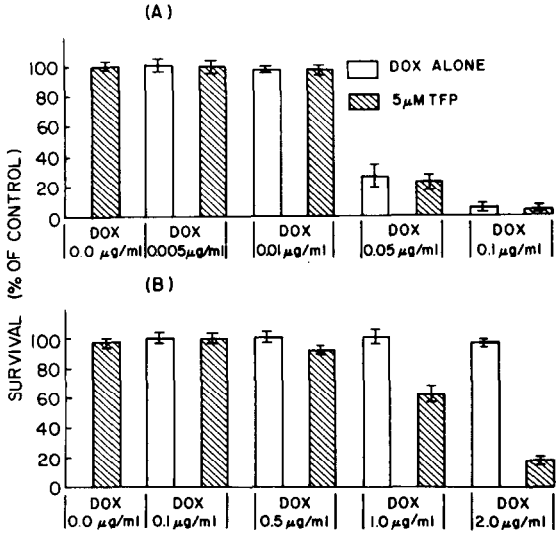


*Fig. 8. Effect of short-term treatment for 2 hr with DOX or DOX + TFP on the survival in soft agar of P388/S (A) and P388/DOX (B) cells. Values are means from at least triplicate experiments; bars, S.E.*

studies following short-term drug exposure (Figs 6–8).

Efforts to reverse resistance by increasing membrane permeability of the anthracycline-resistant cells with the non-ionic surfactant Tween 80 is reported to enhance only cellular uptake of drug but not to alter the pattern of retention, a major determinant of resistance [8, 20]. Skovsgaard [21] found that *N*-acetyl-daunorubicin, which is apparently non-cytotoxic by itself, could behave as a substrate for the efflux of DAU and thereby increase cellular retention and cytotoxic effects. Although various studies have suggested mechanisms and defined characteristics of DOX-resistant cells, it is still not clear what process mediates the important determinant of resistance, *viz.* reduced cellular drug retention. Dasdia *et al.* [22] have shown that in HeLa cells, doxorubicin enhances both calcium content and the rate of $^{45}Ca$ uptake. More recently, Katoh *et al.* [23] demonstrated that in heart cytosol, doxorubicin inhibits both calmodulin- and phospholipid-sensitive $Ca^{2+}$-dependent protein phosphorylation. Increasing cellular DOX levels and thereby cytotoxicity by using calmodulin inhibitors (results from present study and [12]), calcium channel blockers [12, 13] or exposure of cells to drug in media deficient in calcium [24] indicate that attempts to circumvent resistance using these approaches may involve alterations in the levels of cellular calcium and/or inhibition of calcium-dependent cellular processes. Although direct evidence for this proposed mechanism is not available, the requirement of adequate cellular calcium levels for the activation of calmodulin [25] and the phenomenon of active efflux of DOX in the resistant cells [10] suggest that the effects observed with the calmodulin inhibitors is possibly due to inhibition of the calmodulin-mediated processes of calcium transport and the regulation of $Ca^{2+}$-$Mg^{2+}$ ATPase.

In summary, this study demonstrates that in P388/DOX cells there is a good correlation between the potency of calmodulin inhibitors and their effects on cellular levels and cytotoxic effects of doxorubicin. A significant effect of the calmodulin inhibitors in P388/DOX cells was to markedly reduce the heterogeneity in cellular accumulation and retention of DOX. Although a combination of DOX and calmodulin inhibitors did not completely overcome resistance, the >100-fold DOX-resistant cells were only ~10-fold resistant when treated with a combination of DOX and a non-cytotoxic concentration of TFP. The potential application of a combination of DOX and calmodulin inhibitors in chemotherapy to enhance drug sensitivity could be dependent on the mechanism of resistance (reduced uptake and/or enhanced efflux) of the tumor and the schedule of administration of DOX either as a bolus or continuous infusion.

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