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MODULATION OF DOXORUBICIN-INDUCED CHROMOSOMAL DAMAGE BY CALMODULIN INHIBITORS AND ITS RELATIONSHIP TO CYTOTOXICITY IN PROGRESSIVELY DOXORUBICIN-RESISTANT TUMOR CELLS

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Abstract—Modulation of doxorubicin (DOX) cytotoxicity by the calmodulin inhibitor trifluoperazine (TFP) in progressively doxorubicin-resistant L1210 mouse leukemia cells is unrelated to effects on drug accumulation. Based on the clastogenic activity of DOX, the effects of TFP and the selective calmodulin inhibitor 1,3-dihydro-1-[1-[4-methyl-4*H*,6*H*-pyrrolo[1,2-*a*][4,1]-benzoxazepin-4-yl-methyl]-4-piperidinyl]-2*H*-benzimidazol-2-one(1:1) maleate (CGS9343B) on DOX-induced chromosomal damage and its relationship to cytotoxicity were evaluated in sensitive and progressively DOX-resistant L1210 cells. Potentiation of DOX cytotoxicity by CGS9343B (a potent inhibitor of calmodulin which does not inhibit protein kinase C) was related to the level of resistance. Further, for equivalent cytotoxicity, cellular DOX levels in the absence versus the presence of TFP or CGS9343B were markedly higher. Exposure to calmodulin inhibitors following DOX treatment enhanced chromosomal aberrations and cytotoxicity. Maximal effects of calmodulin inhibitors were apparent when used during and after DOX treatment, and potentiation of cytotoxicity was related to modulation of DOX-induced chromosomal aberrations. Results suggest that inhibition of calmodulin-regulated processes is a potential target in the modulation of DNA damage/repair, and could play a pivotal role in the expression of "acquired resistance" to DOX.

Doxorubicin (DOX†, Adriamycin®) is a clinically important antitumor antibiotic widely used in the chemotherapy of hematological malignancies and solid tumors [1]. Although the binding of DOX to nucleic acids may play a key role in cytotoxicity, it is becoming increasingly apparent that cellular effects contributing to a cytotoxic response are clearly multifactorial [2].

Tumor cell resistance to DOX is often encountered, and this is either "intrinsic" or "acquired" following repeated courses of chemotherapy. Although mechanisms of intrinsic resistance are ill-defined, studies in experimental model systems with acquired resistance have implicated a key role for the overexpressed plasma membrane P-glycoprotein [3]. The functional role of P-glycoprotein in the expression of resistance is suggested to involve the active efflux of DOX, based on its homology with bacterial periplasmic transport proteins [4].

However, our studies on cellular pharmacokinetics of DOX in model systems with acquired resistance to DOX have revealed that there is a limited correlation between alterations in drug accumulation and expression of resistance [5, 6]. Further, modulation of DOX resistance by the calmodulin inhibitor trifluoperazine (TFP) is unrelated to restoration of changes in drug accumulation [5, 6].

Although TFP is a well documented inhibitor of calmodulin, its selectivity is limited [7], and the novel calmodulin antagonist 1,3-dihydro-1-[4-methyl-4*H*,6*H* - pyrrolo[1,2 - *a*][4,1] - benzoxazepin - 4 - yl - methyl]-4-piperidinyl]-2*H*-benzimidazol-2-one(1:1) maleate (CGS9343B) has been demonstrated to be a more selective probe to evaluate the role of calmodulin function [7]. Thus, to gain further insights into the mechanisms governing modulation of DOX resistance, in the present study, we have determined the effects of the calmodulin inhibitors TFP and CGS9343B on DOX-induced chromosomal damage and its relationship to cytotoxicity using sensitive and progressively DOX-resistant L1210 mouse leukemia cells. The results suggest a potential role for calmodulin inhibitors in affecting the course of DOX-induced chromosomal damage, and indicate the possible role of this effect as a mechanism governing the efficacy of TFP and CGS9343B in modulating DOX cytotoxicity in resistant cells.

MATERIALS AND METHODS

The parental sensitive (L1210/S) L1210 mouse leukemia was established in culture as previously described [5]. Progressively resistant sublines were developed by culturing sensitive L1210/S(S) cells in increasing concentrations of DOX, and cells adapted to grow in 0.05 μg/mL and 0.25 μg/mL DOX were

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† Abbreviations: DOX, doxorubicin; TFP, trifluoperazine dihydrochloride; CGS, CGS9343B; and FBS, fetal bovine serum.

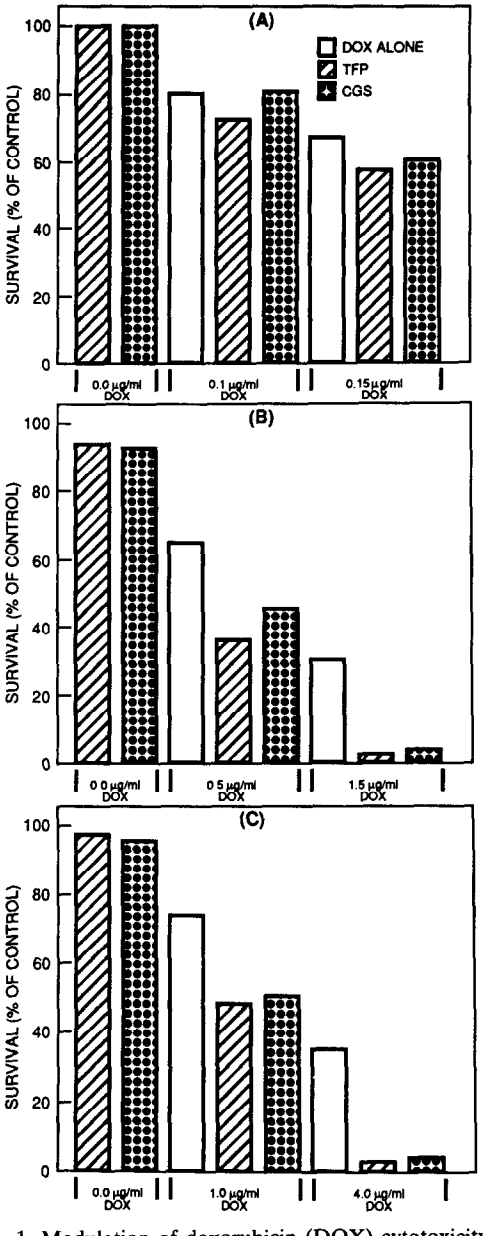

Fig. 1. Modulation of doxorubicin (DOX) cytotoxicity in (A) L1210/S(S), (B) L1210/DOX 0.05(R1) and (C) L1210/DOX 0.25(R2) mouse leukemia cells treated simultaneously with DOX and calmodulin inhibitors TFP or CGS9343B (CGS). Cells were treated for 1 hr with DOX in the absence or presence of the calmodulin inhibitors TFP or CGS and plated in soft agar at a density of 5×10^3 cells per 35×10 mm Petri dish; the colony count (mean ± SE) in the untreated control was 1385 ± 50, corresponding to a plating efficiency of approximately 28%. Each value is the mean from at least triplicate experiments with a standard deviation <15%.

identified as L1210/DOX 0.05(R1) and L1210/DOX 0.25(R2) respectively. Based on *in vitro* concentration–response survival curves, the R1 and R2 sublines were 10- and 40-fold resistant, respectively, to the cytotoxic effects of DOX compared to similarly treated S cells. Conditions for maintenance *in vitro* and other characteristics of the sensitive and progressively DOX-resistant sublines are similar to those described earlier [5]. TFP was provided by Dr. Carl Kaiser, Smith Kline & French Laboratories, Philadelphia, PA. CGS9343B was obtained from Dr. Richard Lovell, Ciba Geigy Corp., Summit, NJ.

Cytotoxicity in vitro. Cytotoxic effects of DOX in the absence or presence of calmodulin inhibitors were determined using a soft-agar colony forming assay [5]. Stock solutions of DOX and calmodulin inhibitors were prepared in sterile glass distilled water and working dilutions in RPMI 1640 supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (HEPES) buffer and 10% fetal bovine serum (FBS). The concentration of DOX selected for treatment was based on previously characterized concentration–response curves which produced a 50% reduction in colony formation (IC_{50}) in the absence or presence of 5 μM TFP. The sensitive (S) and progressively DOX-resistant sublines (R1 and R2) were treated in RPMI 1640 supplemented with 25 mM HEPES and 10% FBS with IC_{50} concentrations of DOX in the absence or presence of 5 μM TFP or CGS9343B for 1 hr. Following treatment, cells were centrifuged (80 *g*) and washed twice with drug-free RPMI 1640 supplemented with 10% FBS and plated immediately or were resuspended in fresh RPMI 1640 supplemented with 25 mM HEPES and 10% FBS in the absence or presence of 5 μM TFP or CGS9343B and incubated for an additional 18 hr prior to plating. Viability of control and treated cells was determined based on trypan blue dye exclusion, and 5×10^3 viable cells were plated in triplicate in 35×10 mm Petri dishes. The plating medium, incubation conditions and analysis of colony growth in soft agar were similar to those described earlier in detail [5].

Cytotoxic effects of DOX in the absence or presence of 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), a "selective" inhibitor of protein kinase C [8], were evaluated by the soft-agar colony assay [5]. Briefly, log-phase cultures of the DOX-resistant subline (R2) were treated with 0.1, 0.5 or 1.0 μg/mL DOX in the absence or presence of 5, 10, 25 and 50 μM H-7 for 3 hr at 37°. Cells were then washed in drug-free medium and plated in soft agar as described earlier.

Cellular accumulation of doxorubicin. Aliquots of 1×10^6 cells from log-phase cultures of sensitive or progressively DOX-resistant (R1 and R2) sublines treated with DOX alone or calmodulin inhibitors TFP or CGS during and/or after DOX exposure were washed twice with ice-cold 0.85% sodium chloride solution. The cell pellet following washing was resuspended in 50% ethanol/0.3 N hydrochloric acid, vortexed and centrifuged. The supernatant fraction was analyzed for DOX content fluorimetrically as described earlier [5] and cellular DOX levels expressed in ng/10^6 cells. Previous studies [5] have established that DOX is not metabolized by the sensitive or DOX-resistant cells in the absence or presence of calmodulin inhibitors.

Cytogenetic analysis for chromosomal aberrations. Aliquots of control and treated cells from experimental protocols outlined for cytotoxicity studies

Table 1. Effect of post-treatment with calmodulin-inhibitors on doxorubicin-induced chromosomal aberrations in sensitive and progressively doxorubicin-resistant L1210 mouse leukemia cells

Cell line	DOX* (μg/mL)	TFP or CGS† (5 μM)	CX‡	B‡	G‡	Aberration score§	Survival‖ (% of control)
L1210/S(S)	0.0		0	0	1	1	100
	0.0	TFP	0	0	2	2	97
	0.0	CGS	0	0	2	2	95
	0.1		0	0	8	8	74
	0.1	TFP	4	0	5	17	42
	0.1	CGS	1	1	7	12	69
	0.15		0	1	1	3	60
	0.15	TFP	7	0	16	37	23
	0.15	CGS	25	5	6	91	36
L1210/DOX 0.05(R1)	0.0		0	0	1	1	100
	0.0	TFP	0	0	0	0	97
	0.0	CGS	0	0	2	2	92
	0.5		0	0	2	2	76
	0.5	TFP	2	2	12	22	46
	0.5	CGS	3	0	1	10	71
	1.5		1	0	1	4	50
	1.5	TFP	9	0	2	29	15
	1.5	CGS	12	0	4	40	36
L1210/DOX 0.25(R2)	0.0		0	0	1	1	100
	0.0	TFP	0	0	1	1	96
	0.0	CGS	0	0	1	1	98
	1.0		0	0	1	1	89
	1.0	TFP	2	0	3	9	65
	1.0	CGS	0	0	3	3	96
	4.0		0	0	1	1	75
	4.0	TFP	4	0	20	32	28
	4.0	CGS	11	3	11	50	54

* Cells were treated for 1 hr with the indicated concentrations of DOX and subsequently exposed to calmodulin inhibitors.

† Controls and cells treated with DOX were incubated subsequently for 18 hr with 5 μM TFP or CGS (CGS9343B) and then processed for chromosomal aberrations or survival in soft agar.

‡ Chromosomal aberrations: CX = complex rearrangements, quadriradials and triradials; B = rings and chromosomal breaks; and G = chromatid breaks and gaps. Data are from a representative experiment which has been replicated at least twice.

§ Aberration score was the computed sum of total aberrations with CX, B and G being scored as 3, 2 and 1 respectively.

‖ Each value is the mean of triplicate experiments with a standard deviation <15%. Survival is based on colony counts. Cells were plated at a density of 5×10^3 cells per 35 × 10 mm Petri dish; the colony count (mean ± SE) in the untreated control was 1385 ± 50 corresponding to a colony-forming efficiency of 28%.

were exposed to 0.1 μg/mL Colcemid® (Gibco Laboratories) for 30–60 min at 37°. Samples were then processed for preparation of G-banded metaphase spreads by standard methods [9, 10]. Twenty metaphases from each sample were analyzed for the total number of aberrations [11], and an "aberration score" was summed. Complex rearrangements, quadriradials and triradials were scored as "3", rings and chromosome breaks were scored as "2" and chromatid breaks and gaps were scored as "1."

The relationship between the variables of cellular doxorubicin levels, chromosomal aberration score and survival was examined using the Pearson correlation coefficient.

RESULTS AND DISCUSSION

A comparison of the effects of CGS9343B and TFP in modulating the cytotoxicity of DOX in the sensitive and progressively DOX-resistant sublines is outlined in Fig. 1. Similar to our earlier results with TFP, equimolar concentrations of CGS9343B were relatively non-cytotoxic (<10% kill). The key observations were that CGS9343B, a selective calmodulin inhibitor, was as effective as TFP in modulating the cytotoxicity of DOX and that its efficacy was dependent on the level of the resistance.

Well documented cellular effects of DOX are damage to DNA [12] and clastogenic activity [13]. Based on our previous observations [14] demonstrating the efficacy of long-term treatment with TFP on DOX cytotoxicity, we evaluated the effects of treatment with calmodulin inhibitors during and/or after DOX exposure. Calmodulin inhibitors affect DNA repair [15] and may be involved in the expression of DOX resistance. The effect of treatment with calmodulin inhibitors TFP and CGS9343B following DOX exposure is shown in Table 1. The

Table 2. Effect of pre- and/or post-treatment with calmodulin inhibitors on doxorubicin-induced chromosomal aberrations in sensitive and progressively doxorubicin-resistant L1210 mouse leukemia cells

Cell line	DOX* (μg/mL)	TFP or CGS† (5 μM) Pre-	Post-	CX‡	B‡	G‡	Aberration score§	Survival‖ (% of control)
L1210/S(S)	0.0			0	0	1	1	100
	0.0	TFP	TFP	0	0	2	2	97
	0.0	CGS	CGS	0	0	2	2	95
	0.1	TFP		0	0	9	9	74
	0.1	CGS		0	1	2	4	76
	0.1	TFP	TFP	5	1	10	27	34
	0.1	CGS	CGS	2	0	10	16	52
L1210/DOX 0.05(R1)	0.0			0	0	1	1	100
	0.0	TFP	TFP	0	0	0	0	97
	0.0	CGS	CGS	0	0	2	2	92
	0.5	TFP		0	0	4	4	51
	0.5	CGS		9	3	6	39	64
	0.5	TFP	TFP	9	1	15	44	8
	0.5	CGS	CGS	16	3	2	55	45
L1210/DOX 0.25(R2)	0.0			0	0	1	1	100
	0.0	TFP	TFP	0	0	1	1	96
	0.0	CGS	CGS	0	0	1	1	98
	1.0	TFP		0	0	1	1	61
	1.0	CGS		2	0	3	9	70
	1.0	TFP	TFP	45	1	5	142	27
	1.0	CGS	CGS	0	0	2	2	61

* Cells were treated for 1 hr with the indicated concentrations of DOX in the presence of calmodulin inhibitors and incubated subsequently in the absence or presence of the calmodulin inhibitors.

† Controls and cells treated with DOX in the presence of calmodulin inhibitors were incubated subsequently for 18 hr in the absence or presence of 5 μM TFP or 5 μM CGS (CGS9343B) and processed for chromosomal aberrations or survival in soft agar.

‡ Chromosomal aberrations: CX = complex rearrangements, quadriradials and triradials; B = rings and chromosome breaks; and G = chromatid breaks and gaps. Data are from a representative experiment which has been replicated at least twice.

§ Aberration score was the computed sum of total aberrations with CX, B and G being scored as 3, 2 and 1 respectively.

‖ Each is the mean of triplicate experiments with a standard deviation <15%. Survival is based on colony counts. Cells were plated at a density of 5×10^3 cells per 35 × 10 mm Petri dish; the colony count (mean ± SE) in the untreated control was 1385 ± 50 corresponding to a colony-forming efficiency of 28%.

post-treatment with calmodulin inhibitors potentiated DOX cytotoxicity, and this was more pronounced in the resistant than in the sensitive cells. Chromosomal aberrations in cells treated with DOX and subsequently exposed to calmodulin inhibitors substantiated cytotoxicity data; chromosomal aberrations were increased in DOX-treated cells subsequently incubated in the presence versus the absence of calmodulin inhibitors. Further, TFP and CGS9343B were also of similar potency in enhancing DOX-induced chromosomal aberrations and cytotoxicity.

Based on these findings indicating the efficacy of calmodulin inhibitors in modulating DOX-induced chromosomal aberrations, we then sought to identify the efficacy of TFP or CGS9343B when used during and/or after DOX exposure. Results from these studies on the modulation of DOX cytotoxicity are shown in Table 2. A remarkable observation was that although TFP and CGS9343B were equally effective when used simultaneously with DOX, in cells treated with the calmodulin inhibitors during and after DOX treatment, TFP produced markedly greater cytotoxicity than CGS9343B. Chromosomal aberrations in cells treated with calmodulin inhibitors during and/or after DOX treatment were in agreement with the cytotoxicity data, demonstrating that the calmodulin inhibitors were most effective in modulating clastogenic activity when used during and after DOX exposure.

Since calmodulin inhibitors do affect drug accumulation in multidrug resistant cells [3, 5, 6], cellular DOX accumulation following treatment with calmodulin inhibitors (TFP or CGS9343B) during and/or after DOX exposure was determined. The results are presented in Fig. 2, and analysis of the data revealed a correlation of 0.45 ($P = 0.11$), −0.26 ($P = 0.37$) and −0.42 ($P = 0.13$) in the L1210/S, L1210/DOX 0.05(R1) and L1210/DOX 0.25(R2), respectively, between cellular DOX levels and cytotoxicity. The relationship between cellular DOX levels and chromosomal aberration score indicated a correlation of 0.64 ($P = 0.05$), 0.38 ($P = 0.28$) and 0.38 ($P = 0.28$) in the L1210/S, L1210/DOX 0.05(R1) and L1210/DOX 0.25(R2) cells respectively.

Studies with H-7 were carried out to evaluate the possible effects of TFP on protein kinase C and consequent modulation of DOX cytotoxicity. The results presented in Fig. 3 with the L1210/DOX 0.25(R2) resistant subline in which maximal effects

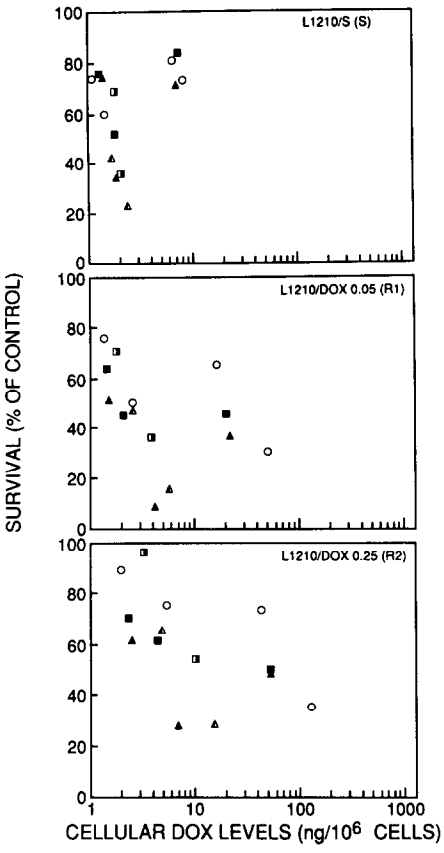

Fig. 2. Relationship between cellular DOX levels and survival in soft agar of L1210/S(S), L1210/DOX 0.05(R1) and L1210/DOX 0.25 (R2) cells. The cells were treated with DOX alone (○); 5 μM TFP for 18 hr following DOX treatment (▲); 5 μM TFP during (1 hr), or during (1 hr) and after (18 hr) DOX treatment (▲); 5 μM CGS9343B for 18 hr following DOX treatment (□); 5 μM CGS9343B during (1 hr), or during (1 hr) and after (18 hr) DOX treatment (■). The extracellular DOX concentrations during the 1-hr treatment were 0.1 and 0.15 μg/mL DOX for L1210/S(S) cells, 0.5 and 1.5 μg/mL DOX for L1210/DOX 0.05(R1) cells, and 1.0 and 4.0 μg/mL DOX for the L1210/DOX 0.25(R2) cells. Cells were plated at a density of 5×10^3 cells per 35 × 10 mm Petri dish; 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 of replicate determinations from at least duplicate experiments, with a standard deviation <15%.

of TFP or CGS9343B are apparent, indicate that unlike the calmodulin inhibitors, H-7 had limited effects in modulating DOX cytotoxicity. Further, enhanced DOX cytotoxicity with H-7 was apparent wherein H-7 alone was markedly cytotoxic. No remarkable modulation of DOX cytotoxicity by H-7 in L1210/S cells was apparent (data not shown).

Resistance to DOX appears clearly to be multifactorial, given the diverse cellular targets involved in eliciting a cytotoxic response [2]. A major mechanism of resistance which has gained considerable emphasis is the role of drug efflux mediated by P-glycoprotein [3, 4]. Although some alterations in DOX accumulation are apparent in the resistant cells, a noteworthy observation from the present results and our previous studies [5] is the requirement for higher cellular DOX levels in the resistant versus sensitive cells for equivalent cytotoxicity. Additionally, we have also reported that in progressively DOX-resistant L1210 cells, alterations in DNA cleavage activity are not merely due to changes in catalytic activity of topoisomerase II [16]. The results from this study demonstrate that the efficacy of calmodulin inhibitors in modulating DOX cytotoxicity is related to alterations in damage to DNA and the subsequent chromosomal aberrations, based on a pronounced correlation of -0.7 ($P < 0.01$), -0.71 ($P < 0.01$) and -0.75 ($P < 0.01$) in the L1210/S, L1210/DOX 0.05(R1) and L1210/DOX 0.25(R2) cells respectively. The comparative studies with TFP and CGS9343B also suggest the involvement of a calmodulin-regulated process based on the "selectivity" of CGS9343B as a calmodulin antagonist and not an inhibitor of protein kinase C. The studies with H-7 (Fig. 3), a "selective" and potent inhibitor of protein kinase C [8], demonstrate that this agent is a weak modulator of DOX cytotoxicity in the resistant cells. Further, lack of TFP-induced inhibitory activity on the protein kinase C system as a major mechanism governing modulation of DOX resistance is also supported by the differential effects of CGS9343B or TFP versus H-7 on DOX cytoxicity (Figs. 1 and 3). Although CGS9343B is reported to be more potent than TFP as a calmodulin inhibitor [7], such differences in potency between the two agents were not apparent in the present study, and may be related to possible differences in the cellular pharmacokinetics between these agents.

Similar to earlier results with TFP [5], the efficacy of CGS9343B on cytotoxicity was unrelated to remarkable increases in DOX accumulation. Overall, for equivalent cytotoxicity, cellular levels of DOX in the presence of TFP or CGS9343B were similar, and significantly lower (2- to 3-fold) than with DOX alone, in the resistant sublines. The present results suggest that calmodulin inhibitors seem to exert their effects by potentiating DOX-induced damage to a key cellular target, viz. DNA, and the results on chromosomal aberrations underscore that modulation of DOX-induced DNA damage may be a potential target for the efficacy of these agents. Other studies on modulation of DNA damage by antineoplastic agents have also suggested a role for calmodulin inhibitors [15, 17].

In summary, modulation of DOX resistance in the L1210 cells appears to be selective for inhibitors of calmodulin and suggests a role for their effects on DOX-induced chromosomal damage in governing cytotoxic response. Although the DOX-resistant L1210 sublines overexpress P-glycoprotein, efficacy of the calmodulin inhibitors is possibly not due directly to interaction with this membrane pump, since there was limited correlation with effects on alterations in cellular DOX levels [5, 16]. Thus, inhibition of calmodulin-regulated processes appears to be a potential target in affecting DNA damage/chromosomal aberrations induced by DOX and could play a pivotal role in understanding mechanisms governing modulation of "acquired" DOX resistance by calmodulin inhibitors.

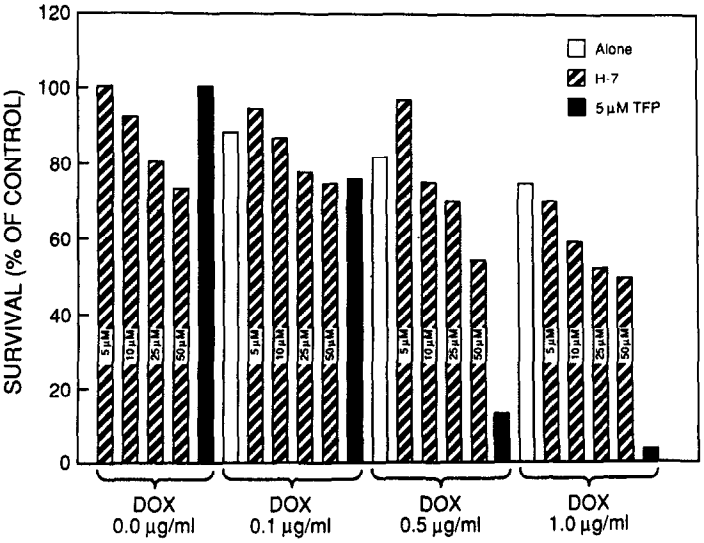

Fig. 3. Effects of H-7 and TFP on the survival in soft agar of 40-fold DOX-resistant L1210/DOX 0.25(R2) cells treated for 3 hr at 37°. Cells were plated at a density of 5×10^3 cells per 35×10 mm Petri dish; the colony count (mean ± SE) in the untreated control was 1385 ± 50, corresponding to a plating efficiency of approximately 28%. Each value is the mean from replicate determinations from duplicate experiments; with a standard deviation <15%.

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