Calmodulin inhibitor trifluoperazine in combination with doxorubicin induces the selection of tumour cells with the multidrug resistant phenotype

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Summary Trifluoperazine (TFP) is effective in modulating DNA damage/repair in doxorubicin (DOX) treated cells. In the present study we have characterised the resistance phenotype of parental sensitive L1210 mouse leukaemia cells (L1210/S) adapted to grow in the presence of 0.017 μM DOX + 5 μM TFP (L1210/DT). Although with prolonged exposure, 0.017 μM DOX alone adapted <35% cell kill in L1210/S cells, similar cytotoxicity was achieved at 0.43 μM DOX in L1210/S cells selected in the presence of 0.017 μM DOX + 5 μM TFP. L1210/DT cells were >30-fold resistant to DOX following a 3 h exposure of TFP. In contrast, DOX sensitivity in cells adapted to grow in 5 μM TFP alone was comparable to L1210/S cells. Resistance to other inhibitors of topoisomerase II in L1210/DT cells was >30-fold to etoposide and >6-fold to amsacrine. The levels of the 170 kDa and 180 kDa isoforms of topoisomerase II in an immunoblot were comparable between the L1210/S and L1210/DT cells. Cross resistance to vincristine in the L1210/DT cells was accompanied by the overexpression of plasma membrane P-glycoprotein. Although a 1.5–2-fold decrease in accumulation of etoposide and DOX was observed in the L1210/DT cells, drug levels for equivalent DNA damage in the alkaline elution assay were >5-fold higher in the L1210/DT versus L1210/S cells. No abrogation in the modulating effects of TFP on DOX, VP-16 or amsacrine induced cytotoxicity was apparent in the L1210/DT cells. Results suggest that: (a) TFP in combination with low concentrations DOX can induce the selection of cells with the multidrug resistant phenotype; and (b) characteristics of cells selected for resistance to DOX or DOX plus TFP are comparable.

The antitumour agent doxorubicin (DOX), an anthracycline antibiotic interacts with multiple cellular targets which possibly govern its cytotoxic activity (Riggs, 1992). Although the clinical efficacy of DOX has been documented in a number of tumour types, the development of resistance with repeated courses of chemotherapy is not uncommon (Riggs, 1992). This form of resistance which is 'acquired' has been characterised in a variety of model systems selected by prolonged exposure to DOX (Endicott & Ling, 1989). Since a precise target governing antitumour effects of DOX have not been defined, the mechanisms of resistance have been equally elusive. However, the identification of overexpression of membrane P-glycoprotein (PGP) responsible for cellular efflux of drug and/or alterations in topoisomerase II (TOPO II) in model systems, underscores the significance of these putative targets (Endicott & Ling, 1989; Ganapathi et al., 1989).

The search for agents to modulate chemosensitivity of tumours is of potential importance. Characteristics of agents which modulate the multidrug resistant (MDR) phenotype have been recently reviewed (Ford & Hait, 1990). In general, the interaction of the modulating agents with P-glycoprotein resulting in increased drug accumulation has been an accepted mechanism of action (Endicott & Ling, 1989; Ford & Hait, 1990). While MDR modulating agents invariably increase drug accumulation, a role for such alterations directly contributing to a cytotoxic response are dependent on the type of antitumour agent (Ganapathi et al., 1991b).

Since we originally reported (Ganapathi & Grabowski, 1983) the modulation of DOX cytotoxicity by the calmodulin inhibitor trifluoperazine (TFP), our subsequent studies have demonstrated that the effect of TFP in modulating cytotoxicity of DOX and other inhibitors of topoisomerase II is usually not correlative with the increase in cellular drug levels (Ganapathi et al., 1989; Ganapathi et al., 1991b). Prolonged pulse exposure to DOX plus TFP is also significantly more cytotoxic than DOX alone in both wild-type or resistant tumour cells (Ganapathi et al., 1984). Since the use of MDR modulating agents in initial treatment regimens to prevent emergence of drug resistance has been suggested (Salmon et al., 1991), we have pharmacologically and biochemically characterised L1210 mouse leukaemia cells selected for resistance to a combination of DOX plus TFP. Results from this study suggest that while mechanisms of resistance are qualitatively similar to that observed with cells selected for resistance to DOX alone, minimally cytotoxic concentrations of DOX alone in combination with non-cytotoxic levels of TFP can induce the selection of >30-fold DOX-resistant cells with the MDR phenotype.

Materials and methods

Ascites from a mouse bearing L1210 lymphoid leukaemia was used to establish the in vitro cell line (Ganapathi & Grabowski, 1988). Cells were routinely cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 25 mM N-2-hydroxyethylpiperazine-N-ethanesulfonic acid buffer (M.A. Bioproducts, Walkersville, Maryland), 10% foetal bovine serum (Sterile Systems, Logan, Utah) and 10 μM 2-mercaptoethanol.

The parental sensitive L1210 cell line (L1210/S) was adapted to grow for 24 weeks in the presence of 0.017 μM DOX plus 5 μM TFP for selection of the resistant subline (L1210/DT). Cell kill of L1210/S cells in a soft agar colony assay following short-term (3 h) and prolonged exposure (96 h) to 0.017 μM DOX alone was <10% and <35% respectively. Following this selection period, cells for experiments outlined were maintained in the absence of DOX plus TFP. Another separate subline was simultaneously developed from the parental cells (L1210/S) by continuous exposure for 24 weeks to 5 μM TFP alone (L1210/5 μM TFP). Treatment conditions for isolation of resistant sublines involved exposure of cells to the selecting agent(s) for 5 days followed by regrowth in medium without selection pressure for 2 days in repetitive cycles.

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Cytotoxicity in vitro

Cytotoxic response to doxorubicin (DOX), etoposide (VP-16), vincristine (VCR) or amascline (m-AMSA) in the sensitive and resistant sublines was determined by a soft agar colony assay (Ganapathi & Grabowski, 1988). Briefly sensitive or resistant sublines were treated for 3 h with DOX, VP-16, VCR, or m-AMSA in the absence or presence of 5 μM TFP at 37°C in a humidified 5% CO₂ plus 95% atmosphere. Control and treated cells were subsequently washed with drug-free medium and plated in 35 × 10 mm Petri dishes, incubated at 37°C in humidified 5% CO₂ plus 95% air atmosphere for 96 h and colonies (>50 cells) counted as described earlier (Ganapathi & Grabowski, 1988). The colony forming efficiency of the sensitive and the sublines selected for resistance to DOX plus TFP or TFP alone was approximately 33%.

Drug accumulation and retention in vitro

Log-phase cultures of L1210/S and L1210/DT cells were treated in vitro at 37°C in a humidified 5% CO₂ plus 95% air atmosphere with doxorubicin or [3H]-VP-16 (>95% pure by high performance liquid chromatography) in the absence or presence of 5 μM TFP. Cells following treatment were washed 2–3 times using cold (4°C) 0.85% sodium chloride solution and levels of DOX and VP-16 quantified by spectrofluorimetry (Ganapathi & Grabowski, 1988) and liquid scintillation respectively (Kamath et al., 1991).

Experiments on drug retention were carried out by preloading cells with the IC₅₀ of DOX ± 5 μM TFP for 3 h. Cells were subsequently centrifuged, washed and resuspended in medium in the absence of 5 μM TFP. Samples were retrieved at 15–30 min intervals over 120 min and cellular DOX levels quantified as described earlier (Ganapathi & Grabowski, 1988).

Drug induced DNA damage by alkaline elution

Damage to DNA induced by DOX or VP-16 in the absence or presence of 5 μM TFP was determined by alkaline elution under deproteinising conditions (Kohn et al., 1981). Details of the method for determining DNA single-strand breaks (DNA-SSB) by DOX and VP-16 have been previously described (Ganapathi et al., 1991a; Kamath et al., 1991). Elution with tetrapropylammonium hydroxide-EDTA, 0.1% SDS, pH 12.1 was carried out at a flow rate of 0.03–0.04 ml min⁻¹ with fractions collected at 3 h intervals over 18 h for DOX treated cells, and a flow rate of 0.12–0.16 ml min⁻¹ with fractions collected at 5 min intervals over 30 min for VP-16 treated cells.

Immunoblotting for topoisomerase II

Nuclear extracts from log phase cultures of L1210/S and L1210/DT cells were prepared with 0.35 M NaCl as

![Figure 1](https://example.com/figure1.png)  
**Figure 1** Cytotoxic effects of DOX, VP-16, m-AMSA, and VCR in the absence or presence of 5 μM TFP in L1210/S and L1210/DT cells treated for 3 h. Survival is based on colony counts. Cells were plated at a density of 5 × 10⁵ cells/35 × 10 mm Petri dish and colony count (mean ± standard error) in the untreated control was 1645 ± 474 corresponding to a colony forming efficiency of 33%. Each point is the mean ± standard error of triplicate experiments.

### Table 1 Cytotoxic effects of doxorubicin (DOX) in L1210/S and L1210/5 μM TFP cells

| DOXorubicin (μM) | Survival (% of Control) |
|-----------------|------------------------|
| L1210/S        | L1210/5 μM TFP         |
| 0.017 μM        | 98                     | 94                     |
| 0.017 μM + 5 μM TFP | 97                     | 92                     |
| 0.086 μM        | 60                     | 54                     |
| 0.086 μM + 5 μM TFP | 52                     | 42                     |
| 0.172 μM        | 13                     | 10                     |
| 0.172 μM + 5 μM TFP | 12                     | 5                      |

*Cells were treated with doxorubicin in the absence or presence of 5 μM TFP for 3 h at 37°C.

*Survival was based on inhibition of colony formation compared to the untreated control in a soft-agar colony assay. The data are the mean value from triplicate Petri-dishes in a representative experiment. The range between replicate Petri-dishes was <10%.
Detection of phosphorylated P-glycoprotein

Log-phase cultures of L1210/S and L1210/DT cells in phosphate free RPMI 1640 supplemented with 2 mM L-glutamine and 10% foetal bovine serum were labelled for 2–3 h with carrier-free $^{32}$Porthophosphoric acid (0.83 mCi ml$^{-1}$) at 37°C in a 95% air plus 5% CO$_2$ atmosphere (Ganapathi et al., 1991a). Cells were pelleted, lysed and PGP immunoprecipitated with C-219 monoclonal antibody (Centocor Inc., Malvern, Pennsylvania) as previously described (Anderson & Blobel, 1983; Ganapathi et al., 1991a). Samples were electrophoresed on 5% SDS-polyacrylamide gels. The gels were fixed, dried and autoradiographed at –70°C using prefalsed X-OMAT AR film (Kodak Laboratories, Rochester, NY).

Results

The sensitive (L1210/S) and resistant sublines (L1210/DT & L1210/5μM TFP) proliferated in vitro as single cell suspension cultures with a doubling time of approximately 10–12 h. Based on a periodic determination of the cytotoxic effects of DOX in a soft-agar colony assay, the L1210/DT cells were stably resistant in the absence of DOX plus TFP for at least 3 months (200 doublings) during in vitro culture.

The results with L1210/S and L1210/DT cells evaluating the cytotoxic effects of drugs that belong to the MDR phenotype and/or inhibit topoisomerase II in the absence or presence of 5 μM TFP are outlined in Figure 1. Based on the IC$_{50}$ (concentration required to reduce colony forming ability by 50% compared to the untreated control), following 3 h drug treatment the L1210/DT cells were 30-fold resistant to DOX and VP-16, 6-fold resistant to m-AMSA and completely resistant to VCR (≤20% kill at 0.1–2.0 μM) compared to similarly treated L1210/S cells. Further, while the modulation of DOX (2- to 20-fold), VP-16 (2- to 10-fold), and m-AMSA (3-fold) cytotoxicity by 5 μM TFP was readily apparent in the L1210/DT vs L1210/S cells, the effects on VCR toxicity were modest in the L1210/S (2-fold) and minimal in the L1210/DT cells. In contrast to the DOX resistance observed in L1210/DT cells selected with the combination of DOX + 5 μM TFP, as shown in Table I, no apparent resistant to DOX was observed in cells adapted to grow in the presence of 5 μM TFP alone for time periods comparable to selection of the L1210/DT cells.

The effect of 5 μM TFP on the accumulation of DOX and VP-16 in the L1210/S and L1210/DT cells is shown in Figure 2. Accumulation of DOX in the L1210/S and L1210/DT cells was time dependent (1 h ≤ 3 h) and cellular DOX levels in L1210/DT cells were 10–50% lower than in similarly treated L1210/S cells. Although the effect of 5 μM TFP on DOX accumulation in L1210/S cells was not remarkable, cellular DOX levels in L1210/DT cells were 30–100% higher in the presence vs absence of 5 μM TFP. The magnitude of difference in accumulation of VP-16 in L1210/DT vs L1210/S cells was comparable to that observed with DOX. In contrast, although 5 μM TFP increased VP-16 levels 20–50% in the L1210/S cells, no effect on VP-16 accumulation with L1210/DT cells was observed. Steady state levels of VP-16 are achieved in 1 h. However, in the case of doxorubicin, steady state levels are generally not achieved within 3 h. The significant increase in accumulation of VP-16 in sensitive cells, due to trifluoperazine, is apparent only at 40 μM which is far in excess of the range used for the cytotoxicity experiments. The effect of trifluoperazine on increasing cellular accumulation of VP-16 in sensitive, but not in doxorubicin-resistant sublines has been previously reported by us (Kamath et al., 1991).

The cellular retention of DOX in L1210/S and L1210/DT cells is outlined in Figure 3. The experimental strategy of using different concentrations of doxorubicin for the L1210/S vs L1210/DT cells for treatment with or without trifluoperazine was carried out in order to achieve comparable cellular doxorubicin levels prior to retention experiments. The 3 h uptake followed by retention was carried out in order to mimic the protocol used for cytotoxicity experiments. In L1210/S cells treated with the IC$_{50}$ of DOX, in the absence or presence of 5 μM TFP, DOX retention was 50–70% of that initially accumulated and represented approximately 5 ng DOX 10$^{-6}$ cells. In contrast, while DOX
levels in L1210/DT cells treated with IC₅₀ of DOX plus 5 μM TPF was 2-fold lower than in the cells treated with IC₅₀ of DOX alone, drug retention expressed as a percentage of that initially accumulated was comparable with both treatments. The effect of TPF on DOX and VP-16 induced DNA-SSB in L1210/S and L1210/DT cells is shown in Figure 4. Induction of DNA-single strand breaks by equimolar doses of DOX and VP-16 were 2–4-fold lower in L1210/DT vs L1210/S cells. However, in both L1210/S and L1210/DT cells, DNA strand breaks induced by DOX or VP-16 were potentiated in the presence of 5 μM TPF. The levels of the TOPO II using antisera specific for 170 kDa and 180 kDa isoforms of TOPO II are shown in Figure 5. The results show that the levels of the two isoforms of TOPO II (170 kDa and 180 kDa) in the L1210/S and L1210/DT cells are comparable. The amount of P-glycoprotein in L1210/S and L1210/DT cells following metabolic labeling with [³²P]orthophosphoric acid is shown in Figure 6. Results demonstrate the absence of any phosphorylated PGP in L1210/S cells which is consistent with the lack of overexpression of PGP in these cells (Ganapathi et al., 1991a). However, in the L1210/DT cells, the overexpression and phosphorylation of PGP is readily apparent.

Discussion

Drug resistance in the chemotherapy of cancer continues to be a constant challenge. Although mechanisms of resistance could be specific for a given class of agents, expression of resistance to drugs with different mechanistic basis for cytotoxicity has been a subject of considerable interest (Endicott & Ling, 1989). The phenomenon of broad cross-resistance referred to as the multidrug resistant phenotype is typified by the overexpression of a 150–180 kDa membrane glycoprotein, termed P-glycoprotein which is responsible for efflux of accumulated drug (Endicott & Ling, 1989). Numerous hydrophobic compounds have been demonstrated to modulate MDR, and an accepted mechanism for their efficacy is based on interaction with PGP (Endicott & Ling, 1989; Ford & Hait, 1990). Since the occurrence of resistant tumour cells is not uncommon, the use of the modulating agents in combination treatment has been suggested to prevent emergence of multidrug resistant cells (salmon, et al., 1991). Among the modulation agents, verapamil has been the most widely studied (Endicott & Ling, 1989; Ford & Hait, 1990). Our previous studies have focussed on trifluoperazine as a modulating agent and while its efficacy in affecting vinca alkaloid cytotoxicity was dependent on enhancing cellular drug levels, the potentiation of cytotoxicity with inhibitors of TOPO II was not correlated with corresponding increases in drug accumulation (Ganapathi et al., 1991b).

In this report, we demonstrate that the inclusion of TPF with minimally cytotoxic concentrations of DOX can induce the selection of cells with the MDR phenotype. While the L1210/DT cells were selected following exposure of L1210/S cells to 0.017 μM DOX + 5 μM TPF, to achieve comparable levels of resistance, L1210/S cells in previous experiments had to be progressively exposed with up to 20-fold higher concentrations of DOX (Ganapathi & Grabowski, 1988).

The results in Figure 1 demonstrate that the L1210/DT cells are cross resistant to inhibitors of TOPO II and vincristine. No cross-resistance to VCR in L1210/DT cells was observed (data not shown). The expression of resistance to VCR in L1210/DT cells was not surprising based on the overexpression of PGP and this is consistent with the relationship between overexpression of PGP and VCR resistance (Ganapathi et al., 1991b). The reduced accumulation and retention of DOX in the L1210/DT cells
suggests a role for PGP overexpression. However, the data in Figure 3 simulating treatment conditions for cell survival data in Figure 1 suggest the following: (a) At the IC₅₀ of DOX alone, DOX retention at near steady state is 5-fold higher in the L1210/DT vs L1210/S cells; (b) in L1210/DT cells treated with the IC₅₀ of DOX ± 5 μM TFP, the percent of DOX retained is comparable; and (c) cellular DOX levels for equivalent kill in L1210/DT cells were 3-fold higher in absence vs presence of TFP. The requirement of higher DOX levels for equivalent cell kill is also apparent in the data on induction of DNA-SSB by DOX and VP-16 (Figure 4). The reduced induction of DNA-SSB is not correlative with corresponding changes in drug levels and may be related to selective alterations in drug stimulated DNA cleavage activity without reduction in levels (Figure 5) or unknotting activity of P4 DNA (data not shown) of TOPO II. The requirement of low cellular DOX levels for equivalent DNA-SSB or cell kill in the presence vs absence of TFP is comparable to our observations in other model systems of DOX resistant cells (Ganapathi et al., 1991a) and possibly not related to cellular drug redistribution.

Our previous studies on the exposures of sensitive or DOX-resistant cells with 5 μM TFP following DOX treatment demonstrated enhanced chromosomal aberrations and cell kill suggestive of inhibition in DNA repair (Ganapathi et al., 1990). It thus may be possible that the induction of resistance following selection with a lower concentration of DOX in the presence of TFP is a consequence of alterations in DNA repair. The reduced TOPO II mediated DNA strand breaks and overexpression of PGP in L1210/DT cells are different from other reports using a combination of DOX and verapamil, since in these cells no overexpression of PGP was observed, and alterations in TOPO II included a decrease in both levels and catalytic activity (Bellamy et al., 1990; Chen et al., 1990). The absence of DOX resistance in cells adapted to grow in TFP alone is comparable to that reported with verapamil alone (Twentyman et al., 1990), demonstrating that these mechanistically different agents based on pharmacological effects may not affect putative targets of DOX cytotoxicity when used alone. Further, the continued ability of TFP to modulate DOX resistance in L1210/DT cells, while surprising, suggest that targets involved in modulation are possibly not compromised.

In summary, results from this study demonstrate that non-cytotoxic concentrations of 5 μM TFP in combination with 0.017 μM DOX can induce selection of >30-fold DOX-resistant cells with the MDR phenotype. The mechanisms of resistance which involve alterations in TOPO II mediated DNA strand breaks and PGP overexpression in cells selected with DOX + TFP are comparable to that observed in cells following selection with DOX alone. Although a potentiation in the development of resistance clinically needs to be carefully assessed, a combination of DOX plus verapamil in tumour bearing mice has also been suggested to lead to the rapid development of resistance (Formelli et al., 1988).

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The abbreviations used are: DOX, doxorubicin, TFP, triflouromethane phosphorothioate; VP-16, etoposide; VCR, vincristine; m-AMSA, amacrine; FBS, fetal bovine serum; MDR, multi-drug resistant.
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