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RAPID COMMUNICATION

EFFECT OF THE CALMODULIN INHIBITOR TRIFLUOPERAZINE ON PHOSPHORYLATION OF P-GLYCOPROTEIN AND TOPOISOMERASE II: RELATIONSHIP TO MODULATION OF SUBCELLULAR DISTRIBUTION, DNA DAMAGE AND CYTOTOXICITY OF DOXORUBICIN IN MULTIDRUG RESISTANT L1210 MOUSE LEUKEMIA CELLS

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Doxorubicin (DOX) is a potent antitumor antibiotic widely used in the clinical management of hemopoietic malignancies and solid tumors [1]. Recent studies suggest that DNA damage induced by DOX involves interaction with a key nuclear enzyme, topoisomerase II (TOPO II) [2]. Identification of the membrane P-glycoprotein (PGP) in multidrug-resistant cells has also suggested its involvement in the efflux of DOX as a mechanism governing resistance [3].

Our studies [4] with progressively DOX-resistant L1210 mouse leukemia cells have identified changes in TOPO II function as well as the overexpression of PGP. In this model system, efficacy of the calmodulin inhibitor trifluoperazine (TFP) in modulating DOX sensitivity is dependent on the level of resistance, and expression of resistance does not appear to be directly related to alterations in cellular drug levels [5]. Both PGP and TOPO II are phosphoproteins [3,6], and their functions may determine cytotoxicity of DOX. To gain a better understanding of the mechanisms governing expression and modulation of DOX resistance, we have determined the effect of TFP on subcellular distribution and DNA damage of DOX, as well as phosphorylation of PGP and TOPO II, using the sensitive and progressively DOX-resistant L1210 mouse leukemia model system.

MATERIALS AND METHODS

The isolation and characteristics of the sensitive (S), 10-fold DOX-resistant (R1), and 40-fold DOX-resistant (R2) L1210 mouse leukemia cells have been described previously [4,5]. Cell lines were routinely cultured in RPMI 1640 medium supplemented with N-(2-hydroxyethyl)piperazine-N-2-ethanesulfonic acid buffer, 2 mM L-glutamine (M.A. Bioproducts, Walkersville, MD), 10% fetal bovine serum (Sterile Systems Inc., Logan, UT) and 10 μM 2-mercaptoethanol (hereafter referred to as buffered RPMI 1640 medium). The IC_{50} values of DOX (concentration that produces a 50% reduction in colony formation compared with the untreated control in a soft-agar colony assay following treatment for 1 hr at 37°) in the absence of 5 μM TFP in S, R1 and R2 cells were 0.26, 2.59, and 6.9 μM, respectively [7]. The IC_{50} values of DOX in the presence of 5 μM TFP in S, R1 and R2 cells were 0.17, 0.86 and 1.72 μM, respectively [7].

Cellular and nuclear drug levels were determined by treating sensitive and resistant sublines with the IC_{50} of DOX in the absence or presence of 5 μM TFP for 1 hr in buffered RPMI 1640 medium followed by 1 hr in drug-free buffered RPMI 1640 medium at 37° in a 5% CO_2 plus 95% air atmosphere. Cells following treatment were washed twice with cold (4°) 0.85% sodium chloride solution and recovered by centrifugation at 200 *g*. Nuclei were isolated by rupturing cells with 40 strokes in a Dounce homogenizer using hypotonic lysis buffer (10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl) followed by recovery of nuclei by centrifugation (1000 *g*) in hypotonic lysis buffer - 0.25 M sucrose. Analysis of DOX levels using daunorubicin as the internal standard was carried out by HPLC following extraction of cells or nuclei as previously described [8]. The eluting solvent was acetonitrile - pH 4.0 ammonium formate buffer at a flow rate of 3.5 mL/min. Actual run conditions for HPLC with a μBondapak phenyl column (Waters Associates, Milford, MA) were a linear gradient from 32% acetonitrile - 68% pH 4.0 ammonium formate buffer to 68% acetonitrile - 32% pH 4.0 ammonium formate buffer achieved in 6 min, with final conditions held for 4 min before resetting to initial conditions for the next run. Detection was by an FL-750 flow fluorimeter (McPherson Instrument, Acton, MA) at excitation and emission wavelengths of 490 and 590 nm, respectively. Integration of peak areas to quantify DOX levels was carried out with an HP3396A integrator (Hewlett Packard Co., Palo Alto, CA).

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The effect of TFP on DOX-induced DNA single-strand breaks (DNA-SSB) was determined using the alkaline elution method under deproteinizing conditions [9]. The DNA from sensitive or resistant cells was labeled with [2-^{14}C] thymidine for 18-24 hr at 37°, and [methyl-^{3}H] thymidine labeled cells were used as the internal standard. Briefly, ^{14} C-labeled cells were treated with 0.26 μM DOX ± 5 μM TFP for S cells or the IC_{50} of DOX in the absence or presence of 5 μM TFP for the R1 and R2 cells at 37° for 1 hr. Following treatment, cells were deposited on polycarbonate filters (2 μm pore diameter, Nucleopore Corp., Pleasanton, CA) and eluted with tetrapropylammonium hydroxide-EDTA-0.1% sodium dodecyl sulfate (SDS), pH 12.1, at a flow rate of 0.03 to 0.04 mL/min; fractions were collected at 3-hr intervals [9]. The DNA-SSB induced by DOX were compared to those induced by γ-ray and expressed as rad-equivalents [9].

The catalytic activity and levels of TOPO II were determined by the P4 unknotting assay and immunoblotting, respectively [10].

Cells (3 x 10^6) were washed and incubated in phosphate-free RPMI 1640 supplemented with 10% fetal bovine serum for 1 hr at 37° in humidified 5% CO_2 plus 95% air. Labeling (0.83 mCi/mL) with carrier-free [^{32}P]orthophosphoric acid for 2 hr at 37° was followed by the addition of 5 μM TFP for an additional 30 min. Cells were pelleted and lysed and PGP was immunoprecipitated with C219 [3] monoclonal antibody (Centocor Inc., Malvern, PA) using a modification of the method described by Anderson and Blobel [11]. Polyclonal antibodies against human TOPO II polypeptide were prepared from an expression plasmid p56zII-1.8 [12] provided by Dr. Leroy Liu, and immunoprecipitation was carried out as previously described [10]. Protein A-PGP complex was dissociated at room temperature for 30 min in Laemmli buffer [13]. Protein A-TOPO II complex was dissociated by boiling in Laemmli buffer [13]. Samples were electrophoresed on 5% SDS-polyacrylamide gels. The gels were fixed, dried and autoradiographed at -70° using preflashed X-OMAT AR film (Kodak Laboratories, Rochester, NY). Phosphorylation of PGP and TOPO II was quantified by densitometric scanning.

RESULTS AND DISCUSSION

Cellular and nuclear accumulation of DOX in the S, R1 and R2 cells treated with the IC_{50} of DOX in the absence or presence of 5 μM TFP is shown in Table 1. Based on the differences in the IC_{50} of DOX with and without 5 μM TFP in the sensitive and resistant sublines, cellular accumulation of DOX in the R1 and R2 cells was higher than in the S cells. Notably, for equivalent cytotoxicity, DOX levels in whole cells or nuclei were significantly higher ($p<0.05$) in the R1 and R2 versus the S cells. However, in contrast to S cells, in the R1 and R2 sublines, DOX levels in whole cells or nuclei, when treated with the IC_{50} of DOX in the presence of TFP, were significantly lower ($p<0.05$) than in the absence of TFP. No metabolites of DOX were apparent in HPLC chromatograms of extracts from whole cells or nuclei from S, R1 or R2 cells treated with the IC_{50} of DOX ± 5 μM TFP.

TABLE 1. Effect of trifluoperazine on cellular and nuclear accumulation of doxorubicin in sensitive and progressively doxorubicin-resistant L1210 mouse leukemia cells

Cell Line	Doxorubicin* IC_{50} (μM)		Doxorubicin † (ng/10^6 cells or nuclei)			
			- TFP		+ TFP	
	- TFP	+ TFP	W	N	W	N
L1210/S (S)	0.26	0.17	2.8 ‡	1.4 ‡	3.2 ‡	1.1 ‡
L1210/DOX0.05 (R1)	2.59	0.86	25.7	13.1	10.8	7.6
L1210/DOX0.25 (R2)	6.90	1.72	48.9	30.8	14.8	10.6

* IC_{50} based on 50% reduction in colony formation compared with the untreated control in a soft agar assay after treatment for 1 hr at 37° [7]. Survival of S, R1 or R2 cells treated with 5 μM TFP alone was >95% [7].

† Doxorubicin levels in whole cells (W) or nuclei (N) following treatment with the IC_{50} of DOX in the absence or presence of 5 μM TFP for 1 hr at 37° followed by efflux in drug-free medium for 1 hr at 37°.

‡ Doxorubicin levels were determined by HPLC in whole cells or nuclei. Values are means from at least triplicate experiments.

The effect of TFP on DOX induced DNA-SSB in sensitive and DOX-resistant cells is presented in Fig. 1. The elution characteristics of DNA from S, R1 or R2 cells treated with 5 μM TFP alone were similar to those of the

untreated control (data not shown) and DNA-SSB in sensitive or resistant cells treated with or without TFP were DOX concentration dependent. Induction of DNA-SSB by DOX in the sensitive cells was between 15 and 30 rad equivalents and not significantly different in the absence or presence of TFP. In contrast, DNA-SSB in R1 and R2 sublines treated with the IC_{50} of DOX ± 5 μM TFP were significantly higher ($p<0.05$) than in S cells. Although DNA-SSB in the R1 and R2 cells treated with the IC_{50} of DOX ± 5 μM TFP were not significantly different, at the IC_{50} of DOX alone, DNA strand breaks in the R2 subline were about 40% higher.

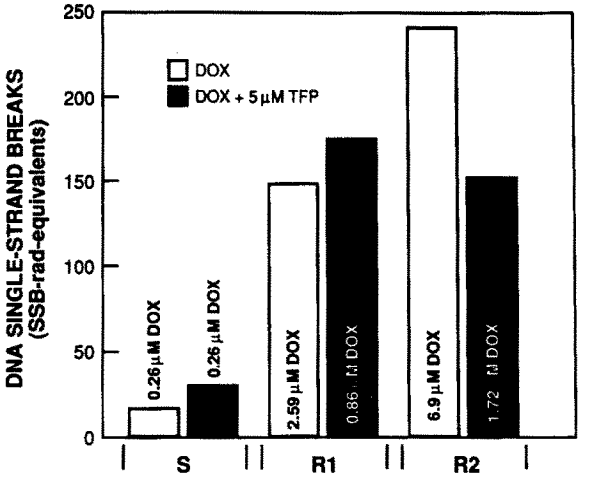

Fig. 1. Modulation of DOX-induced DNA-SSB by TFP in sensitive and progressively DOX-resistant L1210 mouse leukemia cells treated for 1 hr. Values are means from at least triplicate experiments.

Densitometric scans of the immunoblotting data (Fig. 2) revealed a <15% difference in the 170 kDa TOPO II protein level between the S, R1 and R2 cells. The P4 unknotting assay data (Fig. 3) indicated similar enzyme activity (<16% difference as determined by densitometry) between the S, R1 (data not shown) and R2 cells. Treatment of sensitive or resistant cells with TFP had no effect on the unknotting activity of TOPO II (data not shown). Overall, changes in levels or catalytic activity of TOPO II between sensitive and resistant sublines were inadequate to explain the level of DOX resistance.

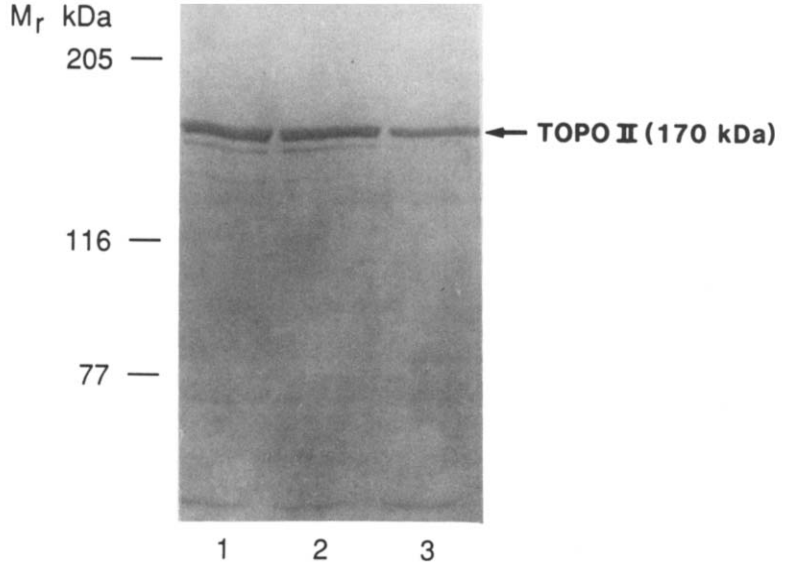

Fig. 2. Detection of TOPO II in sensitive and progressively DOX-resistant L1210 mouse leukemia cells. Lanes 1, 2 and 3 are S, R1 and R2 cells, respectively. Each lane contained 75 μg protein from nuclear extracts.

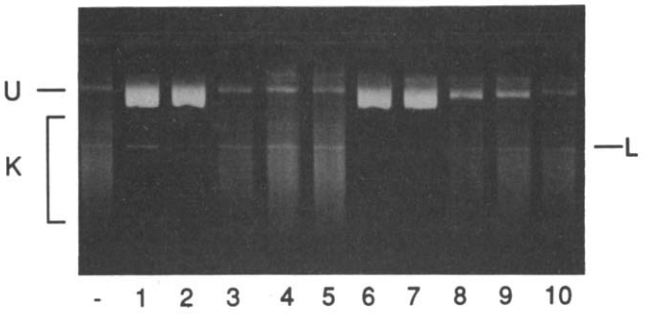

Fig. 3. TOPO II activity of extracts from S (lanes 1-5) and R2 (lanes 6-10) L1210 mouse leukemia cells detected with the P4 unknotting assay. Extracts were serially diluted to contain the following amounts of protein: Lanes 1 and 6, 400 ng; lanes 2 and 7, 200 ng; lanes 3 and 8, 100 ng; lanes 4 and 9, 50 ng; lanes 5 and 10, 25 ng; and — no enzyme (negative control). K, U and L are knotted, unknotted and linear P4 DNA, respectively.

The effects of TFP on phosphorylation of PGP and TOPO II in the sensitive and DOX-resistant cells are shown in Figs. 4 and 5. Consistent with earlier results, no PGP was detectable in the S cells, and in the R1 and R2 cells the levels and thus phosphorylation of PGP were dependent on the level of resistance [4]. Although TFP had no effect in the R1 cells, a 40% increase in phosphorylation of PGP in R2 cells was observed.

Phosphorylation of TOPO II in the resistant sublines was lower than in the sensitive cells. Although TFP had no effect on phosphorylation of TOPO II in the sensitive cells (data not shown), a 20-50% increase in phosphorylation of TOPO II in the resistant sublines was observed.

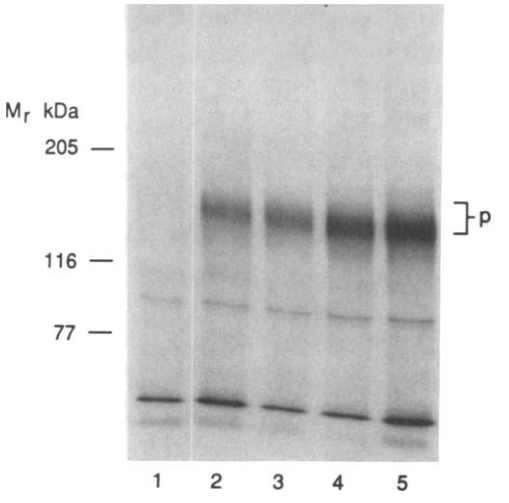

Fig. 4. Phosphorylation of PGP (P) in sensitive and progressively DOX-resistant L1210 mouse leukemia cells. Lane 1, S; lane 2, R1; lane 3, R1 + TFP; lane 4, R2; and lane 5, R2 +TFP. Areas of the PGP band in S, R1, R1 + TFP, R2 and R2 + TFP lanes are 0.9, 9.7, 9.6, 15.8 and 22.2 units, respectively. Data from a representative experiment are shown.

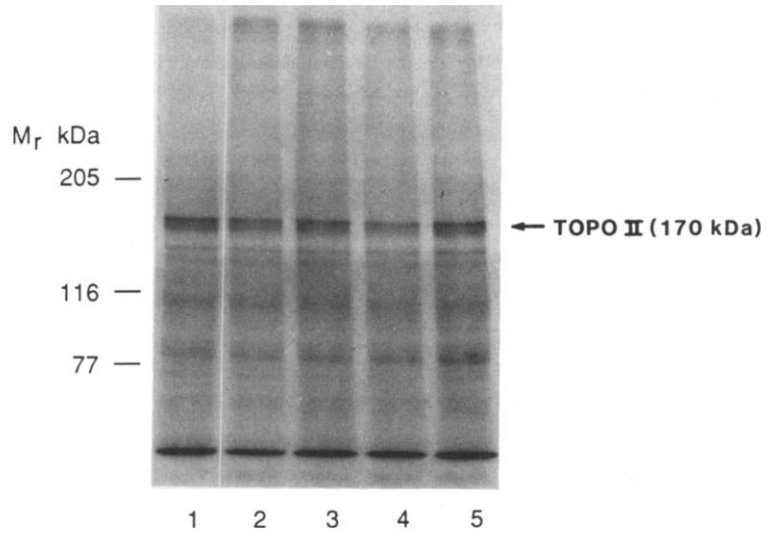

Fig. 5. Phosphorylation of TOPO II in sensitive and progressively DOX-resistant L1210 mouse leukemia cells. Lane 1, S; lane 2, R1; lane 3, R1 + TFP; lane 4, R2; and lane 5, R2 + TFP. Areas of the TOPO II band in S, R1, R1 + TFP, R2, and R2 + TFP lanes are 7.2, 5.9, 6.7, 5.6 and 8.5 units, respectively. Data from a representative experiment are shown.

Results from the present study demonstrate that cellular and nuclear levels of DOX were markedly higher in the resistant sublines than in sensitive cells when treated with equitoxic drug concentrations. Although the precise mechanism governing DOX-induced cytotoxicity is not defined, a correlation between DOX-induced chromosomal aberrations and cell kill exists [7]. The DNA damage data demonstrate that for equivalent cytotoxicity, DNA strand breaks in sensitive cells are lower than in resistant sublines. Modulation of DOX-resistance by TFP does not appear to be due to drug redistribution to the nucleus, since for comparable DNA strand breaks in the resistant sublines nuclear DOX levels were 2- to 3- fold higher in the absence versus the presence of TFP. The elevated DNA strand breaks for equivalent cytotoxicity in the resistant versus the sensitive cells may be related to higher levels of DOX accumulation. Further, at least in the R2 subline, the higher level of DNA-SSB when treated with the IC_{50} of DOX alone may be due to the high concentration of DOX and enhanced repair of DNA lesions [7]. Ongoing studies with the R2 subline also suggest that within 30 min > 40% of DNA-SSB are rejoined following treatment with DOX alone, compared to < 7% following exposure to DOX plus TFP (data not shown).

The effect of modulating agents on phosphorylation of PGP is suggested to regulate its activity [14]. Enhanced phosphorylation of PGP by TFP in R2 versus R1 cells is consistent with our earlier data demonstrating that significant effects of TFP on accumulation and/or retention of DOX are apparent in R2 versus R1 cells. The requirement in the resistant cells for higher nuclear DOX levels in the absence versus the presence of TFP to produce comparable DNA strand breaks and cytotoxicity does not appear to be related to alterations in levels or catalytic activity of TOPO II. However, an increase in the basal level of phosphorylation of TOPO II in the presence of TFP may be responsible for an increase in DNA strand breaks at lower DOX levels in the resistant sublines. The present data on phosphorylation measured only global effects, but they warrant a detailed investigation of possible alterations in the phosphorylation sites of TOPO II induced by TFP.

In summary, the results from the present study using the sensitive and progressively DOX resistant L1210 model system demonstrated that the effects of TFP are not due to redistribution of DOX to the nucleus, and modulation of cytotoxicity is related to effects on DOX-induced DNA strand breaks. Although TFP affects phosphorylation of PGP and TOPO II (R2 > R1), the comparable DNA strand breaks at lower DOX levels with TFP in the resistant sublines suggest that modulation of TOPO II function related to drug-induced DNA damage by calmodulin-mediated events may be an important mode of action.

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