Circumvention of Atypical Multidrug Resistance with Tumor Necrosis Factor

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Some "multidrug-resistant" (MDR) cell lines are not associated with a defect in drug accumulation or with the overexpression of P-glycoprotein. These cell lines are defined as "atypical MDR" (at-MDR) and they often express altered or mutated topoisomerase II. We investigated the ability of tumor necrosis factor to reverse at-MDR (in the human ovarian cancer cell line A2780 DX3) on the basis of its efficacy in potentiating *in vitro* topoisomerase II-targeted drugs, and because there is convincing evidence that the synergy is due to an increased number of topoisomerase-associated strand-breaks as well as to an increased level of extractable topoisomerase

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Many cell lines selected for resistance to doxorubicin (Doxo),[1-3] epipodophyllotoxins,[4-6] mitoxantrone,[7] m-AMSA[8] and 9-hydroxyellipticine[9] express multidrug resistance (MDR) that is not associated with a defect in drug accumulation or overexpression of P-glycoprotein (Pgp). Such cell lines are defined as atypical MDR (at-MDR) because they express altered and/or mutated topoisomerase II activity. The distinctive features of at-MDR have been reported in recent reviews.[10, 11] Recently we have characterized a human ovarian cancer cell line, A2780-DX3, which displays at-MDR. In this resistant subline the cleavage activity of topoisomerase II is about 40% of the level present in the parental cell line, while the catalytic activity of topoisomerase II is only about 15% lower than that in the parental cell line (manuscript in preparation).

The *in vitro* potentiation of topoisomerase II-targeted drugs by tumor necrosis factor (TNF) has been documented.[12-17] There is convincing evidence that the synergy between TNF and DNA topoisomerase II-targeted drugs is related to the ability of TNF to increase topoisomerase-associated strand-breaks as well as to increase the level of extractable topoisomerase catalytic activity.[14, 17]

The ability of TNF to reverse at-MDR was evaluated in human ovarian cancer cell line A2780-DX3 in the range of 0.1–10000 U/ml. A2780-DX3 cells are completely resistant to TNF cytotoxicity over the broad concentration range of TNF tested (0.1–10000 U/ml) [IC25 (25% inhibitory concentration) = 10000 U/ml] while the wild type line (A2780 WT) is very sensitive to TNF [IC50 (50% inhibitory concentration) = 8.8 U/ml] (cells were incubated with TNF for 20 h).

Since there is a correlation between TNF resistance and constitutive production of TNF,[18] we measured TNF in unconcentrated culture supernatant using a sensitive radioimmunoassay (sensitivity 0.5 fmol). A2780-DX3 cells do not constitutively produce TNF (<1 fmol in 10 ml of culture medium over a period of 4 days with 2.5×10^6 cells in a 25 cm^2 culture flask). A2780 WT and A2780-DX3 have the same number of receptors (data not shown). Perhaps responsiveness to TNF is not related to the number or affinity of TNF-binding sites[19]; if this is the case, there may be an important postreceptor mechanism controlling the cellular TNF response.

Because 1000 U/ml TNF is the most active dosage in terms of VP-16-induced SSB potentiation in A2780-DX3 cells (Table I) we chose this concentration to study the ability of TNF to reverse the drug resistance in A2780-DX3 cells.

Fig. 1 shows that when cells were incubated with TNF plus doxorubicin [(Doxo) panel a] or mitoxantrone [(Mito) panel b] or VP16 (panel c), a very significant enhancement of cytotoxicity to A2780-DX3 cells was observed. The IC50 values of Doxo, Mito and VP16 in WT cells are 0.07 μM, 0.002 μM and 0.42 μM, respectively. In A2780-DX3 cells the IC50 of Doxo was 10 μM; for Mito and VP16 we determined only the IC25, since the dose-response curve showed a maximum in the range of concentrations giving a cellular survival above 50%. The IC25 of Mito was 3.42 and that of VP16 was 37.30. When A2780-DX3 cells were incubated for 20 h with Doxo or Mito or VP16 in the presence of 1000 U/ml TNF, the IC50 values were 2.40 μM, 2.07 μM, and 15.01 μM, respectively. In A2780-DX3 cells the sensitivity to Doxo, Mito or VP16 was partially restored by TNF.

To define the role of TNF in the reversal of drug resistance, the effects of TNF on the production of

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protein-DNA single strand breaks (SSB) induced in A2780-DX3 cells by Doxo or Mito or VP16 was determined. Drug-induced DNA-SSB was measured by alkaline elution, under deproteinizing conditions, according to Russo *et al.*[19] A2780-DX3 cells were very resistant to these drugs in comparison to the parental cell line (A2780). In WT cells the drug concentration (μM) required to produce 600-rad equivalent SSB in A2780

(WT) is 15.42 for Doxo, 67.43 for Mito and 0.62 for VP16. On the other hand, in A2780-DX3 cells the drug concentration (μM) required to produce 600-rad equivalent SSB is 677.8 for Doxo, 1122.0 for Mito and 274.1 for VP16. When A2780-DX3 cells were incubated simultaneously with 1000 U/ml TNF and different concentrations of drug for 1 h, they became more sensitive in terms of induction of SSB by Doxo, Mito or VP16 (Table I).

Table I. DNA Single Strand Breaks (SSBs) Produced in A2780 DX3 Cells by Doxo or Mito or VP16 with and without TNF

Drugs (μM)		SSB (rad-equivalents)			
		−TNF	+TNF		
			1000 U/ml	100 U/ml	10 U/ml
Doxo	1	32.7±1.8	17.0±2.8	—	—
	5	98.0±3.6	371.7±11.3	—	—
	10	142.1±10.1	656.7±18.4	—	—
	100	151.7±14.7	—	—	—
Mito	1	0	57.45±2.3	—	—
	10	59.0±10.8	230.0±8.7	—	—
	100	65.0±4.9	421.2±10.1	—	—
VP16	0.1	0	29.7±1.2	—	—
	1	0	154.2±12.5	104.7±8.7	—
	10	40.2±7.7	733.2±18.4	358.2±11.6	47.0±0.8

Cells were incubated with the indicated drug concentrations for 1 h, then assayed by means of the alkaline elution technique, under deproteinizing conditions.[18] Data represent the mean±SE (standard error) of at least three independent experiments performed in triplicate.
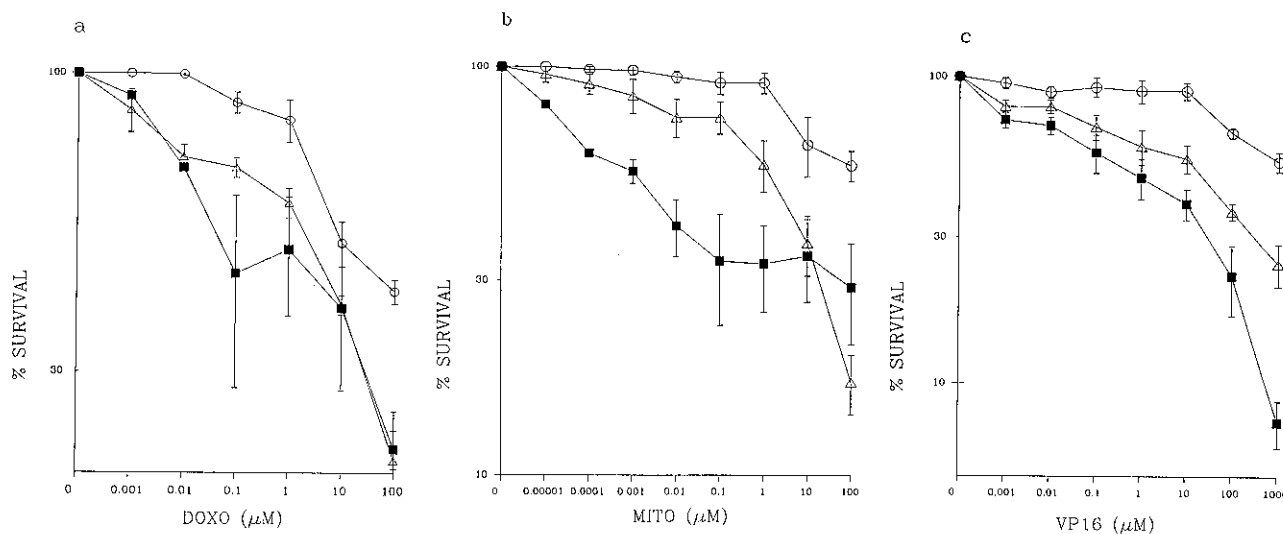

Fig. 1. Effect of TNF on doxorubicin (panel a) or mitoxantrone (panel b) or VP16 (panel c) cytotoxicity toward A2780 (WT) (■) and A2780 DX3 cells (○). Cells were exposed, 24 h after plating, to various concentrations of drug or drug+TNF for 20 h. A2780 DX3+TNF (△). TNF was obtained from Knoll-BASF (Ludwigshafen, Germany). A stock solution of TNF, containing 0.1 mg/ml of protein was stored at −80°C. Specific activity was 8.74×10^{-6} U/mg protein (48 h L929 bioassay without actinomycin D, as determined in the Knoll-BASF laboratories). Drugs were diluted in RPMI 1640 with 10% serum to achieve appropriate final concentrations, and immediately used. Cytotoxicity was monitored with the crystal violet assay, as described by Valenti *et al.*[16]

Table II. Doxo or Mito or VP16 Concentration (μM) Required to Produce 600 rad Equivalent SSB in A2780 (WT) and A2780-DX3 Cells in the Presence (1000 U/ml) or in the Absence of TNF

	A2780 (WT)	A2780 DX3	
		−TNF	+TNF
Doxo	15.42	677.8	8.93
Mito	67.43	1122.0	147.57
VP16	0.12	276.1	8.04

The values are extrapolated from data in Table I.

Table II depicts the drug concentration (μM) required to produce 600-rad equivalent SSB with each drug without and with TNF. As shown in Table II, TNF completely restored the sensitivity to Doxo and almost completely restored the sensitivity to Mito and VP16.

In order to better define the role of TNF in restoring sensitivity to topoisomerase II-targeted drugs in A2780-DX3 cells, the formation of covalent complexes between topoisomerase II (contained in 0.35 M NaCl nuclear extracts)-radiolabeled SV40 DNA at various concentrations of VP16 was quantified by a filter binding assay. This assay measures the initial stage (covalent binding to the 5′ termini of DNA breaks) of the topoisomerase II-catalyzed strand breakage and reunion reaction.[20] Experiments were carried out with nuclear extract obtained from A2780 WT cells and from A2780-DX3 cells previously treated for 5 min with 0 or 1000 U/ml TNF. The results (Fig. 2) show that at equimolar VP16 concentrations, topoisomerase II from A2780-DX3 cells did not induce more covalent binding than did the enzyme from A2780 cells. When the same experiments were performed with nuclear extract prepared from A2780-DX3 cells previously incubated with 1000 U/ml TNF for 5 min, high levels of covalent binding were obtained. Thus, there is an increased formation of topoisomerase II-DNA complexes with the extract obtained after TNF treatment.

There is a good relationship between the data obtained *in vivo* (cells) and in the purified system. Taken together, the results described above strongly support the hypothesis that TNF is able to reverse at-MDR by altering the activity of DNA topoisomerase II. Considering the importance of DNA topoisomerase II-inhibitors (Doxo, Mito and VP16) in the treatment of human ovarian cancer and considering that this cancer often reappears in a form which is resistant to a wide range of drugs, there is an urgent need for new effective treatments. An alternative therapeutic approach to human ovarian cancer, in patients who have relapsed after a standard chemotherapy, might be the use of TNF in combination with topoisomerase II inhibitors.
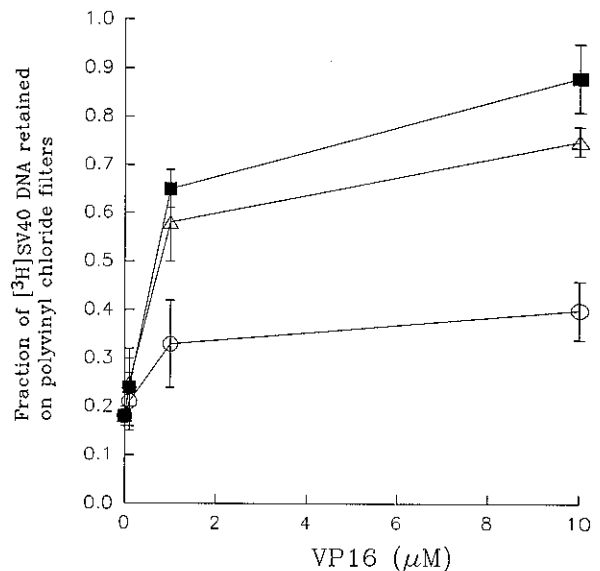

Fig. 2. DNA-binding activity in nuclear extracts from wild type and DX3 A2780 cells. Samples were assayed according to Pommier *et al.*[21] Covalent binding was assayed as the fraction retained after a 3 ml wash of the filter with LS10. Cleavable complex formation was quantified by determining the covalent binding of topoisomerase II to ³H-labeled SV40 DNA, evaluated as: convalent binding = filter/filter + LS10 fraction + EDTA fraction, where filter, LS10 fraction, and EDTA fraction represent the cpm of filter, processed as previously described,[19] the LS10 fraction (0.2% Sarkosyl, 40 mM EDTA, 2 M NaCl, pH 10) and the EDTA fraction (20 mM EDTA, washing fraction), respectively. In the absence of topoisomerase II the background value was less than 2%. Nuclear extract was prepared according to Drake *et al.*[9] and Valenti *et al.*[16] The extracts were incubated with ³H-labeled SV40 DNA together with various concentrations of VP16 at 37°C for 30 min. The reactions were stopped by the addition of 20 mM EDTA., pH 10, and the mixture was applied to a PVC filter. A2780 WT (■), A2780 DX3 (○), A2780 DX3 +TNF (△).

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