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Relationship between cytotoxicity, drug accumulation, DNA damage and repair of human ovarian cancer cells treated with doxorubicin: modulation by the tiapamil analog RO11-2933*

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Summary. The effect of *N*-(3,4-dimethoxyphenyl) *N*-methyl-2-(naphthyl)-*m*-dithiane-2-propylamine hydrochloride (RO11-2933), an analog of the calcium channel blocker tiapamil, on doxorubicin (DOX)-induced cytotoxicity and DNA damage in human ovarian cancer cells sensitive and resistant to DOX was investigated. A2780-DX2, A2780-DX3, and A2780-DX6 cell sublines were characterized by 7-, 26-, and 48-fold resistance after 2 h DOX exposure and 30-, 50-, and 500-fold resistance after 72 h DOX exposure, respectively. Increased drug efflux resulting in a lower intracellular drug accumulation, decreased DOX-induced DNA single-strand breaks (DNA SSBs), and rapid DNA repair correlated with the degree of resistance. In addition, DNA SSBs were rapidly repaired within 8 h in A2780-DX3 cells, whereas no significant repair of DNA SSBs was observed in sensitive cells. In comparison with verapamil, RO11-2933 was found to reverse DOX resistance at lower and nontoxic concentrations (2 μM as compared with 10 μM verapamil). This reversion was complete in cells with a low degree of resistance (A2780-DX1 and A2780-DX2) but partial in highly resistant cells (A2780-DX3 and A2780-DX6), and continuous exposure to RO11-2933 was essential for optimal reversal of drug resistance. Interestingly, RO11-2933 was found to inhibit the repair of DNA SSBs induced by DOX but not those induced by X-ray. These results suggest that the potentiation of DNA SSBs and the specific inhibition of DNA repair by RO11-2933 in multidrug-resistant cells could be of particular value in overcoming MDR in the clinic.

Introduction

Although doxorubicin (DOX) is a key chemotherapeutic agent widely used in the clinical management of ovarian cancer [7, 23, 24, 40], its efficacy is often hampered by the development of tumor cells resistant to this agent [28, 39] and cross-resistant to a variety of chemotherapeutic agents with different structures and mechanisms of action [4, 39]. Studies of multidrug resistance (MDR) in a variety of systems indicate that the mechanism of action of DOX is not restricted to its interaction with the DNA helix [1, 25] but may also involve other cellular targets. DOX has been demonstrated to maintain its cytotoxic activity without entering the nucleus [33, 35]. On the other hand, other mechanisms have been reported, such as: (1) the alteration of membrane integrity [12, 21] and (2) the induction of free radicals that lead to lipid peroxidation of intracellular membranes as well as direct macromolecular damage [2, 5, 22]. These multiple targets increase the likelihood that resistance to DOX can be a multifactorial event. Thus, although reduced drug uptake by resistant cells is an important determinant of resistance to DOX, its initial demonstration was not well correlated with the development of resistance.

In an effort to understand this phenomenon, we focused on the identification of determinants involved in resistance to DOX in human ovarian cancer cells. These studies included drug accumulation and retention and DNA damage and repair. The effects of RO11-2933, a calcium channel blocker, on these parameters were also investigated.

Materials and methods

Drugs and chemicals. Doxorubicin HCl was purchased from Farmitalia Carlo Erba (Milan, Italy). RO11-2933 (Fig. 1) was kindly supplied by Hoffmann-LaRoche AG (Basel, Switzerland). [^{14}C]-Thymidine (sp. act., 56 mCi/mmol) and [^{3}H]-thymidine (sp. act., 91 Ci/mmol) were purchased from Moravek Biochemicals, Inc., and Amersham. Proteinase K and tetrapropylammonium hydroxide (TPOH) were obtained from Bethesda Research Laboratory and Fisher Scientific Company, respectively.

Selection of DOX-resistant sublines. Human ovarian cancer cell line A2780 was established by Rogan et al. from cells from an untreated patient [30]. Selection of the resistant A2780-DX1, -DX2, -DX3, and -DX6 sublines was accomplished as follows. A2780-DX1 cells were obtained by the exposure of sensitive cells to 0.01 μM DOX (IC_{20}) for 14 days. A2780-DX2 and -DX3 were selected from A2780-DX1 by increasing the concentration of DOX in

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Abbrevations: RO11-2933, *N*-(3,4-dimethoxyphenethyl)-*N*-methyl-2-(2-naphthyl)-*m*-dithiane-2-propylamine hydrochloride; DOX, doxorubicin-HCl; SSBs, single-strand breaks; MDR, multidrug resistance

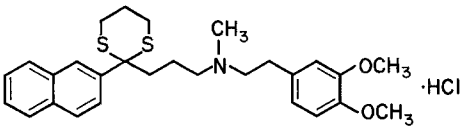

Fig. 1. Structure of N-(3,4-dimethoxyphenethyl)-*N*-methyl-2-(naphthyl)-*m*-dithiane-2-propylamine hydrochloride (RO11-2933)

CELL LINES		RELATIVE FOLD OF RESISTANCE	
		2h exposure	72h exposure
A2780		1	1
14 days	10^{-2} μM DOX		
A2780-DX1		4	5
14 days	5×10^{-2} μM DOX		
A2780-DX2		7	30
14 days	10^{-1} μM DOX		
A2780-DX3		26	53
21 days	5×10^{-1} μM DOX		
A2780-DX4		31	77
21 days	1 μM DOX		
A2780-DX5		35	241
35 days	5 μM DOX		
A2780-DX6		48	526

Fig. 2. In vitro selection of ovarian cancer cell sublines resistant to DOX. The relative degree of resistance was calculated as the ratio of the IC_{50} of resistant cells to that of sensitive A2780 cells, following 2 or 72 h in vitro exposure to DOX

the culture medium up to 0.1 μ*M*. Using further exposure to DOX at concentrations of 0.5, 1.0, and 5.0 μ*M*, we obtained the sublines A2780-DX4, -DX5, and -DX6 (Fig. 2). After selection, the resistant A2780-DX cells were maintained in culture under continuous exposure to the selected drug concentrations.

Both parental and DOX-resistant cell lines were cultured in monolayers in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 m*M* glutamine, penicillin and streptomycin (100 IU/ml and 100 μg/ml, respectively) in a humidified atmosphere containing 5% CO_2 at 37° C. Although the cell lines under investigation were frequently checked for their level of resistance to DOX, cells were taken out of the bank every 3 months.

Cytotoxicity studies. Sensitivity to different drugs was determined by measuring the inhibition of cell growth after 2 and 72 h exposure. Approximately 5×10^4 cells were seeded in 24-well Falcon plates for 24 h before drug treatment to enable the attachment of cells and exponential growth. At the end of treatment, the medium was removed and cells were washed with phosphate-buffered solution (PBS), detached with trypsin-EDTA, and counted using a Coulter Counter (Model ZBI, Coulter Electronics, Hialeah, Fla). The IC_{50} concentration was defined as the drug concentration producing 50% inhibition of cell growth compared with that in nontreated controls. The relative pattern of the degree of cross-resistance was determined as the ratio of the IC_{50} of each resistant subline to that of the sensitive parental cell line.

Cellular DOX accumulation and retention. Approximately 2.5×10^5/ml A2780 parental or DOX-resistant cells were seeded in 6-well plates for 24 h before drug treatment. Cells were exposed to 2 μ*M* DOX in the absence or presence of 2 μ*M* RO11-2933. At different times, cells were washed twice with ice-cold PBS, trypsinized, counted, and processed for evaluation of DOX accumulation.

For studies on DOX retention, cells were treated with 2 μ*M* DOX in the absence or presence of 2 μ*M* RO11-2933 for 2 h, then washed with ice-cold PBS and reincubated in DOX-free medium in the absence of presence of 2 μ*M* RO11-2933. At different times, cells were harvested and total DOX equivalents were determined using a modification of the method described by Finkel et al. [10]. Cells were lysed in 0.05% sodium dodecyl sulfate (SDS) at −20° C for approximately 24 h. Proteins were precipitated by 3.3% $AgNO_3$ before the addition of 4.0 ml *n*-butyl alcohol. After centrifugation at 3,500 *g* for 10 min, the organic layer was removed and the total fluorescence of the extracts was measured at 588 nm emission and 480 nm excitation in a spectrofluorometer (Aminco Bowman, Silver Spring, Md). A standard curve was prepared with fresh DOX dilutions in water-saturated *n*-butyl alcohol; it was linear in the concentration range 0.02–10.0 μg/ml, with a correlation coefficient of >0.998.

DNA SSB assay. DNA SSBs were quantified by the alkaline elution assay according to methods previously described by Kohn [17], Kohn et al. [18], and Bradley and Kohn [6]. Approximately 0.125×10^6/ml sensitive or resistant A2780 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum containing either 0.025 μCi/ml [^{14}C]-thymidine or 0.5 μCi/ml [^{3}H]-thymidine. After 20 h, the labeled cells were washed twice with cold PBS and the radioactivity was chased into high-molecular-weight DNA by an incubation in medium containing 10^{-5} *M* thymidine for 4 h. DOX-treated [^{14}C]-labeled cells mixed with an equal number of [^{3}H]-labeled cells as an internal standard were deposited on polycarbonate (PC) filters (Nucleopore) and lysed for 1 h prior to elution at room temperature with 2% SDS and 0.025 *M* EDTA (pH 9.7) in the presence of proteinase K (0.25 mg/ml). Filters were washed with 5 ml 0.02 *M* EDTA (pH 10.3) and elution was carried out in a dark environment at 2 ml/h with a solution of TPOH/EDTA and 0.1% SDS at pH 12.1.

Drug-induced DNA SSB frequencies (P_{BD}) were calculated according to the following formula [18]:

$$P_{BD} = \frac{\log (rl/ro)}{\log (Ro/ro)} P_{BR},$$

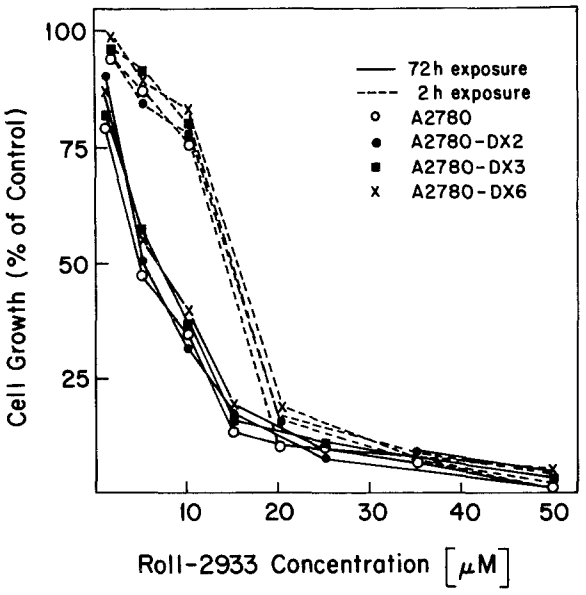

Fig. 3. Dose-response curves of RO11-2933 in sensitive and resistant A2780 cell lines. Exponentially growing cells were treated with RO11-2933 for 2 and 72 h. Each point reported corresponds to the mean of at least three experiments; each varied by no more than 10%

where P_{BR} is the DNA break frequency produced by 300-rad X-ray-irradiated cells (external standard cells), r_1 and r_0 are the DNA retention in drug-treated and control [^{14}C]-labeled cells, respectively, and Ro is the DNA retention of 300-rad X-ray-irradiated cells.

DNA repair assay. The repair assay of drug-induced DNA SSBs was carried out on [^{14}C]-labeled cells previously exposed for 4 h to DOX alone or DOX plus 2 μ*M* RO11-2933. After treatment, the cells were washed with PBS and postincubated for 2, 4, and 8 h at 37° C in the absence or presence of RO11-2933. At the end of the culture, [^{14}C]-labeled cells were mixed with an equal number of [^{3}H]-labeled internal-standard cells and deposited on PC filters for alkaline elution as described above. The fraction of rejoined DNA strand breaks was calculated using the formula described by Bradley and Kohn [6].

$$\text{Fraction of DNA rejoined} = \frac{c - b}{a - b} \times 100,$$

where a is the fraction of DNA retained by untreated controls, b is the fraction retained by 0-time postincubation of drug-treated cells, and c is the fraction retained after postincubation for a specified time.

Results

Cytotoxicity of RO11-2933

Figure 3 demonstrates the cytotoxic dose-response curve of RO11-2933 alone following 2 and 72 h exposure. Under these conditions, RO11-2933 exhibited a similar cytotoxic effect on A2780 and its resistant cell lines. The IC_{50} was approximately 14 and 5 μ*M* for 2 and 72 h exposure, respectively. In resistance-reversal studies of cell viability in the presence of RO11-2933, we used the concentration of 2 μ*M* RO11-2933, which yielded about 6% and 26% of cell growth inhibition after 2 and 72 h exposure, respectively.

Table 1. Characteristics of sensitive and resistant A2780 cell lines

	A2780	A2780-DX2	A2780-DX3	A2780-DX6
Doubling time (h)	24± 1	28±2	25± 1	30± 4
Modal chromosome number	46	46	46	46
Relative cell volume[a]	1	1.1	1	1.0
Protein level[b] (μg/l × 10^6 cells)	106±15	141±0	124±16	113±14

[a] Determined with a channelizer connected to a Coulter counter. Polystyrene microspheres with a diameter of 9.85 μm were used as size reference. The relative cell volume was evaluated as the volume ratio of each resistant line to that of the sensitive line
[b] Determined by the Lowry method [20] (mean ± SD)

Table 2. Cytotoxicity of DOX alone or in combination with RO11-2933 against sensitive and resistant A2780 cell lines

Experimental groups[a]	Treatment	IC_{50} [μ*M*][b]:			
		A2780	A2780-DX2	A2780-DX3	A2780-DX6
A	DOX	0.12±0.04	0.89±0.03	3.15±0.25	5.78±0.90
B	DOX	0.01	0.30	0.50	5.00
C	DOX+RO11-2933	0.01	0.34	0.05	0.07
D	DOX+RO11-2933	0.08±0.02	0.09±0.01	0.45±0.22	1.02±0.09
E	DOX+RO11-2933	0.07±0.04	0.07±0.01	0.15±0.01	0.35±0.11

[a] A: treatment with DOX for 2 h, followed by 70 h incubation in drug-free medium; B: treatment with DOX for 72 h; C: treatment with DOX in combination with 2 μ*M* RO11-2933 for 72 h; D: treatment with DOX in combination with 2 μ*M* RO11-2933 for 2 h, followed by 70 h incubation in drug-free medium; E: treatment with DOX in combination with 2 μ*M* RO11-2933 for 2 h, followed by 70 h incubation in DOX-free medium containing 2 μ*M* RO11-2933
[b] IC_{50} was determined from the mean value of at least three experiments (mean ± SD)

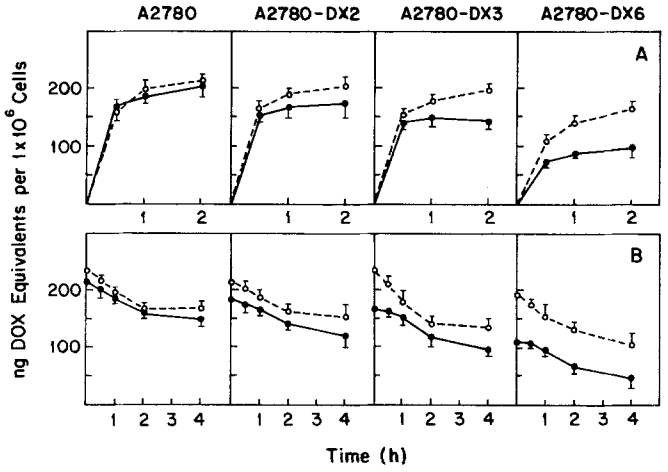

Fig. 4. DOX accumulation and retention in sensitive and resistant A2780 cell lines. **A** DOX accumulation: exponentially growing cells were incubated for 0.5, 1, and 2 h with 2 μ*M* DOX alone (———) and in combination with 2 μ*M* RO11-2933 (– – – –). **B** DOX retention: exponentially growing cells were incubated with 2 μ*M* DOX alone or in combination with 2 μ*M* RO11-2933. After 2 h exposure, cells treated with DOX alone were postincubated in drug-free medium (———) and those treated with DOX in combination with RO11-2933 were postincubated in DOX-free medium containing 2 μ*M* RO11-2933 (– – – –). After 1, 2, and 4 h, cells were extracted for quantitation of DOX equivalence as indicated in *Materials and methods* (mean ± SD)

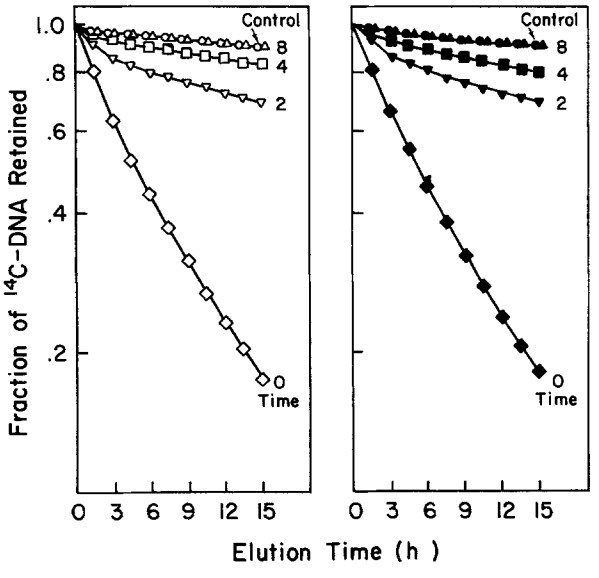

Fig. 5. Effect of RO11-2933 on the rejoining of DNA SSBs induced by X-ray: 1×10^6 [^{14}C]-labeled A2780-DX3 cells were exposed to 2 μ*M* RO11-2933 at 37° C for 4 h prior to irradiation at 300 rad and then allowed to repair at 37° C for 2, 4, and 8 h in the presence of 2 μ*M* RO11-2933 (*right panel*). Control cells (*left panel*) were exposed to 300 rad alone

Characterization of DOX-sensitive and -resistant cell lines

As outlined in Fig. 2, resistant cell lines were characterized according to their doubling time, modal chromosome number, cell volume, protein content, and in vitro sensitivity; the results are summarized in Tables 1 and 2. The data in Table 1 indicate that although sensitive and resistant cell lines had a modal chromosome number of 46 as well as identical cell volume and protein content, the doubling times increased with increasing degree of resistance.

Data in Table 2 summarize the in vitro cytotoxicity to A2780 cells of DOX alone and in combination with the modulator, RO11-2933. These data indicate that the relative degree of resistance to DOX of A2780-DX2, -DX3, and -DX6 cells were 7-, 26-, and 48-fold, respectively, for the 2-h exposure (group A) and 30-, 50-, and 500-fold, respectively, for the 72-h exposure (group B). DOX was about 10 times more cytotoxic to sensitive cells exposed for 72 h. Data in Table 2 also indicate that reversal of DOX resistance by RO11-2933 was highly dependent on the degree of resistance as well as the conditions of exposure to the modulator. For example, exposure for 2 h to the modulator was sufficient to reverse the resistance of -DX2 cells completely. In contrast, partial restoration of in vitro sensitivity to -DX3 and -DX6 was achieved when these cells were exposed to the modulator for 2 or 72 h (groups D and E). Even under continuous-exposure conditions, -DX3 and -DX6 cells were still 2- and 6-fold resistant (group E). It is also noteworthy that since continuous exposure to DOX produced a higher degree of resistant colonies (group B), continuous exposure to DOX and the modulator (group C) was sufficient for a complete restoration of -DX2 sensitivity.

DOX accumulation and retention

DOX accumulation and retention in sensitive and resistant cells were investigated; the results are summarized in Fig. 4. Compared with that in sensitive cells, DOX accumulation decreased by 23%, 30%, and 52%, respectively, in A2780-DX2, -DX3, and -DX6 sublines (Fig. 4A). In the presence of 2 μ*M* RO11-2933, DOX accumulation in sensitive and resistant cells was similar. The data in Fig. 4B indicate that whereas DOX was retained well in sensitive cells, its rapid loss from resistant cells was evident. In comparison with that in sensitive cells, DOX retention after drug removal decreased by 13%, 25%, and 56% in A2780-DX2, -DX3, and -DX6, respectively. In the presence of 2 μ*M* RO11-2933, DOX efflux was reduced in resistant cells and the net drug accumulation in sensitive and resistant cells became equal.

Effect of RO11-2933 on the repair of X-ray induced DNA SSBs

DNA elution was carried out on A2780, A2780-DX2, -DX3, and -DX6 cells exposed to 150, 300, 450, and 600 rad X-ray. The X-irradiation produced nearly identical numbers of DNA SSBs in both sensitive and resistant cells, and the relationship between the logarithm of the DNA fraction retained on the filters at a fixed elution time and the radiation dose was linear.

The effects of RO11-2933 on the rapir of X-ray-induced DNA SSBs are shown in Fig. 5. The data indicate that the DNA in resistant cells sustained extensive damage from X-irradiation (Fig. 5, *left panel*), which was rapaired within 8 h after X-ray exposure. The continuing presence of RO11-2933 prior to and after X-irradiation did not potentiate the degree of DNA damage or repair. Results

Table 3. Frequency of DNA SSBs in sensitive and resistant A2780 cells treated with DOX alone and in combination with RO11-2933

DOX (μM)	RO11-2933 (μM)	DNA SSB frequencies[a] (rad equivalents):			
		A2780/S	A2780-DX2	A2780-DX3	A2780-DX6
0	0	14± 4	12± 2	14± 3	11± 2
1.0	0	479± 69	ND	56±21	ND
1.0	1.0	439±109	ND	161±10	ND
3.7	0	1,095±185	396±27	209±19	144±20
3.7	2.0	1,022± 94	491±61	496±82	361±43

[a] DNA SSB frequencies were calculated according to Kohn et al. [18] (mean±SD)
ND, not done

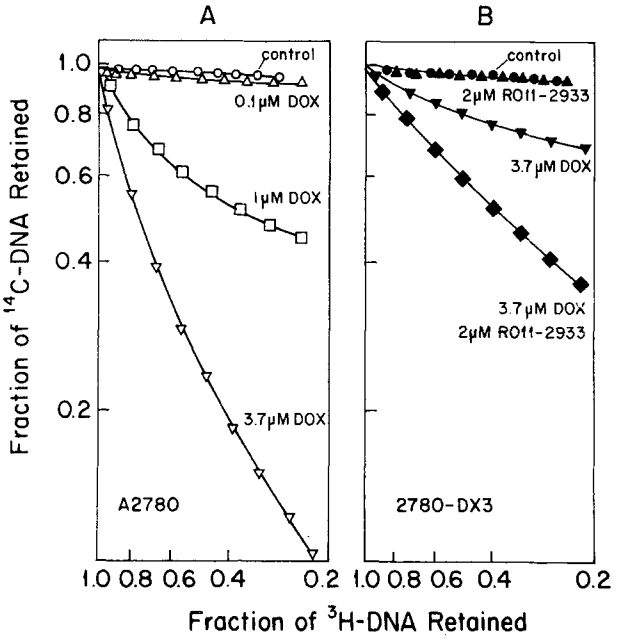

Fig. 6. DNA SSBs induced by DOX alone or in combination with RO11-2933 in **A** sensitive and **B** resistant A2780 cells. Cells containing [^{14}C]-DNA were incubated with drug for 4 h. Alkaline elution was carried out using DOX-treated cells and plotted against the simultaneous elution of [^{3}H]-DNA as an internal standard

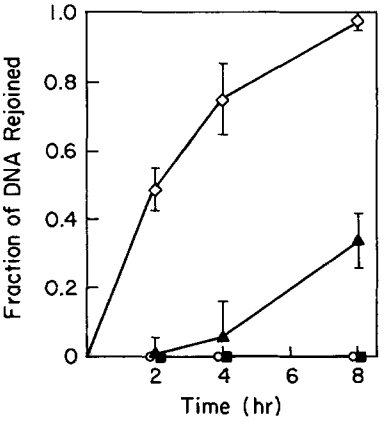

Fig. 7. Effect of RO11-2933 on the rejoining of DNA SSBs induced by DOX. Following exposure to DOX alone and in combination with RO11-2933, the cells were allowed to repair at 37° C for 2, 4, and 8 h in the absence and presence of RO11-2933. Symbols: rate of repair of SSBs in A2780 cells, (○); A2780-DX3 cells exposed to 3.7 μM DOX alone, (◇); 3,7 μM DOX in combination with 2 μM RO11-2933 for 2 h, (△); and A2780-DX3 cells exposed to 3.7 μM DOX plus 2 μM RO11-2933 and postincubated in the presence of 2 μM RO11-2933, (■)

also indicate that sensitive A2780 and multidrug-resistant cells were equally sensitive to X-irradiation, which produced comparable DNA SSBs (data not shown).

Effect of RO11-2933 on DOX-induced DNA SSBs

The frequency of DNA SSBs induced in A2780 and A2780-DX3 cells by treatment with 0.1, 1.0, and 3.7 μM DOX is shown in Table 3 and Fig. 6. The results obtained can be summarized as follows: (1) in sensitive A2780 cells, there was a direct relationship between the dose of DOX and the amount of DOX-induced SSBs; (2) in A2780 cells, the DNA SSB frequency was approximately 8-fold higher than in the A2780-DX3 cells exposed to 1 μM DOX alone; and (3) in the resistant cells, RO11-2933 in combination with DOX promoted a significant increase in DNA SSB frequency. In fact, the 56±21 and 209±19 rad equivalents determined in A2780-DX3 cells after 1.0 and 3.7 μM DOX exposure, respectively, increased to 161±10 and 496±82 rad equivalents in the presence of 1.0 and 2.0 μM RO11-2933, respectively (Table 3). In addition, the amount of DNA SSBs induced in resistant cells with 3.7 μM DOX in the presence of the modulator (Fig. 6B) was equivalent to that obtained in sensitive cells treated with 1 μM DOX alone (Fig. 6A).

Effect of RO11-2933 on repair of DOX-induced DNA SSBs

The effect of RO11-2933 on the repair of the DNA SSBs induced in sensitive and resistant cells (-DX3) were investigated; the results are shown in Fig. 7. During the 8-h period following treatment with either DOX or DOX and RO11-2933, the fraction of rejoined DNA was minimal in the sensitive A2780 cells. In contrast, repair of the induced SSBs in -DX3 cells was complete after treatment with DOX alone. In the presence of 2 μM RO11-2933 for 2 h, a small fraction of the SSBs were repaired within 8 h. On the other hand, when resistant cells were maintained in the continuous presence of 2 μM RO11-2933, no significant repair of DNA SSBs were observed up to 8 h after DOX removal from the culture medium.

Discussion

The acquisition of multi-drug resistance (MDR) represents a major limitation to successful antitumor chemotherapy. During recent years, efforts have been dedicated to the

identification of mechanisms leading to acquisition of MDR as well as the definition of new treatment strategies to circumvent resistance. In the present investigation, DOX resistance was induced in human ovarian cancer cells by stepwise increases in drug concentration. This procedure enabled the selection of cells with a low level of resistance, e.g., A2780-DX2 (7-fold resistance), and higher levels of resistance, e.g., A2780-DX3 and -DX6 (26- and 48-fold, respectively). The procedure outlined in Fig. 2 indicates that the acquisition of resistance was initially rapid but became progressively slower with increasing drug concentration, which suggests that at least one modification occurs in the process of acquiring a highly resistant phenotype. Moreover, the selective procedure induced an atypical MDR. In fact, the A2780-DX sublines were also cross-resistant to cisplatin, navelbin, and colchicine, to a degree that increased as the cell population became more resistant to DOX (data not shown). This evidence suggests that common mechanism(s) of resistance may be shared by these drugs, probably as the result of the expression of a modified single gene (or genes) under coordinated regulation.

Previous investigations have demonstrated that an active outward drug transport, resulting in lower intracellular drug accumulation, is partly responsible for DOX resistance [9, 19, 30–32, 37]. In the A2780-DX sublines, impaired accumulation and retention were observed. As shown in Fig. 4, the degree of drug accumulation and retention decreased as the degree of resistance increased. These data are in agreement with those reported in other cell lines. Overexpression of *p*-glycoprotein, PG170, was detected in A2780-DX3 and -DX6 (data not shown). In these cell lines, lower intracellular DOX accumulation and retention correlated with the overexpression of P-170. However, differences in the amount of DOX accumulation and retention could not account for the differences in resistance level, since the drug level in A2780-DX3 cells was 33% lower than that in the sensitive A2780 line (Fig. 3), although the former were about 50-fold resistant (Fig. 2). Moreover, in A2780-DX6 cells, the drug level was only 52% lower than that in A2780 cells (Fig. 3), whereas the former were 526-fold resistant after long-term exposure (72 h, Fig. 2).

Since the major biological activity of DOX is thought to reside in its ability to interact with DNA [14], studies on damage and repair were also carried out. Our results showed an induction of DNA SSBs in sensitive and resistant cells treated with 1.0 or 3.7 μM DOX. However, the extent of DNA SSBs decreased with increasing drug resistance (Table 3). The results are in agreement with previously reported findings in other cell lines [29, 34, 41]. In addition, DNA SSB repair was in resistant cells almost complete within 8 h, whereas no significant DNA repair was observed in sensitive cells. These data suggest that the mechanism(s) of resistance to DOX are multifactorial events associated with reduced drug accumulation and retention as well as a modification of the drug's interaction with DNA that results in decreased SSB induction and increased repair capacities.

We also investigated the effects of RO11-2933 on DOX resistance. Previous studies have shown that verapamil, a calcium channel blocker, can successfully potentiate DOX cytotoxicity in resistant cells [8, 13, 15, 24, 30, 36]. The clinical use of verapamil in combination with DOX is limited by the relatively high verapamil dose required to achieve the plasma drug concentration necessary for in vitro reversal of DOX resistance without unacceptable host toxicity. In addition, Rabkin et al. [26] have shown that pretreatment with verapamil may potentiate the myocardial toxicity caused by conventional DOX treatment [26, 27]. For these reasons, new calcium-channel-blocker analogs with greater selectivity were identified by Kessel and Wilberding [16].

Among the agents evaluated, RO11-2933 was found to be more potent than verapamil (1 μM vs 10 μM) [3, 27, 38] but less toxic in vivo (data not shown). Results of these studies revealed that restoration of drug sensitivity was complete in cells with a low resistance level (A2780-DX1 and -DX2) and correlated with greater drug accumulation and retention as well as increased DNA damage. In contrast, we found that RO11-2933 could only partially reverse DOX resistance in highly resistant cells (A2780-DX3 and -DX6), even when they were continuously exposed to the optimal concentration of the modulator.

As shown by our cytotoxicity experiments (Table 2), a residual 2- and 5-fold resistance was observed in A2780-DX3 and -DX6, respectively, when 2 μM RO11-2933 (the maximally tolerated concentration) was added to the incubation medium. Furthermore, RO11-2933 effectively retarded the repair of DOX-induced DNA SSBs in the resistant cells, and this effect was strictly related to the treatment schedule. In fact, A2780-DX3 cells treated with 3.7 μM DOX alone showed complete DNA repair that decreased to 33% when the drug was combined with RO11-2933 and to approximately 0 when 2.0 μM RO11-2933 was maintained in the posttreatment medium.

The evidence that RO11-2933 completely reverses cell sensitivity to DOX in cells with a lower degree of resistance (A2780-DX1 and -DX2) has some clinical value, since only low-level resistance may be expected to occur in clinical situations. Furthermore, inhibition of DNA repair by the modulator in resistant cells may offer an additional selective advantage for the clinical use of this agent over other calcium channel blockers.

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