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ANTHRACYCLINE-INDUCED DNA BREAKS AND RESEALING IN DOXORUBICIN-RESISTANT MURINE LEUKEMIC P388 CELLS*

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Abstract—Energy-dependent drug efflux is believed to be a major factor in cellular resistance to doxorubicin (DOX). However, recent studies have shown that decreased retention alone cannot account for anthracycline resistance, and possibly other factors, such as drug metabolism, free radical scavengers, and altered DNA damage/repair, may be involved. We have measured DOX-induced DNA damage and its repair in P388 cells sensitive (P388/S) and resistant (P388/R) to DOX. Our studies show 2- to 5-fold less DNA damage, measured as protein-associated single-strand DNA breaks, in P388/R cells when compared to similarly treated P388/S cells. The repair of DNA in whole cells, expressed as percent DNA rejoined, was complete in 4 hr in P388/R, whereas no repair was seen in P388/S cells until 20 hr. No difference in repair of DNA lesions was observed when nuclei were used in repair experiments. The absence of repair in sensitive whole cells may be due to high retention or slow drug efflux. Increase of cellular DOX retention by exposure of cells to trifluoperazine (TFP) or verapamil (VPL) did not result in the increase of DNA damage in P388/R cells. DOX analogs, *N*-trifluoroacetyladriamycin-14-valerate (AD 32), 4′-*O*-tetrahydropyranyladriamycin (THP-adriamycin), and *N*-benzyladriamycin-14-valerate (AD 198), induced 2- to 4-fold more DNA damage than DOX in resistant cells. There was no difference in the poly(ADP-ribose) synthesis of P388/S and P388/R cells exposed to DOX or AD 32. Since ADP-ribose polymer synthesis is associated with free radical-induced DNA damage and is indicative of DNA repair by an excision–repair mechanism, data from these studies suggest that DNA breaks in anthracycline-exposed cells may not be due to free radical production but rather to other mechanisms, such as inhibition of DNA topoisomerase II activity. The present studies, in addition to emphasizing the role of DNA damage in resistance, also underscore the relative importance of DNA topoisomerase II function in anthracycline cytotoxicity.

Energy-dependent rapid efflux of DOX‖ and daunomycin has been shown to be a major cause for drug resistance in tumor cells [1, 2]. Phenothiazines and verapamil by blocking drug efflux enhance DOX retention and chemosensitivity in resistant cells [3–8]. However, several recent studies [9–11] suggest that cellular retention alone does not always correlate with anthracycline cytotoxicity and suggest that mechanisms other than rapid efflux may also be associated with cellular resistance to anthracyclines. Studies from our laboratory [12], as well as those of Kessel and Wilberding [13], show that DOX-resistant P388 leukemic cells continue to be resistant to daunomycin and the DOX analog AD 32 in spite of retaining cytotoxic drug levels.

Among the several mechanisms for anthracycline cytotoxicity [14], intercalation of DOX to DNA is well-established [15], and DNA damage is observed in DOX-exposed cells [16–20]. Since DNA damage is closely linked to anthracycline cytotoxicity, it is reasonable to suspect that DNA damage and its consequent repair may play an important role in cellular resistance. The present studies, along with those reported earlier [21, 22], emphasize the importance of DNA damage in cellular anthracycline resistance and implicate the enzyme DNA topoisomerase II as being mechanistically involved in this resistance [23, 24].

MATERIALS AND METHODS

Drugs. DOX (Adriamycin) hydrochloride was obtained from Adria Laboratories, Columbus, OH. THP-adriamycin [25] was a gift from the late Dr. Hamao Umezawa, formerly Director, Institute of Microbial Chemistry, Tokyo, Japan. The prep-

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‖ Abbreviations: DOX, doxorubicin; AD 32, *N*-trifluoroacetyladriamycin-14-valerate; THP-adriamycin, 4′-*O*-tetrahydropyranyladriamycin; AD 198, *N*-benzyl-adriamycin-14-valerate; PBS, 20 mM sodium phosphate, pH 7.2, containing 150 mM NaCl; P388/S and P388/R, P388 cells sensitive and resistant, respectively, to doxorubicin; SSB, single-strand breaks; DSB, double-strand breaks PA-SSB, protein-associated single-strand breaks; TFP, trifluoperazine; VPL, verapamil; poly(ADP-ribose), poly(adenosine diphosphoribose); NAD^+, nicotinamide adenine dinucleotide; TCA, trichloroacetic acid; and EGTA, ethyleneglycolbis(amino-ethylether)tetra-acetate.

aration and biological properties of AD 32 and AD 198 have been described previously [26–28].

Cells and cytotoxicity assay. Log-phase cultures of the murine leukemic P388 cell line and its DOX-resistant subline, P388/R [8, 29], were maintained in Minimal Essential Medium (Catalog No. 320-1385, Grand Island Biological Co., Long Island, NY) supplemented with 10% fetal-bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and 2-mercaptoethanol (10 mM). For growth inhibition assays, cells (10^6/ml) incubated with different drug concentrations for 1 hr at 37° were centrifuged, washed twice with ice-cold medium, and resuspended in prewarmed fresh medium for 24 hr. Cells were counted for viability by the trypan blue dye exclusion procedure.

Incorporation of radioisotopes. Log-phase cells (0.6×10^6/ml) were grown in medium containing either 1 μCi/ml of [*methyl*-^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA) or 0.08 μCi/ml of [2-^{14}C]thymidine (56 mCi/mmol; Amersham, Arlington Heights, IL). After one doubling time (approximately 12 hr), the medium was removed and the cells were washed with Ca^{2+}, Mg^{2+}-free PBS. Radioactivity was chased by a 2-hr post-incubation in fresh medium containing 10 μM non-radioactive thymidine. The amount of radioactivity incorporated into DNA was about 2.2×10^5 dpm for [^{3}H] and 1.2×10^5 dpm for [^{14}C] per 10^6 cells in both cell lines.

Preparation of nuclei. ^{3}H-Labeled cells were washed once with buffer A (1 mM KH_2PO_4, 5 mM $MgCl_2$, 150 mM NaCl, and 1 mM EGTA, pH 6.4), resuspended in 1 ml buffer A, and lysed with 9 ml of buffer B (buffer A plus 0.3% Triton X-100, Eastman Kodak Co., Rochester, NY). After 20 min at 4°, 30 ml of buffer A was added, and the nuclei were sedimented by centrifugation at 200 *g* for 10 min. The nuclear preparation was checked by phase-contrast microscopy after staining with trypan blue.

γ-Irradiation of cells. ^{3}H-Labeled cells (1.0 to 1.2×10^6) in conical polystyrene tubes were chilled in an ice-water bath for 30 min prior to and during γ-irradiation. The tubes were exposed to 425 Ci of [^{60}Co]-radiation at a distance of 25 cm. A linear dose-related increase in single-strand breaks (SSB) was seen in both P388/S and P388/R cells exposed to [^{60}Co]-radiation. P388/R cells had between 20 and 30% less DNA damage than P388/S cells. This observation was used for converting drug-induced

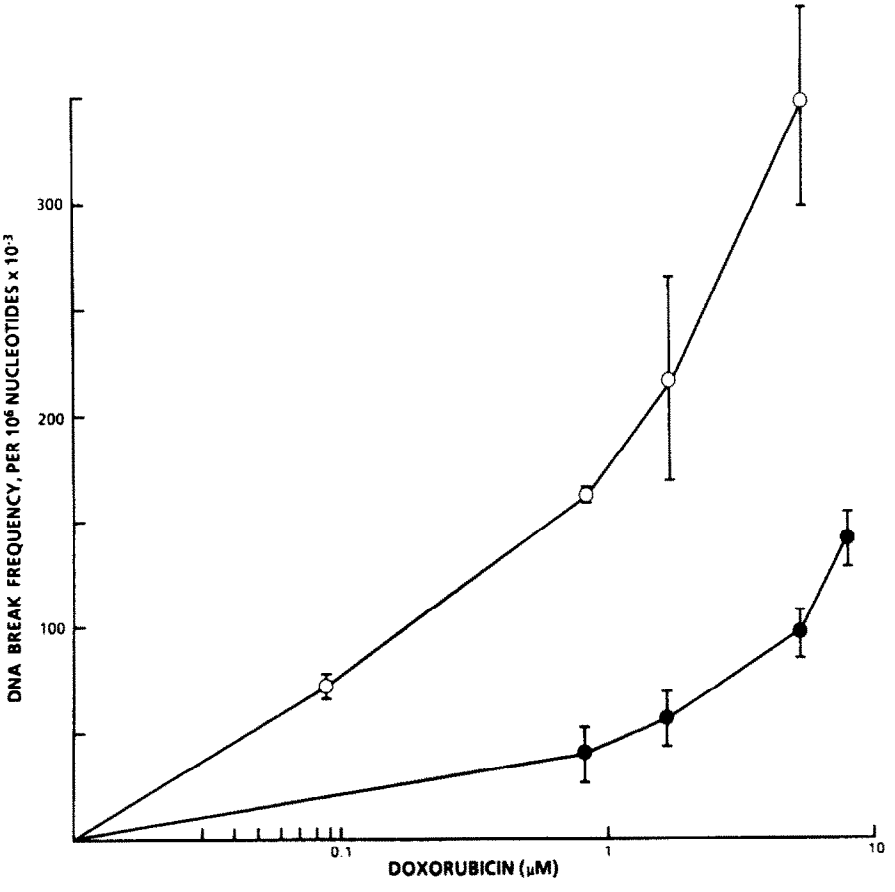

Fig. 1. DOX-induced DNA damage, measured as PA-SSB, in P388/S (○) and P388/R (●) cells. After exposure to indicated drug concentrations for 1 hr at 37°, cells (1×10^6) were washed with cold PBS, and alkaline elution was carried out according to the procedure of Kohn *et al.* [30] at pH 12.1. Cell lysates were treated with proteinase K (0.25 mg/ml) prior to elution with pH 12.1 buffer. Values are means ± SD, $N \geq 4$.

DNA breaks into radiation equivalents, and break frequency was calculated from the reported value of 0.9 breaks per rad per 10^9 nucleotides [30].

Drug treatment. Stock solutions of DOX (1.7 mM) and THP-adriamycin (1.5 mM) were prepared in PBS. AD 32 (1.4 mM) and AD 198 (0.72 mM) were dissolved in absolute ethyl alcohol and diluted with PBS. For alkaline elution analysis, ^{14}C- or ^{3}H-labeled cells were exposed to different anthracycline concentrations at 37° for 1 hr and washed with PBS. For cellular drug retention analysis, 7–10 ml of unlabeled P388/S and P388/R cells (10^6 cells/ml) were incubated with various drug concentrations. After 1 hr of incubation at 37°, 1.0-ml aliquots were removed for laser flow cytometric quantitation of cellular drug fluorescence, and the rest of the sample was used for fluorometric analysis. For the later assay, cells were centrifuged at 200 *g* for 10 min, and washed twice with chilled PBS. The cell pellet was resuspended in 1.0 ml of ethanol–1 N HCl–water (100:57:33), sonicated, and centrifuged at 800 *g* for 10 min. The drug fluorescence in the supernatant solution was measured in an Aminco–Bowman spectrofluorometer, using the excitation and emission wavelengths of 485/590, 470/570, 476/560, and 470/590 nm for DOX, THP-adriamycin, AD 32, and AD 198 respectively.

Alkaline elution of DNA. The alkaline (pH 12.1) filter elution procedure developed by Kohn *et al.* [30] was followed to estimate DNA damage in γ-irradiated or drug-treated cells. Equal numbers of drug-treated ^{14}C-labeled and "reference" ^{3}H-labeled cells were pooled in a glass tube at ice-water temperature and then filter-eluted on a 2-μM polyvinyl filter (BSWP-02500, 25 mm diameter, Millipore Corp., Bedford, MA). The total number of cells per filter was about 1×10^6. The steps followed for filter elution, proteinase K digestion and radioactivity measurement of the filter eluates and the filter are reported in our earlier publication [31]. A computer program developed by Dr. Robert C. Duncan of The University of Miami Cancer Center was used for estimating the radioactive DNA fractions retained on the filter based on the values from the scintillation counter printout.

Assay for DNA repair in DOX-treated cells and nuclei. The ^{14}C-labeled cells, after exposure to DOX for 1 hr at 37°, were washed twice and resuspended in growth medium, and aliquots (1×10^6/ml) of cells were reincubated for specified time periods at 37°. Similarly, nuclei after drug exposure were diluted 30-fold with buffer A (used in nuclear preparation) and reincubated for specified time periods at 37°. The ^{14}C-labeled "test" cells or ^{14}C-labeled nuclei were mixed with an equal number of ^{3}H-labeled "reference" cells, and alkaline elution of the cell or nuclei mixture was carried out at pH 12.1. The percent of DNA rejoined was calculated [32] from the fraction of DNA left on the filter at the end of 7.5 hr of elution by the formula $[(c - b)/$

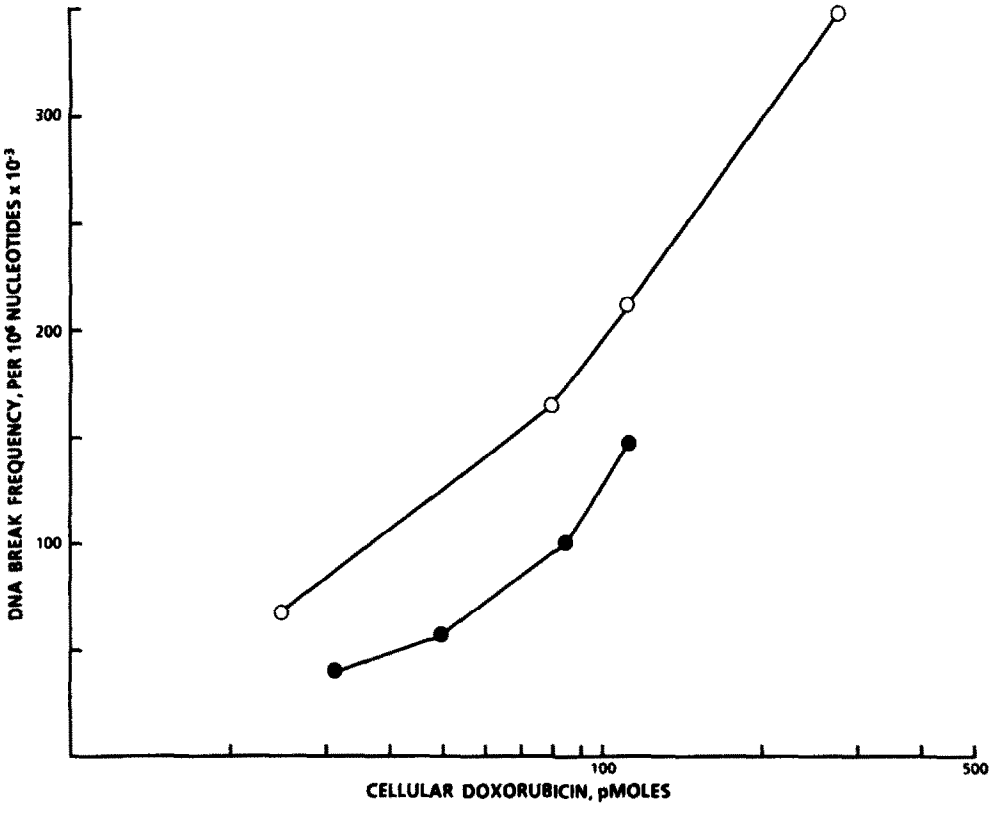

Fig. 2. Frequency of PA-SSB relative to cellular DOX retention in P388/S (○) and P388/R (●) cells. PA-SSB in drug-treated cells were measured by alkaline elution assay [30]. The fluorometric procedure followed for measurement of cellular drug content is given in Materials and Methods.

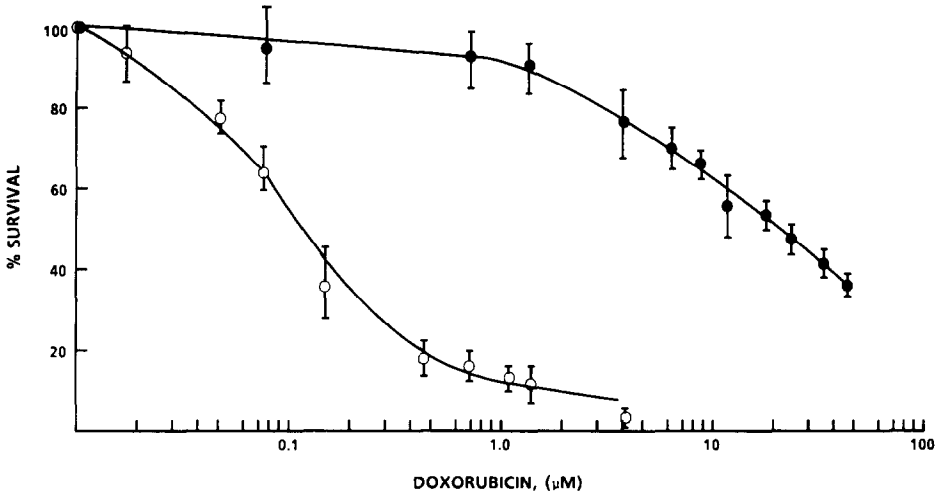

Fig. 3. Cytotoxicity of DOX in P388/S (○) and P388/R (●) cells. Cells (1×10^6/ml) exposed to different drug levels for 1 hr at 37° were washed twice with growth medium, resuspended in prewarmed medium, allowed to grow for 24 hr, and counted for viability by trypan blue exclusion. Values are means ± SD, $N \geq 4$.

$(a - b)] \times 100$, where a is the fraction of DNA retained from no-drug treated controls, b is the 0-min, post-drug treated control, and c is the fraction retained after post-incubation for a specified time.

Poly (ADP-ribose) synthesis. This determination was carried out according to the procedure of Benjamin and Gill [33]. Cells (2.5×10^7) were washed twice with PBS, and the cell pellet was suspended in a reaction mixture (5 ml) consisting of 5% dextran T-150, 10 mM $MgCl_2$, 0.05% Triton X-100, 30 μM non-radioactive NAD^+ and [^{3}H]NAD^+ (1 μCi/ml), and 40 mM Tris–HCl, pH 8.0. After the pellet was dispersed with a pasteur pipette, the reaction mixture was incubated at 35°. Aliquots (4×10^6 cells/ml) were withdrawn at 0, 5, 10, 20, and 30 min, and 2 ml of 10% TCA were added to stop the reaction. The TCA precipitate, collected on a Whatman GF/A filter, was washed three times with 10% TCA, dried and mixed with 10 ml Aqualyte for measurement of radioactivity in a Beckman model 7000 scintillation counter.

RESULTS

DOX-induced DNA breaks. Figure 1 shows the extent of DNA damage, determined as PA-SSB, in P388/S and P388/R cells exposed to DOX. In both cell lines, DNA break frequencies were dose-dependent. However, the amount of PA-SSB in P388/R cells was 4- to 5-fold less than those observed in the P388/S cells. Since low drug retention is a characteristic feature of DOX-resistant cells, it was important to check whether reduced DNA breaks observed in P388/R cells were due to lower cellular drug retention. Figure 2 shows that, although a correlation exists between PA-SSB and cellular DOX retention, P388/R cells generally have a 2-fold lower DNA break frequency than P388/S cells at any given cellular DOX level.

DOX cytotoxicity in P388/S and P388/R cells. Figure 3 compares the growth inhibitory effects of DOX on P388/S and P388/R cells. The IC_{50} values for DOX-sensitive and -resistant cells were 0.15 and 25 μM respectively. P388/R cells were about 170-, 60-, 46-, and 20-fold resistant to DOX and the analogs, AD 32, THP-adriamycin and AD 198 respectively.

DNA breaks by DOX analogs. The amount of PA-SSB induced by different DOX analogs is shown in Fig. 4. Although THP-adriamycin, AD 32 and AD 198 induced less DNA breaks in P388/R than in P388/S cells (effects similar to DOX), in P388/R cells the amount of DNA breaks caused by the analogs were 2- to 3-fold greater than those induced by DOX.

Cellular anthracycline retention and induction of PA-SSB. The cellular retention of DOX, THP-adriamycin, AD 32 and AD 198 in P388/S and P388/R cells exposed to different drug concentrations for 1 hr at 37° was measured by laser-excited flow cytometric analysis of intact cells and by fluorometric quantitation after ethanol–HCl extraction. Cellular retention of DOX and THP-adriamycin was 3- to 5-fold less in P388/R than in P388/S cells, whereas retention of DNA non-intercalators, AD 32 and AD 198, was similar in both sensitive and resistant cells (Fig. 5). The amount of PA-SSB was 2- to 3-fold less in P388/R than in P388/S cells (Table 1), regardless of whether the anthracycline was a DNA intercalator or not. AD 32 and AD 198, in spite of their similar retention in P388/S and P388/R cells, induced more PA-SSB in P388/R cells than did DOX.

We reported earlier that co-incubation of P388/R cells with either TFP or VPL increases both cellular DOX retention and chemosensitivity [8]. To test whether increasing drug retention, by blocking efflux, would result in increased DNA damage, we co-incubated P388/R cells with 5.16 μM DOX and 15 μM TFP or 15 μM VPL and correspondingly measured cellular drug levels and DNA damage as PA-SSB. The results of these experiments are shown in Table 2 and Fig. 6. Co-incubation with TFP or

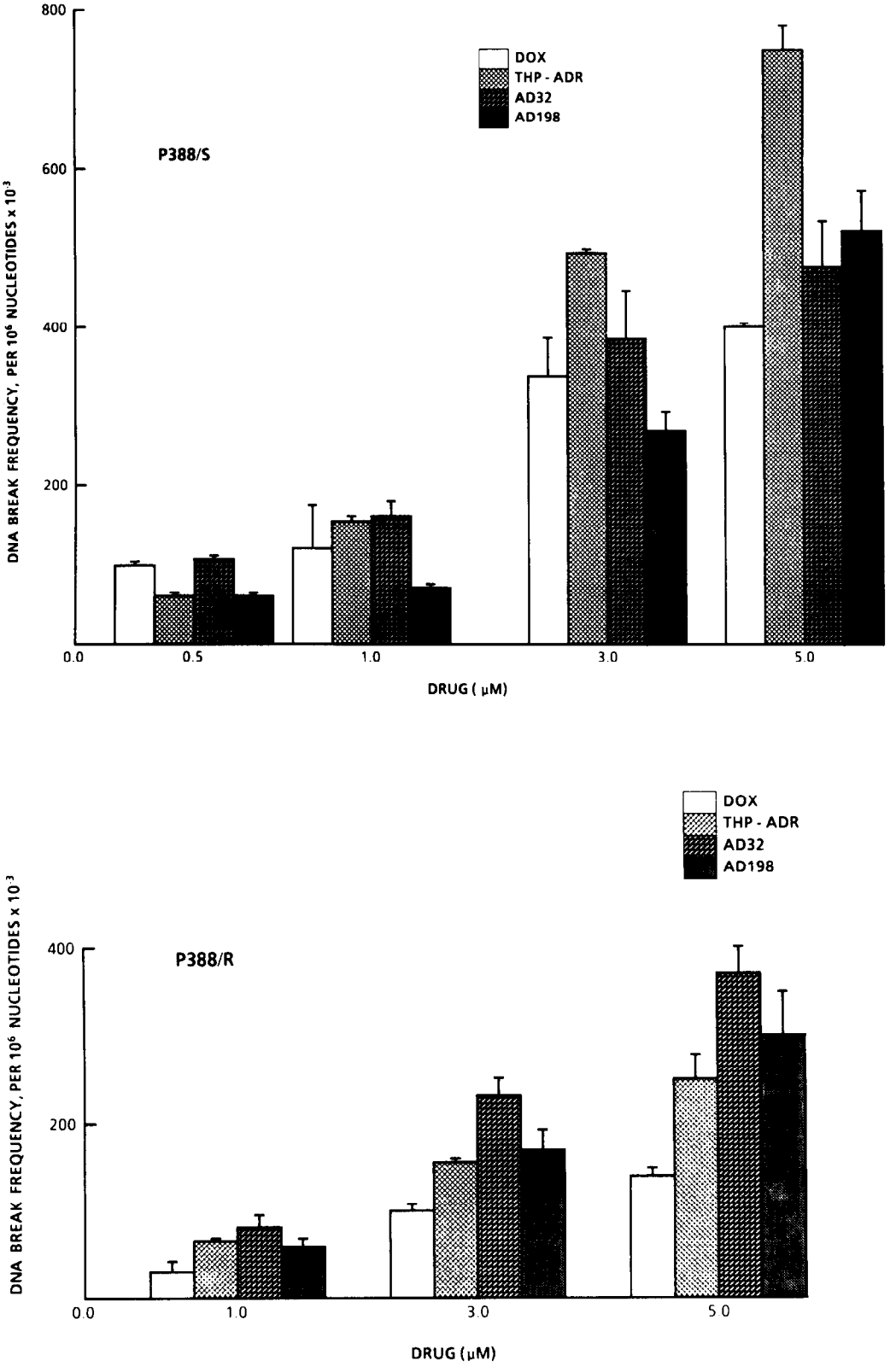

Fig. 4. Effects of DOX and its analogs on PA-SSB frequencies in P388/S and P388/R cells. Cells (1×10^6) were incubated with different concentrations of DOX, THP-adriamycin, AD 32, and AD 198 for 1 hr at 37°. After washing twice with ice-cold PBS, the cells were lysed with Sarkosyl on filters, digested with proteinase K (0.25 mg/ml), and eluted with pH 12.1 buffer [30]. Values are means ± SD, $N \geq 4$.

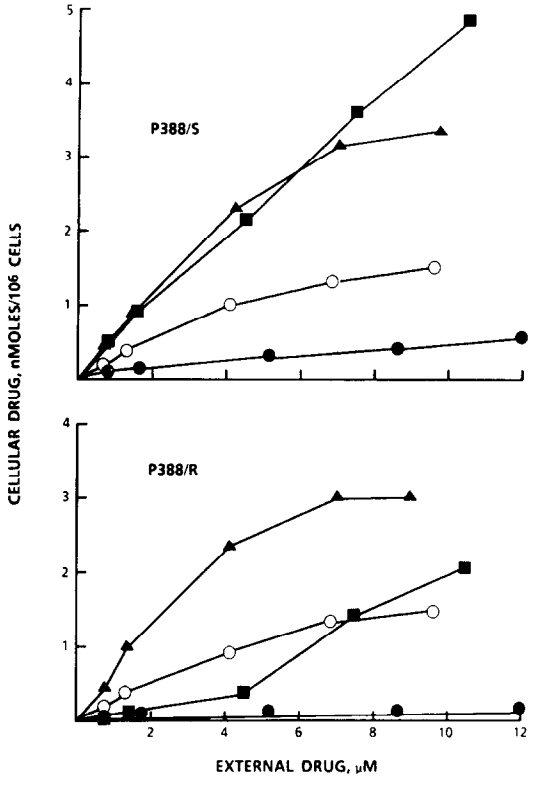

Fig. 5. Retention of DOX and its analogs in P388/S and P388/R cells. Cells (1×10^6/ml) after exposure to drug for 1 hr at 37°, were washed twice with ice-cold PBS and pelleted at 200 *g*. The cell pellet was resuspended in an ethanol–1 N HCl–water (100:57:33, by vol.) mixture, sonicated, and spun at 800 *g* for 10 min. The drug fluorescence in the supernatant solution was measured at excitation and emission wavelengths (nm) of DOX, 485/590 (●); THP-adriamycin, 470/570 (■); AD 32, 476/560 (○); and AD 198, 470/590 (▲) respectively.

VPL increased DOX retention 3- and 2-fold respectively, only in P388/R cells. Although exposure to DOX and TFP resulted in a 2-fold increase of PA-SSB, co-incubation with VPL did not increase PA-SSB in resistant cells (Fig. 6). The increased amount of PA-SSB seen in DOX + TFP treated P388/R cells did not reach the level seen in P388/S cells incubated with DOX alone. It should be noted, however, that treatment of P388/R cells with 15 μM TFP alone will cause DNA damage, equivalent to 85 rads, whereas VPL alone has no such effects on PA-SSB. This was taken into consideration while calculating net DNA damage in cells exposed to either of the DOX efflux blockers.

Repair of DOX-induced DNA damage. Figure 7 shows the amount of DNA repaired (expressed as percent DNA rejoined) in DOX-treated P388/S and P388/R whole cells and isolated nuclei. The DNA repair was completed in resistant cells within 4 hr, and was not dependent on the extent of DNA damage. In contrast, very little DNA repair occurred in P388/S cells even after exposure to as low as 0.086 μM DOX. Treatment of isolated nuclei from P388/S and P388/R cells with 1.72 μM DOX produced PA-SSB. A 1:30 dilution of drug-treated nuclei in drug-free nuclei buffer and later reincubation at 37° for 2 hr resulted in complete repair of DNA lesions in both cell lines.

Poly(ADP-ribose) synthesis in P388/S and P388/R cells. Data on the synthesis of poly(ADP-ribose) in P388/S and P388/R cells exposed to DOX or AD 32 are shown in Fig. 8A. There was no difference in the rate or amount of polymer synthesis in the sensitive and resistant cell lines exposed to either DOX or AD 32. Irradiation of P388/S and P388/R cells with [^{60}Co], however, resulted in an increased polymer synthesis (Fig. 8B). We observed a 1- and 2-fold increase in poly(ADP-ribose) synthesis in cells exposed to 3000 and 6000 rads and a significant

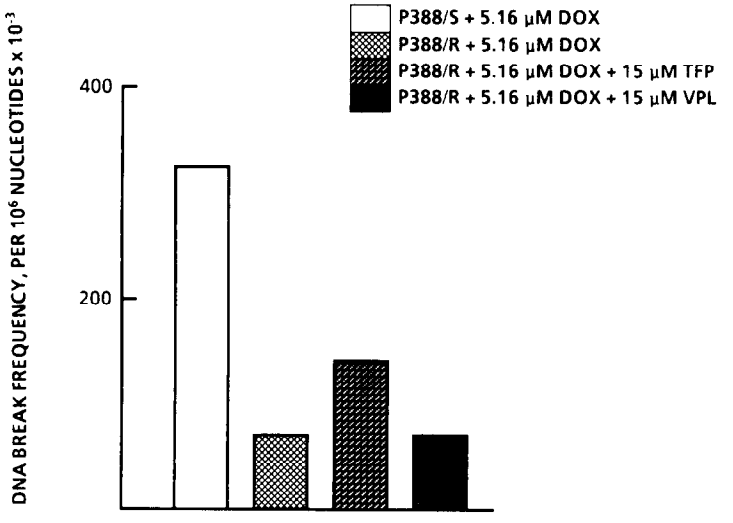

Fig. 6. Effects of TFP and VPL on DOX-induced DNA damage in P388/R cells. Cells (1×10^6) labeled with [^{3}H]thymidine were exposed to DOX (5.16 μM) or together with TFP (15 μM) or VPL (15 μM) for 1 hr at 37°. After washing cells twice with PBS, PA-SSB were determined by alkaline elution at pH 12.1 according to Kohn *et al.* [30].

Table 1. Cellular drug content relative to PA-SSB in P388/S and P388/R cells exposed to DOX and its analogs

Drug	Cellular drug content (pmol/10^6 cells)		DNA breaks in rad equivalents	
	P388/S	P388/R	P388/S	P388/R
DOX	285	86	386	111
THP-adriamycin	2190	400	495	155
AD 32	995	970	422	212
AD 198	2470	2430	256	161

Cells were treated with drug (3.0 μg/ml) for 1 hr at 37° and were washed twice with PBS at 200 *g*. The cell pellet was resuspended in an ethanol–1 N HCl–water (100:57:33, by vol.) mixture, sonicated and spun at 800 *g* for 10 min. Anthracycline fluorescence in the supernatant solution was measured by spectrofluorometry.

increase of polymer synthesis in cells irradiated at doses which equalled drug effects. But the synthesis of the ADP-ribose polymer was always marginal in drug-treated cells. In untreated controls, P388/R cells synthesized 2-fold more poly(ADP-ribose) than the P388/S cells and the significance of increased polymer synthesis in resistant cells is not clear at the present time.

DISCUSSION

The anthracycline antibiotic DOX is effective in the treatment of a broad spectrum of human tumors [34, 35]. However, certain tumors exhibit natural resistance to this drug, while other initially responsive tumors become resistant with therapy. *In vitro*, tumor cell lines selected for their acquired resistance to DOX typically display broad cross-resistance to other chemotherapeutic agents, such as vinca alkaloids and actinomycin D [36–39]. These multidrug resistance phenotypes have been characterized by the presence of markers, such as the 100–200 kD P-glycoprotein [29, 40–42], amplified genes [43, 44], and the presence of double minute and marker chromosomes [45, 46]. Several studies propose defective drug transport (reduced retention due to enhanced energy-dependent efflux) to be the major mechanism for multidrug resistance [1–8]. On the other hand, recent studies show that cellular retention alone cannot account for anthracycline resistance [12, 13]. Increased drug metabolism, high cellular content of free radical scavengers (such as glutathione and α-tocopherol), and reduced DNA damage with or without altered repair can contribute to cellular resistance.

Table 2. DOX retention in P388/S and P388/R cells treated with TFP or VPL

Treatment	DOX* (pmol/10^6 cells)	
	P388/S	P388/R
5.16 μM DOX	285 ± 016†	110 ± 020
5.16 μM DOX + 15 μM TFP	320 ± 010	330 ± 060
5.16 μM DOX + 15 μM VPL	300 ± 020	206 ± 050

* Quantitated by fluorometric assay.
† Mean ± SD of ≥ 3 experiments.

Several cellular targets such as DNA [14, 47, 48] and membranes [49–51] have been identified as responsible for DOX toxicity. DOX intercalation to DNA is well established [15] and DNA damage, determined as single-strand, double-strand and pro-

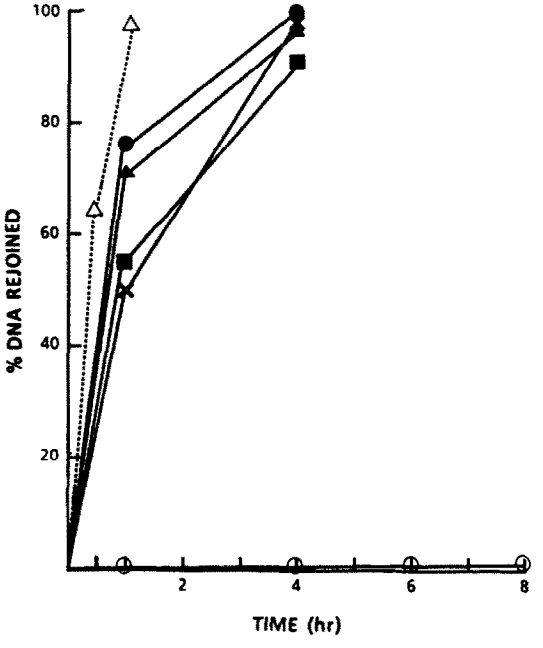

Fig. 7. Repair of DNA in DOX-treated P388/S (open) and P388/R (closed) cells. Cells (1 × 10^6/ml) were exposed to 1.72 (○, ●), 5.16 (▲), 8.6 (■) and 17.2 (×) μM DOX at 37° for 1 hr, washed twice with PBS, diluted with fresh growth medium and incubated at 37° for different time intervals. PA-SSB in cells were quantitated by alkaline elution at pH 12.1. Percent DNA rejoined was calculated according to the formula given in Materials and Methods. Since there was low DNA repair and points overlapped in sensitive cells, data obtained at 5.16 and 8.6 μM are not given. The DNA break frequencies of drug-treated zero hr controls were: 0.07, 0.166, 0.213, 0.347 and 0.428 in P388/S cells and 0.01, 0.036, 0.056, 0.100 and 0.149 in P388/R cells at 0.086, 0.86, 1.72, 5.16 and 8.6 μM DOX respectively. Repair experiments were also carried out with isolated nuclei from P388/S and P388/R cells. Nuclei were treated with 1.72 μM DOX (△---△) for 1 hr at 37°, diluted 1:30 with buffer A, and incubated for different time intervals before alkaline elution. Due to overlapping of the points, data for resistant nuclei are not shown.

tein-associated breaks, is observed in DOX-treated cells [16–20]. Since DNA damage appears to be involved in DOX cytotoxicity, it is reasonable to assume that DNA damage and repair may be a factor in DOX resistance. Recently published data [21, 22] clearly show decreased DNA damage in DOX-resistant P388 cells. Goldenberg *et al.* [21] have reported that, whereas the amount of SSB varies with the degree of DOX-resistance, DOX cytotoxicity correlates well with the amount of DSB rather than SSB; this is because SSB formed under the experimental conditions are attributable to DSB. Although DOX is capable of inducing all three types of DNA breaks, our studies as well as those of Ross *et al.* [19] show predominance of PA-SSB in cells exposed to lower concentrations of drug, closer to DOX IC_{50} (<0.4 μM). In the present study, we have given importance to the anthracycline effects on PA-SSB in P388/S and P388/R cells, and our data show a 2-fold decrease in PA-SSB in P388/R cells when compared to similarly treated P388/S cells. Furthermore, these breaks were lower in number in resistant than in sensitive cells at any given cellular DOX concentration. DOX analogs AD 32, AD 198, and THP-adriamycin also caused low PA-SSB in P388/R when compared to P388/S cells, although in the former the protein-associated breaks induced by DOX analogs were 2- to 3-fold more than those induced by DOX.

Our studies clearly show that an increase in cellular DOX retention, either by exposure of cells to high DOX levels or by blocking DOX efflux with VPL or TFP, did not result in increased PA-SSB in P388/R cells to the levels seen in P388/S cells. The data would suggest that the induction of PA-SSB is independent of cellular drug retention. The increased toxicity of DOX in the presence of efflux blockers, therefore, may not be related to DNA damage, but rather to some other as yet undefined mechanism.

Since there was no difference in the rate of repair of drug-induced DNA lesions in both DOX-resistant and -sensitive cells (slow repair seen in P388/S can be attributable to slow efflux of drug), our studies suggest that DNA repair may not be a factor in DOX resistance.

DOX-induced DNA damage can be attributed to either the generation of free radicals [52–54] or the inhibition of DNA topoisomerase II activity [23, 24]. If DNA damage is due to free radical production, then the repair of DNA lesions should be mediated by an excision–repair pathway similar to the one contingent in cells treated with alkylating agents or irradiated with X, γ or UV rays. Poly(ADP-ribose) polymerase is a chromatin-bound enzyme the activity of which is increased when: (a) DNA is damaged by free radicals or alkylating agents, and (b) DNA lesions are repaired by the excision–repair mechanism [55, 56]. The analyses for poly(ADP-ribose) content in DOX- or AD 32-treated P388/S and P388/R cells show no difference in the polymer synthetic activity, whereas irradiation of cells induced higher polymer synthesis in both cell lines. These data suggest that in P388/S and P388/R cells, at the anthracycline levels used in our experiments, DNA damage is not mediated by free radicals and DNA repair is not via an excision–repair pathway. A similar conclusion was reached in earlier studies involving DOX-treated L1210 cells under euoxic and hypoxic conditions and in the presence or absence of antioxidants or free radical scavengers [57, 58].

The present hypothesis for DOX-induced DNA damage and cytotoxicity involves formation of ternary "cleavable" DNA–drug–topoisomerase II complex and inhibition of DNA replication due to

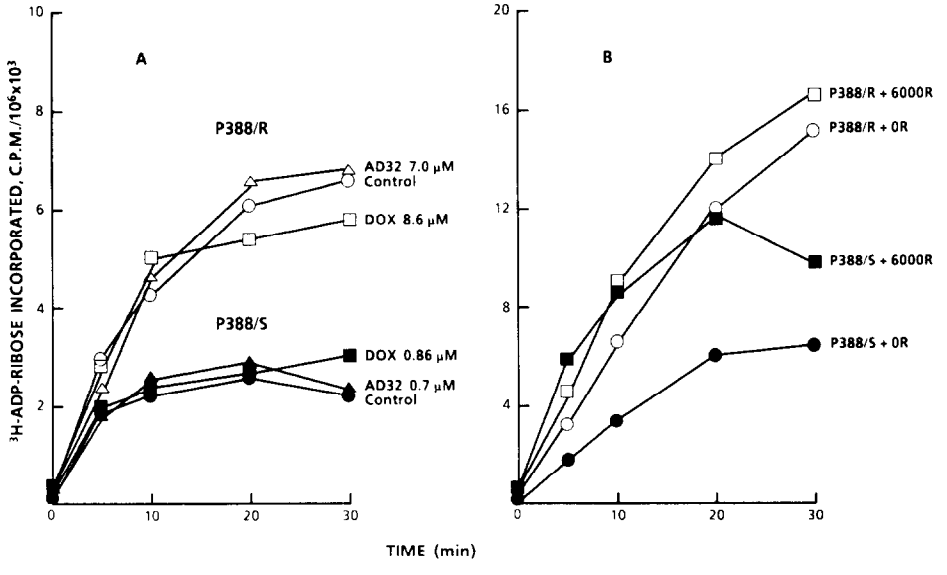

Fig. 8. Poly(ADP-ribose) synthesis in drug-treated (A) and γ-irradiated (B) P388/S (closed) and P388/R (open) cells. Cells (1×10^6) after exposure to DOX or AD 32, for 1 hr at 37°, or 6000 rads of γ-irradiation, were washed twice with ice-cold PBS and incubated at 35° with [^{3}H]NAD^+ (1 μCi/ml) and unlabeled NAD^+ (30 μM) for 0, 5, 10, 20, and 30 min. After precipitating poly(ADP-ribose) with 10% TCA, the precipitates were collected on GF/A filters and washed three times with 10% TCA; the radioactivity on the filters was measured in a scintillation counter.

trapping of the enzyme in the "cleavable" complex [59–61]. Indirect evidence in support of this hypothesis can be obtained from the present studies. First, we see production of protein-associated DNA strand breaks in cells and nuclei exposed to DOX and its analogs and, second, removal of DOX from drug-treated P388/R cells and P388/S and P388/R nuclei results in faster resealing of DNA lesions. Furthermore, our studies also show that anthracycline intercalation with DNA may not be a primary requisite for inhibition of topoisomerase II mediated DNA replication reactions. This is because AD 32, a DNA non-intercalator, also induced PA-SSB and inhibited topoisomerase II activity (data not shown) similar to that seen in cells exposed to epipodophyllotoxins [62].

In conclusion, the present study emphasizes the importance of DNA damage in anthracycline resistance. Results presented here are consistent with other studies reported in DOX-resistant murine leukemia sublines [21, 22] and with human small cell lung carcinoma cells [63]. The present report is additionally significant in that the results on intracellular drug retention and DNA damage by alkaline elution assay with DOX are compared with similarly obtained data for DOX analogs which differ in their abilities to interact directly with DNA. With respect to the question of resistance, further studies on the formation and stability of the DNA–drug–topoisomerase II complexes in sensitive and resistant cell lines are essential in order to correlate topoisomerase II function with anthracycline action.

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