In Vitro Cytotoxicity, Cellular Pharmacology, and DNA Lesions Induced by Annamycin, an Anthracycline Derivative with High Affinity for Lipid Membranes

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ABSTRACT

Annamycin (AN) is an anthracycline antibiotic with high affinity for lipid membranes which is being developed for clinical studies formulated in liposomes. We studied the in vitro cytotoxicity, cellular pharmacology, and DNA damage induced by AN in P388 cells sensitive and resistant to doxorubicin (DOX). AN was as cytotoxic as DOX against P388-sensitive cells and about 50 times more cytotoxic than DOX against P388-resistant cells (resistance index 5 for AN versus 250 for DOX). Cellular uptake of AN by sensitive cells was 2-3-fold higher than that of DOX. In resistant cells, cellular uptake of AN and DOX was approximately 65% and 30%, respectively, of the cellular uptake in sensitive cells. As a result, cellular uptake of AN by resistant cells was higher than uptake of DOX by sensitive cells. DOX was fully retained in sensitive cells while it was effluxed rapidly from resistant cells. In contrast, efflux of AN was similar in sensitive and resistant cells, thus suggesting that it is not mediated by P-glycoprotein. AN was more effective than DOX in inducing single DNA breaks, double DNA breaks, and DNA-protein cross-links, both in sensitive and resistant cells, although DNA damage was lower in resistant cells than in sensitive cells. DNA lesions induced by AN in resistant cells were similar to or greater than those induced by DOX in sensitive cells. These studies indicate that the lack of cross-resistance between DOX and AN appears to be related, at least in part, to the relatively higher cellular uptake of AN compared with DOX and is associated with the ability of AN to induce significant DNA damage in resistant cells.

INTRODUCTION

DOX\(^3\) is one of the most effective antitumor agents (1-4). DOX has been shown to interact with several intracellular biochemical targets, thus triggering a variety of cellular effects. The cytotoxic activity of DOX is mostly thought to be related to its interaction with nuclear topoisomerase II (5-7). However, alternative or additional mechanisms of action involving the cell membrane or the generation of free radicals have also been proposed (8-12). One of the major side effects of DOX is chronic cardiotoxicity (13). There is some evidence that cardiotoxicity and cytotoxicity may be mediated by interaction with different targets, thus making possible the dissociation of both effects (14). Tumor cells originally sensitive to the cytotoxic effects of DOX become resistant by developing an active drug efflux system that reduces the intracellular drug accumulation (15-17) and/or by alteration of topoisomerase II, thus reducing the interaction of the drug with the enzyme (18-21).

During the last few years, numerous efforts to develop DOX analogues with reduced cardiotoxic potential and lack of cross-resistance have been undertaken. Several non-cross-resistant analogues have been described (22-25). These compounds appear to be able to overcome multidrug resistance as a result of an increased cellular accumulation (22, 25), formation of interstrand cross-linking with DNA (24), or a combination of both.

Given the emerging evidence that the cell membrane may play an important role in the cytotoxicity and resistance to DOX, we became interested in studying anthracycline antibiotics with a high affinity for the lipid portion of the cell membrane. We hypothesized that because of such affinity, these compounds should display significant differences from the parent compound in drug uptake, efflux, and subcellular distribution that might result in favorable biological effects. AN is an anthracycline antibiotic selected for its exquisite affinity for lipid membranes. AN resembles idarubicin, an anthracycline approved for the treatment of acute leukemia, in the demethylation of the aglycone at position 4.

AN has been shown to be significantly more active than DOX in vivo against L-1210 leukemia and liver metastases of M-5076 reticulosarcoma (26). To elucidate the cellular mechanisms of action of AN, we decided to study the in vitro cytotoxicity, cellular uptake and efflux, and DNA damage of AN and DOX in P388 cells sensitive and resistant to DOX. A preliminary report of this study has been presented elsewhere (27).

MATERIALS AND METHODS

Chemicals. DOX was purchased from Ben Venue Laboratories, Inc. (Bradford, OH) and dissolved in normal saline solution. AN was synthesized and purified as described in a previous report (28), and the stock solution was prepared in 10% dimethyl sulfoxide saline. \([^{13}C\]Thymidine (2.11 GBq/nmol) was obtained from Amersham International Co. (Arlington Heights, IL). Tetra-n-propyl ammonium hydroxide was purchased from RSA Corp. (Ardsley, NY). Other chemicals unless specifically indicated were obtained from Sigma Chemicals (St. Louis, MO).

Cell Culture. Murine P388 leukemia cells sensitive and resistant to DOX were maintained in RPMI 1640 supplemented with 10% inactivated fetal calf serum at 37°C in a 5% CO\(_2\)/95% air incubator. P388/DOX cells cultured in the absence of DOX for up to 3 months showed no change in drug sensitivity.

Cytotoxicity Assay. Exponentially growing cells (1 x 10\(^6\) cells/ml) were exposed to various concentrations of drugs at 37°C for 1 h, and the treated cells were centrifuged, washed twice with PBS (pH 7.4), resuspended in drug-free medium, and cultured for 72 h. The cell survival was assessed by counting viable cells with 0.2% trypan blue dye exclusion or by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test as described by Hansen et al. (29). The drug effects were expressed as percentage survival compared to control cells, and the ID\(_{50}\) were determined graphically.

Determination of Drug Influx and Efflux. In the uptake experiments, P388-sensitive and -resistant cells were exposed to various concentrations of AN and DOX at 37°C for 1 h. The cells were centrifuged at 4°C and washed twice with cold PBS. The intracellular drugs were extracted with 0.3 N HCl, 50% ethanol as described by Spath et al. (30) and measured with a fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT). The wavelengths of excitation and emission, respectively, were 500 and 560 nm for AN, and 490 and 590 nm for DOX. The amount of intracellular drug was calculated from a standard curve and expressed as pmol/10\(^6\) cells. Standard curves were prepared by

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3 The abbreviations used are: AN, doxorubicin; AM, annamycin (2'-ido-3'-hydroxy-4'-epi-4-demethoxydoxorubicin); PBS, phosphate-buffered saline; ID\(_{50}\), concentration resulting in 50% inhibition of cell growth; DNA-SSB, DNA single-strand breaks; DNA-DSB, DNA double-strand breaks; DPC, DNA-protein cross-linked breaks.
measuring the fluorescence intensity of different DOX and ANN standards dissolved in 0.3 N HCl, 50% ethanol. Fluorescence intensity was linear at a concentration range of 5–100 ng/ml.

In efflux experiments, sensitive and resistant P388 cells were exposed to 1 µg/ml of AN and DOX at 37°C for 1 h. The drugs were then removed, and the cells were washed three times with cold PBS at 4°C and resuspended in drug-free medium. This concentration was chosen since it corresponds approximately to the ID₅₀ of both drugs against P388-sensitive cells. After postincubation in drug-free medium for the indicated time, aliquots of culture were taken, and the drugs retained in cells were extracted and determined as described above.

Isolation of Nuclei. The procedure for isolating nuclei from P388 cells was carried out as described by Taudou et al. (31). Briefly, pelleted cells were suspended in 10 volumes of ice-cold RBS buffer (10 mM Tris-HCl, pH 7.4; 10 mM NaCl; 1.5 mM MgCl₂) and placed on an ice bath for 10 min. Following the addition of 0.1% Triton X-100, the swollen cells were homogenized with 10 up and down strokes in a Dounce homogenizer, and nuclei were separated by centrifugation at 1200 rpm for 5 min at 4°C. The isolated nuclei were washed and resuspended in nuclear buffer at 10⁶ nuclei/ml.

Alkaline Elution Technique. The procedures for alkaline elution were essentially as described by Kohn et al. (32). Three types of DNA damage were evaluated in the present study, including DNA-SSB, DNA-DSB, and DPC. Prior to drug treatment, P388 and P388/DOX cells were labeled for 24 h with ¹⁴C]thymidine (0.1 µCi/ml) in RPMI 1640 containing 10% fetal calf serum. The labeled cells were washed twice with PBS, resuspended in fresh medium, and chased for 3 h.

To assess DNA-SSB, the labeled cells (0.5 × 10⁶) were exposed to various concentrations of drugs for 60 min, washed twice with PBS and diluted in cold PBS buffer, layered on 25-mm diameter, 2-µm pore size polycarbonate filters (Nucleopore, Pleasanton, CA), lysed with 5 ml lysis buffer containing 2% sodium dodecyl sulfate, 25 mM disodium EDTA (pH 9.6), and then incubated with 0.5 mg/ml of proteinase K at room temperature for 1 h. DNA on the filter was eluted with 30 ml alkaline elution solution (pH 12.1) containing 0.1% sodium dodecyl sulfate, 20 mM EDTA, and tetra-n-propyl ammonium hydroxide for 15 h. The elution rate was 0.03–0.04 ml/min. The frequencies of DNA-SSB were expressed as rad-equivalents using the following formula:

\[ \text{Rad equivalents} = \frac{\log (r/r_o)}{\log (R/R_o)} \times 15,000 \text{ rads} \]

where \( r \) is the retention of [¹⁴C]thymidine-labeled DNA from drug-treated cells; \( r_o \) is the retention of [¹⁴C]labeled DNA from control cells; and \( R \) is the retention of labeled DNA from control cells exposed to 1500 rads of ¹³¹Cs irradiation.

For the determination of DNA-DSB, the procedures for cell treatment and DNA elution were essentially similar to those used for DNA-SSB, with the exception that the pH of the elution solution was 9.6 instead of 12.1.

The measurement of DPC was performed according to the method described by Ross et al. (33). Briefly, [¹⁴C]thymidine-labeled control and drug-treated cells were irradiated on an ice bath with 3000 rads of ¹³¹Cs X-rays. The cells were layered on 25-mm diameter, 2-µm pore size polystyrene filters (Omega Specialty Instrument Co., Chemsford, MA) and lysed with lysis buffer without proteinase K and DNA was eluted from the filters with pH 12.1 alkaline elution without a sodium dodecyl sulfate detergent solution. The elution rate was the same as described above. The frequencies of DPC were calculated according to the formula:

\[ \text{Rad equivalents} = [(1 - r) \times (1 - r_o)] \times 3000 \text{ rads} \]

where \( r_o \) and \( r \) are the rates of retention of [¹⁴C]thymidine-labeled DNA on the filter from control and drug-treated cells, respectively.

**Statistical Analysis.** Student's t test was used, and a P value of ≤0.05 was considered significant.

**RESULTS**

Cytotoxicity Studies. The chemical structures of DOX and AN are presented in Fig. 1. We determined the cytotoxic effect of AN and DOX against murine leukemia P388-sensitive and -resistant cells, using the method of trypan blue dye exclusion to count viable cells or the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The relative cytotoxic potency of these drugs was essentially similar by the two methods. Fig. 2 shows the cytotoxic effect of AN and DOX against P388-sensitive and -resistant cells. The dose-response curves indicate that AN had a similar effect against both parental and resistant cell lines. The ID₅₀ values for AN against P388-sensitive and -resistant cells are 0.8 and 4 µg/ml, respectively. DOX showed significant cytotoxicity against P388 parental cells but little effect against P388-resistant cells. The ID₅₀ values for DOX in P388-sensitive and -resistant cells are about 1 and 250 µg/ml, respectively. Based upon the ratio of ID₅₀ between resistant and sensitive cells, the calculated resistance indexes (ID₅₀ DOX-resistant:ID₅₀ DOX-sensitive) for AN and DOX are approximately 5 and 250, respectively. These results indicate that AN is partially not cross-resistant with DOX.

**Cellular Pharmacokinetics.** Initially, we measured the rate of cellular uptake of AN and DOX at 1 µg/ml concentration in P388-sensitive and -resistant cells during a 40-min incubation period. As shown in Fig. 3, the cellular accumulation by P388 parental and resistant cells was biphasic, with an initial phase of rapid uptake (first 10 min) followed by a steady-state phase. AN accumulation by either sensitive or resistant cells was markedly greater than that of DOX throughout the 40-min incubation period. Uptake of DOX by P388-resistant cells was approximately 30% of that in sensitive cells. In the
and the intracellular amounts of AN or DOX in sensitive (•, A) and resistant (O, A) cells were measured as described in "Materials and Methods." Points represent the average of two independent experiments with about 10% variability.

Cell suspensions (1 x 10^6/ml) were exposed to 1 μg/ml of AN and DOX at 37°C. Following the indicated period of incubation, aliquots of cells were taken and the intracellular amounts of AN or DOX in sensitive (•, A) and resistant (O, A) cells were measured as described in "Materials and Methods." Points represent the average of two independent experiments within about 10% of variability.

Following the indicated period of incubation, aliquots of cells were taken and the intracellular amounts of AN or DOX in sensitive (•, A) and resistant (O, A) cells were measured as described in "Materials and Methods." Points represent the average of two independent experiments within about 10% of variability.

The intracellular accumulation of AN and DOX in sensitive cells (•, A) and resistant cells (O, A) was measured by fluorescence spectrophotometer as described in "Materials and Methods." Points, average values from at least three separated experiments; bars, SD.

DOX accumulation in resistant cells decreased quickly to about 50% within 15 min postincubation, indicating a rapid drug efflux, which is a typical multidrug resistance phenotype feature. In the case of AN, the retention curves in sensitive and resistant cells were similar, with an initial phase of rapid efflux lasting 30 min followed by a steady-state phase until 120 min postincubation, thus suggesting that efflux of AN from resistant cells may not be mediated by P-glycoprotein.

Assessment of DNA Breaks in Sensitive and Resistant Cells. We determined different types of DNA damage, i.e., DNA SSB, DNA DSB, and DPC induced by AN and DOX. Results are presented in Fig. 6. At the same concentration, the extent of DSB in sensitive cells produced by AN was about 10 times higher than that of DOX (P < 0.01). Although the amount of DSB caused by AN in resistant cells was lower than that in sensitive cells, it was still similar to or greater than that of DOX in sensitive cells. No DSB was observed in resistant cells after treatment with DOX (Fig. 6A). The extent of SSB induced by AN in sensitive cells was about 2 times higher than that of DOX (P < 0.01), and the amount of SSB generated by AN at lower concentrations (0.5–1 μg/ml) in resistant cells was about 10 times lower than that in sensitive cells, whereas at a higher concentration (5 μg/ml), it was only 2 times lower in resistant cells than in parental cells. DOX produced only a small amount of SSB in resistant cells (Fig. 6B). In the case of drug-induced DNA-protein cross-links, the results were similar to those of DSB in both cell lines. Interestingly, the extent of DPC induced by AN in resistant cells was similar to or greater than that of DOX in sensitive cells (Fig. 6C). The data obtained indicate that AN, at the same concentration, is more effective in inducing DNA lesions in P388-sensitive cells than DOX. Resistance to DOX in P388 is associated with a reduced ability of DOX in inducing DNA strand breaks. The lack of cross-resistance to AN may be related to its ability to induce DNA lesions in resistant cells.

Since several investigators have reported that drug transport and cytoplasm metabolism in whole cell systems could influence the formation of drug-induced DNA lesions (34), we used isolated nuclei to determine whether AN- and DOX-induced DNA breaks were related to drug uptake and metabolic factors. The results showed that the extent of DNA DSB and DPC in isolated nuclei from P388 cells exposed to 0.5–5 μg/ml of AN and DOX for 60 min was directly

Fig. 3. Time course for intracellular accumulation of AN and DOX in P388-sensitive and -resistant cells. Cell suspensions (1 x 10^6/ml) were exposed to 1 μg/ml of AN and DOX at 37°C. Following the indicated period of incubation, aliquots of cells were taken and the intracellular amounts of AN or DOX in sensitive (•, A) and resistant (O, A) cells were measured as described in "Materials and Methods." Points represent the average of two independent experiments within about 10% of variability.

Fig. 4. Concentration curve of intracellular accumulation of AN and DOX in murine leukemia P388-sensitive and -resistant cells. Cells (1 x 10^6) were exposed to various concentrations of drugs at 37°C for 60 min, harvested, and washed three times with PBS. The intracellular accumulation of AN and DOX in sensitive (•, A) and resistant cells (O, A) was measured by fluorescence spectrophotometer as described in "Materials and Methods." Points, average values from at least three separated experiments; bars, SD.

Fig. 5. Drug efflux from P388-sensitive and -resistant cells. Exponentially growing leukemia P388-sensitive and -resistant cells were centrifuged, washed three times with cold PBS, and resuspended in drug-free medium. At different times postincubation, aliquots of cell suspensions were taken and the intracellular retention of AN and DOX in sensitive cells (•, A) and in resistant cells (O, A) was determined by fluorescence spectrophotometer as described in "Materials and Methods." Points, mean values from three independent experiments; bars, SD.
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Related to drug concentration but was markedly reduced as compared with those found in the whole cell system (data not shown). These results are consistent with the reports by Capranico et al. (35).

Persistence of DNA Strand Damage. Since resealing or persistence of DNA breaks produced by anthracyclines may be linked to the cell killing mechanism (36), we also studied the alteration in the extent of DNA DSB following drug removal. P388 cells were exposed to 1 μg/ml of AN and DOX for 60 min, centrifuged, washed three times with cold PBS, and resuspended in drug-free medium and continuously incubated at 37°C for different time periods. As shown in Fig. 7, within 1–4 h postincubation, the DNA DSB produced by DOX in drug-free medium progressively increased up to 1.41- to 2.11-fold as compared to DNA damage at the initial time point. Under the same experimental conditions, DNA DSB produced by AN slightly decreased, reaching a steady state within 4 h postincubation. Even at 12 h postincubation, the amounts of DSB were 76% and 96% of DSB at an initial time point in AN- and DOX-treated cells, respectively. These data indicate that anthracycline compounds are able to tightly bind to the DNA molecule and cause persistent damage to DNA strands.

Correlation of DNA Breaks and Cytotoxicity. The relationship between DNA strand breaks and the cytotoxic effect of AN and DOX in P388-sensitive and -resistant cells is presented in Fig. 8. At equitoxic concentrations, AN induced about 4-fold higher DSB and 2.5-fold higher DPC than DOX in P388 cells (Fig. 8, A and C). In resistant cells, very little DNA damage induced by DOX was detected, even at higher concentrations. Nevertheless, the extent of DNA damage induced by DOX in resistant cells at equitoxic concentrations was strikingly lower than that produced by AN (Fig. 8, B and D). The results suggest that there is no relationship between anthracycline-induced DNA damage and cytotoxicity in this experimental system, and these data are consistent with the reports by other investigators (16, 34).

DISCUSSION

The results of this study indicate that the lipophilic anthracycline analogue AN is not cross-resistant with DOX in an in vitro cellular system and that the lack of cross-resistance of AN is associated with

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**Fig. 6. DNA lesions produced by AN and DOX in P388-sensitive and -resistant cells.** Exponentially growing cells were treated with the indicated concentrations of drugs at 37°C for 60 min. The DNA damage caused by AN and DOX was determined by alkaline elution technique as described in “Materials and Methods.” A, DNA-DSB induced by AN and DOX in sensitive cells (•, △) and resistant cells (○, △). B, drug-induced DNA-SSB. C, AN- and DOX-induced DPC. Points, mean values from at least three separate experiments; bars, SD. *P (0.05; **P (0.01; AN versus DOX in P388-sensitive and resistant cells.

**Fig. 7. Persistence of DSB in P388 cells treated with AN and DOX.** P388 cells were exposed to 1 μg/ml of AN (●) and DOX (○) at 37°C for 60 min, the drugs were removed, and the cells were washed three times with cold PBS and reincubated in drug-free medium for different periods of time prior to determination of DSB. DSB were determined by alkaline elution technique as described in “Materials and Methods.” Points, average values from three independent experiments; bars, SD.
its ability to achieve relatively high levels of cellular accumulation and to induce significant DNA damage in resistant cells.

In sensitive cells, the cellular uptake of AN was 1.5–2-fold higher than that of DOX; in resistant cells, the uptake of AN was reduced by 20–30% while that of DOX was reduced by 60–70%. The net result was a 3-fold higher drug accumulation of AN than DOX in resistant cells. The results of DNA damage show similar trends; in sensitive cells, AN was about 2, 10, and 3 times more effective than DOX in producing single-strand breaks, double-strand breaks, and DNA-protein cross-links, respectively. In resistant cells, the ability of AN to produce such DNA lesions was decreased by 2- to 10-fold while that of DOX was decreased by 10- to 300-fold, the net result being a marked increase of the difference in the ability to produce such DNA lesions in resistant cells between AN and DOX. The cellular uptake and DNA damage results obtained in our experiments with DOX are very similar to those reported previously by other authors using the same cell line (35, 38-40). The decrease in DNA damage induced by DOX in P388-resistant cells was manyfold higher (10–300-fold) than the decrease in cell accumulation (2–3-fold). This was also true for AN (2–10-fold reduction of DNA damage for a 30% decrease in cell accumulation). The lack of proportionality between changes in drug accumulation and DNA damage at the concentration range tested indicate that other factors, nuclear or cytoplasmic, must play a role in the mechanisms of drug resistance. This is further stressed by the reported evidence that reversal of DOX efflux and increased cellular accumulation as a result of treatment with calcium channel blocker do not always result in increased DNA damage (38, 41).

The extent of DNA damage did not correlate with cytotoxicity. At equitoxic levels, AN induced 2–4-fold higher DNA damage than DOX. The cellular accumulation and amount of DNA damage produced by AN in resistant cells was very similar to those produced by DOX in sensitive cells when the same concentrations were used, thus indicating that the differences in DNA damage between the two drugs may be mostly explained by the differences in cell accumulation (17, 36, 38, 42). However, in spite of similar DNA damage, the DOX ID_{50} against sensitive cells was 4 times lower than that of AN against resistant cells. Increased DNA damage with anthracycline analogues with some structural features of AN (4-demethoxylation) has been previously reported (35).
This difference in the amount of DNA damage needed to achieve the same degree of cytotoxicity with the two drugs may be due to the following possible mechanisms: (a) DNA damage induced by the two drugs is qualitatively different; (b) other mechanisms of cytotoxicity apart from DNA damage are involved; (c) DNA repair is faster in the case of AN. Since the differences in kinetics of double-stranded DNA breaks induced by the two drugs in sensitive cells were minor, DNA repair does not seem to be a major factor in the relative higher cytotoxicity of DOX when corrected for the amount of DNA damage produced (43–45). As suggested by other investigators, a qualitatively different interaction between drug-DNA and topoisomerase II is possible (46). Studies of sequence specificity may reveal differences in the site of DNA breaks induced by both drugs that may be biologically relevant (45, 47).

The resistance associated with the P388 cells used in these experiments has been found to be multifactorial, involving both cell membrane and nuclear mechanisms. In our experiments, DOX was found to be effluxed from resistant cells but not from sensitive cells, thus supporting the speculation that a P-glycoprotein-mediated efflux is operative in the resistant cells (15, 16, 38). However, in the case of AN, the efflux patterns from sensitive and resistant cells were similar, thus suggesting that efflux from resistant cells is not related to P-glycoprotein.

Studies with isolated nuclei showed an almost complete and remarkable abrogation of the increased ability of AN to induce DNA lesions compared with DOX. The results of DOX-induced DNA damage in nuclei are very similar to those reported previously by other investigators (35). This abrogation of the increased DNA damage induced by AN when whole nuclei were used indicates that membrane or cytoplasmic effects are necessary for AN to exert its DNA-damaging effects. Based on the exquisite affinity of AN for lipid bilayers, it is reasonable to hypothesize that most of the cellular uptake of AN probably represents drug bound to intracytoplasmic membrane structures, which may act as a slow intracytoplasmic drug release system.

In summary, our studies indicate that AN is a non-cross-resistant analogue of DOX. The studies to date suggest that the lack of cross-resistance is mainly related to an increased drug accumulation and secondarily an increased ability to induce DNA damage. However, differences in the interaction of the two drugs with DNA may also play a role and deserve to be investigated in the future. Because of its exquisite affinity for lipid membranes, AN can be formulated in a wide variety of liposomes. By exploiting its non-cross-resistance properties and optimizing its pharmacokinetics and tumor uptake by using the appropriate liposomes, liposomal AN may prove to have significant potential in the treatment of human cancer.

REFERENCES

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