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# DNA damage and cytotoxicity of mitoxantrone and doxorubicin in doxorubicin-sensitive and -resistant human colon carcinoma cells

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**Summary.** The effects of mitoxantrone (Mx) and doxorubicin (Dx) on cytotoxicity and DNA damage as assayed by alkaline elution were studied in two human colon adenocarcinoma cell lines sensitive (LoVo) and resistant (LoVo/Dx) to doxorubicin. Mx was more cytotoxic than Dx to LoVo cells and was partially cross-resistant in LoVo/Dx. In LoVo cells, Mx produced about 5 times more DNA single-strand breaks (DNA-SSB) than Dx, but both drugs caused an equal number of DNA double-strand breaks (DNA-DSB). In LoVo/Dx cells, the number of DNA-DSB was very low for both Dx and Mx, but DNA-SSB were about 20 times higher for Mx. In LoVo cells, the number of DNA-DSB and protein-associated SSB were similar at equitoxic concentrations. For LoVo/Dx, the partial cross-resistance of Mx might be explained by the much higher number of DNA-SSB produced by this drug.

## Introduction

Mitoxantrone (Mx) is an anthracenedione that has shown a broad spectrum of activity in several experimental mouse tumors [10]. Clinical data indicate its safety, and it is indicated for the therapy of leukemia in cases where it does not appear to be cross-resistant to anthracyclines [7, 14, 18].

Like other intercalating agents, Mx causes DNA breakage, probably because of stabilization of the DNA-topoisomerase II cleavable complexes that are converted to DNA breaks upon treatment with denaturing agents [15]. However, the mechanism of action of Mx could be different from that of Dx because, although Mx, like Dx, binds to DNA by intercalation, the hydroxyl groups of Mx side chains can also participate in hydrogen bonding with phosphate oxygens of another chain, thereby cross-linking DNA helices [1]. Moreover, Mx causes non-protein-associated DNA-SSB [5]. These differences could help to explain the greater cytotoxicity of Mx, as compared with Dx, observed in several cell lines [11].

LoVo/Dx cells were obtained after their prolonged exposure to Dx and have been shown to be resistant to vinka alkaloids, epipodophyllotoxins and m-AMSA as well [6]. This "pleiotropic" drug resistance to different classes of drugs seems to be due to a decrease in intracellular drug retention, resulting in a lower level of DNA damage [2, 6]. However, in some cases, induction of protein-associated DNA-SSB has been found to be independent of cellular drug retention [17], and other investigators [19] have shown that drug-induced cell killing does not correlate with cellular Dx content in sensitive and resistant cells.

The aim of the present study was to correlate differences in DNA damage with cytotoxicity and drug resistance to Mx and Dx in a Dx-sensitive and a Dx-resistant human colon carcinoma cell line.

## Materials and methods

*Drugs.* Mx was obtained from Cyanamid Italia (Catania, Italy) and Dx was obtained from Farmitalia-Carlo Erba (Milano, Italy); both drugs were in the hydrochloride form. Compounds were dissolved at a concentration of 1 mg/ml in sterile, distilled water and stored in a dark environment at −20° C. Dilutions were made in growth medium immediately before use.

*Cell cultures.* LoVo cells [8, 9] were grown at 37° C in a humidified $CO_2$ atmosphere. The culture medium was Ham's F 12 (Gibco; Grand Island, NY) supplemented with 15% fetal calf serum (Flow Laboratories), 1% vitamins (BME vitamin solution 100×, Gibco), 100 IU/ml penicillin and 100 μg/ml streptomycin. The doxorubicin-resistant line (LoVo/Dx) was obtained as previously described [6] and maintained in the same growth medium in the presence of 0.1 μg/ml Dx, which was withdrawn 24 h before the experiments.

*Labelling conditions and drug treatment for alkaline elution.* Cell monolayers were grown in 25-$cm^2$ flasks (Costar; Cambridge, Mass). Cultures used for experiments were in exponential growth, with a doubling time of 30 h. Uniform labelling of DNA was obtained with methyl [$^3$H]-thymidine (0.05 μCi/ml, 30 mCi/mmol) (New England Nuclear; Boston, Mass). After 24 h, tritiated thymidine was removed and chased with fresh medium for about 16 h. Cells ($0.5-1 \times 10^6$) were treated for 4 h at 37° C by the addition of the appropriate dilution of the stock solution.

*Alkaline elution.* DNA damage was detected by the alkaline elution technique previously described by Kohn

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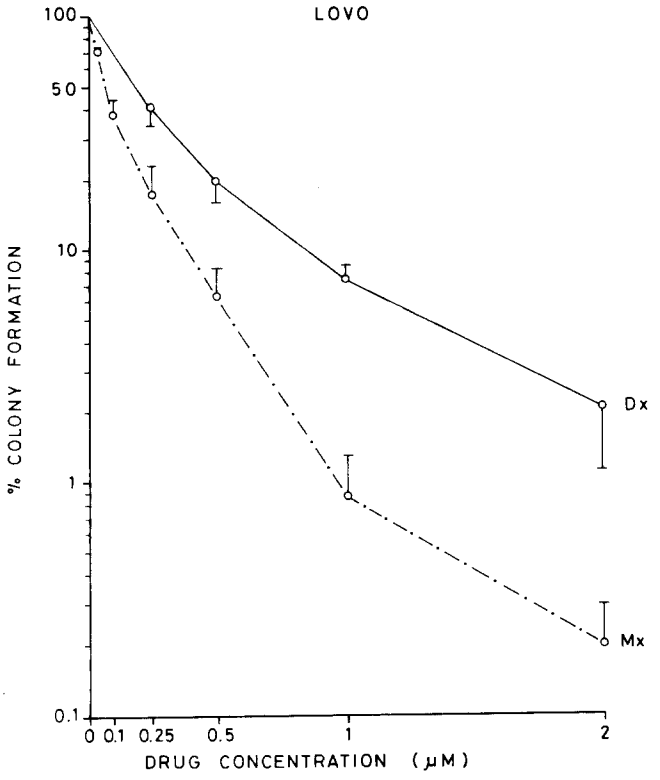


**Fig. 1.** Effects of mitoxantrone (*Mx*) and doxorubicin (*Dx*) on the colony-forming ability of LoVo cells. Points represent the mean of at least three experiments and bars, the SE

et al. [13]. For the measurement of DNA-SSB, the cells were washed twice with 5 ml cold phosphate-buffered solution (PBS) (4° C) at the end of treatment, scraped with a rubber policeman and resuspended in 10 ml PBS. Cells were loaded onto polycarbonate filters [0.8-μm pore size and 25-mm diameter (Nucleopore; Pleasanton, Calif)] and lysed with 5 ml solution containing 2% sodium dodecyl sulfate (SDS), 0.02 *M* ethylenediaminetetraacetate (EDTA) and 0.1% glycine (pH 10) (lysis solution). After the outlet of the filter holders were connected to the pumps, 2 ml of 0.5 mg/ml proteinase K (Merck; Darmstadt, FRG) dissolved in the lysis solution were added to a reservoir over the filters and pumped for approximately 1 h at 0.035 ml/min. DNA was eluted from the filters by pumping 20 m*M* EDTA solution adjusted to pH 12.2 with tetrapropylammonium hydroxide (Fluka, FRG) containing 0.1% SDS (elution buffer) at approximately 2 ml/h for 15 h. Before the measurement of DNA damage, fractions were mixed with Aquasure (New England Nuclear; Boston, Mass) containing 0.7% acetic acid. Untreated cells were irradiated with 300 rad before they were loaded onto the filters.

For DNA-DSB assay, cells were loaded onto the pump-connected filters immediately after PBS elution, after which 2 ml lysis solution with proteinase K was added; DNA was then eluted with the elution buffer adjusted to pH 9.6. To report DNA-SSB as rad equivalents, calculations were made according to Kohn et al. [13]; DNA-DSB were calculated against a calibration curve obtained after irradiation of LoVo and LoVo/Dx cells with different amounts of radiation.

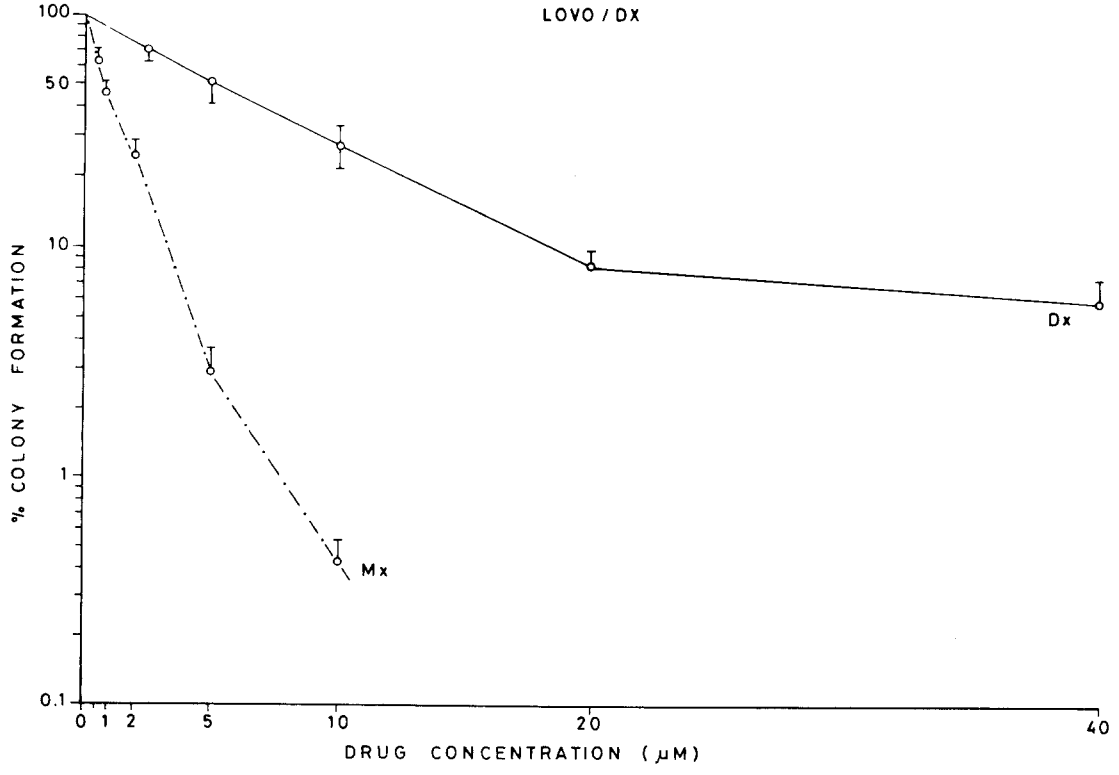


**Fig. 2.** Effects of mitoxantrone (*Mx*) and doxorubicin (*Dx*) on the colony-forming ability of LoVo/Dx cells. Points represent the mean of at least three experiments and bars, the SE

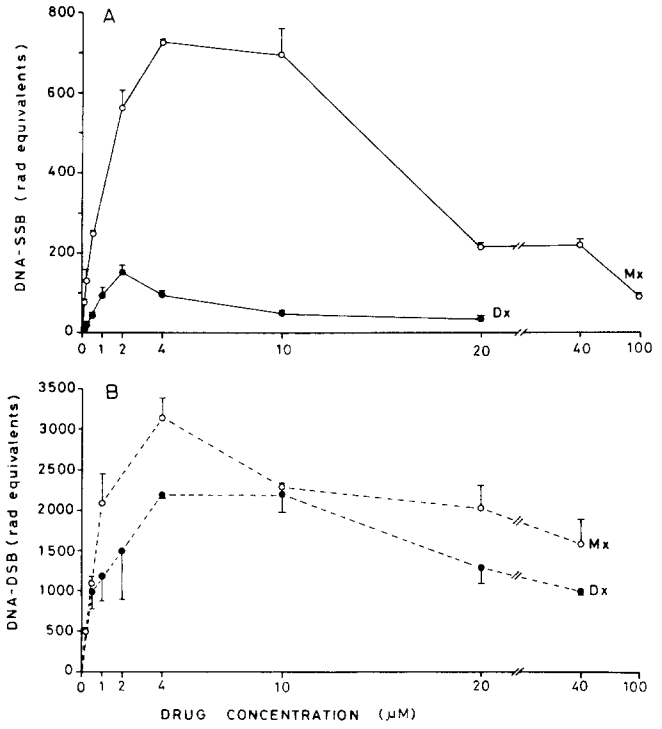


**Fig. 3.** Single- and double-strand breaks (*SSB, DSB*) in DNA of LoVo cells after treatment with mitoxantrone (*Mx*) and doxorubicin (*Dx*) for 4 h at 37° C. SSB and DSB are expressed in rad equivalents, i. e. the amount of radiation required to produce equivalent DNA damage. Points represent the mean of at least two experiments, each carried out in duplicate; bars indicate the SE

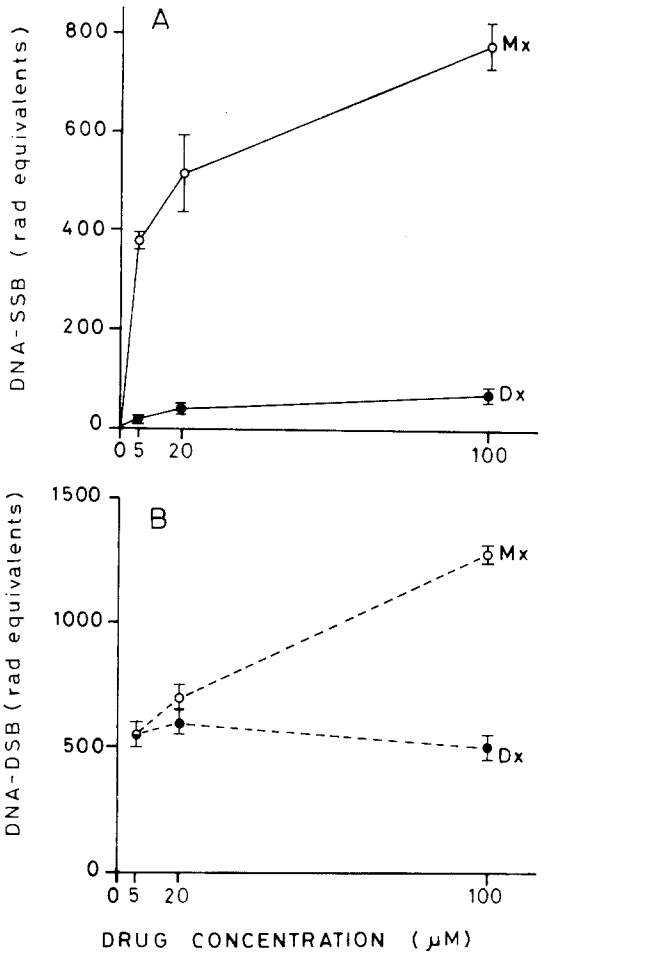


**Fig. 4.** Single- and double-strand breaks (*SSB, DSB*) in DNA of LoVo/Dx cells after treatment with mitoxantrone (*Mx*) and doxorubicin (*Dx*) for 4 h at 37° C. SSB and DSB are expressed in rad equivalents, i. e. the amount of radiation required to produce equivalent DNA damage. Points represent the mean of at least two experiments, each carried out in duplicate; bars indicate the SE

**Table 1.** Cross-resistance pattern of LoVo and LoVo/Dx cells treated with mitoxantrone and doxorubicin

| Agent | $IC_{50}$ (μ*M*): | | Relative resistance[a] |
|---|---|---|---|
| | LoVo | LoVo/Dx | |
| Mitoxantrone | 0.086 | 0.8 | 9 |
| Doxorubicin | 0.22 | 5.2 | 23 |

[a] $IC_{50}$ (LoVo)/$IC_{50}$ (LoVo/Dx)

*Cytotoxicity assay.* Cells ($10^3$ and $0.5 \times 10^3$, respectively, for treated and untreated samples) were plated in 35-mm wells of a 6-multiwell culture plate (Falcon) at 24 h before treatment. After 4 h treatment with the drugs, wells were washed with 2 ml PBS and filled with fresh medium. On day 10 after plating, colonies were stained with 1% crystal violet in 20% ethanol. Colony growth inhibition was evaluated by counting colonies with >50 cells. Data represent the means of three experiments, each carried out with six replicates per drug concentration.

**Table 2.** Relationship between cytotoxicity and DNA damage (SSB and DSB) after treatment of LoVo and LoVo/Dx cells with mitoxantrone and doxorubicin

| | | Colony formation | DNA-SSB (total) | DNA-SSB (non-protein-associated) | DNA-DSB (rad equivalents) |
|---|---|---|---|---|---|
| LoVo | Mx 0.5 μ*M* | 7 | 250 | 150 | 1,200 |
| | Dx 1.0 μ*M* | 7 | 105 | ND | 1,225 |
| LoVo/Dx | Mx 5.0 μ*M* | 3 | 383 | 257 | 550 |
| | Dx 17 μ*M* | 10 | 40 | ND | 600 |

ND, Not detectable

## Results

The cytotoxic effect of 4 h exposure to Mx and Dx was determined by the colony-forming assay. The dose-response curves are shown in Fig. 1 for LoVo and in Fig. 2 for LoVo/Dx cells. Mx was more cytotoxic than the anthracycline in the sensitive cell line, and the resistant line showed lower resistance to Mx than to Dx (Table 1).

Figure 3 reports the formation of DNA-SSB and DNA-DSB in LoVo cells after Mx and Dx treatment. Mx produced more DNA-SSB than did Dx, but both drugs caused almost the same amounts of DNA-DSB. As shown in Table 2, Mx caused a significant amount of non-protein-associated DNA-SSB (i. e. obtained in the absence of proteinase K), whereas Dx did not.

Mx produced a larger number of DNA-SSB than did Dx in LoVo/Dx cells. DNA-DSB remained at a background level for Dx at all concentrations (500 rad equivalents correspond to about 94% of the DNA on the filter), whereas for Mx the level of DNA-DSB increased over the concentration range but was much lower than that observed in LoVo cells (Fig. 4).

## Discussion

In the present study we investigated the relationship between DNA breakage and cytotoxicity induced by Mx and Dx in the LoVo cell line and in a LoVo subline (LoVo/Dx) selected for resistance to Dx. Both drugs induced DNA-SSB and DNA-DSB, conceivably due to the ability of these drugs to induce the formation of DNA-topoisomerase II cleavable complexes that are converted to DNA breaks upon treatment with protein denaturants and proteinase K. Both drugs have, in fact, a demonstrated ability to induce cleavage of DNA in vitro in the presence of ATP and topoisomerase II [20], although the size of the fragments are different for Dx and Mx.

Comparison of DNA-SSB and DNA-DSB induced in LoVo and LoVo/Dx cells by Mx and Dx revealed clear differences. When these cells were exposed to equimolar concentrations of each drug, the number of DNA breaks, particularly DNA-SSB, were much higher for Mx. When DNA breaks were investigated as a function of drug concentration, there was an initial increase followed by a plateau and then by a decline in DNA lesions. This biphasic behaviour, which has previously been described for anthracyclines [3] but not for anthracenediones, is probably due to the occurrence of a change in DNA structure when a high level of intercalation of the drugs is present in DNA, altering the DNA-topoisomerase II interaction.

In addition to the protein-associated DNA breaks, Mx caused a marked number of non-protein-associated DNA breaks, whereas Dx did not. Previous studies have demonstrated this finding in another cell type [5], but the mechanism of these DNA lesions has not been elucidated. For Dx, DNA breaks not associated with proteins were shown to result from reactive oxygen intermediates produced by reduction of the drug to semiquinone free radical, mediated by NADPH cytochrome P-450 reductase [4]. In our case, Dx did not produce detectable non-protein-associated DNA-SSB (Table 2); on the other hand, Mx has been shown to be activated less than Dx by this enzyme [12]. Therefore, the free DNA-SSB produced by Mx in LoVo cells should be due to some alternative mechanism.

It may be that non-protein-associated DNA breaks are due to excision repair induced by Mx binding to DNA, this binding being different from that of anthracyclines [1]. In fact, it has been shown that Mx can induce changes in DNA and chromatin structure different from those produced by anthracyclines because of the presence of side chains bearing basic amino groups that can bind electrostatically to the phosphate groups of DNA [1, 16]. However, it should be noted that the protein-associated DNA breaks appear to be better related to the cytotoxicity of Mx in LoVo and LoVo/Dx cells. In these cell lines we found that equitoxic doses of Mx and Dx produced similar amounts of protein-associated DNA breaks (Table 2).

We did not investigate Mx retention in LoVo and LoVo/Dx cells; it is therefore possible that the lower level of resistance of LoVo/Dx to Mx as opposed to Dx is at least in part due to a relatively higher intracellular concentration of Mx. However, the much higher number of DNA-SSB produced by Mx in both LoVo and LoVo/Dx cells support the view that there are differences in the mechanism of cytotoxicity between Mx and Dx; these differences probably also justify the lack of complete cross-resistance.

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