Cancer Chemother Pharmacol (1990) 27: 47–51

Cancer Chemotherapy and Pharmacology

© Springer-Verlag 1990

Comparative effects of doxorubicin and 4′-epi-doxorubicin on nucleic acid metabolism and cytotoxicity in a human tumor cell line*

Orazio Cantoni[1], Piero Sestili[1]*, Flaminio Cattabeni[1], Cristina Geroni[2], and Fernando Giuliani[2]

[1] Istituto di Farmacologia e Farmacognosia and Centro di Farmacologia Oncologica Sperimentale, Universita' degli Studi di Urbino, Urbino, Italy
[2] Farmitalia Carlo Erba Research Center, Nerviano, Milano, Italy

Summary. The effects of 4′-epi-doxorubicin (4′E-Dx) and doxorubicin (Dx) on cell survival were investigated after treatments for 1 or 24 h. It was found that after short-term exposure, 4′E-Dx causes a higher level of killing than Dx, whereas a similar cytotoxic response is detectable after 24 h. Under the same experimental conditions (treatment for 1 or 24 h) at equimolar concentrations, the two anthracyclines were equally inhibitory to DNA or RNA synthesis. Both the degree and the rate of induction of DNA strand breakage over a 1-h drug exposure were higher for 4′E-Dx than for Dx. These data were related to results of experiments carried out to investigate the rates of uptake and egress of the anthracyclines. From this study we concluded that no relationship can be established between the cytotoxicity of 4′E-Dx and Dx and their ability to inhibit DNA and RNA synthesis. In addition, the different DNA-damaging action of the two drugs does not appear to be solely dependent on the different rates of cellular uptake.

Introduction

4′-Epi-doxorubicin (4′E-Dx) is an epimer of doxorubicin (Dx) that has been shown to effectively inhibit the growth of various types of murine tumors [1, 6]. The major advantage of 4′E-Dx over Dx seems to be its lower toxicity, which has been demonstrated in laboratory animals in which greater inhibition of tumor growth was achieved by increasing the dose [5]. A comparison between the cytotoxicity and DNA breakage produced by 4′E-Dx vs Dx was made in our previous work [4] in attempts (a) to connect these two phenomena and (b) to investigate the differences underlining the action of the two drugs at the cellular and molecular level. Conclusions on the first point were not reached, although a higher degree of toxicity for 4′E-Dx (1-h treatment) as compared with Dx was accompanied by a higher induction of protein-concealed DNA single-strand breaks. However, the major problem in relating these effects was that DNA lesions produced by 4′E-Dx were repaired (on post-incubation in a drug-free medium), whereas those generated by Dx persisted. The different kinetics of DNA-lesion removal is certainly the most striking difference observed following cell exposure to the two anthracyclines. Since this effect appeared to be related to the pharmacodynamic characteristics of the drugs, we performed a detailed study on the relationships between DNA breakage, the effects on DNA/RNA synthesis or cell survival and the actual amount of drug responsible for producing such effects.

Materials and methods

Materials. Dx and 4′E-Dx were supplied by Ricerca Chimica (Farmitalia Carlo Erba Research Laboratories, Milano, Italy). [methyl-^{14}C]-Thymidine (0.25 mCi/mM), [6-^{3}H]-thymidine (5 mCi/mM) and [6-^{3}H]-uridine (1 mCi/mM) were purchased from New England Nuclear Corp. (Boston, Mass., USA). Free acid ethylenediaminetetraacetic acid (EDTA), disodium EDTA, tetrasodium EDTA and sodium dodecyl sulfate were obtained from Sigma Chemical Co. (St. Louis, Mo., USA). Tetraethylammonium hydroxide was purchased from Merck-Schuchardt GmbH (München, FRG). Proteinase K was obtained from Boehringer Mannheim (Mannheim, FRG), and polycarbonate filters, from Nuclepore (Pleasanton, Calif., USA). Minimum essential medium (MEM, Hanks'), fetal bovine serum (FBS) and trypsin were purchased from Gibco (Grand Island, N.Y., USA).

Cells and radioactive labeling. HeLa cells were maintained in plastic culture flasks with MEM supplemented with 10% FBS, 50 IU/ml penicillin and 50 IU/ml streptomycin. Experimental cultures for alkaline elution were plated in 60-mm tissue-culture dishes, labeled overnight with [methyl-^{14}C]-thymidine (0.05 μCi/ml) and then chased for 6 h in label-free medium. Cells for cytotoxicity, uptake/efflux or DNA/RNA synthesis studies were plated in 60-mm dishes 1 day before the experiment.

Drugs and drug treatment. Dx and 4′E-Dx were freshly dissolved in distilled water and their concentrations were assessed spectrophotometrically.

* Recipient of a fellowship from the Associazione Italiana per la Ricerca sul Cancro

Offprint requests to: O. Cantoni, Istituto di Farmacologia e Farmacognosia, Universita' di Urbino, I-61029 Urbino, Italy

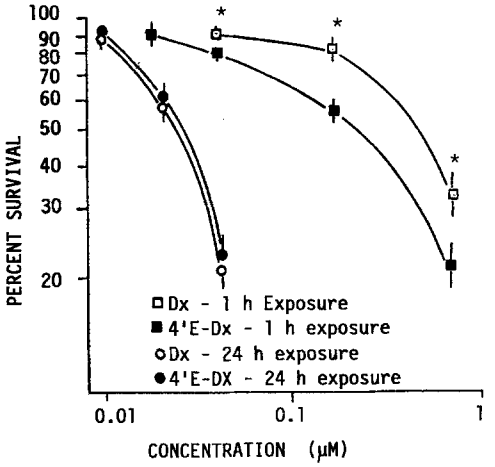

Fig. 1. Comparative cytotoxic effects as measured from the cloning efficiency of HeLa cells after 1 or 24 h incubation with Dx or 4′E-Dx. Cells were treated in the log phase. Points represent the mean ± SEM of 4 experiments, each performed in duplicate. Differences between treatments with the same concentrations of Dx and 4′E-Dx were determined by Student's *t*-test (* $P < 0.05$)

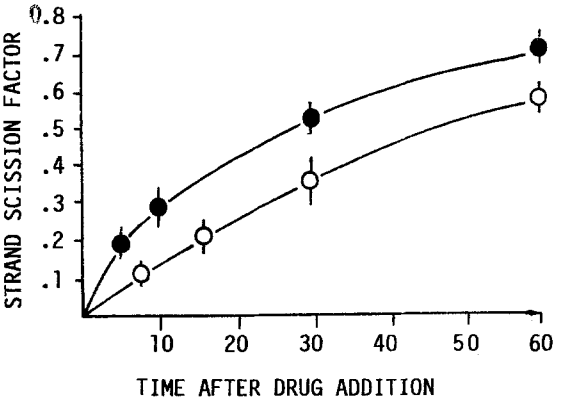

Fig. 2. Formation of DNA single-strand breaks in HeLa cells after the addition of 3.45 μM Dx or 1.72 μM 4′E-Dx. Data are expressed as the strand scission factor and points represent the mean ± SEM of 3 independent experiments. ●——●, 4′E-Dx; ○——○, Dx

Cytotoxicity. Cells were inoculated at a density of 5×10^5 cells/60-mm dish; 24 h later, monolayers were exposed for 1 or 24 h to various concentrations of each drug and rinsed with Saline A (0.14 M NaCl, 5 mM KCl, 4 mM $NaHCO_3$ and 5 mM glucose). Survival was determined by the cells' colony-forming ability, a colony being defined as >50 cells in close proximity. The plating efficiency of drug-treated plates was always standardized to that of the untreated controls.

Intracellular accumulation and retention. Immediately after exposure to the drugs or at specific intervals thereafter, cell monolayers were washed three times with 5 ml ice-cold Saline A and then scraped into 7.5 ml ice-cold 50% ethanol/0.3 *N* HCl to extract the anthracyclines. The suspension was centrifuged at 2,000 *g* for 15 min at 4° C and the anthracyclines were assayed in the supernatant by spectrofluorometric analysis (excitation and emission wavelengths, 479 and 593 nm, respectively).

DNA and RNA synthesis. A total of 5×10^5 cells were plated in 60-mm dishes; 24 h later, cells were exposed for 1 or 24 h to various concentrations of Dx or 4′E-Dx. The medium was removed from cultures that had been treated with the drugs for 1 h and cells were rinsed twice with 5 ml Saline A and then post-incubated in a complete culture medium containing 0.5 μCi/ml [6-^{3}H]-thymidine for 30 min at 37° C. Tritium-labeled precursors were added during the last 2 h of drug exposure to the medium of cells undergoing a 24-h treatment. Uptake of [6-^{3}H]-thymidine and [6-^{3}H]-uridine by DNA and RNA, respectively, was estimated by the following two methods: (1) cell monolayers were rinsed twice with 5 ml Saline A and macromolecules were precipitated with 5 ml ice-cold 10% trichloroacetic acid (TCA). This step was repeated twice. The TCA-precipitable fraction was dissolved in 2 ml 0.4 *N* NaOH, and aliquots (1 ml) were collected into counting vials, neutralized with 0.4 ml 1 *N* HCl and, after the addition of 10 ml Lumagel, counted in a Beckman LS 1800 scintillation counter. (2) Cell monolayers were scraped and cell suspension was injected into 2 vol. ice-cold 10% TCA; the precipitate was then collected on glass-fiber filters and the filters were washed three times with 5 ml ice-cold 10% TCA, dried and placed in scintillation vials with 10 ml Aqualuma Plus. Data obtained using the two procedures gave comparable results.

Alkaline elution. Cells containing [^{14}C]-DNA were incubated for various intervals with increasing concentrations of Dx or 4′E-Dx at 37° C in an atmosphere containing 5% CO_2. Immediately after drug treatment the dishes were placed on ice and rinsed three times with ice-cold Saline A. Cells were removed from the dishes by trypsinization (1%) at 0° C for 5 min. Alkaline elution was carried out by a procedure virtually identical to that described by Kohn et al. [12], with minor modifications [2, 3].

Briefly, 7×10^5 cells were gently loaded onto a 25-mm (2-μm pore) polycarbonate filter and then rinsed twice with 10 ml ice-cold Saline A containing 5 mM EDTA (disodium salt). Cells were then lysed with 5 ml 2% sodium dodecyl sulfate, 0.025 M EDTA (tetrasodium salt; pH 10.1), and lysates were incubated for 60 min in the lysis solution containing 0.5 mg/ml proteinase K. Lysates were rinsed with 7 ml 0.02 M EDTA (tetrasodium salt; pH 10.1) and the DNA was eluted overnight in a dark environment with 1.5% tetraethylammonium hydroxide/0.02 M EDTA (free acid)/0.1% sodium dodecyl sulfate (pH 12.1) at a flow rate of ca. 45 μl/min. Fractions of approximately 5 ml were collected and counted in 7 ml Lumagel containing 0.7% glacial acetic acid.

DNA remaining on the filters was recovered by heating for 1 h at 60° C in 0.4 ml 1 *N* HCl, followed by the addition of 0.4 *N* NaOH (2.5 ml), and radioactivity was determined by scintillation counting. DNA was also recovered from the interior of the membrane holder after vigorous flushing with 3 ml 0.4 *N* NaOH. This solution was processed for scintillation counting as described above. Strand scission factors (SSF) were calculated from the resulting elution profiles by determination of the absolute log of the ratio of the percentage of DNA retained in filters in the drug-treated sample to that retained in the untreated control sample (both after 9 h elution).

Results

The effect of 1 or 24 h exposure to 4′E-Dx or Dx on the cloning efficiency of HeLa cells is shown in Fig. 1. A log-log scale was used to accomodate all survival curves on the same graph, since the drugs differed widely in their abiiity to cause cell lethality after 1 or 24 h exposure. 4′E-Dx caused greater cell killing than Dx after 1 h treatment, whereas the toxicity of the two drugs was similar after 24 h. Shoulderless survival curves (straight lines) were obtained for both compounds when the cloning efficiency data shown in Fig. 1 were plotted vs drug concentration on a linear scale (not shown). The effect of varying the exposure time on DNA strand breakage induced after treatment with a single drug concentration (3.45 μM Dx and 1.72 μM 4′E-Dx) is shown in Fig. 2. 4′E-Dx-induced DNA breakage was not a linear function of exposure time; in fact, the rate of strand-break induction continuously

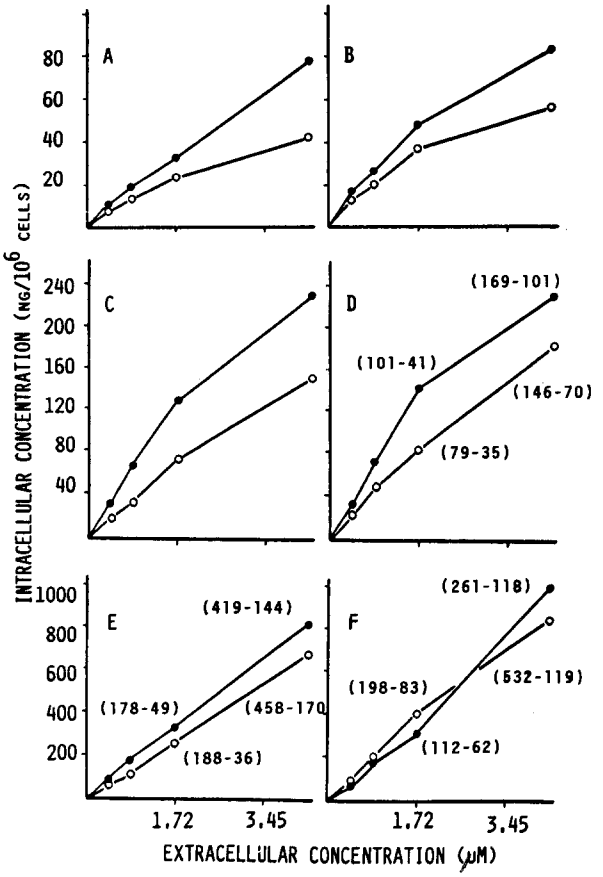

Fig. 3 A–F. Cellular uptake of Dx (○——○) and 4′E-Dx (●——●) by HeLa cells. Cells were treated with concentrations of 0.43, 0.86, 1.72 and 4.3 μM of the drugs and their levels were measured immediately after treatment for **A** 5 min, **B** 10 min, **C** 30 min, **D** 60 min, **E** 240 min and **F** 24 h. Also shown in **D**, **E** and **F** is the intracellular concentration of the anthracyclines after recovery in drug-free media (numbers in parentheses: the first and second numbers refer to the intracellular content after 1 and 24 h, respectively). Each value represents the mean of duplicate determinations

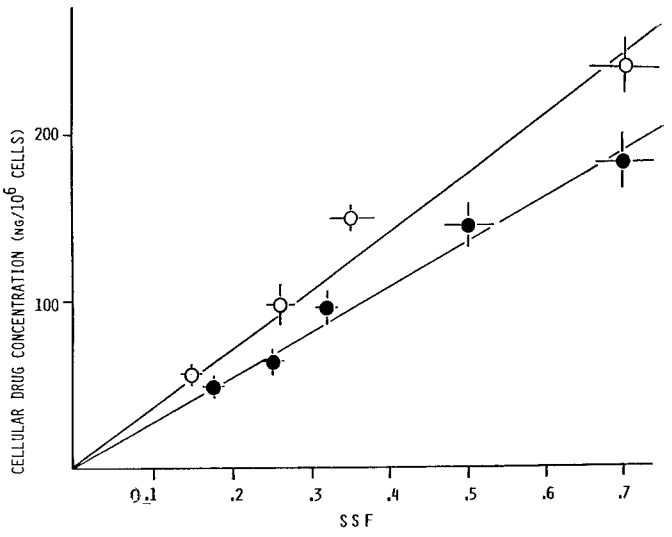

Fig. 4. Relationships between the induction of DNA single-strand breakage and the intracellular levels of 4′E-Dx (●——●) or Dx (□——□). Cells were treated with various concentrations of the anthracyclines for different intervals and were then assayed either for DNA damage or for their content of 4′E-Dx and Dx. Results represent the mean ± SEM calculated from at least 3 separate experiments

decreased with time. In contrast, the rate of formation of DNA single-strand breaks appeared to be slower and more dependent on the time of exposure in cells treated with Dx. Since the concentration of Dx used in these experiments was twice that of 4′E-Dx, data reported in Fig. 2 also indicate that Dx is a less potent inducer of DNA damage than is its epimer.

Intracellular accumulation of 4′E-Dx or Dx after various times of exposure to increasing concentrations is shown in Fig. 3. The intracellular content of anthracyclines increased continuously over the 24-h treatment period. Levels of 4′E-Dx were considerably higher than those of Dx after 5, 10, 30, 60 or 240 min drug treatment, whereas they were approximately the same following 24 h exposure. Figure 3 also shows the efflux of Dx and 4′E-Dx from cells treated for 1, 4 or 24 h and then post-incubated in drug-free medium for an additional 1 or 24 h. Although the differences were not striking, it would seem that 4′E-Dx egresses faster than Dx.

We also attempted to relate the cyto- and genotoxic effects produced by the anthracyclines with their intracellular concentrations. It should be noted, however, that at concentrations that resulted in a cytotoxic response, it was not possible to obtain a reliable measurement of the intracellular content of the drugs and we therefore could not directly relate these two phenomena. Nevertheless, since the concentrations used in DNA-damage experiments result in intracellular levels of drugs that can be measured with accuracy, these two parameters were compared and the results are shown in Fig. 4. SSF values are those resulting from Fig. 2 and from additional experiments in which cells were treated for 1 h with various other concentrations of 4′E-Dx and Dx, whereas intracellular concentrations are those shown in Fig. 3. Data depicted in Fig. 4 suggest that the higher DNA-damaging action of 4′E-Dx as compared with Dx is not solely dependent on a faster rate of uptake.

Finally, we investigated the effects of 4′E-Dx and Dx on DNA and RNA synthesis. Logarithmically growing HeLa cells were exposed to tritiated thymidine or uridine for 30 min either after 1 h treatment with various concentrations of Dx or 4′E-Dx or during the last 2 h of a 24-h treatment. Incorporation of radiolabeled precursors in the TCA-insoluble fraction was determined as a measure of DNA or RNA synthesis. Figure 5 shows that inhibition of DNA (A) and RNA (B) synthesis by 4′E-Dx and Dx was very similar. The two drugs inhibited the incorporation of radiolabeled thymidine or uridine in the TCA-precipitable fraction to approximately the same extent after either 1 or 24 h exposure.

Discussion

The data presented in this paper indicate that 4′E-Dx is more toxic than Dx to HeLa cells treated for 1 h, whereas similar levels of killing were generated by the two drugs after exposure for 24 h (Fig. 1). In addition, either the degree or the rate of induction of DNA strand breakage

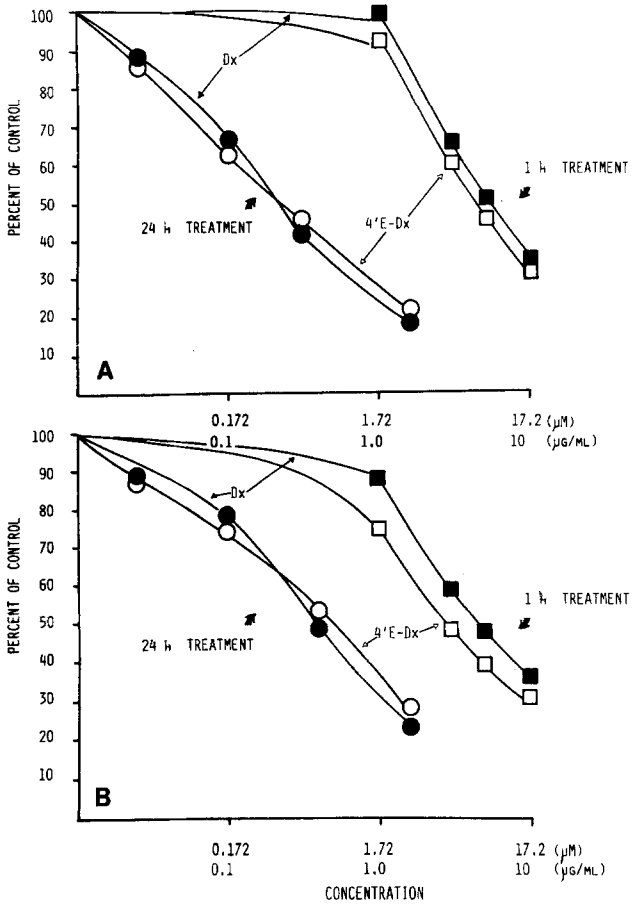

Fig. 5 A, B. Inhibition of **A** DNA and **B** RNA synthesis by Dx or 4′E-Dx. HeLa cells in the logarithmic growth phase were exposed for 1 or 24 h to various concentrations of each drug. Uptake of [^{3}H]-thymidine or [^{3}H]-uridine was determined as detailed in Materials and methods. Results represent the isotopes incorporated by the drug-treated cells as a percentage of the control cell value. Points represent the mean of 2 triplicate experiments

over a 1-h period of drug exposure were higher for 4′E-Dx than for Dx (Fig. 2). Taken together, these results suggest that in comparison with its epimer, Dx has a lower cyto- and genotoxic potential after short times of incubation. This lessened reactivity could be explained by the fact that the cells may take up less Dx, i.e. the uptake of the latter compound may be slower than that of 4′E-Dx. This would account for the differences in cytotoxicity and DNA-damaging action over the 1st h of incubation and for the almost superimposable cytotoxic response elicited by the two drugs after treatment for 24 h. Indeed, we have found that the uptake of 4′E-Dx is more rapid than that of Dx following treatment for 5, 10, 30 or 60 min and that uptake of the two drugs is almost identical after 4 or 24 h (Fig. 3). In addition, 4′E-Dx seems to egress more slowly than Dx on post-treatment incubation in a drug-free medium. However, data shown in Fig. 4 clearly indicate differences in the extent of DNA breakage at similar intracellular concentrations of the anthracyclines tested, thus suggesting that the effects of 4′E-Dx and Dx at the DNA level are not identical.

Therefore, at least two factors contribute to the higher induction of DNA breakage by 4′E-Dx as compared with Dx: a faster rate of uptake and a higher ability to produce DNA lesions. It is known that the anthracyclines, as well as other intercalating agents, stabilize a DNA-topoisomerase II complex, which then results in the production of DNA single-strand breaks and DNA-protein cross-links [15, 17]. We have recently suggested [4] that it is unlikely that 4′E-Dx and Dx might possess different degrees of specificity for the enzyme, since we found approximately the same ratio between DNA single- and double-strand breaks [4]. An alternative hypothesis, which we have also previously presented [4], is that 4′E-Dx and Dx may stabilize the DNA-topoisomerase complex in a different fashion. This would be consistent with the different ability of the two drugs to produce DNA lesions and with the different rates that characterize the removal of 4′E-Dx- and Dx-induced DNA single-strand breaks [4].

Cell lethality produced by the anthracyclines has been correlated with their ability to inhibit DNA and RNA synthesis [7, 14]. However, recent findings [9, 10, 16] suggest the lack of a relationship between these two parameters, and our data support this hypothesis. Although 4′E-Dx was more toxic than Dx after 1 h exposure, the two drugs caused approximately the same level of DNA and RNA synthesis inhibition. Our data contrast with previous reports by Glazer et al. [8] and Kim and Kim [11], who indicated that Dx has a greater inhibitory effect on DNA than it does on RNA synthesis in human cells. However, other authors [7, 13, 18] found that in cultured rodent tumor cells, the sensitivity of total RNA synthesis to the inhibitory action of Dx was slightly higher or equal to that of DNA synthesis. Glazer et al. [8] have suggested that human cells may metabolize Dx differently than rodent cells. Our data suggest differences between cell lines rather than a species-related difference in drug metabolism.

In conclusion, the data presented in this paper suggest that the higher toxicity exerted by 4′E-Dx as compared with Dx after short times of exposure is mainly dependent on a faster rate of uptake, although other factors (i.e. stabilization of the DNA-topoisomerase II complex) may be relevant in this regard. In addition, since the two drugs inhibited either DNA or RNA synthesis to approximately the same extent, it can also be concluded that the process of inhibition of nucleic acid synthesis is not related to the different cytotoxicity of Dx and 4′E-Dx.

References

1. Arcamone F, Penco S, Vigevani A, Radaelli S, Franchi G, Di Marco A, Casazza AM, Dasdia T, Formelli F, Soranzo C (1975) Synthesis and antitumor properties of new glycosides of daunomycinone and Adriamycinone. J Med Chem 18: 703–707
2. Cantoni O, Costa M (1983) Correlations of DNA strand breaks and their repair with cell survival following acute exposure to mercury (II) and X-rays. Mol Pharmacol 24: 84–89
3. Cantoni O, Sestili P, Cattabeni F (1985) Detection of protein associated DNA breaks by the alkaline elution technique utilizing polycarbonate and polyvinylchloride membranes. IRCS Med Sci 13: 248–249
4. Cantoni O, Sestili P, Cattabeni F, Geroni G, Grandi M, Giuliani FC (1989) Cellular and molecular pharmacology of 4′-epidoxorubicin in HeLa cells: comparison with its parent drug, doxorubicin. J Cancer Res Clin Oncol 115: 373–378

5. Casazza AM, Di Marco A, Bertazzoli C, Formelli F, Giuliani FC, Pratesi G (1978) Antitumor activity, toxicity and pharmacological properties of 4′-epi-Adriamycin. In: Siegenthaler W, Luthy R (eds) Current chemotherapy. American Society for Microbiology, Washington, D.C., pp 1257–1260
6. Casazza AM, Di Marco A, Bonadonna G, Bonfante V, Bertazzoli C, Bellini O, Pratesi G, Sala L, Ballerini L (1980) Effects of modifications in position 4 of the chromophore, or in position 4′ of the aminosugar, on the antitumor activity and toxicity of daunorubicin and doxorubicin. In: Crooke ST, Reich SD (eds) Anthracyclines: current status and new development. Academic Press, New York, pp 405–430
7. Crooke ST, Duvernay WH, Galvan L, Prestayko AW (1978) Structure-activity relationships of anthracyclines relative to effects on macromolecular synthesis. Mol Pharmacol 14: 290–298
8. Glazer RI, Hartman KD, Richardson CL (1982) Cytokinetic and biochemical effects of 5-iminodaunorubicin in human colon carcinoma cells in culture. Cancer Res 42: 117–121
9. Johnston JB, Glazer RI (1983) Pharmacological studies of 3′-(4-morpholinyl)-3-deaminodaunorubicin in human colon carcinoma cells in vitro. Cancer Res 43: 1044–1048
10. Johnston JB, Glazer RI (1983) Cellular pharmacology of 3′-(4-morpholinyl) and 3′(4-methoxy-1-piperidinyl) derivatives of 3′-(deaminodaunorubicin) in human colon carcinoma cells in vitro. Cancer Res 43: 1606–1610
11. Kim SH, Kim JH (1972) Lethal effect of Adriamycin on the division cycle of HeLa cells. Cancer Res 32: 323–325
12. Kohn KW, Ewig RAG, Erickson LC, Zwelling LA (1981) Measurements of strand breaks and cross-links by alkaline elution. In: Friedberg EC, Hanawalt P (eds) DNA repair: a laboratory manual of research procedures. Marcel Dekker, New York, pp 379–401
13. Meriwether WD, Bachur NR (1972) Inhibition of DNA and RNA metabolism by daunorubicin and Adriamycin in L 1210 mouse leukemia. Cancer Res 32: 1137–1142
14. Monparler RL, Karon M, Siegel SE, Avitia F (1976) Effect of Adriamycin on DNA, RNA and protein synthesis in cell-free systems and intact cells. Cancer Res 36: 2891–2897
15. Pommier Y, Schwartz RE, Kohn KW, Zwelling LA (1984) Formation and rejoining of deoxyribonucleic acid double-strand breaks induced in isolated cell nuclei by antineoplastic intercalating agents. Biochemistry 23: 3194–3201
16. Siegfred JM, Sartorelli AC, Tritton TR (1983) Evidence for the lack of relationship between inhibition of nucleic acid synthesis and cytotoxicity of Adriamycin. Cancer Biochem Biophys 6: 137–142
17. Tewey KM, Rowe TC, Yang L, Halligan BD, Liu LF (1984) Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. Science 226: 466–468
18. Tong GL, Henry DW, Acton EM (1979) 5-Iminodaunorubicin. Reduced cardiotoxic properties in an antitumor anthracycline. J Med Chem 22: 36–39