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# *IN VITRO* DNA STRAND SCISSION AND INHIBITION OF NUCLEIC ACID SYNTHESIS IN L1210 LEUKEMIA CELLS BY A NEW CLASS OF DNA COMPLEXERS, THE ANTHRA[1,9-*cd*]PYRAZOL-6(2*H*)-ONES (ANTHRAPYRAZOLES)

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**Abstract**—CI-937 and CI-942 belong to a new class of DNA complexers, the anthra[1,9-*cd*]pyrazol-6(2*H*)-ones (anthrapyrazoles), and are being further developed as antitumor drugs based on their curative properties against murine solid tumour models. The biochemical effects of these agents were studied in L1210 leukemia in relation to other clinically used intercalators. After a 1-hr exposure, CI-937 and CI-942 reduced the cloning efficiency of L1210 cells by 50% at $3.0 \times 10^{-8}$ and $1.5 \times 10^{-7}$ M respectively. Based on an ethidium displacement assay, these drugs bound strongly to DNA, reducing the fluorescence of an ethidium–DNA complex by 50% at concentrations of 23 and 33 nM for CI-937 and CI-942 respectively. This was comparable to mitoxantrone at 15 nM, but much more potent than Amsacrine which required over 1.3 μM. A distinct property of the anthrapyrazoles was a much more potent inhibitory effect on whole cell DNA synthesis than on RNA synthesis. After L1210 cells were exposed to drug for 2 hr the concentration needed to inhibit DNA synthesis by 50% was 0.33 and 0.57 μM for CI-937 and CI-942, respectively, whereas 2.0 and 11.3 μM were required to inhibit RNA synthesis by the same extent. This was in contrast to Adriamycin and mitoxantrone which inhibited both activities equally at similar concentrations. It was apparent that the inhibition of these processes was not due to substrate depletion since intracellular ribonucleoside and deoxyribonucleoside triphosphates either remained constant or were elevated after a 2-hr exposure to 1 or 10 μM drug. A similar discriminatory effect was observed on DNA and RNA polymerase in permeabilized cells, and the inhibition of nucleic acid synthesis in this system could be reversed by exogenously added DNA. Since the high incidence of cardiotoxicity associated with the administration of anthracyclines has been related to the formation of reactive oxygen species, the ability of the anthrapyrazoles to augment superoxide dismutase sensitive oxygen consumption was observed in a rat liver microsomal system. CI-937 and CI-942 induced 5- and 10-fold less oxygen consumption than Adriamycin, producing rates of 12.4, 24.2 and 138.9 nmoles/min/mg microsomal protein, respectively, at a drug concentration of 0.5 mM. The anthrapyrazoles were similar to other known intercalators in that they caused both single- and, to a lesser extent, double-strand DNA breaks that were tightly associated with protein in a concentration-dependent manner. The rates at which these lesions formed, however, were quite different between drugs. CI-937 at a concentration of 1 μM produced maximum strand breakage after 30 min, whereas CI-942 was much slower requiring at least 3 hr. If L1210 cells were treated with the anthrapyrazoles such that substantial strand breaks occurred and the drug removed, the breaks were repaired very slowly over the first 30 min but then additional lesions occurred thereafter for at least 2 hr. Additional DNA damage after drug was removed was also observed with Adriamycin and mitoxantrone but not Amsacrine with which 90% of the strand breaks disappeared within 30 min.

Intercalating agents currently occupy a prominent position in the treatment of malignant diseases and thus the antitumor and biochemical effects of these compounds continue to be the subject of intensive research. The most widely used drugs of this class are the anthracyclines [1, 2]. However, since a significant number of patients treated with these agents develop an accumulative and irreversible cardiomyopathy which severely limits dosage [1–3], there has been a considerable effort to develop structural analogues or other classes of DNA binders which may circumvent or at least reduce this disadvantage. Despite the large number of chemical modifications that have been made, no anthracycline analogue as yet has demonstrated therapeutic efficacy equal to Adriamycin while at the same time showing a reduced tendency to cause cardiotoxicity [2]. Among the more recently synthesized classes of intercalators that have demonstrated usefulness in the clinic are the aminoacridine derivative Amsacrine [4, 5] and the anthracenedione mitoxantrone [6, 7] and, while the overall incidence of cardiac failure appears to be lower with these drugs, results are too preliminary to draw firm conclusions on this aspect.

The present paper describes the biochemical effects of a new class of DNA complexers, the anthra[1,9-*cd*]pyrazol-6(2*H*)-ones (hereafter referred to as the anthrapyrazoles). These agents were synthesized with the rationale that chromophore modification of the anthracenedione nucleus might diminish cardiotoxicity by reducing the potential to form semiquinone free radicals [8, 9]. Two of these

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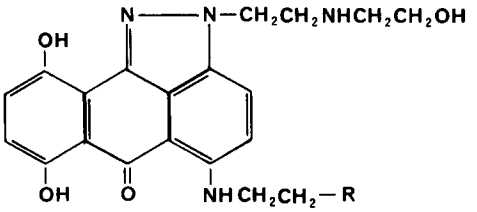


| | R |
|---|---|
| CI-937 | $NHCH_3$ |
| CI-942 | $CH_2NH_2$ |

Fig. 1. Molecular structures of CI-937 and CI-942.

agents, CI-942 and CI-937 (structures are shown in Fig. 1), have been selected for further development based on their curative activity in murine solid tumor models [10]. Data are provided describing the effects of CI-942 and CI-937 on macromolecular synthesis in intact and permeabilized L1210 cells, the formation of DNA strand breaks, relative binding affinity for DNA and production of superoxide radical. The biochemical activities of these agents are compared with other clinically used DNA binders, which include Adriamycin, mitoxantrone and Amsacrine.

## MATERIALS AND METHODS

*Chemicals.* Purine and pyrimidine nucleotides, native calf thymus DNA, poly(deoxyadenylate-deoxythymidylate), Adriamycin, ethidium bromide and superoxide dismutase were purchased from the Sigma Chemical Co., St. Louis, MO. DNA polymerase (*Escherichia coli*) was obtained from PL Biochemicals, Inc., Milwaukee, WI. Tetrapropylammonium hydroxide was from the Eastman Kodak Co., Rochester, NY. [*Methyl*-$^{14}$C]Thymidine, [*methyl*-$^{3}$H]thymidine, [5-$^{3}$H]uridine, L-[4,5-$^{3}$H]leucine, deoxy[8-$^{3}$H]adenosine 5′-triphosphate, [*methyl*-$^{3}$H]thymidine 5′-triphosphate and [5-$^{3}$H]-uridine 5′-triphosphate were purchased from Amersham, Arlington Heights, IL. CI-942 and CI-937 were synthesized as previously described [8] and supplied by Dr. Hollis Showalter (Warner-Lambert Co.). Amsacrine (*N*-[4-(9-acridinylamino)-3-methoxyphenyl]methanesulphonamide) was provided by the Warner-Lambert Co., Ann Arbor, MI. Mitoxantrone (1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthracenedione dihydrochloride) was from Lederle, Pearl River, NY.

*Cell culture.* All experiments employed L1210 mouse leukemia cells grown as a suspension culture in RPMI 1640 medium supplemented with 5% fetal bovine serum and 50 μg/ml gentamycin. Clonogenic assays were performed by a modification of the procedure described by Himmelfarb *et al.* [11]. Noble agar (Difco Laboratories, Detroit, MI), 2.5% in sterile water, was melted and diluted 1:8 with RPMI 1640 containing 15% fetal calf serum. Cells were exposed to various concentrations of drug for 1 hr, washed by centrifugation, and resuspended in fresh medium at 100–500 cells/ml. One milliliter of drug-treated or untreated cells was placed in sterile 12-ml plastic tubes and mixed with 4 ml of diluted agar. The agar was allowed to gel into a semi-solid state, and the tubes were incubated upright at 37°. Colonies were counted after 7–11 days.

*Incorporation of radioactive precursors into macromolecules.* Incorporation of radiolabeled precursors into DNA, RNA and protein was monitored by exposing logarithmically growing cells to either [*methyl*-$^{3}$H]thymidine, [5-$^{3}$H]uridine or L-[4,5-$^{3}$H]-leucine, respectively, at a concentration of 1 μM and a specific activity of 1 μCi/nmole. At regular intervals the cells from a 1-ml aliquot were injected into 2 vol. of ice-cold 15% trichloroacetic acid (TCA), and the precipitate was collected on glass fiber filters. The filters were washed five times with 2-ml aliquots of ice-cold 15% TCA, dried, and placed in scintillation vials along with 10 ml of Ready-Solv (Beckman, Irvine, CA). Radioactivity was determined in a Beckman LS 6800 scintillation counter.

*Deoxyribonucleoside triphosphate assay.* Approximately $5 \times 10^7$ cells were extracted with 60% aqueous methanol as described previously [12]. dCTP and dGTP were measured enzymatically utilizing *E. coli* DNA polymerase along with calf thymus DNA as a template/primer [13]. dATP and TTP were determined in an identical manner except that poly(deoxyadenylate-deoxythymidylate) was employed as a template/primer [14]. Standard curves for each deoxyribonucleoside triphosphate were included in all assays.

*Ribonucleoside triphosphate analysis.* Approximately $10^7$ cells were extracted with 0.5 ml of ice-cold 0.7 M perchloric acid. Extracts were centrifuged to remove precipitated protein, neutralized with solid potassium bicarbonate, and centrifuged once more to remove potassium perchlorate. Fifty microliters of the supernatant fraction was analyzed by anion-exchange chromatography utilizing a Perkin–Elmer series 4 liquid chromatograph equipped with a Whatman Pellicular anion exchange precolumn (0.4 × 6 cm) and a Whatman Partisil PSX 10/25 SAX column (0.46 × 25 cm). Nucleotides were resolved with an ammonium phosphate gradient starting with 5 mM, pH 2.8, and ending at 0.5 M, pH 4.8. Precise details of the elution procedure are given elsewhere [15]. Peaks were detected using a Kratos spectroflow 773 variable wavelength detector and electronically integrated using a Perkin–Elmer Sigma 15 integrator calibrated against known standards.

*DNA and RNA polymerase assays.* DNA and RNA polymerase activities were determined in permeabilized L1210 cells by a modification of the method of Berger [16]. Cells were suspended for 30 min at a concentration of $2 \times 10^6$/ml in ice-cold permeabilizing buffer containing 10 mM Tris, pH 7.8, 10 mM EDTA, 4 mM $MgCl_2$, and 30 mM 2-mercaptoethanol. The cells were collected by centrifugation and resuspended into permeabilizing buffer at $10^7$ cells/ml. Substrates for the assay were dissolved in reaction buffer containing 100 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid) (Hepes), pH 7.8, 20 mM $MgCl_2$ and 150 mM NaCl at the following concentrations: DNA polymerase–15 mM ATP, 0.3 mM dATP, 0.3 mM dCTP, 0.3 mM

dGTP, and 3 μM [*methyl*-$^3$H]TTP, 5 μCi/nmole; RNA polymerase–15 mM ATP, 0.3 mM CTP, 0.3 mM GTP, and 3 μM [5-$^3$H]UTP, 5 μCi/nmole. The reaction was started by mixing two parts cell suspension with one part substrate mixture. After a 15-min incubation at 37° the reaction was terminated with 2 ml of ice-cold 15% TCA, and the precipitate was collected and processed as described in the incorporation studies. In studies where activated DNA was exogenously supplied in the assay, 0.05% Triton X-100 was included in the final reaction mixture to allow access of macromolecules to the polymerases as previously described [14]. Activated calf thymus DNA was prepared by the method of Setlow [17].

*Ethidium fluorescence assay.* The relative affinity of these drugs for DNA was determined by the reduction of fluorescence of an ethidium–DNA complex in the presence of drug as previously described [18, 19]. In brief, 3 ml of buffer (9.4 mM NaCl, 10 μM EDTA and 2 mM Hepes, pH 7.0) containing 1.26 μM ethidium bromide was placed in a quartz cuvette to which 20 μl of calf thymus DNA (150 μM in nucleotides, $E_{260}$ = 6600) was added, and the relative fluorescence before and after the addition of DNA was determined in an SPF-500 ratio spectrofluorometer (American Instrument Co., Silver Spring, MD) equipped with a 250 W xenon lamp. The excitation wavelength was 546 nm and the emission 595 nm. Repeated aliquots of the test drug (10 μl) were introduced into the cuvette, and the relative fluorescence was determined after each addition. The concentration of drug needed to reduce fluorescence of the ethidium–DNA complex by 50% ($C_{50}$) was determined graphically.

*Oxygen consumption measurements.* Microsomes were prepared from rat liver by the following method. All procedures were performed at 4°. Approximately 150 g of liver was rinsed in ice-cold 0.15 M KCl and homogenized for 1 min with a Waring blender in 3 vol. of the same solution. The homogenate was centrifuged at 15,000 *g* for 15 min, the pellet discarded, and the supernatant fraction centrifuged for 1 hr at 100,000 *g*. The pellets were resuspended in 60 ml of 0.15 M KCl plus 1 mM EDTA (pH 7.0) and centrifuged at 100,000 *g* for 1 hr. The pellets were washed once more by resuspension into 0.1 M potassium phosphate, pH 7.5, and centrifuged at 100,000 *g* for an additional hour. Finally, the washed microsomes were resuspended in 50 ml of 50% glycerol in 0.1 potassium phosphate, pH 7.5, at a protein concentration of 32 mg/ml and stored at −20°. The microsomes were stable for at least 1 year when stored in this manner. Protein was determined by the method of Peterson [20].

Oxygen consumption was monitored with a model 53 oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, OH) equipped with a YSI 5331 oxygen probe. All measurements were made in a closed, water-jacketed and continuously stirred reaction compartment at 37°. The 2-ml reaction mixture consisted of 0.2 M potassium phosphate, pH 8.0, previously aerated by bubbling with filtered air for 10 min, 0.5 to 2.5 mg microsomal protein, 0.5 mM NADPH and $5 \times 10^{-5}$–$5 \times 10^{-4}$ M drug. Microsomes were solubilized with 2% Triton X-100 prior to measurement to reduce endogenous activity [21]. All components except drug were added to the compartment, the oxygen probe was inserted, and an endogenous rate of oxygen consumption was determined over approximately 2 min. Drug was then added via polyethylene tubing inserted in the groove of the probe, and rates were determined over a period of 2–10 min. Endogenous activity was subtracted from these rates, and data are expressed as initial rates in nmoles oxygen/min calculated from first-order rate constants determined by the method of Ito and Yamamoto [22], assuming an initial concentration of dissolved oxygen of 210 μM.

*DNA strand scission assays.* DNA single-strand breaks were measured by alkaline elution as described by Kohn *et al.* [23]. L1210 cells in early log were grown in [*methyl*-$^{14}$C]thymidine, 1 μM at a specific activity of 0.02 μCi/nmole, for 24 hr. The cells were exposed to drug at a specified concentration and time interval, after which $10^6$ cells were placed on polycarbonate filters (2 μm pore diameter, Nucleopore Corp., Pleasanton, CA) in a 25-mm filter holder with a 50-ml funnel (Millipore, Bedford, MA) and washed by gravity with three 5-ml aliquots of ice-cold phosphate-buffered saline. The cells were lysed by passing 5 ml of 2% sodium dodecyl sulfate–25 mM EDTA, pH 9.7, through the filter. Two milliliters of the same solution containing 0.5 mg proteinase K/ml (Sigma) were layered over the cells and pumped through the filter at 20 μl/min for 1 hr, collecting the eluate into scintillation vials. The funnel was then filled with eluting buffer (tetrapropylammonium hydroxide–25 mM EDTA–0.1% sodium dodecyl sulfate, pH 12.1), and this solution was pumped through the filter at 20 μl/min with 1-hr fractions being collected for at least 16 hr. At the end of the run the filters were placed in scintillation vials and incubated with 0.4 ml of 1 N HCl at 60° for 1 hr followed by 1 ml of 1 N NaOH for 1 hr at room temperature. Residual radioactivity in the filter holders and tubing was pumped into scintillation vials with an additional 2 ml of eluting buffer. All radioactivity was incorporated into Ready-Solv (Beckman) containing 0.7% acetic acid. Data were graphed on a semilogarithmic plot of [$^{14}$C] retained on the filter versus elution time in hours. Since these plots were linear, the data could also be expressed numerically as elution rate constants representing the negative slopes of the elution curves.

Elution rates for double-stranded breaks were determined as described by Bradley and Kohn [24] for neutral elution; the method was identical to the procedure for single-strand breaks except that the eluting buffer was at pH 9.6.

The presence of DNA–protein cross-linking was assessed as described by Kohn *et al.* [23], and the method again was identical to the alkaline elution procedure except for the following. The cells were placed on 2 μm pore polyvinyl chloride filters (Millipore), and the lysis solution contained 2 M NaCl, 0.2% sodium dodecyl sarcosine and 50 mM EDTA, pH 10. After lysis, residual lysis solution was removed by washing the filter with 3 ml of 40 mM EDTA, pH 10. Treatment with proteinase K was omitted, and DNA elution was carried out with tetrapropylammonium hydroxide, 25 mM EDTA at pH 12.1.

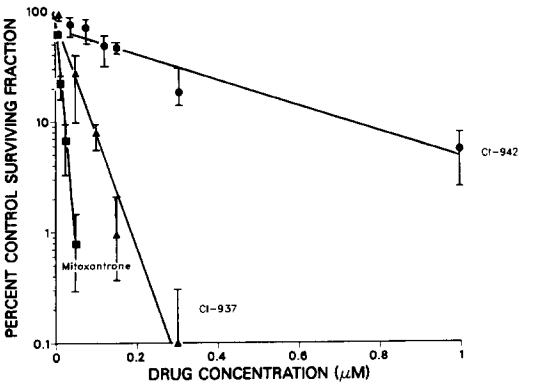


Fig. 2. Survival of L1210 cells treated for 1 hr with various concentrations of CI-937, CI-942 or mitoxantrone. Points represent the mean of two to three experiments and bars are the range.

Repair of single-strand breaks was assessed by first incubating cells with drug for 1 hr, washing the cells twice in an excess of 37° medium, and resuspending the cells into 10 ml of medium at 37°. Cells were then incubated at 37°, and aliquots ($10^6$ cells) were periodically removed and lysed on filters following the procedure for single-strand breaks.

## RESULTS

*Clonogenic assays.* The cytotoxicity of CI-942 and CI-937, as assessed in a colony forming assay using L1210 cells, is shown in Fig. 2 and compared to that of mitoxantrone. Cell kill after a 1-hr exposure was concentration dependent, and the dose response was quite steep creating a narrow range between that concentration of drug producing no effect and total inhibition. CI-937 was 5-fold more potent than CI-942, requiring concentrations of $3.0 \times 10^{-8}$ and $1.5 \times 10^{-7}$ M, respectively, to inhibit survival by 50%. Mitoxantrone was comparable to CI-937 having an $IC_{50}$ of $1.2 \times 10^{-8}$ M.

*Inhibition of nucleic acid synthesis.* Table 1 shows the concentrations of CI-942 and CI-937 necessary to inhibit by 50% thymidine, uridine or leucine incorporation into the TCA-insoluble fraction in L1210 cells after a 2-hr exposure, in comparison to Amsacrine, Adriamycin and mitoxantrone. The anthrapyrazoles were very potent inhibitors of DNA synthesis, similar in potency to mitoxantrone and Amsacrine and four to six times more potent than Adriamycin. RNA synthesis was much less sensitive to the anthrapyrazoles requiring six and twenty times more CI-937 and CI-942, respectively, to produce a 50% inhibition than that required for DNA synthesis. The preferential inhibition of DNA synthesis was also observed with Amsacrine but not with Adriamycin or mitoxantrone which inhibited DNA and RNA synthesis to the same extent at similar concentrations. All drugs inhibited protein synthesis only at very high concentrations.

Table 1. Inhibition of whole cell DNA, RNA and protein synthesis in L1210 cells*

| Drug | $IC_{50}$ (μM) | | |
|---|---|---|---|
| | DNA | RNA | Protein |
| CI-942 | 0.59 ± 0.11 | 11.3 ± 1.6 | 15.0 ± 5.0 |
| CI-937 | 0.33 ± 0.13 | 2.0 ± 0.3 | 5.0 ± 0.1 |
| Amsacrine | 0.34 ± 0.24 | 26.0 ± 1.0 | 20.0 ± 4.6 |
| Adriamycin | 2.05 ± 0.52 | 2.1 ± 0.5 | 40.0 ± 9.9 |
| Mitoxantrone | 0.50 ± 0.27 | 0.6 ± 0.1 | 5.5 ± 1.5 |

* Cells were incubated for 2 hr at 37° with various concentrations of drug and then exposed to tritiated thymidine, uridine or leucine for 30 min as described in Materials and Methods. Data are expressed as the micromolar concentration of drug needed to inhibit 50% and represent the mean of three experiments ± standard error.

Table 2. Inhibition of DNA and RNA polymerase by CI-942 and CI-937 in permeabilized L1210 cells*

| Drug | $IC_{50}$ (μM) | |
|---|---|---|
| | DNA | RNA |
| CI-942 | 15.3 ± 1.8 | 127.0 ± 8.6 |
| CI-937 | 13.3 ± 7.7 | 49.0 ± 3.5 |

* Procedures for permeabilizing cells and measuring DNA and RNA polymerase are described in Materials and Methods. Data are expressed as the micromolar concentration of drug needed to inhibit 50% and represent the mean of three experiments ± standard error.

A permeabilized cell system was employed to study the effect of the anthrapyrazoles on DNA and RNA polymerase. As shown in Table 2, the preferential inhibition of DNA synthesis versus RNA synthesis was also prevalent in this system although from 25- to 40-fold more drug was required than in viable cells. The effect of exogenously supplied, activated DNA on the inhibition of DNA and RNA polymerase is shown in Table 3. A constant amount of CI-942 or CI-937 was included in the reaction mixture such that the polymerase activity was approximately 30% of control in the absence of exogenous DNA. The inhibition of both processes could be reversed by DNA bringing the activities to approximately 90% of the control.

*Effects of CI-942 and CI-937 on the intracellular concentrations of ribonucleoside and deoxyribonucleoside triphosphates in L1210 cells.* Cells exposed for 2 hr to 1 μM CI-942 or CI-937 showed no significant changes in the intracellular pools of ribonucleoside triphosphates (Table 4). Increasing the concentration to 10 μM resulted in an increase in these substrates especially in the CTP and GTP pools. These results were compared to other intercalators (mitoxantrone, Adriamycin and Amsacrine) and were qualitatively similar. Likewise little effect or a slight decrease was observed on deoxyribonucleoside triphosphate pools in cells exposed to a 1 μM concentration of the anthrapyrazoles (Table 5). Again this was similar to the control drugs with the exception of an increase in dCTP and dATP in the presence of Adriamycin and Amsacrine.

*Ethidium fluorescence assay.* The reduction in fluorescence of an ethidium–DNA complex when exposed to a drug has been shown to be indicative

Table 3. Effect of exogenous activated DNA on the inhibition of DNA and RNA polymerase by CI-942 and CI-937 in permeabilized L1210 cells*

| | Percent control activity | | | |
|---|---|---|---|---|
| | DNA polymerase | | RNA polymerase | |
| Exogenous DNA (μg/ml) | CI-942 (20 μM) | CI-937 (20 μM) | CI-942 (150 μM) | CI-937 (150 μM) |
| 0 | 31.3 ± 5.3 | 29.3 ± 3.5 | 28.7 ± 7.7 | 28.7 ± 10.7 |
| 50 | 46.7 ± 4.1 | 43.0 ± 6.6 | 28.7 ± 5.0 | 25.3 ± 5.8 |
| 100 | 62.3 ± 9.9 | 57.0 ± 14.6 | 29.3 ± 7.4 | 32.3 ± 14.4 |
| 200 | 82.3 ± 8.4 | 76.7 ± 13.3 | 46.0 ± 3.1 | 39.0 ± 2.7 |
| 300 | 84.5 ± 6.5 | 80.0 ± 3.0 | 80.5 ± 19.5 | 81.5 ± 18.5 |
| 400 | 92.0 ± 4.0 | 87.0 ± 7.0 | 86.0 ± 14.0 | 90.0 ± 10.0 |
| 500 | 89.5 ± 3.5 | 88.5 ± 10.5 | 85.5 ± 14.5 | 95.5 ± 4.5 |

* Experimental procedures are described in Materials and Methods. Data are expressed as a percentage of the control activity in the absence of drug and represent the mean of three experiments ± standard error.

of binding to DNA and can be used to assess relative affinities between drugs [18]. Figure 3 shows the reduction in fluorescence caused by CI-937 and CI-942 in comparison to mitoxantrone. CI-937 was about 30% more potent than CI-942 having $C_{50}$ values of 23 and 33 nM respectively. Although actual association constants cannot be calculated from this data, it indicates a very high affinity for DNA similar to mitoxantrone which had a $C_{50}$ of 15 nM. The acridine analog, Amsacrine, was much less potent, having a $C_{50}$ of 13,667 nM (data not shown).

*Oxygen consumption studies.* The capacity for these drugs to form superoxide radical was assessed by measuring the rate of oxygen consumption in the presence of rat liver microsomes and NADPH. As shown in Fig. 4, CI-937 and CI-942 stimulated oxygen consumption under these conditions which was proportional to the amount of microsomal protein. The rates of oxygen consumption with 0.5 mM drug and 1 mg microsomal protein are compared to Adriamycin in Table 6. Oxygen consumption in all three cases was inhibited by superoxide dismutase, indicating that the activity was related to production of superoxide radical. The anthrapyrazoles, however, caused five to ten times less oxygen consumption thao Adriamycin. The rates of oxygen consumption induced by the three drugs were saturable and, assuming simple Michaelis–Menten kinetics, a $K_m$ and $V_{max}$ were calculated using the weighted method of Wilkinson [25]. The major kinetic difference between Adriamycin and the anthrapyrazoles was a decrease in the $V_{max}$ for the latter which was sufficient to account for the observed reduction in oxygen consumption. Since the intracellular concentrations of drug *in vivo* would most likely be far less than the $K_m$ values determined in this study, the catalytic specificity ($V_{max}/K_m$) is also listed in Table 6 which approximates a first-order rate constant at very low drug concentrations. The values for CI-942 and CI-937 were similar, whereas that for Adriamycin was 8-fold higher.

*Formation of DNA strand breaks by CI-942 and CI-937.* The formation of DNA strand breaks and their rates of repair and association with protein were assessed in L1210 cells exposed to the anthrapyrazoles by filter elution techniques [23, 24]. Figure

Table 4. Effects of CI-942 and CI-937 on the intracellular levels of ribonucleoside triphosphates in L1210*

| Drug | CTP | UTP | ATP | GTP |
|---|---|---|---|---|
| | (pmoles/$10^6$ cells) | | | |
| Control | 268 | 504 | 1719 | 345 |
| CI-942 (1 μM) | 287 (107) | 599 (119) | 1796 (104) | 364 (106) |
| CI-942 (10 μM) | 384 (143) | 642 (127) | 1954 (114) | 494 (143) |
| CI-937 (1 μM) | 371 (138) | 634 (126) | 1957 (114) | 464 (134) |
| CI-936 (10 μM) | 604 (225) | 670 (133) | 1916 (111) | 715 (207) |
| Mitoxantrone (1 μM) | 397 (148) | 705 (140) | 2053 (119) | 488 (141) |
| Mitoxantrone (10 μM) | 580 (216) | 701 (139) | 2001 (116) | 694 (201) |
| Adriamycin (1 μM) | 373 (139) | 564 (112) | 1863 (108) | 411 (119) |
| Adriamycin (10 μM) | 518 (193) | 735 (146) | 1959 (114) | 615 (178) |
| Amsacrine (1 μM) | 290 (108) | 533 (106) | 2029 (118) | 410 (119) |
| Amsacrine (10 μM) | 280 (104) | 477 (95) | 1814 (106) | 388 (112) |

* Cells were incubated with the indicated drug concentrations for 2 hr. Data are expressed as pmoles/$10^6$ cells and are the mean of two experiments. Numbers in parentheses are the experimental values expressed as a percentage of control.

Table 5. Effects of CI-942 and CI-937 on the intracellular levels of deoxyribonucleoside triphosphate in L1210*

| Drug | dCTP | TTP | dATP | dGTP |
|---|---|---|---|---|
| | | (pmoles/$10^6$ cells) | | |
| Control | 5.2 | 13.2 | 8.1 | 13.5 |
| CI-942 | 4.8 (92) | 9.6 (72) | 7.8 (96) | 12.6 (93) |
| CI-937 | 5.7 (109) | 10.5 (79) | 7.3 (90) | 11.6 (86) |
| Mitoxantrone | 5.2 (100) | 9.5 (72) | 6.6 (82) | 11.3 (84) |
| Adriamycin | 8.5 (163) | 14.1 (106) | 12.2 (150) | 12.0 (89) |
| Amsacrine | 7.5 (144) | 17.2 (130) | 13.4 (165) | 15.3 (113) |

* Cells were incubated with 1 μM drug for 2 hr. Data are expressed as pmoles/$10^6$ cells and are the mean of two experiments. Numbers in parentheses are drug-treated values expressed as a percentage of control.

5 shows alkaline and neutral elution profiles in cells exposed for 1 hr to various concentrations of drug. Both drugs caused single-strand breaks in a concentration-dependent manner (Fig. 5, A and B), and it was apparent that after a 1-hr exposure CI-937 was about ten times more potent than CI-942. Under elution conditions which indicate double-strand breaks, i.e. pH 9.6 (Fig. 5, C and D), substantially higher concentrations for both drugs were required to induce significant elution rates, and again CI-937 was five to ten times more potent than CI-942. Since the concentrations of drug required to produce substantial double-strand breaks are at least an order of magnitude higher than those which produce significant cell kill (Fig. 2), it is unlikely that this type of lesion is relevant with regard to cytotoxicity.

Figure 6A shows a comparison of alkaline elution rates produced by the anthrapyrazoles and three control drugs in L1210 cells exposed to equimolar doses (1 μM) for 1 hr. CI-937 and mitoxantrone were similar and were most potent in producing single-stranded breaks followed by Amsacrine. CI-942 produced a much lower elution rate followed by Adriamycin which was the least potent. In the double-strand break assay (Fig. 6B) in which cells were exposed to 20 μM drug for 1 hr, the pattern of elution rates changed somewhat in that CI-942 and Adriamycin produced identical elution rates which were very slow and Amsacrine appeared to be the most potent under these conditions. CI-937 and mitoxantrone were again similar and of intermediate potency. A portion of the elution rate under neutral conditions, however, could be due to coincident single-strand breaks on opposite strands.

Recent data suggest that DNA strand breaks caused by intercalators may be associated with endonuclease or topoisomerase activity [26–28]. An indication of this interaction is evident by the formation of covalent linkages between DNA and protein when cells are treated with these drugs. DNA linked to protein can be detected by alkaline elution experiments performed in the absence of proteinase treatment and under conditions in which protein is retained on the filter. Thus a reduction in elution rate for the DNA under these conditions would suggest a tight association with protein. Figure 7 shows that, in samples treated with proteinase, substantial elution rates were obtained as shown before. Elution rates for cells not treated with proteinase, however, were equal to or less than the control, indicating a strong association with protein similar to what has been reported previously for other intercalators [29–31].

Some of the differences in potency may be attrib-

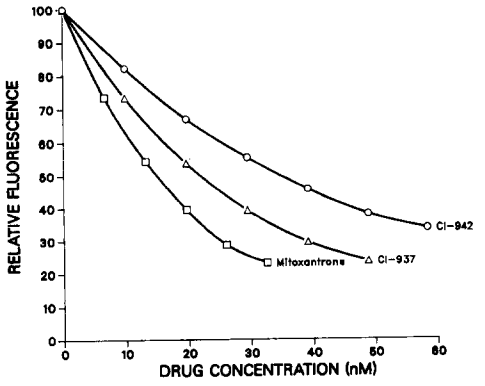


Fig. 3. Fluorescence of an ethidium–DNA complex in the presence of various concentrations of CI-937, CI-942 or mitoxantrone. Fluorescence was measured at 595 nm with an excitation at 546 nm. The concentration of calf thymus DNA was 1.0 μM and ethidium was 1.26 μM.

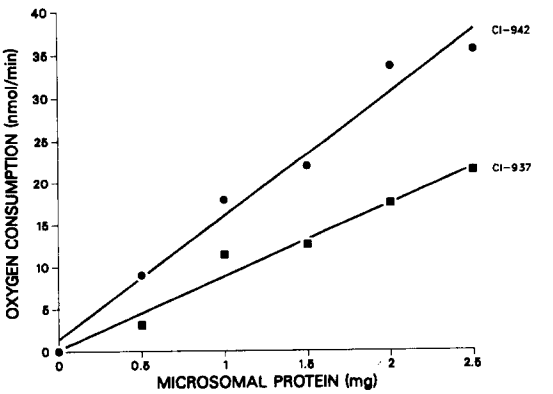


Fig. 4. Dependence of augmented oxygen consumption on microsomal protein. Assay procedures are described in Materials and Methods. Drug concentrations are 0.5 mM.

Table 6. Stimulation of oxygen consumption in rat liver microsomes by CI-942, CI-937 and Adriamycin*

| Drug | Oxygen consumption† −SOD | Oxygen consumption† +SOD | $K_m$ (μM) | $V_{max}$ (nmoles/mg protein/min) | $V_{max}/K_m$ |
|---|---|---|---|---|---|
| CI-942 | 24.2 ± 2.2 | 9.1 ± 3.1 | 740 ± 10 | 57.8 ± 3.5 | 0.078 |
| CI-937 | 12.4 ± 1.9 | 8.0 ± 2.6 | 258 ± 11 | 18.1 ± 2.6 | 0.070 |
| Adriamycin | 138.9 ± 5.4 | 60.1 ± 7.8 | 394 ± 21 | 242.3 ± 14.7 | 0.614 |

* Procedures for measuring oxygen consumption are described in Materials and Methods. Data are expressed as the mean of three experiments ± standard error.

† Rates of oxygen consumption (nmoles/min) in the presence of 1 mg of microsomal protein, 0.5 mM NADPH, 0.5 mM drug with or without 100 μg superoxide dismutase (SOD).

uted to the exposure time required for maximum strand breakage. Figure 8 shows that the formation of single-stranded breaks was dependent on the time of exposure. CI-937 attained maximal effects within 30 min, whereas strand breakage by CI-942 occurred more slowly and was linear for nearly 3 hr. After cells were exposed to drug for 3 or more hours, differences in the amount of DNA strand breakage caused by both drugs were minimal.

Finally, a comparison of the rates of repair of DNA strand breaks in L1210 exposed for 1 hr to equally potent concentrations of the drugs is shown in Fig. 9. As reported previously [31, 32], Amsacrine breaks were repaired very rapidly and the elution rate had decreased by 70% within 15 min after removal of the drug and 90% by 30 min. The repair rates in cells exposed to both anthrapyrazoles were similar to Adriamycin and mitoxantrone in that repair proceeded very slowly over the first 30 min; however, additional strand breakage occurred thereafter over at least 90 min. Further strand breakage was most prevalent in cells treated with Adriamycin or CI-937.

## DISCUSSION

DNA complexers have been established as among the most effective classes of antitumor agents in clinical use today and are widely used against a number of malignant diseases [1–7]. Accumulative cardiotoxicity, however, has severely limited the prolonged use of some of these agents [1–3] to an extent that considerable efforts have been made to eliminate this problem through structure modification [1, 2, 33] and development of other classes of intercalating agents such as the aminoanthraquinones

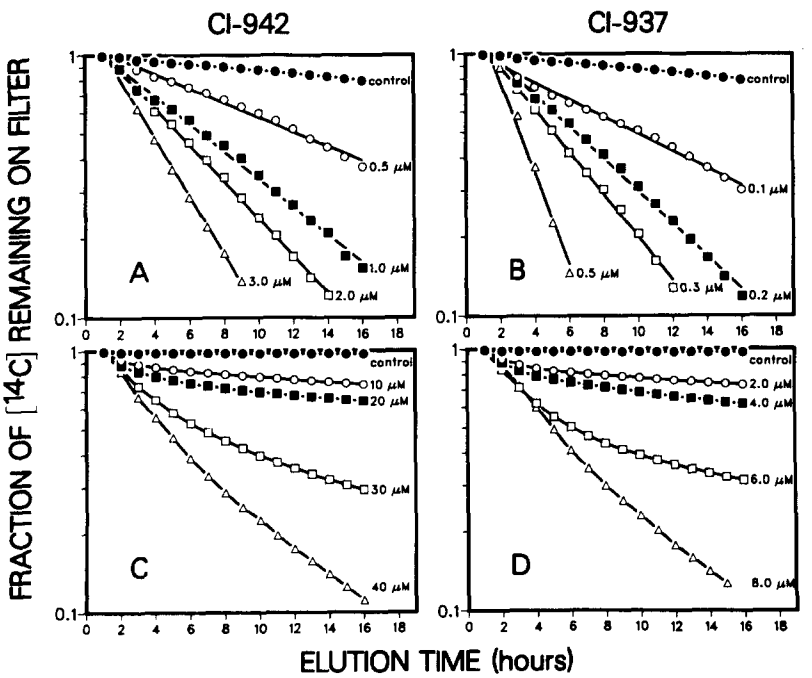


Fig. 5. Alkaline (A and B) and neutral (C and D) elution profiles of L1210 cells treated with various concentrations of CI-942 (A and C) and CI-937 (B and D). Cells containing [$^{14}$C]DNA were exposed to the indicated concentrations of drug for 1 hr. Elution procedures are described in Materials and Methods.

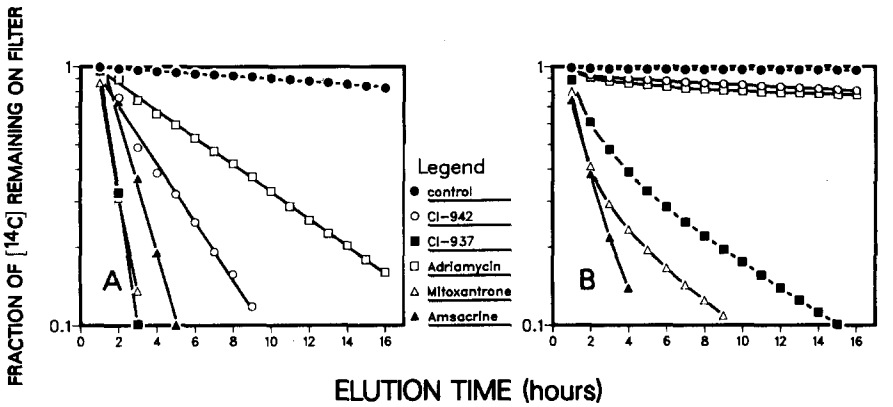


Fig. 6. Alkaline (A) and neutral (B) elution profiles of L1210 treated for 1 hr with either 1 $\mu$M (A) or 20 $\mu$M (B) drug. Elution conditions are described in Materials and Methods.

[6, 7, 34] and acridine derivatives [35]. The present paper has described some aspects of the biochemical pharmacology of a new class of DNA binders, the anthrapyrazoles. These compounds were synthesized with the rationale that a specific modification of the anthracenedione nucleus might tend to reduce cardiotoxicity via a limited tendency to form semiquinone free radicals [8, 9]. These agents have exhibited broad spectrum antitumor activity being curative in a number of murine solid tumor models [10].

The anthrapyrazoles appear to interact strongly with DNA as indicated by a potent reduction in the fluorescence of an ethidium–DNA complex in the presence of drug. The $C_{50}$ values were in the same range of that obtained with mitoxantrone which has a reported association constant of $1.8 \times 10^6\ M^{-1}$ [36] and 2–3 orders of magnitude lower than Amsacrine which is a relatively weak binder at $3.7 \times 10^5\ M^{-1}$ [37]. This strong association with DNA may account for the rapid cytotoxic effect and potency of these compounds in the clonogenic assay. CI-942 and CI-937 were quite cytotoxic to L1210 cells after a 1-hr exposure and exhibited a very steep dose response, with CI-937 being about 5-fold more potent than CI-942. This difference in potency has also been described with regard to antitumor studies *in vivo* [10].

A salient characteristic of the anthrapyrazoles was a much more potent inhibition of DNA synthesis than RNA synthesis. After L1210 cells were exposed to drug for 2 hr, 20-fold less CI-942 and 6-fold less CI-937 were required to inhibit DNA synthesis by 50% than RNA synthesis. This is similar to what was observed with Amsacrine but different from Adriamycin and mitoxantrone which inhibited both activities to the same extent at similar concentrations. It was apparent that the inhibition of these processes was not due to substrate depletion since intracellular ribonucleoside and deoxyribonucleoside triphosphates either remained constant or were elevated in the presence of these drugs. The same pattern of inhibition was also evident in the polymerase assays using permeabilized cells and appeared to result entirely from the interaction with

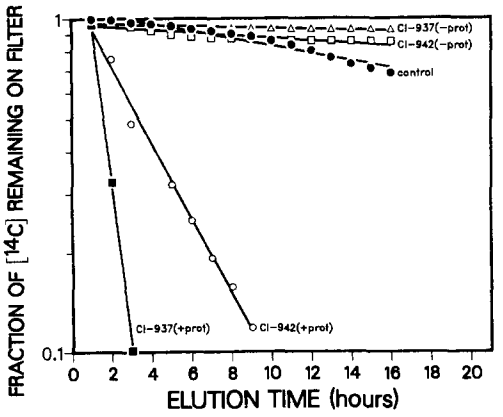


Fig. 7. Alkaline elution profile of L1210 cells treated for 1 hr with 1 $\mu$M CI-937 or CI-942 with (+prot) or without (−prot) proteinase treatment. Elution conditions are described in Materials and Methods. Filters used in the +prot assay with 2 $\mu$m pore polycarbonate and those in the −prot were 2 $\mu$m pore polyvinyl.

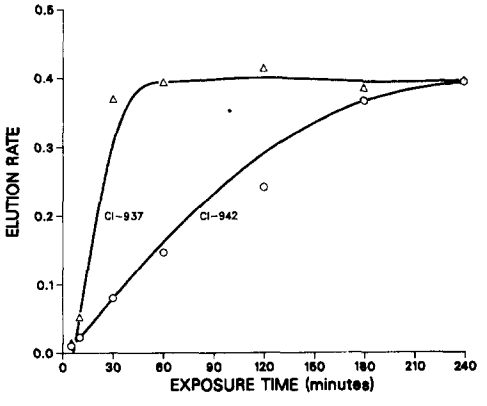


Fig. 8. Effect of drug exposure time on the production of single-strand breaks in L1210 cells. Cells containing [$^{14}$C]-DNA were exposed to 1 $\mu$M drug at zero time, and aliquots ($10^6$ cells) were removed periodically and injected into ice-cold saline. The cells were collected and lysed on filters, and alkaline elution was performed as described in Materials and Methods. Elution rate represents the negative slope of the line calculated by least squares from a plot of the fraction of DNA retained on the filter versus the elution time in hours.

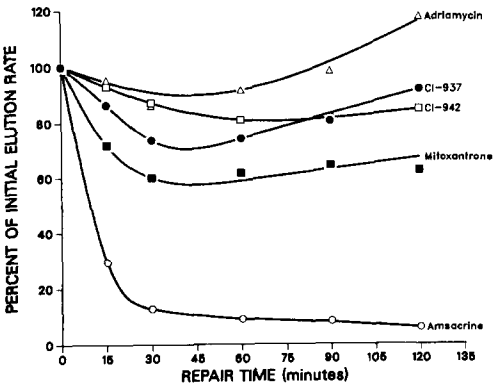


Fig. 9. Repair of drug-induced DNA strand breaks in L1210 cells. Ten milliliters of cells ($5 \times 10^5$) containing [$^{14}$C]DNA was exposed to equally potent concentrations of the indicated drugs, washed two times in 10 ml of 37° medium, and resuspended into 25 ml of 37° medium. Five milliliters ($10^6$ cells) was removed periodically and subjected to alkaline elution as described in Materials and Methods. Data are expressed as percentage of the elution rate (see Fig. 8) immediately following drug exposure. The concentrations of the drugs were: CI-937, 0.5 μM; CI-942, 5 μM; Adriamycin, 10 μM; mitoxantrone, 0.5 μM; and Amsacrine, 2 μM.

the DNA template since this effect could be reversed by addition of exogenous primer DNA. Since little difference in the ability to displace ethidium from DNA existed between mitoxantrone and the anthrapyrazoles, the discriminative effect on DNA and RNA synthesis of these two classes of drugs most likely derives from properties distinct from differences in binding affinity. Selective inhibition of one or the other process by other intercalators has also been reported previously. With regard to the anthracyclines, preferential inhibition of DNA or RNA synthesis is strongly influenced by modifications in the chromophore and side groups. Conversion of daunorubicin to 5-iminodaunorubicin, for example, markedly reduces the potency to inhibit RNA synthesis yet inhibition of DNA synthesis remains unaltered [38]. Crook *et al.* [39] described two types of anthracyclines in which one was equally inhibitory toward both processes and the other which inhibited RNA synthesis at about a 10-fold lesser concentration than DNA synthesis. These differences appeared to depend on a carbomethoxy group at the 10 position and a glycosidic chain with two or three sugars. While not understood, some evidence indicates that the observed differences may be related to the dissociation kinetics of the drug–DNA complex [40]; however, orientation of the bound molecule and interaction with DNA or RNA polymerases [41–43] may also influence these activities.

The biochemical basis for the high incidence of cardiotoxicity observed with anthracyclines has received much attention in the last several years. Although still not fully understood, evidence is beginning to implicate the production of reactive oxygen species as a contributing factor including superoxide radical, hydroxyl radical, hydrogen peroxide and lipid peroxides [44]. The capacity for many quinone anticancer agents to be reduced to semiquinone free radicals by intracellular reductive enzymes has been supported by electron spin resonance studies [45, 46]. Reports showing superoxide dismutase sensitive oxygen consumption in the presence of liver microsomal or heart sarcosomal preparations [47, 48] and the production of lipid peroxidation products [49, 50] suggest that semiquinone free radicals are capable of donating electrons to molecular oxygen thus forming superoxide radical or to unsaturated lipids producing lipid peroxides. In this context, the anthrapyrazoles induced far less (5- to 10-fold) superoxide dismutase sensitive oxygen consumption than Adriamycin in the rat liver microsomal system, a property that may be indicative of a lesser cardiotoxicity. Further evidence that these compounds are less cardiotoxic than Adriamycin has also been suggested by studies employing a mouse fetal heart model and electron microscopy [51].

Although the precise mechanism by which intercalators produce their cytotoxic or antitumor effect is unknown, a common result in cells treated with these agents is the appearance of protein-linked single- and double-strand DNA breaks [29–31, 52]. Recent evidence indicates that these breaks may be produced by topoisomerase II in response to DNA intercalation [26–28] or interaction of the enzyme with the drug itself [53]. Whereas correlation of cytotoxicity with these DNA lesions has been low [29, 31], consistent results may be complicated by continued production of breaks after the drug is removed or the fact that most correlative studies have used drugs of different classes which may induce breaks at different specific sites on the DNA [54] with correspondingly different degrees of lethality to the cell. The anthrapyrazoles were similar to other known intercalators in that they caused both single- and, to a lesser extent, double-stranded DNA breaks in a concentration-dependent manner which appeared to be tightly associated with protein. A marked difference in the rate of formation of these breaks, however, was observed between CI-937 and CI-942. At a concentration of 1 μM, maximum DNA breakage was attained within 30 min with CI-937 whereas at least 3 hr were required in the presence of CI-942. The differences in the rates of DNA strand scission between CI-937 and CI-942 could account for the difference is cytotoxic potency *in vitro* and may reflect different transport rates into the intracellular compartment. An analogous situation exists between the rate of DNA scission caused by Amsacrine and Adriamycin with the former requiring only 10–30 min to attain a maximum single-stranded break frequency but the latter causing a linear increase in breaks for over 4 hr [30, 31]. Likewise, transport studies have shown that mAMSA equilibrates across the membrane within 10 min [31], whereas Adriamycin requires much longer to attain a steady state [55].

The cytotoxicity of a drug may also be related to the inability of a cell to recover from or repair the DNA breaks induced by intercalators rather than the quantity of these lesions. Strand breaks by the anthrapyrazoles were repaired very slowly over 30 min after drug removal, and additional lesions occurred thereafter. This pattern was similar to

Adriamycin and mitoxantrone but quite different from Amsacrine from which the cell recovered very quickly. The additional breaks occurring after drug removal may be due to drug sequestered within the cell or within membranes as has been suggested for Adriamycin and ellipticine [56]. Excessive dilution of the cells, however, did not accelerate repair of anthrapyrazole-induced DNA breaks.

In conclusion, the anthrapyrazoles produce biochemical effects similar in certain respects to other intercalators since they are potent inhibitors of nucleic acid synthesis and induce protein-associated DNA strand breaks. It is apparent, however, that these drugs have a unique pattern of biochemical effects in that they exhibit a preferential inhibition of DNA synthesis versus RNA synthesis (similar to Amsacrine) but cause apparently irreversible protein-associated DNA strand breaks (as do mitoxantrone and Adriamycin). The advantages to these particular biochemical properties, although not readily apparent, may be reflected in their unique spectrum of antitumor activity.

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