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Cytokinetic and Biochemical Effects of 5-Iminodaunorubicin in Human Colon Carcinoma in Culture

Robert I. Glazer,[1] Kathleen D. Hartman, and Carol L. Richardson

Applied Pharmacology Section, Laboratory of Medicinal Chemistry and Biology, National Cancer Institute, Bethesda, Maryland 20205 [R. I. G, K. D. H.]; and Meloy Laboratories, Springfield, Virginia 22151 [C. L. R.]

ABSTRACT

The semisynthetic anthracycline, 5-iminodaunorubicin (IM), was investigated to see whether modification of the benzoquinone moiety to produce a drug with low free radical potential would alter the cytotoxic and biochemical characteristics of this drug in comparison to Adriamycin (ADR), an agent with high free radical potential. Cell viability was measured in human colon carcinoma (HT-29) cells by soft-agar cloning. Upon exposure of either log-phase or early plateau-phase cells for 2 hr to IM or ADR, a threshold exponential cell lethality curve was obtained. Prolonging drug exposure to 24 hr produced an exponential decline in cell survival and a marked reduction in viability of both log-phase and early plateau-phase cells. Inhibition of DNA synthesis in log-phase cells after 2 and 24 hr of exposure to IM and ADR paralleled the increased cell lethality produced by the drugs. In contrast, total RNA synthesis was not inhibited by IM, whereas ADR impaired both RNA and DNA synthesis. Nuclear rRNA synthesis was not significantly inhibited following 24 hr of exposure to 10^{-7} M ADR or IM but was inhibited by 85 and 35% at 10^{-6} M ADR or IM, respectively. The affinity of IM and ADR for HT-29 DNA was measured *in vitro* by displacement of acridine orange binding and was found to be similar for both analogs. These studies suggest that the cytotoxicity of IM and ADR results from the interactions of these drugs with DNA.

INTRODUCTION

The anthracycline antibiotic, ADR,[2] is one of the most active anticancer drugs in clinical use (2, 3, 20). One of its major limitations, apart from its myelotoxicity, is the increased risk of congestive heart failure associated with an excessive cumulative dose of drug (19). Recently, a partial dissociation of the cardiotoxicity and antitumor activity of the anthracycline, daunorubicin, was achieved by modification of the benzoquinone moiety to form IM (17). IM was as potent as daunorubicin and ADR against P388 leukemia *in vivo* but was far less acutely cardiotoxic than ADR in rats *in vivo* (17). In addition, IM was more than 10-fold less cardiotoxic than ADR upon isolated rat myocytes (16) and did not induce ultrastructural damage to the nucleolus of rat myocardium *in vivo* as did ADR (15). The mechanism for the reduced cardiotoxic effects of IM is believed to result from strong intramolecular hydrogen bonding to obviate the reduction of the quinone function and the subsequent generation of superoxide anion and free radicals (13).

[1] To whom requests for reprints should be addressed, at National Cancer Institute, Building 37, Room 6D28, Bethesda, Md. 20205.

[2] The abbreviations used are: ADR, Adriamycin (doxorubicin); IM, 5-iminodaunorubicin; dThd, thymidine; RPMI Medium 1640, Roswell Park Memorial Institute Tissue Culture Medium 1640; SDS, sodium dodecyl sulfate; IC_{50}, concentration of drug required to inhibit 50%.

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Because of the strong clinical potential of IM due to its lesser dose-limiting myocardial and myelosuppressive toxicities, we examined its chemotherapeutic activity and mode of action in parallel with ADR against a human colon carcinoma in culture, and these results form the basis for this report.

MATERIALS AND METHODS

Materials. [5-*methyl*-^{3}H]dThd (20 Ci/mmol) and [*U*-^{14}C]uridine (522 mCi/mmol) were purchased from New England Nuclear, Boston, Mass. ADR·HCl was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute, and IM·HCl was kindly provided by Dr. E. Acton, Stanford Research Institute. RPMI Medium 1640 was purchased from Hem Research, Inc., Rockville, Md.; heat-inactivated fetal calf serum was purchased from Grand Island Biological Co., Grand Island, N. Y.; and gentamicin was purchased from Flow Laboratories, McLean, Va.

Tissue Culture. HT-29 cells originally derived from a human colon carcinoma (7) were obtained from Dr. L. Erickson, National Cancer Institute. Cells were grown under 5% CO_2:air in RPMI Medium 1640 supplemented with 10% fetal calf serum and gentamicin (50 µg/ml). Cell inoculums were 0.83×10^5 cells/10 ml medium in 25-sq cm plastic flasks.

Drug Treatment. Log-phase (3 day) cells or early plateau-phase (5 day) cells were treated with 10^{-8}, 10^{-7}, and 10^{-6} M ADR or IM dissolved in dimethylformamide for either 2 or 24 hr. Control flasks received an equivalent concentration (0.1%, v/v) of dimethylformamide alone, which was without effect on cell growth, cell viability, and nucleic acid synthesis. After drug treatment, cells were harvested by trypsinization with 0.05% trypsin in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} and containing 0.02 M EDTA. The growth medium was decanted, and cell monolayers were first rinsed with 3 ml of trypsin solution followed by incubation for 10 min at 37° with 0.3 ml of trypsin solution. Trypsinization was terminated by the addition of 10 ml of RPMI Medium 1640 containing 10% fetal calf serum and gentamicin (50 µg/ml).

Cell Viability Determinations. After trypsinization, cell dilutions of 200, 1,000, and 10,000 cells were prepared in RPMI Medium 1640 containing 10% fetal calf serum and gentamicin (50 µg/ml). Soft agar cloning was performed as described by Vistica *et al.* (18) except that RPMI Medium 1640 was used. Duplicate 6-cm plastic Petri dishes were plated with 200, 1,000, and 10,000 cells for each drug-treated and control flask. After 14 days, colonies were fixed with 95% ethanol and stained with 0.01% gentian violet in 1% acetic acid. Cell viability is expressed as the number of colonies produced by drug-treated cells divided by the number of colonies produced by control cells (corrected for cloning efficiency) × 100. Cloning efficiency ranged from 60 to 90%.

DNA and RNA Determinations. Following the addition of ADR or IM, cells were pulse labeled during the last hr of drug treatment with 1 µCi [5-*methyl*-^{3}H]dThd and 1 µCi [^{14}C]uridine. After trypsinization, the cells were centrifuged at 400 × *g* for 10 min at 4° and washed once with ice-cold phosphate-buffered saline (5.6 mM Na_2HPO_4:1.1 mM KH_2PO_4:0.154 M NaCl (pH 7.4). DNA and RNA were extracted by the addition of 3 ml of 1% SDS:0.1 M Tris-HCl (pH 8.0):0.01 M EDTA followed by 1.5 ml of phenol mixture [phenol:*m*-cresol:water (7:2:2; v/v) containing

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0.1% 8-hydroxyquinoline] and 1.5 ml of chloroform. After vigorously vortexing for 5 min, the emulsion was clarified by centrifugation at 10,000 × *g* for 10 min. The supernatant was removed and precipitated with 3 volumes of 2% (w/v) potassium acetate in 95% ethanol at −20° overnight. DNA and RNA were collected by centrifugation at 10,000 × *g* for 20 min and dissolved in 0.2 ml water. The sample was divided into two 0.1-ml aliquots. DNA was obtained by incubating a 0.1-ml sample with 1 ml 0.01 M Tris-HCl (pH 7.4):0.2 M NaCl:0.01 M EDTA, 20 µg RNase A, and 20 units RNase T_1 at 37° for 2 hr. RNA was obtained by incubating a 0.1-ml sample with 1 ml 0.01 M Tris-HCl (pH 7.2):0.5 M NaCl:0.01 M $MgCl_2$ and 10 µg DNase I (electrophoretically pure) at 37° for 2 hr. At the end of each incubation, 3 volumes of 2% potassium acetate in 95% ethanol were added, and samples were precipitated at −20° overnight. RNA and DNA were collected by centrifugation at 10,000 × *g* for 20 min, and aliquots were removed to determine radioactivity and absorbance at 260 nm.

Agarose Electrophoresis of rRNA. Cells were grown at 10-fold the number used in the cell viability experiments (8.3 × 10^5 cells/100 ml medium in 150-sq cm flasks) and pulse labeled for 1 hr with 10 µCi [^{14}C]uridine. Cells were washed once with 200 ml of phosphate-buffered saline, and nuclei were isolated as described previously (8). rRNA was extracted with 3 ml 0.1% SDS:0.14 M NaCl:0.02 M sodium acetate (pH 5.1) and 3 ml phenol mixture by vigorously vortexing for 5 min. The aqueous phase was removed after centrifugation at 10,000 × *g* for 10 min, and RNA was precipitated with 3 volumes of 95% ethanol at −20° overnight. RNA was electrophoresed in 2% agarose: urea:iodoacetate gels as described by Locker (11). Gels were sliced into 2-mm sections, dissolved in 0.2 ml 70% perchloric acid, and mixed with 10 ml Aquasol, and the radioactivity was determined. Approximately 0.5 A_{260} unit of RNA was applied per gel.

DNA Binding *in Vitro*. The relative affinities of IM and ADR for HT-29 DNA were determined by displacement of acridine orange binding using the fluorescent polarization assay described previously (16). The assay was modified with microcuvets, such that the final volume was 0.4 ml. DNA was obtained from HT-29 cells as described above or from calf thymus (Sigma Chemical Co., St. Louis, Mo.). The concentrations of acridine orange (5 × 10^{-7} M) and DNA (0.5 µg) remained constant at each drug concentration used.

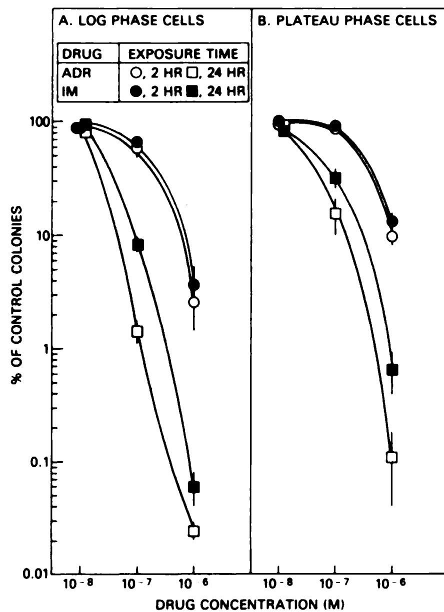

Chart 1. Viability of log- and plateau-phase cells after exposure to ADR and IM. Log- and plateau-phase cells were exposed for 2 and 24 hr to 10^{-8}, 10^{-7}, and 10^{-6} M ADR or IM. After drug exposure, cells were grown in soft agar, and colony formation was used as a measure of cell viability. Results are expressed as a percentage of colonies formed from drug-treated cells *versus* control cells taken as 100%. *Points*, mean of 6 to 8 determinations; *bars*, S.E.

RESULTS

Cell Viability. To test the proliferation dependence of IM and ADR cytotoxicity, log-phase cells and early plateau-phase cells were exposed to IM and ADR for 2 or 24 hr, and cell viability was determined by soft agar cloning (Chart 1). A 2-hr exposure of either cell population to the 2 drugs produced a threshold exponential decrease in cell survival. IM and ADR were equipotent under these conditions. Prolonging the duration of drug

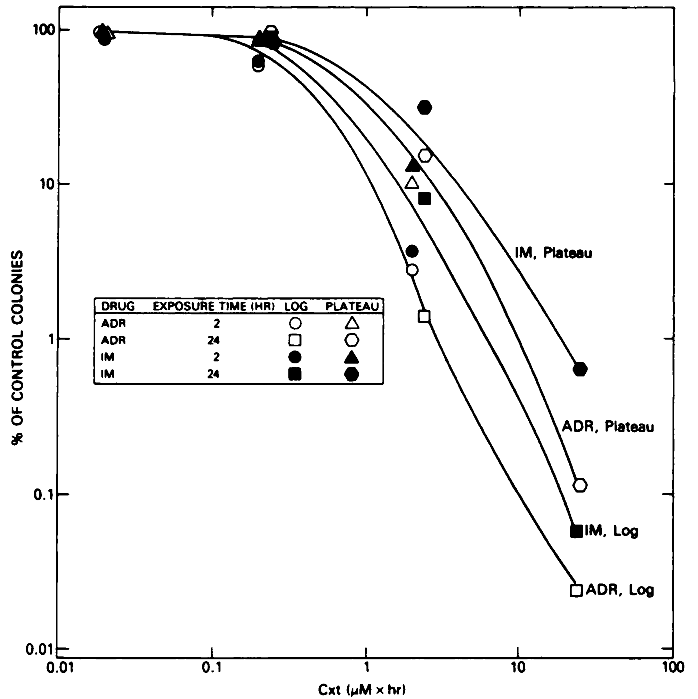

Chart 2. Concentration × time (*Cxt*) effects of ADR and IM on HT-29 cells. The cell viability of HT-29 cells as measured by colony formation in soft agar is expressed as a function of concentration × time (µM × hr) following incubation of cells for 2 or 24 hr with 10^{-8}, 10^{-7}, and 10^{-6} M ADR or IM. Results were calculated from the data presented in Chart 1.

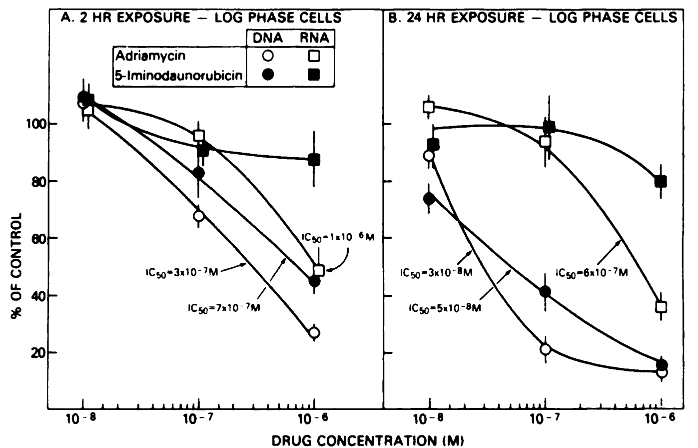

Chart 3. Incorporation of [^{3}H]dThd into DNA and [^{14}C]uridine into RNA in log-phase cells after exposure to ADR and IM. Log-phase cells were exposed for 2 and 24 hr in 10^{-8}, 10^{-7}, and 10^{-6} M ADR or IM. During the last hr of drug exposure, cells were incubated with 1 µCi [^{3}H]dThd and 1 µCi [^{14}C]uridine, and DNA and RNA were isolated as described in "Materials and Methods." Results are expressed as a percentage of the incorporation of either [^{3}H]dThd or [^{14}C]uridine into DNA and RNA, respectively, in drug-treated cells *versus* control cells taken as 100%. Control values (dpm/10^6 cells) were: *A*, DNA, 120,200 ± 6,000; RNA, 28,000 ± 2,000. *B*, DNA, 126,400 ± 10,000; RNA, 30,500 ± 3,000. *Points*, mean of 4 to 6 determinations; *bars*, S.E.

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exposure to 24 hr produced a more rapid exponential reduction in cell survival, and IM was 20 and 50% as potent as ADR at 10^{-7} and 10^{-6} M concentrations, respectively.

To determine whether a continuity existed between the effects of the drugs on cell viability and the duration of drug exposure at varying drug concentrations, cell lethality was expressed as a function of concentration × time (Chart 2). The results clearly indicate that cell lethality was a continuous function of concentration × time with the following decreasing order of sensitivity: ADR-treated log-phase cells > IM-treated log-phase cells > ADR-treated plateau-phase cells > IM-treated plateau-phase cells. Moreover, it appears that, unless a concentration × time (μM × hr) value of one is exceeded, no cell lethality will result from drug treatment.

DNA and RNA Synthesis. To obtain a measure of DNA and RNA synthesis, log-phase cells were pulse labeled for 1 hr with [^{3}H]dThd and [^{14}C]uridine, and the effects of IM and ADR were determined (Chart 3). DNA synthesis was inhibited to the greatest extent by both drugs, and this effect was more pronounced with increasing drug exposure intervals. In contrast, RNA synthesis was less sensitive to ADR and was virtually insensitive to IM. The IC_{50} of ADR for DNA synthesis was approximately 50% lower than the IC_{50} of IM after 2 or 24 hr of drug exposure. However, of greater significance was the order of magnitude reduction in the IC_{50} of both ADR and IM for DNA synthesis upon increasing the drug exposure interval from 2 to 24 hr. Under similar conditions, the IC_{50} of ADR for RNA synthesis was decreased less than 2-fold.

To determine whether the lesser sensitivity of total RNA synthesis to IM and ADR was indicative of a particular class of RNA, nuclear rRNA was measured after 24-hr drug treatment, since this species of RNA has been reported to be particularly sensitive to inhibition by anthracyclines (5) (Chart 4). IM inhibited rRNA synthesis less than 10% at 10^{-7} M, but it reduced 36S, 28S, and 18S rRNA synthesis by 35 to 50% at 10^{-6} M. Low-molecular-weight 4S RNA was not affected. In contrast, 10^{-7} M ADR inhibited 36S, 28S, and 18S rRNA by 15 to 20%, and at 10^{-6} M, it reduced all species of rRNA by 65 to 85% as well as 4S RNA by 40%. Neither drug affected the processing of rRNA.

DNA Binding *in Vitro*. In order to estimate the relative affinities of IM and ADR for DNA, HT-29 cell DNA was isolated, and

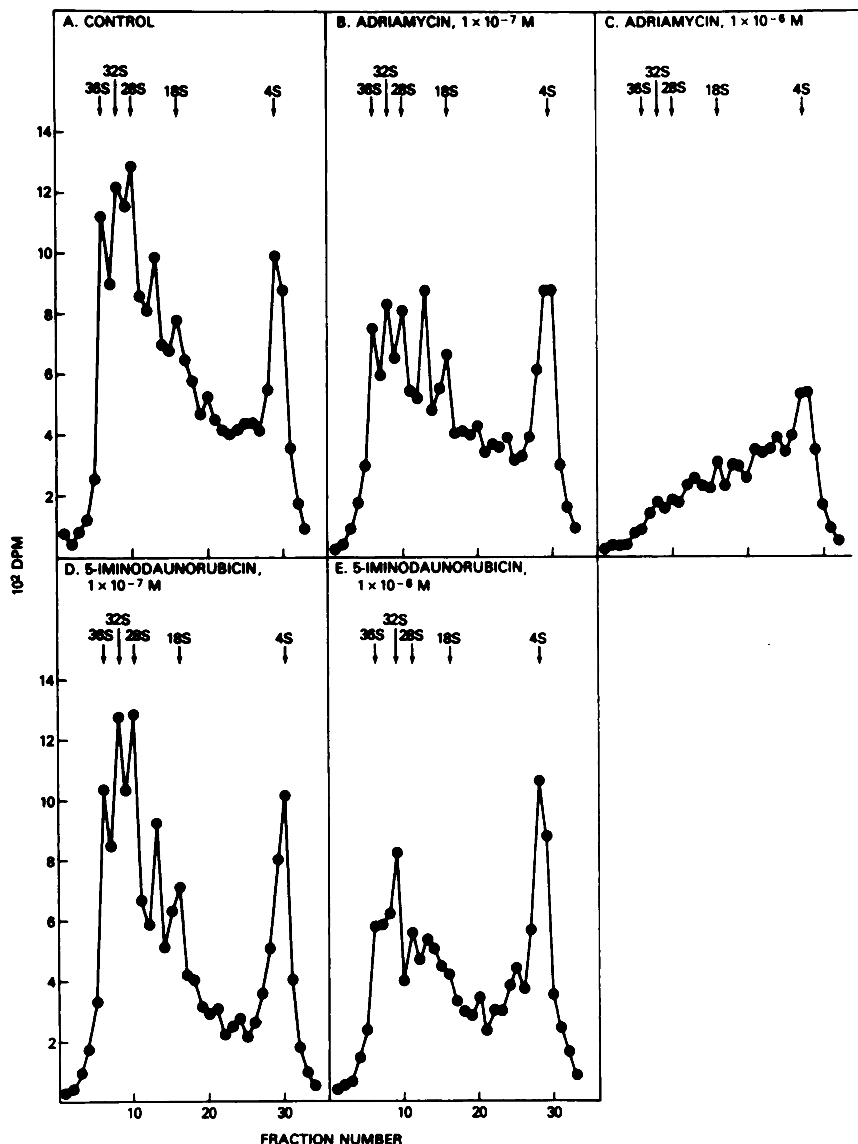

Chart 4. Agarose electrophoresis of nuclear rRNA from log-phase cells after 24-hr exposure to ADR and IM. Log-phase cells were exposed for 24 hr to 10^{-7} and 10^{-6} M ADR or IM. During the last hr of drug exposure, cells were incubated with 10 μCi [^{14}C]uridine, nuclear rRNA was isolated, and 0.5 A_{260} units of RNA were electrophoresed as described in "Materials and Methods." Control values (dpm/A_{260}) were 37,500 ± 3,500 for 5 determinations.

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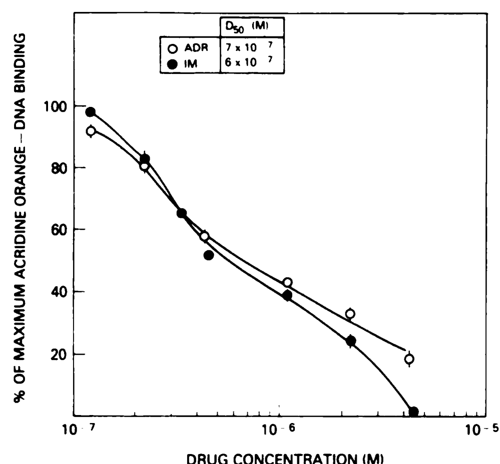

Chart 5. Displacement of acridine orange from HT-29 DNA by ADR and IM. Varying concentrations of ADR or IM were mixed with 0.01 M sodium cacodylate buffer (pH 6.7) containing 0.5×10^{-6} M acridine orange. HT-29 DNA (0.5 μg) was added, and acridine orange displacement by ADR or IM was measured by fluorescent polarization as described in "Materials and Methods." *Bars*, S.E.

competitive displacement assays of acridine orange binding were carried out (Chart 5). The concentration of drug required to displace 50% of the acridine orange bound to DNA of IM and ADR was 6×10^{-7} M and 7×10^{-7} M, respectively, indicating that both drugs have similar affinities for the intercalating sites in HT-29 cell DNA occupied by acridine orange. In parallel studies with calf thymus DNA, the concentration of drug required to displace 50% of the acridine orange bound to DNA was 2×10^{-7} M for both IM and ADR (results not shown).

DISCUSSION

The present investigation of the anthracycline analog, IM, indicates that modification of the quinone moiety may produce significant changes in its effects on nucleic acid synthesis without greatly compromising its chemotherapeutic activity. Although IM was slightly less cytotoxic than ADR, it reduced the viability of HT-29 cells by approximately the same order of magnitude. The kinetics of cell lethality produced by IM was also similar to ADR in that there appeared to be a continuity between the drug concentrations producing lethality and the duration of drug exposure for both log-phase and early plateau-phase cells. A characteristic feature of the cell lethality *versus* drug concentration curves for a 2-hr exposure interval to either drug was the threshold exponential survival of log-phase and plateau-phase cells. This effect may be indicative of the differing sensitivities in a mixed population of asynchronous cells. An increase in the duration of drug exposure to 24 hr produced a proportional enhancement in cell lethality and the expected simple exponential lethality curve, suggesting that the duration of drug exposure appears to be a crucial determinant for allowing all cells to progress through the most sensitive stage of the cell cycle. ADR has been found previously to produce maximum cytotoxicity during early to late S phase in Chinese hamster ovary and human tumor cells (1, 4, 6, 9, 10) and to yield cytokinetic (6, 9) and biochemical effects (9) similar to those in the present study. Since the cytokinetic effects produced by IM were similar to those of ADR, these data suggest a similar cell cycle dependency.

The greater inhibitor effect of ADR on DNA *versus* RNA synthesis in HT-29 cells and in HeLa cells (9) contrast with earlier results obtained with rodent tumor cells *in vitro*. It was generally observed that total RNA synthesis was slightly more or equally as sensitive as DNA synthesis to the inhibitory effects by ADR in Novikoff hepatoma (5) and L1210 leukemia (14, 17) cells in culture. These results may reflect differences in the metabolism of ADR and IM in tumor cells if the high free radical potential of ADR (13) is responsible for its inhibitory effects on RNA synthesis. Of particular interest is the fact that IM, which has a low free radical potential (13), also showed greater specificity for inhibiting DNA synthesis. These data suggest that this intrinsic property of the drug is related to its greater DNA specificity and its lesser side effects on peripheral tissues than ADR (12, 13, 17).

Analysis of nuclear rRNA indicated that the synthesis of 36S precursor rRNA was slightly more sensitive to inhibition by ADR than to total RNA or 4S RNA. However, there was still no preferential inhibition of nucleolar RNA versus DNA synthesis, a result similar to that observed with ADR in Novikoff hepatoma cells (5). In contrast, IM was not inhibitory to nuclear rRNA synthesis except at 10^{-6} M and even at the latter concentration was far less inhibitory than ADR.

Thus, in comparison to the action of ADR, IM has little or no inhibitory effect on RNA synthesis at equipotent cytotoxic concentrations. Both drugs inhibit DNA synthesis and have similar binding affinities for DNA, as demonstrated in the acridine orange displacement assay. Clearly, the benzoquinone-modified analog, IM, appears to possess interesting pharmacological properties which merit further experimental and clinical evaluation.

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