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COMPARISON OF DNA SCISSION AND CYTOTOXICITY PRODUCED BY ADRIAMYCIN AND 5-IMINODAUNORUBICIN IN HUMAN COLON CARCINOMA CELLS

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Abstract—The quinone-modified anthracycline, 5-iminodaunorubicin, which does not spontaneously generate free radicals, was compared to Adriamycin on the basis of DNA-protein crosslink-associated single-strand breakage, cell lethality, and the pharmacokinetics of drug uptake and efflux in human colon carcinoma cells in culture. At equivalent cytocidal concentrations, 5-iminodaunorubicin produced more single-strand breakage of DNA than Adriamycin after a 2-hr treatment interval, but the DNA scission produced by 5-iminodaunorubicin rapidly disappeared after drug removal. The kinetics of DNA breakage correlated with the rapid rates of uptake and efflux of 5-iminodaunorubicin in comparison to Adriamycin. These data emphasize the importance of the cellular pharmacokinetics of anthracyclines in relation to their cytocidal and DNA damaging properties. Moreover, the induction of equivalent single-strand breakage of DNA by similar intracellular concentrations of both drugs suggests that the free radical properties of Adriamycin are not involved in DNA scission.

Among the most clinically useful intercalating agents against human malignancy are the anthracyclines, as exemplified by ADR†. One of the newer anthracyclines now in preclinical testing is the benzoquinone-modified analog, IM [1]. The iminoquinone moiety of IM confers upon the drug a negligible capacity to form semiquinone radicals *in vitro*, in contrast to the spontaneous generation of ADR semiquinone-free radicals [2]. IM, after reductive activation *in vitro*, produces single-strand nicking of supercoiled closed circular PM2 DNA at one-tenth the rate of daunorubicin [3]. The unusual stability of reduced IM against reoxidation relates to its reduced capacity to generate reactive oxygen species [3, 4] and its lesser acute cardiotoxic [1, 5, 6] and mutagenic [1] effects in comparison with ADR.

Recent investigations comparing ADR with IM have shown the latter compound on a molar basis to be almost as cytocidal to human colon carcinoma cells as ADR, but to possess more specificity for inhibiting DNA in comparison to RNA synthesis [7]. Because of these differences, it was of interest to examine the ability of these agents to produce SSB in DNA and to compare this phenomenon with their respective toxicities and pharmacodynamics in human colon carcinoma HT-29 *in vitro*.

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† Abbreviations: ADR, Adriamycin (doxorubicin); IM, 5-iminodaunorubicin; PBS, phosphate-buffered saline (6.3 mM Na_2HPO_4, 0.8 mM KH_2PO_4, 0.154 M NaCl, pH 7.4); SSB, single-strand breakage; HPLC, high pressure liquid chromatography; and dThd, thymidine.

MATERIALS AND METHODS

Materials. ADR·HCl and IM·HCl were obtained from Dr. Edward Acton, Stanford Research Institute, Menlo Park, CA. These drugs were determined to be 100% pure as assessed by HPLC described below.

Cell culture. HT-29 cells were grown at 37° under 5% CO_2:air in RPMI medium 1640 containing 10% heat-inactivated fetal calf serum and gentamicin, 50 μg/ml. Cells were initially plated at 0.83×10^5 cells/10 ml medium in 25 cm^2 plastic flasks. Colony formation in soft agar was determined as previously described [7].

Drug treatment. Log phase cells were prelabeled with 1 μCi [^{14}C]dThd for 2 days followed by a 1-day chase with [^{14}C]dThd-free medium. Cells were treated on day 3 for 2 hr with 1 or 2.5 μM ADR or IM as indicated. In those experiments in which the time course of DNA break disappearance or drug efflux was determined, the drug-containing medium was replaced with fresh drug-free medium, and incubation was continued at 37° for the indicated time periods.

SSB and DNA-protein crosslinking. Following drug treatment, medium was removed and the cells were rinsed with ice-cold Hanks' balanced salt solution without Ca^{2+} or Mg^{2+} and containing 20 mM EDTA. The cells were then scraped into 2 ml of the medium and dispersed by vigorous pipetting. The quantitation of SSB and DNA-protein crosslinking

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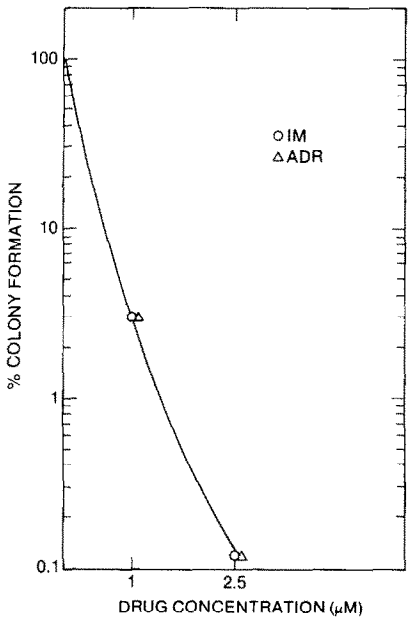

Fig. 1. Cell lethality of IM and ADR. HT-29 cells were treated for 2 hr with 1 and 2.5 μM IM or ADR, and colony formation was determined as described in Materials and Methods.

was carried out by the technique of alkaline elution in the presence and absence of proteinase K [8, 9].

Drug efflux and metabolism. At the specified time intervals after 2 hr of drug treatment, cells were washed three times with ice-cold PBS and extracted twice with 5 ml chloroform–methanol (3:1, v/v). The chloroform–methanol extracts were combined and evaporated under vacuum, and the residue was dissolved in 0.3 ml methanol and used immediately for analysis by HPLC. HPLC was performed using an Altex Ultrasphere ODS 5 μM column (0.46 × 25 cm) and isocratic elution at 1.5 ml/min with 50 mM KH_2PO_4 (pH 3.8)–methanol (35:65, v/v) for ADR or 50 mM KH_2PO_4 (pH 3.8)–methanol (40:60, v/v) for IM. The parent drug and metabolites were detected with a Gilson Spectra-Glo fluorometer with excitation wavelengths of 480 and 546 nm for ADR and IM, respectively, and emission wavelengths of 580 and 600–650 nm for ADR and IM, respectively. Recovery was greater than 90% for both drugs, and no spontaneous degradation of drug occurred.

RESULTS

The cytocidal effects of ADR and IM following a 2-hr treatment of HT-29 cells were measured by colony formation in soft agar (Fig. 1). At drug concentrations of 1 and 2.5 μM, ADR and IM both produced a 97 and 99.9% reduction in colony formation.

To determine whether the lethal effects of ADR and IM were associated with DNA strand breakage, DNA-protein crosslink-associated SSB was determined by alkaline elution using incubation conditions identical to those in the cell lethality experiments. At equivalent (equitoxic) drug concentrations, IM produced 3-fold more SSB than ADR (Fig. 2A); however, the disappearance of SSB produced by IM was very rapid with a $t_{1/2}$ of approximately 30 min (Fig. 2B). In contrast, the SSB produced by ADR remained virtually unchanged at 6 hr after drug removal. The ratio of SSB to DNA-protein crosslinking equaled 0.68 and 0.98 for ADR and IM, respectively (data not shown). Under these experimental conditions, rapid cell death or cell lysis did not occur and, hence, could not have accounted for the SSB produced by either drug. Furthermore, more than 95% of the radioactivity applied to the filter was not solubilized after addition of lysing buffer and immediately prior to alkaline elution. Total recovery of labeled DNA following alkaline elution

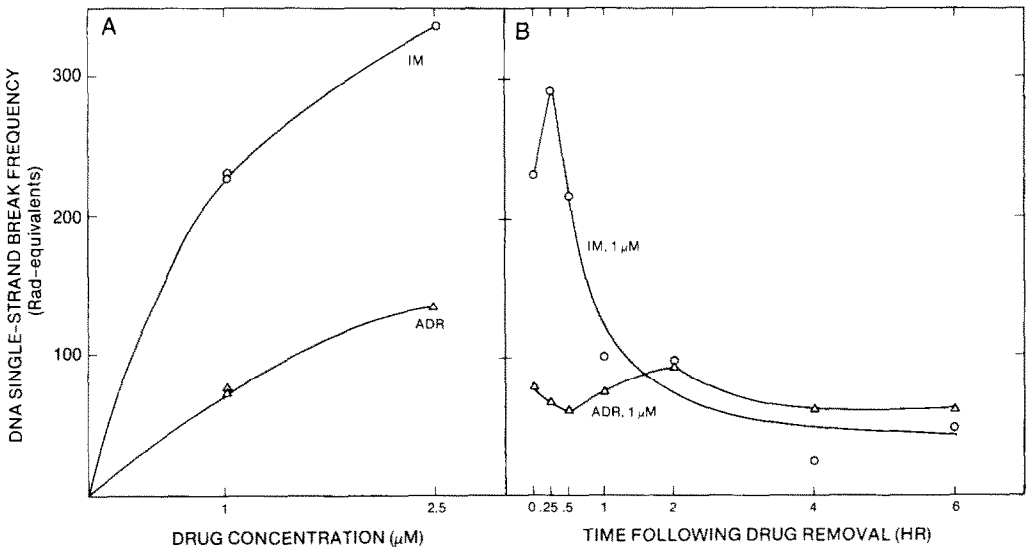

Fig. 2. DNA single-strand breakage in HT-29 cells following exposure to IM and ADR. Cells were treated for 2 hr with 1 and 2.5 μM ADR or IM (A) or for 2 hr with 1 μM ADR or IM followed by drug removal (B). SSB was measured by alkaline elution as described in Materials and Methods.

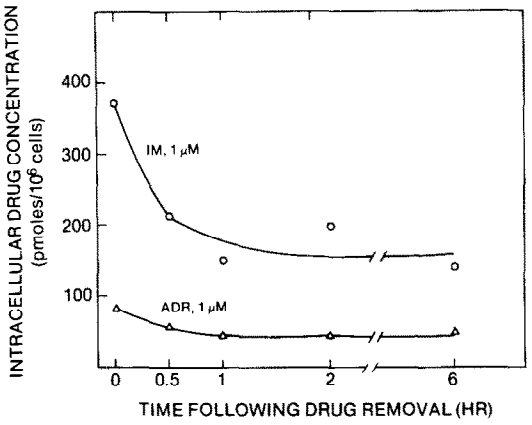

Fig. 3. Drug efflux in HT-29 cells treated with IM and ADR. Cells were treated as described in Fig. 2B, and drug concentrations were measured in chloroform–methanol cell extracts by HPLC as described in Materials and Methods. Each value is the mean of duplicate determinations.

was virtually identical between the control and drug-treated populations of cells.

Since the kinetics of the formation and disappearance of SSB by ADR and IM suggested that these drugs may possess markedly different transport properties, the concentrations of unmetabolized intracellular drugs were determined under the same conditions used for the assessment of cell lethality and SSB (Fig. 3). The initial intracellular drug concentration of IM and ADR 2 hr after addition to the medium indicated that IM reached a level that was about 4-fold greater than ADR. The rate of efflux of IM was rapid with a $t_{1/2}$ of approximately 30–60 min. ADR concentrations decreased by 40% 30–60 min after drug removal and, like IM, did not decrease further over 6 hr.

The metabolism of ADR and IM was assessed by HPLC (Fig. 4). After 2 hr of incubation, a minute trace of alcohol metabolite of IM was detected (as judged by comparison of its retention time with an IM alcohol standard), but no other metabolite was observed (Fig. 4A); 1 hr after drug removal, only unmetabolized IM was present (Fig. 4C). Following similar treatment with ADR, predominantly unmetabolized drug was noted either 2 hr after treatment (Fig. 4B) or 1 hr after drug removal (Fig. 4D).

DISCUSSION

The present study was designed initially to assess the significance of SSB of DNA in human tumor cells treated with equivalent cytocidal concentrations of ADR or IM. The latter drug was of particular interest since it did not spontaneously generate free radicals [2] or readily generate reactive oxygen species [3, 4]. Unexpectedly, IM initially produced three times the SSB of DNA as compared to ADR at equitoxic concentrations after 2 hr of treatment. It appeared from the kinetics of SSB that IM was taken up rapidly and was metabolized or else removed from the cell at a more rapid rate than ADR. The transport kinetics of IM and ADR indicated that the initial increase in SSB frequency produced by IM over that produced by ADR is a result of its rapid transport into the cell. The rapidity by which IM egresses from the cell without being metabolized significantly also suggests an explanation for the similar cytotoxicities produced by IM and ADR. Since colony formation is measured 2 weeks after the plating of drug-treated cells, this time interval allows the intracellular concentrations of each drug to reach the same steady-state level.

IM has a low capacity to spontaneously generate free radicals [2], a property consistent with its lesser acute cardiotoxicity than ADR [1, 6]. The present,

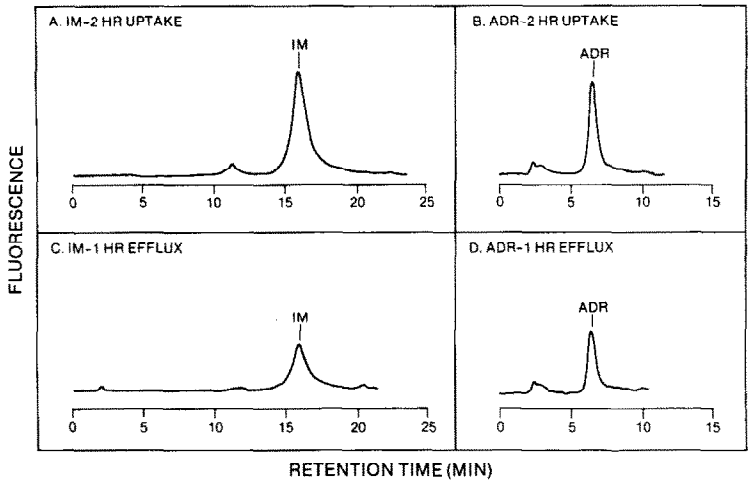

Fig. 4. HPLC of extracts of HT-29 cells treated with IM and ADR. Cells were treated as described in Fig. 2 and extracted 2 hr after drug treatment (A and B) or 1 hr after drug removal (C and D). HPLC was performed as described in Materials and Methods. The peaks eluting at 11 and 16 min in (A) and (C) represent the 13-hydroxy metabolite of IM and unchanged IM, respectively, while the peaks eluting at 2–2.5 and 6 min in (B) and (D) present endogenous fluorescent material present in untreated cell extracts and unchanged ADR respectively.

data suggest that IM has the same capacity to induce SSB in DNA as ADR when intracellular concentrations are taken into account. This phenomenon would imply that SSB is a property of the binding of the drug to DNA and not the generation of free radical intermediates. Indeed, both drugs have equivalent DNA binding characteristics [1, 7]. However, the present study does not distinguish between DNA scission being either a result of, or directly responsible for, drug-related cell death or a general phenomenon of intercalation *per se*. The prominent, rapidly reversing component of SSB produced by IM is similar to that which is produced by intercalators of the acridine and ellipticine classes [8, 10, 11]. It has been speculated previously that the reversible form of DNA scission represents the action of cellular topoisomerases which respond to the helical distortion produced by intercalation [10, 11]. Further kinetic studies will be necessary to demonstrate whether or not this effect is causally associated with cell lethality in these cells.

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