Topoisomerase II Inhibition and Cytotoxicity of the Anthrapyrazoles DuP 937 and DuP 941 (Losoxantrone) in the National Cancer Institute Preclinical Antitumor Drug Discovery Screen

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Background: **The cumulative cardiotoxicity of anthracyclines is thought to result from the generation of free radicals. New DNA topoisomerase II inhibitors less prone to redox reactions, such as mitoxantrone and more recently the anthrapyrazoles, were developed to circumvent this toxicity.** ***Purpose:*** **Two anthrapyrazoles currently in clinical evaluation, DuP 941 (Losoxantrone) and DuP 937, were compared to other topoisomerase II inhibitors with respect to their cytotoxic potency and selectivity and with respect to topoisomerase II inhibition.** ***Methods:*** **Cytotoxicity was tested in the 60 cell lines of the National Cancer Institute preclinical antitumor drug discovery screen (NCI screen). The potency of anthrapyrazoles to inhibit purified topoisomerase II was determined. The specificity of drug-induced topoisomerase II pattern of cleavage, one of the cellular determinants of cytotoxicity, was investigated in human c-myc DNA.** ***Results:*** **Using the COMPARE analysis, we found that the most closely related cytotoxic profiles in the NCI screen were between the anthrapyrazoles and mitoxantrone. Among topoisomerase II inhibitors, the cytostatic potency was by decreasing order: mitoxantrone; doxorubicin, which was slightly greater than DuP 941, azatoxin; DuP 937; and amsacrine, which was much greater than VP-16. The potency of mitoxantrone and anthrapyrazoles to generate DNA double-strand breaks, by induction of the topoisomerase II cleavable complexes in nuclear extracts, was in agreement with cytotoxicity. Sequencing of drug-induced topoisomerase II cleavages in c-myc DNA showed a common cleavage pattern for anthrapyrazoles and mitoxantrone. This pattern was different from the patterns obtained with other topoisomerase II inhibitors.** ***Conclusion:*** **At the molecular and cellular levels, anthrapyrazoles are potent topoisomerase II inhibitors closely related to mitoxantrone.** ***Implications:*** **These results validate the COMPARE analysis using the NCI screen to predict molecular mechanisms of drug action. Anthrapyrazoles, which are unlikely to produce free radicals, might be useful in the same indications as mitoxantrone, especially for patients with cardiac risks, for pediatric patients, and for patients treated with intensified protocols. [J Natl Cancer Inst 86:1239-1244, 1994]**

Anthracyclines are among the most active anticancer drugs. However, their cumulative cardiotoxicity often limits their use. This toxicity has been attributed to redox reactions that generate reactive free radicals [reviewed in (*1*)]. Related compounds less prone to redox reactions, such as mitoxantrone, have been developed to circumvent this problem. The persistence of a reducible dihydroxylnaphthoquinone moiety in the molecule (*see* Fig. 1, part drawn in bold in mitoxantrone structure) was still responsible for some cardiotoxicity (*2*). Since hydroxyl groups were necessary for mitoxantrone activity (*3,4*), a deactivation of the naphthoquinone was obtained through the substitution of one keto by an imino group that was included in a pyrazole ring leading to the anthrapyrazoles (Fig. 1). Three anthrapyrazole compounds (DuP 937, DuP 941, and DuP 942) are under clinical evaluation. They induce cellular DNA damage consistent with DNA topoisomerase II inhibition (*5,6*). Among these derivatives, DuP 941 (Losoxantrone; Du Pont Merck Pharmaceutical Co., Wilmington, Del.) appears to offer the best prospect of development as an anticancer agent in the treatment of advanced breast cancer and shows promising response rates in phase II clinical trials (*7,8*).

In the present study, the cytotoxicity of DuP 941 and DuP 937 was tested, and their cytotoxic potency and selectivity were compared with that of mitoxantrone and other topoisomerase II inhibitors using the 60 cell lines of the National Cancer Institute preclinical antitumor drug discovery screen (NCI screen). Topoisomerase II inhibition was also determined using purified enzyme and DNA.

Materials and Methods

Drugs, Chemicals, and Enzymes

The anthrapyrazoles DuP 937 and DuP 941 were obtained from Dr. Janet G. Dzubow, Du Pont Merck Pharmaceutical Co. Etoposide (VP-16) and teniposide (VM-26) were obtained from Bristol-Myers Co. (Wallingford, Conn.). 5-Iminodaunorubicin, mitoxantrone, and amsacrine were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment (National Cancer Institute, Bethesda, Md.). Azatoxin was synthesized by Dr. T. Macdonald, Department of Chemistry, University of Virginia (Charlottesville). Drug stock solutions were made in dimethyl sulfoxide (DMSO) at 10 m*M*, and further dilutions were made in distilled water immediately before use. The final concentration of DMSO in culture medium or enzymatic reaction did not exceed 1% (vol/vol), a concentration nontoxic to the cells and without detectable effect on in vitro reactions of topoisomerase II.

We purchased human c-myc DNA from the American Type Culture Collection (Rockville, Md.), restriction enzymes and T4 polynucleotide kinase from New England Biolabs (Beverly, Mass.), *Taq* DNA polymerase from Perkin Elmer (Norwalk, Conn.), and polyacrylamide/bis-acrylamide and simian virus 40 (SV40) from Life Technologies, Inc. (Gaithersburg, Md.). [γ-^{32}P]Adenosine 5′-triphosphate and [α-^{32}P]deoxyadenosine triphosphate were purchased from Du Pont NEN (Boston, Mass.). DNA topoisomerase II was purified from mouse leukemia L1210 cell nuclei as described previously (*9*) and was stored at −70 °C in 40% (vol/vol) glycerol, 0.35 *M* NaCl, 5 m*M* $MgCl_2$, 1 m*M* EGTA, 1 m*M* KH_2PO_4, 0.2 m*M* dithiothreitol, and 0.1 m*M* phenylmethanesulfonyl fluoride (pH 6.4). The purified enzyme yielded a single 170-kd band after silver staining of a sodium dodecyl sulfate (SDS)–polyacrylamide gel (*9*). Primer oligonucleotides for polymerase chain reaction (PCR) were prepared using a 392 DNA synthesizer from Applied Biosys-

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See "Notes" section following "References."

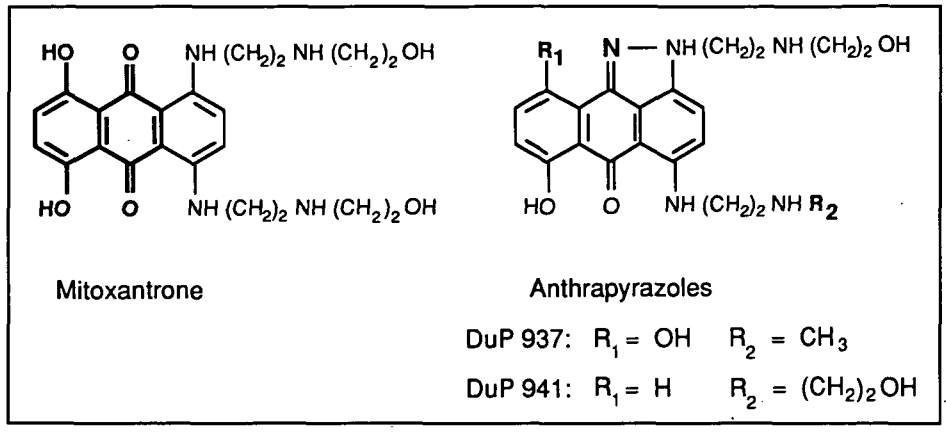

Fig. 1. Chemical structure of mitoxantrone and the anthrapyrazoles DuP 937 and DuP 941 (Losoxantrone). The hydroxylnaphthoquinone moiety of mitoxantrone structure, which is probably involved in cardiotoxicity, is indicated in bold.

tems (ABI; Foster City, Calif.) and purified using oligonucleotide purification cartridges (ABI).

NCI Preclinical Antitumor Drug Discovery Screening Assays

The methodology employed by the NCI screening program has been discussed in detail (*10*). Cellular response was evaluated using a sulforhodamine-B assay (*10*). Briefly, the human tumor cell lines making up the screening panel were grown in RPMI-1640 medium containing 5% fetal bovine serum and 2 m*M* L-glutamine. Cells were plated in 100-µL aliquots into 96-well tissue culture dishes at appropriate cell densities and then incubated at 37 °C for 24 hours prior to the addition of drug. Drugs were solubilized in DMSO and added in sequential 10-fold dilutions. After incubation of the plates at 37 °C for 48 hours, the remaining cells were fixed with trichloroacetic acid (final concentration, 10%), stained with 0.04% sulforhodamine-B for 10 minutes, and washed with 1% acetic acid to remove excess stain. Protein-bound stain was solubilized in 50 mL Tris buffer, and optical density (OD) was read in an automated plate reader at 515 nm. Percent growth inhibition was calculated for each cell line from three different OD measurements: untreated control OD (C), drug-treated test OD (T), and zero time OD (T_0) (OD of untreated cells at time of drug addition). If $T \geq T_0$, the following equation is used: $100 \times [(T - T_0)/(C - T_0)]$. If $T < T_0$, the following equation is used: $100 \times [(T - T_0)/T_0]$.

Preparation and correlation coefficient calculations used in COMPARE have been reported (*11*). Briefly, appropriate data averaging was used for both database and seed compound data when multiple experiments were available over the same concentration range. Seed and database data (as the $\log_{10}$ of the GI_{50}) were then matched by cell line, and the correlation coefficients were calculated over the set of cell lines in common to the seed and each database compound. The method used to calculate the Pearson correlation coefficient was that implemented in the SAS Proc Corr procedure with OUTP option (Version 6) (SAS Institute Inc., Cary, N.C.).

Preparation of End-Labeled DNA Fragments

SV40 DNA was labeled at both termini of the *Bcl* I restriction site using 1 U of *Taq* DNA polymerase and [α-^{32}P]deoxyadenosine triphosphate. Human c-myc DNA fragments were prepared by PCR. The 403–base-pair DNA fragment from the junction between the first intron and first exon was amplified between positions 2671 and 3073, with numbers referring to GenBank genomic positions, using the following oligonucleotides: 5′-TGCCGCATCCACGAAACTTTGC-3′ as sense primer and 5′-CTTGACAAGTCACTTTACCCCG-3′ as antisense primer. Single-end–labeling of the DNA fragment was obtained by 5′-end–labeling of the sense strand primer oligonucleotide. Labeling, PCR conditions, and DNA purifications were as previously described (*12*), except that 0.1 µg of the c-myc DNA digested with restriction enzymes with *Xho* I and *Xba* I was used as a template for the PCR.

Topoisomerase II-Induced DNA Cleavage Reactions

DNA fragments were equilibrated with or without drug in 10 m*M* Tris-HCl (pH 7.5), 50 m*M* KCl, 5 m*M* $MgCl_2$, 0.1 m*M* EDTA, 1 m*M* adenosine 5′-triphosphate, and 15 µg/mL bovine serum albumin for 5 minutes before addition of purified topoisomerase II (40-70 ng) or HL60 nuclear extract, which serves as a source for topoisomerase II (2 µL corresponding to the extract from approximately 10^6 nuclei), in a final reaction volume of 20 µL (*13*). Reactions were performed at 37 °C for 30 minutes and then stopped by adding sodium dodecyl sulfate (SDS) to a final concentration of 1% and proteinase K to 0.4 mg/mL, followed by incubation for 1 hour at 42 °C.

Electrophoresis and Data Analysis

For agarose gel analysis, 3 µL (10×) loading buffer (0.3% bromophenol blue, 16% Ficoll, and 10 m*M* Na_2HPO_4) was added to each sample, which was then heated at 65 °C for 1-2 minutes before loading into an agarose gel made in (1×) TBE [89 m*M* Tris, 89 m*M* boric acid, and 2 m*M* EDTA (pH 8.0)]. Agarose gel electrophoresis was at 2 V/cm overnight. The quantification of drug-induced DNA double-strand breaks in the presence of HL60 nuclear extracts was done as follows: Radioactive gels were scanned using a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). For each lane, the radioactivity was measured in the DNA cleavage products (C) and in the total DNA present in the lane (T). Drug-induced cleavage was expressed as:

$$\text{Percent DNA cleaved} = 100 \times \frac{C/T - C_0/T_0}{1 - C_0/T_0},$$

where C_0 and T_0 are the counts for cleaved and total DNA, respectively, in the presence of nuclear extracts without drug.

For DNA sequence analysis, samples were precipitated with ethanol and resuspended in 2.5 µL loading buffer (80% formamide, 10 m*M* NaOH, 1 m*M* EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue). Samples were heated to 90 °C and immediately loaded into DNA sequencing gels (7% polyacrylamide; 19:1, acrylamide:bis) containing 7 *M* urea in (1×) TBE buffer. Electrophoresis was performed at 2500 V (60 W) for 4 hours. Gels were dried on 3M paper sheets (Minnesota Mining and Manufacturing, St. Paul) and autoradiographed with Kodak XAR-2 film.

Base Preference Analysis

Determination of preferred bases around topoisomerase II cleavage sites was as previously described (*14*).

Results

Cytotoxicity of Anthrapyrazoles in the NCI Preclinical Antitumor Drug Discovery Screen

The anthrapyrazoles DuP 941 and DuP 937 were tested in the in vitro NCI screen under standard conditions (Fig. 2). For comparison, we included results obtained with other topoisomerase II inhibitors, as well as azatoxin, a drug active on both tubulin polymerization and topoisomerase II (*15*), and vinblastine, an inhibitor of tubulin polymerization. The data for mean drug concentrations producing 50% inhibition of cell growth (GI_{50}) are presented according to the NCI mean graph format (*16*). The mean drug concentrations producing GI_{50} are indicated at the top of Fig. 2. The cytostatic potency was by decreasing order, vinblastine >> mitoxantrone > doxorubicin > DuP 941 > azatoxin > DuP 937 > amsacrine >> VP-16. The anthrapyrazoles were most active in the central nervous system cancer cell lines, the leukemia cell lines, and the lung cancer cell lines.

The patterns of cell sensitivity to all the drugs presently in the NCI Standard

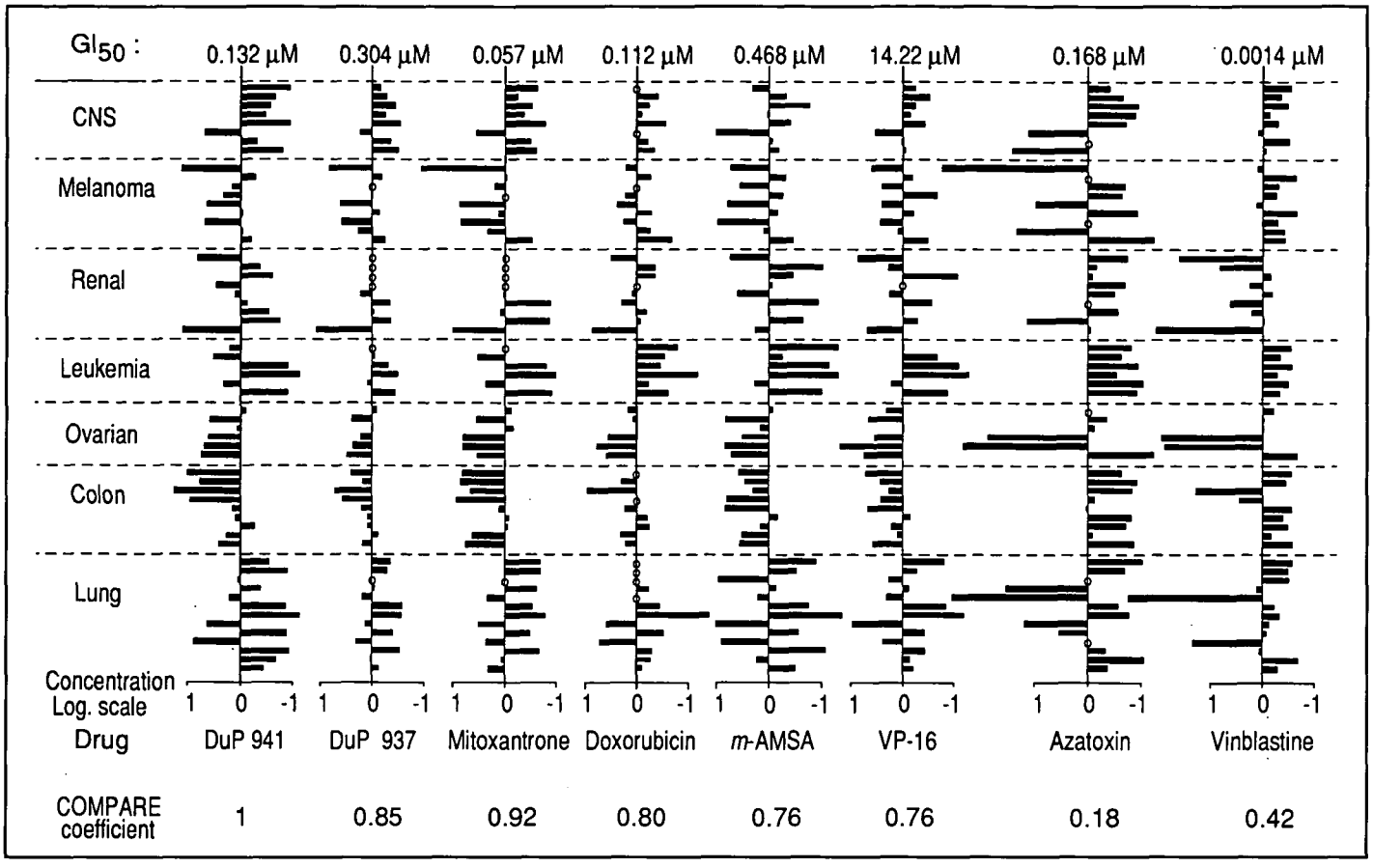

Fig. 2. Comparison of the GI_{50} mean graph profiles for DuP 941 with those of different anticancer agents in the NCI preclinical antitumor drug discovery screen. For each drug, the mean GI_{50} obtained for all the cell lines is indicated at the top of the profile and is represented by a vertical bar through individual plots. Horizontal bars show, in a logarithm scale, the individual GI_{50} for each cell line relative to the mean value. A small circle on the vertical reference bar indicates the absence of available data. Negative values (on the right of the vertical reference) indicate the most sensitive cell lines. At the bottom of the graph are indicated the overall similarities of the GI_{50} profiles between DuP 941 and other compounds, as calculated by the COMPARE software. The NCI screen cell lines are from top to the bottom, central nervous system (CNS): XF 498, SF-539, SF-295, SF-268, U251, SNB-78, SNB-75, and SNB-19; melanoma: UACC-257, UACC-62, M19-MEL, M14, SK-MEL-28, SK-MEL-5, SK-MEL-2, MALME-3M, and LOX IMVI; renal cancer: TK-10, ACHN, 786-0, RXF-631L, RXF-393L, CAKI-1, A498, SN12C, and UO-31; leukemia: SR, RPMI-8226, HL-60 (TB), MOLT-4, K-562, and CCRF-CEM; ovarian cancer: SK-OV-3, IGR-OV-1, OVCAR-8, OVCAR-5, OVCAR-4, and OVCAR-3; colon cancer: KM20L2, KM12, HCT-15, DLD-1, COLO 205, SW-620, HCT-116, HCC-2998, and HT29; small-cell lung cancer: DMS 273 and DMS 114; and non–small-cell lung cancer: LXFL 529, HOP-92, HOP-18, HOP-62, NCI-H460, NCI-H322M, NCI-H226, EKVX, A549/ATCC, NCI-H522, and NCI-H23.

Agent database (171 discrete compounds with well-characterized activity) were computed using the COMPARE software (*16*) and DuP 941 as a reference (bottom of Fig. 2). The best COMPARE correlation coefficients were between the two anthrapyrazoles (.85) and between DuP 941 and mitoxantrone (.92). Comparison of the two anthrapyrazoles with mitoxantrone (Fig. 2) showed a higher correlation coefficient for DuP 941 (.92) than for DuP 937 (.85), suggesting the importance of the side chain for drug activity (*see* Fig. 1). A good correlation was also found with the other topoisomerase II inhibitors (COMPARE coefficients were .80 for doxorubicin and .76 for amsacrine and VP-16), regardless of the action of the drugs on the RNA/DNA synthesis inhibition ratio (*5*). There was no correlation with azatoxin and vinblastine, a finding in agreement with the report that azatoxin preferentially acts first on tubulin and then on topoisomerase II while drug concentrations were increased (*15*). These data suggest that anthrapyrazoles have the same cellular target as mitoxantrone, topoisomerase II.

Induction of Topoisomerase II-Mediated DNA Double-Strand Breaks by Anthrapyrazoles and Mitoxantrone

Drug-induced, topoisomerase II-mediated, double-strand breaks were investigated using nuclear extracts of human myelocytic leukemia-derived cells (HL60) and SV40 DNA, which is a good substrate for topoisomerase II as it is rich in topoisomerase II sites (*13,17*). Mitoxantrone induced twice as many topoisomerase II-mediated DNA double-strand breaks as the two anthrapyrazoles. DuP 941 was slightly more potent than DuP 937 (Fig. 3). Cleavage decreased at the highest doses, a finding consistent with DNA intercalation of these drugs (results not shown). These data demonstrate that anthrapyrazoles are topoisomerase II inhibitors. Their relative potencies in the topoisomerase II assays are consistent with the drug cytotoxic potencies in the NCI screen (*see* Fig. 2).

Sequence Selectivity of Topoisomerase II Cleavage Induced by Anthrapyrazoles

Topoisomerase II inhibitors display considerable variation in activity as ob-

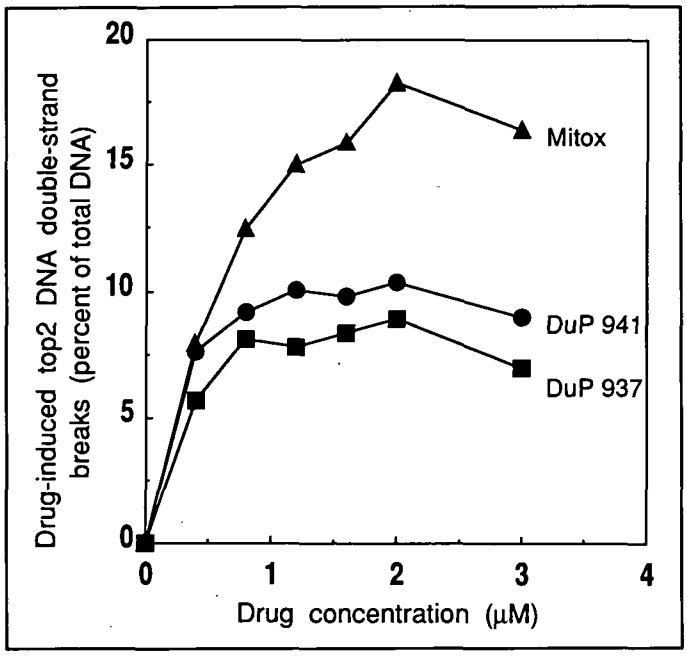

Fig. 3. Potency of DuP 937, DuP 941, and mitoxantrone to induce DNA double-strand breaks in the presence of HL-60 nuclear extracts. ^{32}P-end–labeled SV40 DNA was reacted with the drug and the nuclear extracts for 30 minutes at 37 °C. Reactions were stopped by adding SDS and proteinase K. DNA fragments were separated in 1% agarose gel, and quantification of double-strand breaks was performed using a PhosphorImager.

served clinically. This variability could be related to DNA sequence selectivity of topoisomerase II cleavage sites, which are different for each class of drug (*18*). Topoisomerase II cleavage sites were mapped in the human c-myc proto-oncogene at the junction between the first intron and exon, a region important for transcription regulation (*19*). Fig. 4 shows that anthrapyrazoles and mitoxantrone share a common cleavage pattern. The stronger cleavage intensities observed for mitoxantrone (*see* sites at position 2784-2785) are consistent with the results obtained in SV40 DNA (Fig. 3). The two anthrapyrazoles behaved similarly, and differences were minimal with respect to specificity, e.g., site 2761 for DuP 941. Other topoisomerase II inhibitors, amsacrine (Fig. 4, lane 1) and 5-iminodaunorubicin (Fig. 4, lane 2), showed cleavage patterns very different from those of mitoxantrone (Fig. 4, lanes 3 and 8) and anthrapyrazoles (Fig. 4, lanes 9 and 10). VM-26 induced more cleavage sites than the other topoisomerase II inhibitors. The anthrapyrazole sites appear as a subset of the VM-26 preferred sites. Differences between cleavage patterns are due to different preferences for specific bases flanking the cleavage site and are characteristic of each topoisomerase II inhibitor (*14,18*). For mitoxantrone and anthrapyrazoles, our analysis on 57 sites in the c-myc DNA and 22 strong sites in SV40 DNA (data not shown) showed a strong preference for a cytosine at the 3′-DNA termini of the topoisomerase II cleavage sites.

Discussion

This is the first study evaluating the cytotoxic activity of the anthrapyrazoles, DuP 941 and DuP 937, in the 60 human cell lines of the NCI screen. To the best of our knowledge, it is also the first documented demonstration of topoisomerase II inhibition by these two drugs. The patterns of cytotoxicity in the NCI screen and the topoisomerase II cleavage patterns could provide a reference for comparison with other topoisomerase II inhibitors.

Topoisomerase II inhibition by anthrapyrazoles is consistent with previous alkaline elution data, showing the stimulation of protein-linked DNA strand breaks (*5,6*). Still, anthrapyrazole cytotoxicity could be related to effects on other targets as well. For instance, azatoxin was shown to induce topoisomerase II cleavable complexes both in vitro (*13*) and in cell culture (*15*). However, analysis of NCI screen data using the COMPARE analysis showed that the cytotoxicity profile of azatoxin correlates better with those of tubulin inhibitors than with the profiles of other topoisomerase II inhibitors. Further studies (*15*) confirmed that azatoxin inhibits tubulin polymerization in vitro and that its cytotoxicity involves tubulin inhibition at low doses and topoisomerase II inhibition at higher doses. The COMPARE analysis has previously identified several novel tubulin-binding agents (*20,21*) as well as novel antimetabolites, including the inosine 5′-monophosphate dehydrogenase inhibitor benzamide riboside (*22*) and dihydroorate dehydrogenase inhibitors (Paull KD: unpublished data). A full description of the COMPARE algorithm with many examples of mechanism-based correlations from the NCI Standard Agent database has been prepared (*11*). The COMPARE analysis revealed that the mechanism of drug cytotoxicity for the two anthrapyrazoles correlated well with that of other topoisomerase II inhibitors and best with that of mitoxantrone (Fig. 2), suggesting that the cellular target of anthrapyrazoles leading to cell death is topoisomerase II.

Anthrapyrazoles trap topoisomerase II at the same sites as mitoxantrone (Fig. 4) with preferences for a cytosine at the 3′-DNA termini of the topoisomerase II cleavage site, a more restrictive preference than has been reported (*23*). This selectivity may be due to the preferential binding of anthrapyrazoles and mitoxantrone to GC-rich DNA regions (*24*). These drug–topoisomerase–DNA interactions have been modeled in the stacking model [for review *see* (*18*)]. Among topoisomerase II inhibitors, anthrapyrazoles are most closely related to mitoxantrone, both with respect to topoisomerase II cleavage pattern and to cytotoxicity profiles. Therefore, the sequence selectivity of topoisomerase II cleavage seems to be a major component of the cytotoxicity and selectivity of topoisomerase II poisons (*25*).

The preclusion of free radical formation by elimination of the quinone function was the starting point for anthrapyrazole development. These redox reactions increase oxygen consumption, generate lipid peroxides, and account for the cardiotoxicity and hepatotoxicity of mitoxantrone and anthracyclines (*1,26*). Metals such as iron can be complexed by the hydroxynaphthoquinone function (*1,27*) and may catalyze these adverse reactions. Indeed, ICRF187, a metal chelator, reduces the cardiotoxicity of mitoxantrone and doxorubicin (*1,28*). Alteration of the quinone function, as in 5-iminodaunoru-

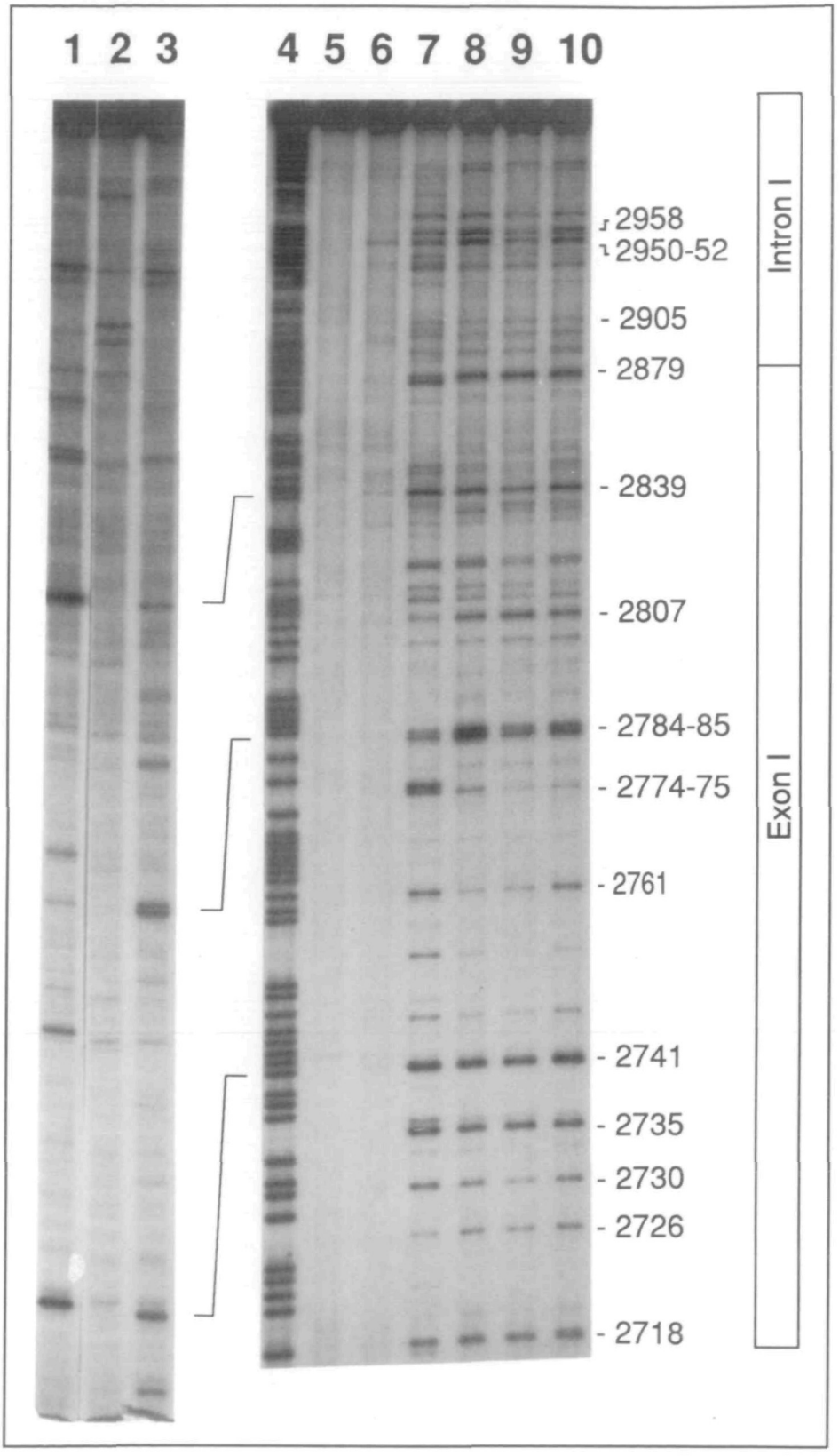

Fig. 4. Topoisomerase II patterns of cleavage in the presence of drugs at the junction between the first exon and first intron of the human c-myc gene. The DNA fragment was prepared by PCR, using a 5′-^{32}P-labeled sense-strand primer. Cleavage reactions were performed at 37 °C for 30 minutes with purified topoisomerase II and stopped by adding SDS and proteinase K. DNA was electrophoresed in 7% denaturing acrylamide gel. Lane 1, +10 μ*M* amsacrine; lane 2, +2 μ*M* 5-iminodaunorubicin; lane 3, +0.05 μ*M* mitoxantrone; lane 4, purine ladder obtained after formic acid reaction; lane 5, DNA control; lane 6, control topoisomerase II; lane 7, +10 μ*M* VM-26; lane 8, +0.2 μ*M* mitoxantrone; lane 9, +0.2 μ*M* DuP 937; lane 10, +0.2 μ*M* DuP 941. Numbers on the right of the autoradiographs correspond to the genomic position of the nucleotide covalently linked to the topoisomerase II. Brackets between the two autoradiographs indicate equivalent cleavage sites for mitoxantrone.

bicin, also reduces the chelation potency and cardiotoxicity of the molecule without affecting topoisomerase II inhibition (*29*). The ability of drugs to produce free radicals has been correlated to their polarographic reduction potential (*30,31*). In contrast to anthracyclines and mitoxantrone, anthrapyrazoles are difficult to reduce. They can even protect against lipid peroxidation (*27*). The doses required for this kind of protection are unlikely to be attained clinically, given the toxic effects and the nature of pharmacokinetics of the anthrapyrazoles (*32,33*). Hence, preclinical studies as well as phase I clinical trials suggest the absence of cardiotoxicity for the anthrapyrazoles (*8*).

Anthrapyrazoles and mitoxantrone share many features, including their cytotoxic profile in the NCI screen, their potency to induce DNA strand breaks (*5,6*), and their sequence selectivity of topoisomerase II cleavages. They are potent intercalators (*24*) and are substrates for mdr–P-glycoprotein pumps (*34*). The only reported difference, besides the absence of free radical generation for anthrapyrazoles, is the stronger inhibition of RNA synthesis by mitoxantrone than by anthrapyrazoles (*5*). Mitoxantrone and DuP 941 behave almost similarly in the in vitro NCI cell screen, which takes into account tissue-specific gene expression. Since the two drugs also share similar pharmacokinetics (*33,35*), it would not be surprising that DuP 941 would exhibit the same anticancer spectrum of activity as mitoxantrone with reduced cardiotoxicity. The point could be of interest in pediatrics and in intensified protocols, especially in the case of associated radiotherapy involving the heart region (*1*).

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Notes

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