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COMPONENTS OF INTRINSIC DRUG RESISTANCE IN THE RAT HEPATOMA

AMY L. ELLIS,* CRAIG E. MUNGER,* RODERICK T. BUNCH,* KAREN E. WOODS,* JOYCE K. RANDOLPH,* LAWRENCE BOISE,* PAUL S. SWERDLOW,* LEONARD A. ZWELLING,† MICHAEL HINDS,† SAUL YANOVICH* and DAVID A. GEWIRTZ*‡

*Departments of Pharmacology/Toxicology and Medicine, Medical College of Virginia, Richmond, VA 23298; and †The University of Texas, M.D. Anderson Cancer Center, Department of Medical Oncology, Division of Medicine, Houston, TX 77030, U.S.A.

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Abstract—A carcinogen-transformed rat hepatoma cell line (Reuber H-35) was utilized as a model system for investigation of the biochemical factors which may limit the effectiveness of chemotherapy in intrinsically resistant tumors such as hepatocellular carcinoma. Northern blotting demonstrated expression of mRNA coding for the P-170 membrane-glycoprotein associated with the multi-drug resistance phenotype, while Western blotting identified the P-170 glycoprotein in the hepatoma cell membrane. Consistent with these observations, tumor cell sensitivity to the vinca alkaloids, vincristine and vinblastine, to the anthracycline antibiotics, Adriamycin® and daunorubicin, and to the demethylepipodophyllotoxin derivative, VM-26, was enhanced by continuous incubation in the presence of the calcium channel antagonist, verapamil. Verapamil produced a minimal change in cell sensitivity to the demethylepipodophyllotoxin derivative, VP-16, and to the aminoacridine, *m*-AMSA. Relatively high detoxification potential via the glutathione metabolic pathway was also observed in the hepatoma cell. The capacity of topoisomerase II in nuclear extracts from the hepatoma cell to mediate cleavable complex formation stimulated by VM-26, VP-16 and *m*-AMSA appeared to be at least comparable to, if not greater than that from drug-sensitive HL-60 cells, suggesting that drug resistance may not occur at the level of this enzyme. Consistent with findings in a number of tumor cell lines resistant to antineoplastic drugs, the antiproliferative activity of the topoisomerase II inhibitors VM-26, VP-16 and *m*-AMSA appeared to be dissociable from the induction of DNA strand breaks, suggesting that such lesions in DNA may fail to fully account for the antiproliferative activity of these agents in the hepatoma cell.

Biochemical factors conferring resistance to antineoplastic drugs have generally been defined in tumor cell lines selected for resistance by exposure of the sensitive parent cell line to chemotherapeutic agents. This approach is quite appropriately based on the clinical development of resistance in tumors initially responsive to chemotherapy, such as in leukemias, lymphomas, and breast and ovarian cancer. Tumor cell lines in which resistance is induced by this selection process frequently demonstrate enhanced drug efflux (for drugs of the natural product class such as the anthracycline antibiotics, the demethylepipodophyllotoxins, the ellipticines and vinca alkaloids) mediated by a specific membrane glycoprotein of molecular weight between 170,000 and 180,000 [1–3]. Expression of this membrane glycoprotein has also been reported in normal host tissue such as the pancreas, liver, and colon epithelium [4].

In addition to the multi-drug resistant phenotype associated with enhanced drug efflux, alternative mechanisms have been proposed to account for drug resistance in the tumor cell. Enhanced levels or activity of detoxification enzymes, particularly those involved in the glutathione metabolic pathway, have been associated with resistance to a variety of antineoplastic agents [5–8]. Alterations in the level, activity or drug-sensitivity of the DNA replicative enzyme, topoisomerase II, are thought to account for resistance to a number of structurally dissimilar topoisomerase II inhibitors [9–14].

The research interests of this laboratory have been focused on the H-35 (Reuber) rat hepatoma, a cell line which was initially generated in the livers of rats exposed to the carcinogen aminoacetyl fluorene [15]. The H-35 rat hepatoma cell line is intrinsically resistant to the anthracycline antibiotics both *in vitro* and *in vivo* [16, 17], and may represent a prototypical carcinogen transformed tumor cell line for investigation of the factors which confer resistance to anthracyclines in tumors of hepatocellular origin.

The studies in this report were designed to probe the basis for intrinsic resistance in the hepatoma cell by: (a) determining the presence of the multi-drug resistance phenotype of the cells by Northern and Western blotting and by the ability of the calcium channel antagonist, verapamil, to enhance the antiproliferative activity of three classes of antineoplastic drugs; (b) assessing cellular detoxification capacity at the level of the glutathione redox metabolic pathways; (c) assessing the drug sensitivity of topoisomerase II extracted from the hepatoma cell; and (d) determining the capacity of select topoisomerase II inhibitors to induce DNA cleavage.

‡ Corresponding author: Dr. David A. Gewirtz, Box 230, Department of Medicine, Medical College of Virginia, Richmond, VA 23298. Tel. (804) 786-9523; FAX (804) 371-8079.

MATERIALS AND METHODS

Materials. VM-26*(4′-demethylepipodophyllotoxin-9-(4,6-*O*-2-thenylidene-β-D-glucopyranoside)) and VP-16 (4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D-glucopyranoside)) were provided by Bristol-Myers Laboratories, Wallingford, CT. 4′-(9-Acridinylamino)methane sulfon-*m*-anisidide (*m*-AMSA) was provided by the Chemical Synthesis Branch of the National Cancer Institute. VM-26, VP-16 and *m*-AMSA were dissolved in dimethyl sulfoxide and generally discarded after 3–4 weeks of freezer storage. Verapamil was provided by the Knoll Pharmaceutical Co., Whippany, NJ. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) for the cell proliferation assay, sodium dodecyl sulfate (SDS), proteinase K, reduced glutathione, oxidized glutathione, NADPH, glutathione reductase, glutathione peroxidase, glutathione transferase, UDP glucuronyl transferase, glycylglycine, L-γ-glutamyl-*p*-γ-nitroanilide, γ-glutamyl transpeptidase, buthionine sulfoximine, H_2O_2 and bisbenzamide trihydrochloride (Hoechst dye 32258) were obtained from the Sigma Chemical Co., St. Louis, MO. Tetrapropyl ammonium hydroxide for the alkaline elution assay and acrylamide were obtained from the Eastman Kodak Co., Rochester, NY. Bisacrylamide was obtained from Bio-Rad Laboratories, Rockville Center, NY. Tris (hydroxyaminomethane) glycine buffer was obtained from the US Biochemical Corp., Cleveland, OH. 3,4-Dichloronitrobenzene, a substrate for the glutathione transferase assay, was obtained from Aldrich Biochemicals, Milwaukee, WI. Covalently closed, supercoiled SV-40 DNA was purchased from either Bethesda Research Laboratories, Gaithersburg, MD, or Lofstrand Lab Limited, Gaithersburg, MD.

Culture conditions and proliferation assay. Reuber H-35 rat hepatoma cells (also known as the H-4-II-E tumor line), obtained from the American Type Culture Collection (Rockville, MD), have a doubling time of approximately 18 hr. MCF-7 cells were provided by Dr. Winnie Chan of the Department of Radiation Oncology at the Medical College of Virginia. For all studies described in this report, cells were subcultured at densities where cells maintained logarithmic growth during the assay procedure. Cells were maintained in monolayer culture in "complete medium" consisting of Dulbecco's minimal essential medium (Hazelton Research Products, Denver, PA) supplemented with glutamine (29.2 mg/100 mL), penicillin/streptomycin (0.5 mg/100 mL), 5% fetal bovine serum (Life Technologies, Grand Island, NY), and 5% defined bovine calf serum (Hyclone Laboratories, Logan, UT) at 37°, under 5% CO_2. Drug effects on cell proliferation were determined as described previously [18]. In the present studies, cells were incubated for either 2 or 72 hr in the presence of the indicated drug before analysis of viable cell number using the MTT tetrazolium dye assay. Studies using verapamil were performed in the continuous presence of both verapamil and the antineoplastic drug in question, unless otherwise indicated.

Northern blotting of mRNA. Northern blotting experiments were performed to determine the expression of mRNA coding for the P-170 membrane glycoprotein in the rat hepatoma, rat hepatocytes and K562 leukemia cells sensitive and resistant to daunorubicin. Rat liver hepatocytes were isolated as described previously [19]. The characterization of K562 cells selected for resistance to daunorubicin is described in a separate report [20].

RNA was isolated from cells as previously described by other investigators [21]. Briefly, cells were harvested and lysed in 4 M guanidine isothiocyanate. RNA was separated by ultracentrifugation through a 5.7 M cesium chloride cushion at 100,000 *g* for 20 hr at 20°. RNA pellets were then washed with 2 vol. of ethanol, dissolved in 0.3 M sodium acetate (NaAc), pH 5.0, and precipitated with ethanol. RNA was recovered by centrifugation at 24,000 *g* for 30 min at 4°. Pellets were resuspended in water.

Ten micrograms of RNA was denatured in 0.02 M morpholino propane sulfonic acid, pH 7.0, 5 mM NaAc, 1 mM EDTA, 2.2 M formaldehyde and 50% formamide. The samples were separated on a 6.6% formaldehyde, 1% agarose gel [22]. Equal loading of RNA in each lane was determined by ethidium bromide staining. Blotting was carried out using the Trans-Vac TE 80 vacuum blotter (Hoefer Scientific Instruments, San Francisco, CA) to Nytran transfer membranes (Schleicher & Schuell). The P-glycoprotein specific probe used in these experiments was the 2.0 kb fragment of the *Hin*dIII/*Eco*RI digest of the plasmid pmdr (a gift of Dr. Jim Croup and Dr. David Housman), a probe which is cross-reactive to rodent and human P-glycoprotein. Filters were hybridized to probes (radiolabeled by nick translation) in the presence of 50 mM sodium phosphate, pH 6.5, 5× Denhardt's solution (0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinyl pyrollidine), 5× SSC (0.75 M NaCl, 0.075 M sodium citrate), 0.1% SDS, yeast RNA (250 μg/mL), 50% formamide and 10% dextran sulfate. Hybridizations were for 16–20 hr at 37°. Filters were washed five times at 37° for 5 min in 2× SSC and 0.2% SDS followed by one wash in 2× SSC and 0.2% SDS at 60° for 40 min and finally one wash at 60° in 0.5× SSC and 0.2% SDS for 40 min before autoradiography. Sizes of RNA species were estimated by comparison to the 18S and 28S rRNA markers.

Western blotting of the P-170 membrane glycoprotein. Plasma membrane preparations were performed as per the instructions of the antibody manufacturer. Briefly, cells were washed three times in phosphate-buffered saline and resuspended in 10 mM KCl, 1.5 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM Tris–HCl, pH 7.4. The cells were broken by ten strokes of a glass homogenizer and the membrane-enriched fractions prepared by ultracentrifugation at 100,000 *g*

* Abbreviations: VM-26, 4′-demethylepipodophyllotoxin-9-(4,6-*O*-2-thenylidene-β-D-glucopyranoside; VP-16, 4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D-glucopyranoside; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PMSF, phenylmethylsulfonyl fluoride; *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; and SDS, sodium dodecyl sulfate.

for 1 hr. The pellet was resuspended in the above buffer and frozen until used.

Gel electrophoresis was by the method of Laemmli [23]. A 7.5% polyacrylamide, 0.2% bisacrylamide gel was run on sample made 1x in sample buffer (1.5% SDS, 125 mM Tris–HCl pH 6.8, 0.35 M β-mercaptoethanol, 5% glycerol) and electrophoresed for 500 V-hr.

Blotting was performed as previously described [24] with the modifications below. Briefly, the gel was soaked in transfer buffer (20% methanol, 0.1% SDS, 190 mM glycine, 25 mM Tris base) for two periods of 40 min each and assembled in a Biorad Transblot strung with extra platinum wire for field uniformity. The transfer buffer was as above but without SDS. Blotting was overnight at 35 V for 660 V-hr total.

After blotting, the filter was probed with monoclonal antibody C219 (Centocor, Malvern, PA) as per the instructions of the manufacturer. Briefly, the antibody was iodinated by the chloramine T method and used to probe blocked filters directly. K562-sensitive (parental) and MDR-resistant cell lines were used as negative and positive controls, respectively.

Glutathione and glutathione-associated metabolic pathways. Levels of glutathione and activity of glutathione reductase, glutathione peroxidase and glutathione transferase were determined in extracts of hepatocytes and H-35 rat hepatoma cells. Cell extracts were prepared by suspension of the cells to a density of approximately 8×10^6 cells/mL in extraction buffer (1 mM EDTA, 30 mM Na_2HPO_4 and 250 mM sucrose) and repeatedly sonicated on ice until no intact cells were visible by microscopy. The cell-lysate was centrifuged at 30,000 *g* for 30 min at 4°, and the supernatant saved for analysis. Cell protein was determined by the method of Bradford [25]. Glutathione levels were assessed by the method of Tietze [26]. Glutathione reductase activity was assessed by monitoring the oxidation of NADPH at 340 nm as described by Worthington and Rosemeyer [27]. Glutathione transferase activity was assessed by the method of Habig *et al.* [28] using 3,4-dichloronitrobenzene as a substrate. Glutathione peroxidase activity was determined using a modification of the procedure of Paglia and Valentine [29] as described by Babson *et al.* [30].

Preparation of nuclear extracts. Nuclear extracts (0.35 M NaCl) from H-35 cells were prepared according to the methodology of Sullivan and colleagues [31] as modified by Danks *et al.* [12]. Nuclear extracts were stored at −80° until needed. Once thawed, extracts were used immediately or discarded. Protein concentration of the extracts was determined by the method of Bradford [25].

Nuclear extracts from HL-60 cells were prepared as described by Zwelling *et al.* [32]. HL-60 cells were washed three times in cold buffer consisting of 150 mM NaCl, 5 mM $MgCl_2$, 2 mM KH_2PO_4, 1 mM ethyleneglycolbis (aminoethylether) tetra - acetate (EGTA), 0.1 mM dithiothreitol, 0.1 mM PMSF and 10% glycerol [33]. Cells were lysed on ice in the same buffer containing 0.3% Triton X-100 for 10 min. Nuclei were pelleted by centrifugation and washed four times with the same buffer without detergent. Nuclei were extracted by adjustment of the buffer to a NaCl concentration of 0.35 M and incubation for 30 min on ice. Nuclei were centrifuged at 100,000 *g* for 60 min at 4°; the supernatant was recovered, adjusted to 50% glycerol, and stored at −20°.

DNA decatenation activity. This assay was used to quantify the amount of topoisomerase II activity in nuclear extracts from the two cell lines, as described previously [32, 34, 35]. [^{3}H]kDNA (approximately 22 μg), used as the substrate in this assay, was isolated from the trypanosome *Crithidia fasciculata* [36, 37]. Reaction buffer was composed of 50 mM Tris–HCl, 85 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM disodium EDTA, 0.03 mg/mL bovine serum albumin and 1 mM ATP, pH 7.6. Reactions were performed for 30 min at 37° and terminated with 1% SDS and 100 μg/mL proteinase K. Reaction products were separated on a 1% agarose gel in an 89 mM Tris-borate buffer system (pH 8) containing 0.5 μg/mL ethidium bromide. Catenated kDNA substrate does not enter the gel; in contrast, decatenated DNA circles enter the gel. DNA in the gels was visualized under ultraviolet light; the gel areas containing catenated and decatenated DNA were excised, placed in a liquid scintillation vial, and melted in a microwave oven; and the amount of DNA was quantified by liquid scintillation spectroscopy. Topoisomerase II activity is expressed as the quantity of (nuclear extract) protein required to decatenate 50% of the kDNA substrate.

Drug-stimulated DNA–protein cross-linking in nuclear extracts. The capacity of H-35 and HL-60 nuclear extracts containing *equal* amounts of topoisomerase II activity to mediate drug-stimulated formation of DNA–protein complexes was determined using SV40 DNA which was uniquely 3′ end-labeled [34, 38]. Reaction buffer was composed of 10 mM Tris, pH 7.4, 50 mM KCl, 50 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 μg/mL bovine serum albumin and 1 mM ATP. Reactions were performed in the presence of various concentrations of topoisomerase II inhibitors for 30 min at 37° using the amount of protein required for decatenation of 50% of the kDNA substrate. Reactions were terminated by addition of 100 μL of a stop-solution containing 0.2 N NaOH, 2% SDS, 2 mM EDTA and 0.5 mg/mL salmon sperm DNA (in a final volume of 150 μL). The DNA–protein complex was precipitated on ice using 50 μL of 0.25 M KCl–0.4 M Tris, pH 8. The precipitate was washed with 200 μL of a solution containing 10 mM Tris, pH 8, 100 mM KCl, 1 mM EDTA, and 0.1 mg/mL salmon sperm DNA, at 65°. Radioactivity in the precipitates was quantitated by liquid scintillation spectroscopy.

Induction of DNA cleavage in intact cells. DNA cleavage induced by incubation with VM-26, VP-16 or *m*-AMSA for 2 hr was determined in H-35 rat hepatoma and L1210 leukemia cells (as controls) using the alkaline unwinding assay of Kanter and Schwartz [39]. DNA cleavage is monitored based on the differential binding of Hoechst dye to single-stranded and double-stranded DNA. Cells were incubated with various concentrations of drug in complete medium for a 2-hr period at 37°, washed

with 0.15 M NaCl buffered to pH 7.4 with mono- and dibasic potassium phosphate, and released from culture flasks utilizing trypsin (0.05 mg/mL) and EDTA (0.02 mg/mL) in saline. Cells were resuspended to a density of 1×10^6 cells/mL and maintained on ice until initiation of the assay (at room temperature). An unwinding period (in alkali) of 10 min was utilized, and fluorescence of the Hoechst dye–DNA complex was determined using a Kratos fluorescence spectrophotometer with excitation at 350 nm and emission at 450 nm. DNA cleavage was quantitated by comparison to that induced by X-irradiation. This assay is sufficiently sensitive to detect changes of between 50 and 100 rad equivalents of DNA cleavage and demonstrates the same extent of DNA cleavage as the alkaline elution assay in L1210 cells exposed to the demethylepipodophyllotoxins. For instance, using alkaline unwinding, we determined that VM-26, at concentrations of 0.1, 0.5 and 1 μM, produced 247, 1508 and 2682 rad equivalents of DNA cleavage, respectively, in L1210 cells. Wozniak and Ross [40], using alkaline elution, reported that VP-16, which is one-tenth as toxic as VM-26 in the L1210 leukemia cell, produces 330, 1600 and 2460 rad equivalents of DNA cleavage at 1, 5 and 10 μM concentrations, respectively.

Induction of DNA–protein cross-links in intact cells. DNA–protein cross-linking in H-35 cells was assessed by a gravity drip filter binding method modified from that described by Oleinick *et al* [41], Bartus *et al.* [42] and Minford *et al.* [33]. Cells in logarithmic growth were labeled with [^{3}H]thymidine at a final specific activity of 1 μCi/nmol for approximately 24 hr, detached from plates using trypsin/EDTA, washed twice with warm phosphate-buffered saline, and resuspended in medium to a density of 2×10^5 cells/mL. Cells were equilibrated in medium under 5% CO_2 in a 37° incubator for 1 hr prior to a 2-hr incubation with VM-26, VP-16 or *m*-AMSA. Cells were pelleted, washed and resuspended in cold phosphate-buffered saline and exposed to 3000 rads of ionizing radiation on ice using a Cesium irradiator.

Gelman Metricel DM800 polyvinyl chloride filters in Swinnex assemblies (Millipore Corp., Beford, MA) were equilibrated with 3 mL of ice-cold 20 mM EDTA (pH 10). All solutions were added into a 5-mL syringe attached to the Swinnex assembly. Cells were deposited gently into the syringes and the EDTA was eluted by gravity flow. Cells on filters were lysed by incubation for 25 min in 0.2% sodium laurylsarcosine/0.05 mM disodium EDTA/2 M NaCl at pH 10. After elution of the pH 10 lysis solution, DNA on the filter was incubated with the same lysis solution at pH 12 for an additional 25 min. The pH 12 detergent solution was eluted and the filter was washed successively with 3 vol. (3 mL each) of the same (pH 12) detergent solution. All eluates were collected by gravity flow. The pH 12 lysate was neutralized with 0.5 mL of 5 N HCl and [^{3}H]DNA associated with the filter quantitated after digestion of the filter in 1 M HCl. The extent of filter retention of [^{3}H]DNA was linear function of the radiation dose between 1000 and 3000 rads. Induction of DNA–protein cross-linking was quantitated by assessing the increase in filter retention of [^{3}H]DNA induced by drug in cells exposed to 3000 rads of ionizing radiation. The capacity of this assay to monitor DNA–protein cross-linking was validated by demonstrating that VM-26 enhanced filter retention of irradiated DNA from L1210 leukemia cells and that proteinase K reversed the enhanced retention induced by VM-26.

Analysis of data. Data were analyzed using the unpaired Student's *t*-test, except where otherwise noted. Drug effects which resulted in P values of 0.05 or below were considered statistically significant.

RESULTS

Determination of the multi-drug-resistant phenotype in the rat hepatoma cell. Figure 1 presents the results of a Northern blotting experiment demonstrating the expression of mRNA coding for the P-glycoprotein in the rat hepatoma cell. Expression of mRNA coding for the P-glycoprotein in the rat liver and in K562 cells with acquired resistance to daunorubicin [20] and lack of expression in K562 cells are presented as controls for the hepatoma cell; it should be noted that it is presently not feasible to predict the quantitative relationship between expression of the P-glycoprotein and the extent of drug resistance. Western blotting experiments also demonstrated high levels of the P-170 glycoprotein in plasma membranes of rat hepatoma cells and drug-resistant K562 cells but not in the membranes of parental K562 cells (data not shown).

Tumor cells expressing the P-glycoprotein would be expected to demonstrate resistance to the anthracycline antibiotics, Adriamycin® and daunorubicin, to the demethylepipodophyllotoxin derivatives, VM-26 and VP-16, and to the vinca alkaloids, vincristine and vinblastine, since the natural products have been shown to exit the tumor cell by a common pathway which limits their cellular accumulation and retention [1–3]. Proliferation studies were performed in the *continuous* presence of the vinca alkaloids, the anthracylcine antibiotics and the demethyl-epiodophyllotoxin derivatives (Table 1). The IC_{50} values for vincristine and vinblastine of approximately 79 and 24 nM, respectively, were significantly higher than those reported for drug-sensitive tumor cell lines (between 1 and 5 nM) [43–46]. The IC_{50} values for VM-26 and VP-16 of 0.57 and 4 μM, respectively, were approximately 15- to 25-fold higher than the range observed for other tumor cell lines such as L1210, CEM, MCF-7, P388 and CHO (approximately 30 nM for VM-26 and 150 nM for VP-16) [44–49]. The IC_{50} values for the anthracycline antibiotics, Adriamycin and daunorubicin of 360 and 116 nM, respectively, were between 4- and 12-fold higher than those reported for such diverse experimental tumors as HeLa, MCF-7, HL-60 and CEM, where the IC_{50} clusters at approximately 30 nM [50–53]. Interestingly, sensitivity to *m*-AMSA in the rat hepatoma (IC_{50} value of 270 nM) was similar to that reported in P388 leukemia and L1210 leukemia [10, 54], suggesting the absence of cross-resistance to this antineoplastic agent.

As the rat hepatoma cell is not derived from a matched "drug-sensitive" parental cell line,

Table 1. Influence of verapamil on sensitivity of H-35 rat hepatoma to antineoplastic drugs

	IC_{50} (nM)			IC_{50} (nM)
	Drug alone (H-35)	Drug + verapamil (H-35)	Ratio of IC_{50} values (Column 1:Column 2)	Drug alone (MCF-7)
Vinblastine	24 ± 5 (3)	2 ± 0.3 (3)	12	0.5
Vincristine	79 ± 9 (4)	8 ± 1 (4)	9.9	
Adriamycin	360 ± 60 (5)	42 ± 6 (4)	8.6	10
Daunorubicin	116 ± 30 (5)	18 ± 1 (4)	6.4	
VM-26	570 ± 160 (4)	90 ± 8 (4)	6.3	10
VP-16	4000 ± 1300 (3)	1830 ± 110 (3)	2.2	
m-AMSA	270 ± 37 (3)	100 ± 129 (3)	1.35	

Hepatoma cells plated at a density of 5×10^3 cells /mL in 96-well microtiter plates were incubated continuously with the indicated drugs in the absence or presence of 1 μM verapamil. Cell proliferation was monitored after 72 hr utilizing the MTT tetrazolium dye assay. Data for H-35 cells are presented as means ± SEM. Values in parentheses indicate the number of independent experiments. MCF-7 breast tumor cells plated at a density of 1×10^4 cells/mL were incubated continuously with the indicated drugs, and cell proliferation was monitored after 7–8 days using the MTT tetrazolium dye assay. Data for MCF-7 cells are presented as means for four independent experiments.

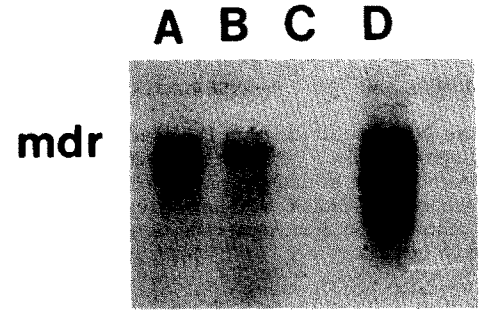

Fig. 1. Northern blotting for *m*-RNA coding for the P-glycoprotein. Northern blot analysis of P-170 glycoprotein expression in H-35 rat hepatoma cells (A), rat liver cells (B), wild-type K562 cells (C) and multi-drug-resistant K562 cells (D).

comparative studies of drug antiproliferative activity were performed using another adherent solid tumor, the MCF-7 breast tumor cell line. Table 1 demonstrates that assessment of the sensitivity of the MCF-7 tumor cell line to drugs from three different classes, Adriamycin, VM-26 and vinblastine, using the MTT tetrazolium dye assay provided values within the range of those reported in the literature [6, 51].

Since drug resistance related to enhanced efflux mediated by the P-170 glycoprotein is generally circumvented by calcium channel antagonists competing for the membrane transport protein [43, 45, 55], studies were designed to assess whether the calcium channel antagonist, verapamil, could enhance sensitivity of the H-35 rat hepatoma cell to Adriamycin, daunorubicin, VM-26, VP-16, the vinca alkaloids and *m*-AMSA. Table 1 presents the alterations in the IC_{50} values of these antineoplastic drugs upon continuous incubation with drugs + verapamil, indicating that verapamil has the capacity to circumvent the drug-resistant phenotype in the hepatoma cell. Of interest is the fact that verapamil produces minimal enhancement of hepatoma cell sensitivity to VP-16 (similar to the finding reported by Politi and Sinha [56] in Adriamycin-resistant MCF-7 tumor cells) and to *m*-AMSA, indicating that these drugs may be poor substrates for the P-170 glycoprotein pump mediating drug efflux.

Glutathione-associated metabolic capacity in the hepatoma cell. The potential detoxification capacity of the rat hepatoma cell was evaluated by assessing the levels of redox enzymes of the glutathione metabolic pathway thought to be associated with resistance to the anthracycline antibiotics [5–8]. Table 2 presents the level of glutathione and the activities of glutathione reductase, glutathione transferase and glutathione peroxidase in the H-35 rat hepatoma cell. Levels of glutathione and glutathione redox enzymes in freshly isolated rat hepatocytes are presented exclusively as positive controls for these assays, since the hepatocyte is a non-proliferating cell which is not a target for antineoplastic drugs. In comparing these values to those reported in the literature for other tumor cell lines, the activity of glutathione peroxidase in the hepatoma cell (136.6 nmol/min/mg) exceeded that reported in a drug-sensitive lung carcinoma (16.6 nmol/min/mg) [57] and a human fibrosarcoma (38.4 nmol/min/mg) [58], and in both Adriamycin-sensitive and -resistant MCF-7 breast cancer cells (4 and 16 nmol/min/mg, respectively) [6]. The activity of glutathione reductase in the hepatoma cell (67.5 nmol/min/mg) also exceeded that reported in the lung carcinoma (10.1 nmol/min/mg) [57] and the fibrosarcoma (5.3 nmol/min/mg) [58]. Glutathione transferase activity also appeared to be significantly higher than that in a number of human tumor cell lines [59]. When glutathione levels in the hepatoma cell were reduced by 85–90% following treatment with buthionine sulfoximine (10 μM) for 24 hr [60], sensitivity of the hepatoma cell to the anthracycline antibiotics was not enhanced (data not shown); this

Table 2. Levels/activity of glutathione and glutathione-redox enzymes

	Hepatocytes	H-35 Hepatoma
Glutathione (nmol/10^6 cells)	50	7.0 ± 1.2 (8)
Glutathione reductase (nmol/min/mg cell protein)	159.2 ± 55.5 (2)	67.5 ± 7.7 (3)
Glutathione peroxidase (nmol/min/mg cell protein)	1529	136.6 ± 10.7 (2)
Glutathione transferase (μmol/min/mg cell protein)	2.65 ± 1.2 (2)	0.51 ± 0.02*† (3)

Values are means ± SEM where the number (N) of experiments (given in parentheses) equals 3 or more. Where N = 2, the mean ± range is given.

* Using 3,4-dichloronitrobenzene as substrate.

† Northern blotting of RNA from the H-35 cell indicates expression of glutathione transferase π (Cowan KH, personal communication, cited with permission).

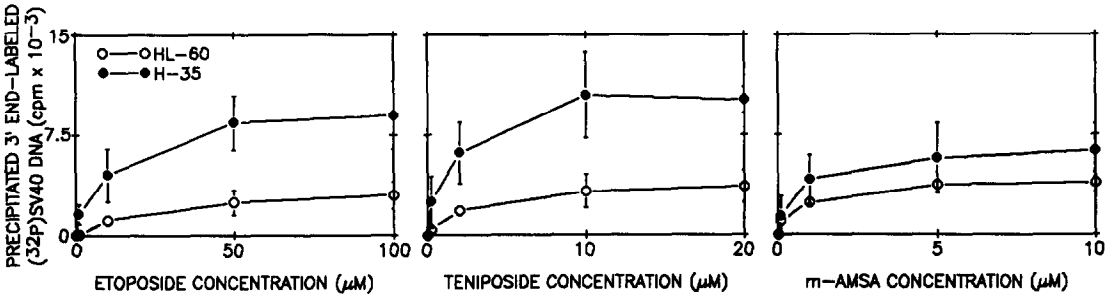

Fig. 2. Drug reactivity of H-35 and HL-60 nuclear extracts. Nuclear extracts from H-35 and HL-60 cells were incubated with the indicated concentrations of etoposide (VP-16), teniposide (VM-26) or *m*-AMSA in the presence of 3′ end-labeled [^{32}P]SV40 DNA for 30 min as described in Materials and Methods. Data are means ± SD of three experiments. Baseline DNA–protein cross-linking (extract + DNA in the absence of drug) was subtracted at each drug concentration. Equivalent catalytic activities of protein were used for both extracts.

latter study supports the idea that the hepatoma cell maintains sufficiently high glutathione redox activity to limit free-radical mediated toxicity of the anthracycline antibiotics even after depletion of the bulk of cellular glutathione.

Drug sensitivity of topoisomerase II from the hepatoma cell. Since it has been demonstrated that alterations in topoisomerase II levels or activity may be associated with resistance to topoisomerase II inhibitors such as *m*-AMSA and the epipodophyllotoxins [9–14], studies were initiated to assess the activity and drug sensitivity to topoisomerase II in *nuclear extracts* from the hepatoma cell. It is difficult, however, to make quantitative comparisons of activity since the hepatoma cell is an intrinsically-resistant tumor cell line without a companion parental cell line. There may be differences in extractable enzyme activity when normalized per unit of extractable protein simply because of the differential protein content of the cell lines studied. When nuclear extracts from the H-35 cells and the well-characterized HL-60 cell line were compared, much more activity per unit protein was observed in the HL-60 cells (i.e. the amount producing decatenation of 50% of the substrate kDNA was 0.113 μg compared to 2.54 μg for the H-35 cells). However, as Fig. 2 indicates, the capacity of VM-26 (teniposide) and VP-16 (etoposide) to stimulate DNA–protein cross-linking using equivalent activities of topoisomerase II was greater for the H-35 cells. The capacity of *m*-AMSA to stimulate DNA–protein cross-linking appeared to be approximately equivalent for both cell lines.

Dissociation between the production of DNA cleavage and the antiproliferative activities of VP-16, VM-26 and m-AMSA. Topoisomerase II inhibitors such as the epipodophyllotoxin derivatives, VP-16 and VM-26, and the aminoacridine, *m*-AMSA, are thought to produce their antiproliferative and cytotoxic activities by the induction of DNA strand breaks and DNA–protein cross-links in tumor cells [33, 61–64]. These effects are observed within the first 1–2 hr after exposure of cells to the antineoplastic drugs, and are reversed upon removal of the cells from the presence of drug [54, 65]. Consequently, studies were performed to assess the influence of a *2-hr* exposure to VP-16, VM-26 and *m*-AMSA on the integrity of hepatoma cell DNA; companion studies were performed to assess the influence of a 2-hr exposure to drug on proliferative capacity of the hepatoma cell.

Figure 3 demonstrates that for VP-16, where inhibition of cell proliferation increased as a function of drug concentration, no DNA damage was detected

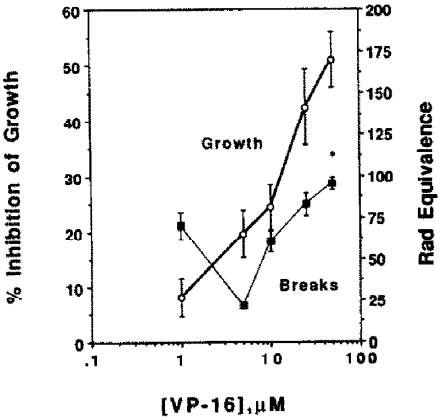

Fig. 3. Effect of VP-16 on growth and DNA integrity in the rat hepatoma. H-35 rat hepatoma cells in logarithmic growth were incubated with the indicated concentrations of VP-16 for 2 hr prior to determination of DNA cleavage using the alkaline unwinding assay. Inhibition of growth was determined using the MTT tetrazolium dye assay. Data are means ± SEM for 3–7 experiments (strand breaks) and 6–13 experiments (growth). Baseline DNA cleavage of 56 ± 1.8 rad equivalents was subtracted at each concentration of VP-16. Key: * $P < 0.01$ vs control.

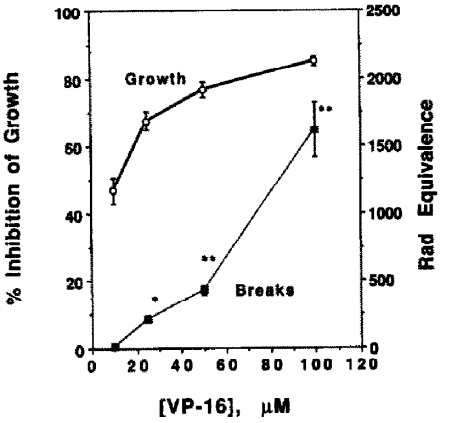

Fig. 4. Influence of verapamil on growth inhibition and induction of DNA strand breaks by VP-16. H-35 rat hepatoma cells were incubated with the indicated concentrations of VP-16 plus 1 μM verapamil for 2 hr prior to determination of DNA cleavage using the alkaline unwinding assay. Inhibition of growth was determined using the MTT tetrazolium dye assay. Data are means ± SEM for 3–6 experiments (strand breaks) and 6–10 experiments (growth). Baseline DNA cleavage of 66 ± 1.82 rad equivalents was subtracted at each concentration of VP-16. Key: * $P < 0.05$, and ** $P < 0.001$ vs control.

in the hepatoma cell at concentrations up through 25 μM ($P < 0.1$), where growth was inhibited by 42.4 ± 6.7%. The paradoxical *increase* in DNA integrity at 5 μM VP-16 does not represent a statistically significant change in DNA damage as compared to control ($P < 0.9$). Furthermore, there was no evidence of induction of DNA–protein cross-links over the concentration range of 10–50 μM VP-16 (Table 3). Neutral elution studies failed to detect induction of any double-strand breaks in DNA by VP-16 over this concentration range (data not shown).

Table 3. DNA–Protein cross-link induction

Drug	Concentration (μM)	% [^{3}]DNA retained on filter	P values
Control		14.1 ± 3.4 (7)	
VP-16	10	14.7 ± 1.8 (5)	<0.9
	25	16.3 ± 3.5 (4)	<0.9
	50	18.2 ± 2.5 (6)	<0.9
Control		10.0 ± 1.1 (14)	
VM-26	1	13.0 ± 1.3 (11)	< 0.1
	5	16.6 ± 2.3 (11)	< 0.01
	10	18.9 ± 2.8 (9)	< 0.01
Control		11.9 ± 1.9 (3)	
m-AMSA	1	18.1 ± 1.5 (3)	< 0.05
	5	22.0 ± 2.9 (3)	< 0.05
	10	37.9 ± 5.4 (3)	< 0.05

DNA–Protein cross-link induction was monitored using the filter-drip asssay described in Materials and Methods. Data are means ± SEM for percent [^{3}H]DNA retained on filters. Values in parentheses indicate the number of replicate experiments. P values were determined using Student's unpaired *t*-test.

Additional studies were performed to determine whether co-incubation with verapamil could modify the relationship between inhibition of growth and the induction of strand breaks by VP-16. Figure 4 demonstrates that, in the presence of verapamil, these DNA strand breaks failed to parallel growth inhibition. For instance, between 0 and 10 μM VP-16, growth was inhibited by 46.1 ± 4.0% without detectable alterations in DNA cleavage. Between 25 and 100 μM VP-16, dramatic increases in DNA breaks (i.e. an increase of approximately 1500 rad equivalents) were accompanied by a small change in growth inhibition (67.6 ± 2.8% at 25 μM VP-16 and 86.21 ± 1.41% at 100 μM VP-16).

Figure 5 presents the results of similar studies comparing inhibition of hepatoma cell growth and induction of DNA strand breaks by the epipodophyllotoxin derivative, VM-26. The IC_{50} value for growth inhibition by VM-26 (after a 2-hr incubation) was approximately *1.5 μM*; however, incubation with VM-26 for 2 hr failed to produce detectable DNA cleavage in the H-35 rat hepatoma cell at drug concentrations up through 5 μM. At 5 μM VM-26, the amount of intact, double-stranded DNA was not different from that under control conditions* ($P < 0.2$). At 10 μM, VM-26 induced

* Control cells demonstrate a baseline level of DNA breakage as a consequence of routine experimental manipulation.

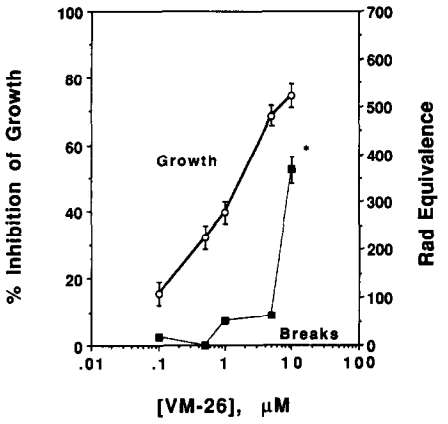

Fig. 5. Influence of VM-26 on proliferation and induction of DNA cleavage in the rat hepatoma. H-35 rat hepatoma cells in logarithmic growth were incubated with the indicated concentrations of VM-26 for 2 hr. Data are mean values ± SEM for 20–30 experiments (proliferation using the MTT tetrazolium dye assay) and 9–13 experiments (DNA strand breaks using alkaline unwinding). Baseline DNA cleavage of 100 ± 2.5 rad equivalents was subtracted at each concentration of VM-26. Key: * $P < 0.01$ vs control.

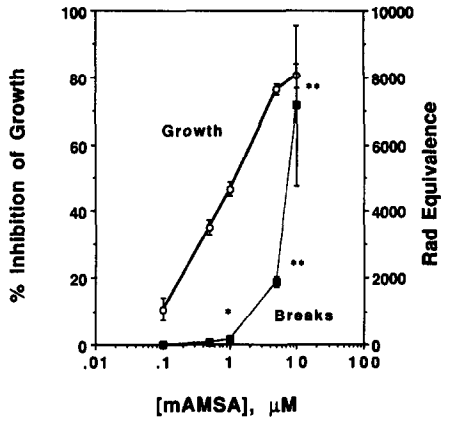

Fig. 6. Effect of *m*-AMSA on growth and DNA integrity in the rat hepatoma. H-35 rat hepatoma cells in logarithmic growth were incubated with the indicated concentrations of *m*-AMSA for 2 hr prior to determination of DNA cleavage using the alkaline unwinding assay. Inhibition of growth was determined using the MTT tetrazolium dye assay. Data are means ± range or SEM for 2–6 experiments (strand breaks) and 4–15 experiments (growth). Baseline DNA cleavage of 91 ± 4.2 rad equivalents was subtracted at each concentration of *m*-AMSA. Key: * $P < 0.01$, and ** $P < 0.001$ vs control.

approximately 367 ± 22 rad equivalents of DNA cleavage in the hepatoma cell ($P < 0.001$).

Table 3 indicates that DNA–protein cross-linking did not differ from control values in the H-35 rat hepatoma cell at a VM-26 concentration of 1 μM (where growth was inhibited by 39.7 ± 3.47%). In this set of experiments, 10.0 ± 1.1% of total DNA which was applied to the filter was retained under control conditions (X-irradiation alone), while 13.0 ± 1.3% of the DNA from hepatoma cells treated with 1 μM VM-26 prior to irradiation was retained; this difference in retention was not statistically significant ($P < 0.1$). At 5 μM VM-26, a small increase in filter retention of [^{3}H]DNA was noted in that 16.6 ± 2.3% of total (X-irradiated) [^{3}H]DNA was retained ($P < 0.01$ compared to control), while at 10 μM VM-26, 18.9 ± 2.8% of total [^{3}H]DNA was retained ($P < 0.01$).

Studies with *m*-AMSA indicate that this drug produces concentration-dependent induction of DNA strand breaks. However, the pattern of strand break production failed to parallel the concomitant inhibition of cell proliferation (Fig. 6). For instance, between 0 and 0.5 μM *m*-AMSA, growth inhibition rose to 35 ± 2.2%, but no DNA cleavage was detected ($P < 0.4$ compared to control), while between 0.5 μM and 5 μM *m*-AMSA, where growth inhibition increased to 76.4 ± 14.6% (a similar incremental increase as between 0 and 0.5 μM), 1905 ± 155 rad equivalents of DNA damage were observed. Table 3 indicates that, in correspondence with the induction of DNA strand breaks, DNA–protein cross-linking was observed at 1, 5 and 10 μM concentrations of *m*-AMSA.*

* Assessment of cell number and viability using the MTT assay immediately after a 2-hr incubation with either VM-26, VP-16 or *m*-AMSA indicated that these agents failed to alter cell integrity.

DISCUSSION

In studies of *acquired resistance*, tumor cell sensitivity to antineoplastic drugs and alterations in select biochemical parameters are compared in a "parent" or wild-type cell line and in a cell line(s) derived from the wild-type by selection in the presence of drug. However, this approach is not feasible when studying an intrinsically resistant cell line such as the H-35 rat hepatoma. In the present analysis, comparative studies of drug sensitivity of MCF-breast tumor cells and the rat hepatoma substantiate the intrinsic drug resistance of the rat hepatoma cell to the anthracycline antibiotics [16, 17]. The H-35 rat hepatoma cell also appears to be intrinsically resistant to the epipodophyllotoxin derivatives and vinca alkaloids but not to *m*-AMSA. The low drug sensitivity in experimental hepatoma is consistent with the relative unresponsiveness of hepatocellular malignancies to chemotherapy [66].

The apparent cross-resistance to three different classes of antineoplastic drugs in the H-35 rat hepatoma cell line is consistent with the presence of the multi-drug resistance phenotype associated with expression of the P-170 membrane glycoprotein which mediates drug efflux [1–3]. As demonstrated in a variety of cell lines [43, 53, 55, 67], resistance to the vinca alkaloids, vincristine and vinblastine, the anthracycline antibiotics, Adriamycin and daunorubicin, and the demethylepipodophyllotoxin derivative, VM-26, was modulated by verapamil in the H-35 hepatoma cell. Verapamil did not significantly lower the IC_{50} value for VP-16 ($P \sim 0.2$), suggesting that this drug may not share the efflux pathway inhibited by verapamil, or that verapamil

may fail to compete effectively with VP-16 for transport out of the cell. Politi and Sinha [56] have previously reported a lack of enhancement of sensitivity to VP-16 by verapamil in the multi-drug-resistant MCF-7 human breast tumor cell line. Verapamil also failed to alter the IC_{50} for *m*-AMSA ($P \sim 0.9$), suggesting that *m*-AMSA is not a substrate for the P-170 efflux pump, a conclusion which would be consistent with the fact that the H-35 hepatoma cells do not appear to be resistant to *m*-AMSA.

Although verapamil lowered the IC_{50} values for the anthracycline antibiotics to a range observed in drug-sensitive cells [50–53], the amount of daunorubicin accumulated by the H-35 cells even in the absence of verapamil (29 pmol/10^6 cells; [16]) exceeded that accumulated in L1210 leukemia cells at a similar concentration (4 pmol/10^6 cells; [68]). This observation suggested that unusually high intracellular concentrations of daunorubicin may be required to inhibit hepatoma cell growth, and implied that other factors, such as the activity of glutathione redox enzymes, may contribute to drug resistance in the H-35 cell.

The levels of glutathione and the activity of glutathione-related enzymes in the rat hepatoma cell were found to be relatively high compared to other tumor cell lines which are either sensitive or resistant to the anthracycline antibiotics such as the MCF-7 breast cancer line, a human fibrosarcoma and human lung cancer cells [6, 46, 57, 58]. The previously reported lack of conversion of anthracyclines to deoxyaglycones in the hepatoma cell [18] suggests the absence of enzyme activities necessary for activation of anthracyclines to free radicals [69, 70]. These observations regarding the glutathione redox pathway, the lack of conversion to deoxyaglycones, and the lack of protection from the antiproliferative activity of daunorubicin and idarubicin by free radical scavengers [18, 71] suggest that the hepatoma cell is not likely to be sensitive to anthracycline-induced injury mediated by free radicals. This conclusion is also supported by the absence of non-protein associated single-strand breaks in hepatoma cells treated with daunorubicin [18].

Recent evidence indicating that the demethyl-epipodophyllotoxins may be converted to free radical intermediates suggests that detoxification capacity via the glutathione redox pathway which protects against free radical mediated injury could potentially protect the tumor cell from the cytotoxic effects of VM-26 and VP-16 as well [72].

In the rat hepatoma cell, the demethylepipodophyllotoxin derivatives, VM-26 and VP-16, inhibit cell growth while producing minimal (essentially undetectable) or relatively low levels of DNA strand breaks and DNA–protein cross-links, the lesions associated with inhibition of topoisomerase II [31, 35, 40, 54, 61, 62, 64, 65]. At the IC_{50} value for VM-26 there was no detectable DNA cleavage or DNA–protein cross-linking. At the IC_{50} value for VP-16, there was no detectable DNA–protein cross-link induction and barely 100 rad equivalents of DNA cleavage. In contrast, in other tumor cell lines, the epipodophyllotoxin derivatives have generally been shown to induce relatively high levels of DNA cleavage in association with expression of their antiproliferative and cytotoxic activities. For instance, in wild-type MCF-7 breast tumor cells, and in K562 human leukemic cells, VP-16 produces 1000 rad equivalents of DNA strand breakage at concentrations below the IC_{50} value [73, 74]. At a concentration close to its IC_{50} value, VP-16 produces approximately 1700 rad equivalents of DNA cleavage in L1210 leukemia cells [40]. In the H-35 rat hepatoma cell, DNA cleavage induced by the aminoacridine, *m*-AMSA, also failed to parallel the inhibition of cell proliferation. These observations suggested that the drug sensitivity of topoisomerase II might also contribute to resistance in the H-35 rat hepatoma cell line.

The amount of extractable topoisomerase II activity in the H-35 cells was considerably lower than in HL-60 cells, which may, in part, account for the low levels of DNA damage induced by the topoisomerase II inhibitors in the H-35 cells. However, when similar amounts of topoisomerase II activity (as quantitated by decatenation) were utilized, VM-26, VP-16 and *m*-AMSA stimulated DNA–protein cross-linking in extracts from the hepatoma cell as well as or better than in extracts from HL-60 cells. Therefore, the topoisomerase II in H-35 cells is unequivocally sensitive to these antineoplastic drugs.

A number of laboratories have reported dissociation between the induction of DNA strand breaks (i.e. bulk DNA damage) and the antiproliferative/cytotoxic effects of antineoplastic drugs. For instance, we have reported previously that the anthracycline antibiotic, daunorubicin, also fails to induce DNA damage in the hepatoma cell at drug concentrations where proliferation is inhibited by up to 80% [18]. While idarubicin *was* observed to induce DNA strand breaks in the hepatoma cell, these breaks failed to correspond *directly* with antiproliferative activity [71]. In terms of tumor cell lines with acquired resistance, Bakic *et al.* [34] demonstrated that the cytotoxicity of *m*-AMSA fails to correspond with induction of DNA damage in an HL-60 tumor cell line resistant to *m*-AMSA. At approximately 1 log cell kill, *m*-AMSA produced more than 1500 rad equivalents of DNA cleavage in HL-60 cells and less than 100 rad equivalents of cleavage in the HL-60/*m*-AMSA line. This was speculated to be due to a non-topoisomerase II-related mechanism of *m*-AMSA-induced cytotoxicity that could only be detected in a cell line with an *m*-AMSA-resistant form of topoisomerase II [14]. In Adriamycin-resistant MCF-7 breast cancer cells, Sinha *et al.* [74] recently reported the absence of DNA strand breaks at toxic concentrations of VP-16. Similarly, in epipodophyllotoxin-resistant L1210 cells, Roberts *et al.* [75] reported minimal DNA strand breaks at the IC_{50} for VM-26. In a drug-resistant Chinese hamster ovary cell line, Spironidis *et al.* [76] recently demonstrated dissociation between cytotoxicity and strand breaks for VP-16.

Although Seneviratne and Goldenberg [77] have recently demonstrated a close correlation between induction of DNA stand breaks and toxicity of Adriamycin in both sensitive and resistant P388 leukemia cells, the dissociation(s) between bulk DNA damage and antitumor activity, as cited,

appears to occur primarily in cells which are drug resistant, probably because, in these cells, the more typical mechanism of cytotoxicity is not operational. The H-35 rat hepatoma cell, a carcinogen-induced tumor cell line, has been shown to be *intrinsically resistant* to the anthracycline antibiotics both *in vitro* and *in vivo* [16, 17]. This resistance is consistent with the studies by Carr and Laishes [78], indicating that cells cultured from carcinogen-induced preneoplastic nodules in liver are resistant to the cytotoxic effects of various chemicals including Adriamycin.

In summary, in the hepatoma cell where resistance to antineoplastic agents appears to be multifactorial, involving high levels of metabolic (detoxification) enzyme activity and presumed limitations on drug accumulation related to expression of the multi-drug resistance phenotype, an apparent dissociation is observed between the antiproliferative activity of select topoisomerase II inhibitors and the induction of bulk damage in DNA (i.e. damage throughout the genome). This dissociation between bulk DNA cleavage and antiproliferative activity suggests that it may be important to assess the capacity of topoisomerase II inhibitors to produce breaks at *discrete* genomic sites, since these lesions may ultimately prove to be more relevant to the mechanism of action of these agents than damage throughout the genome [79].

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