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PERSISTENT INTRACELLULAR BINDING OF MITOXANTRONE IN A HUMAN COLON CARCINOMA CELL LINE

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Abstract—Incubation of human carcinoma cells with mitoxantrone resulted in an intracellular distribution of the drug into cytoplasmic, nuclear and cytoskeletal compartments occurring within 1 min of drug treatment. Incubation of the cells in drug-free medium resulted in an efflux of the drug such that 80% of the intracellular drug was eliminated from the cells by 72 hr. Approximately 20% of the initial intracellular drug concentration remained in the cells after the drug had been removed from the medium. The majority of the persistent intracellular drug was associated with soluble cytoplasmic proteins and fractions enriched in nucleic acid. Approximately 10% of the persistent drug binding was associated with cellular structures that had been depleted of soluble cytoplasmic protein and nucleic acid. During the persistent drug binding, the cells enlarged at least 2-fold as determined by microscopic examination. An increasing percentage of the cells was also observed to contain a DNA content consistent with a G_2 cell cycle arrest. Taken together, these data suggest that the persistent intracellular binding of mitoxantrone results in a G_2 cell cycle arrest and cellular damage.

Mitoxantrone is a chemotherapeutic anthracene drug used for the treatment of a number of cancers including breast cancer, leukemias, and non-Hodgkin's lymphomas [1]. Although it shares structural similarities to the anthracycline doxorubicin, the mechanism(s) of action of the two drugs appears to be quite different. Doxorubicin is a DNA intercalator whose primary target appears to be DNA, although its mechanism of action is not wholly understood [2]. While mitoxantrone binds DNA, it does not appear to be a DNA intercalator, nor do the induced DNA lesions (single-strand breaks, double-strand breaks, and DNA–protein crosslinks) correlate with its cytotoxicity [3]. Alternative biological targets may be involved in the cytotoxic action of mitoxantrone.

The amount of intracellular mitoxantrone accumulation may be important to the drug toxicity, as cell lines either selected for resistance to mitoxantrone directly or acquiring resistance as a result of the multidrug resistance (MDR) phenotype are associated with reduced intracellular drug accumulation [4, 5]. In the case of MDR, the decreased net intracellular drug accumulation is a result of overexpression of P-glycoprotein (P-gp) and enhanced drug efflux. However, others have suggested that drug resistance may involve more than P-gp alone. In some cases, the degree of resistance is not correlated directly with the degree of intracellular accumulation of drug, and MDR cell lines which do not display P-gp have been reported [6–9]. Anthracycline-resistant P388 cells have been reported which possess identical intracellular drug concentrations compared to the drug-sensitive parent cell line [10]. We recently reported a mitoxantrone-resistant human colon carcinoma cell line which had only a slight decrease in mitoxantrone intracellular concentration compared to the drug-sensitive cell line which did not overexpress P-gp, suggesting that other mechanisms may be operating in mitoxantrone resistance [4]. Studies detailing the intracellular binding of a chemotherapeutic agent which is not removed from the cells may provide insight into the cytotoxic mechanism of action of the drug. We investigated the persistent intracellular binding of the chemotherapeutic agent mitoxantrone within biochemical fractions of a human colon carcinoma cell line which is not multidrug resistant, and examined its relationship to cell division and cell morphology.

MATERIALS AND METHODS

Cell cultures, labeling conditions. WiDr, a human colon carcinoma cell line, was obtained from the American Type Culture Collection and was maintained in exponential growth in monolayer culture in RPMI 1640 medium (Gibco Inc., Grand Island, NY) containing 10% fetal bovine serum (Whittaker) and 1% penicillin–streptomycin (Gibco). The cells were maintained in a humidified incubator of 95% air and 5% CO_2. The cell cultures were plated at 0.2×10^6 cells/35 mm dish in 2 ml of medium and were grown for 2 days at 37° prior to the experiments. Cell numbers were determined using an electronic particle counter (Coulter Electronics, Hialeah, FL). Cellular protein was labeled using 0.5 μCi/ml [^{35}S]methionine (*Escherichia coli* hydrolysate labeling agent, sp. act. 1037 Ci/mmol, ICN), in medium containing 10% of the normal concentration of methionine for 18 hr; then the medium was replaced with fresh medium for 4 hr. DNA was labeled using

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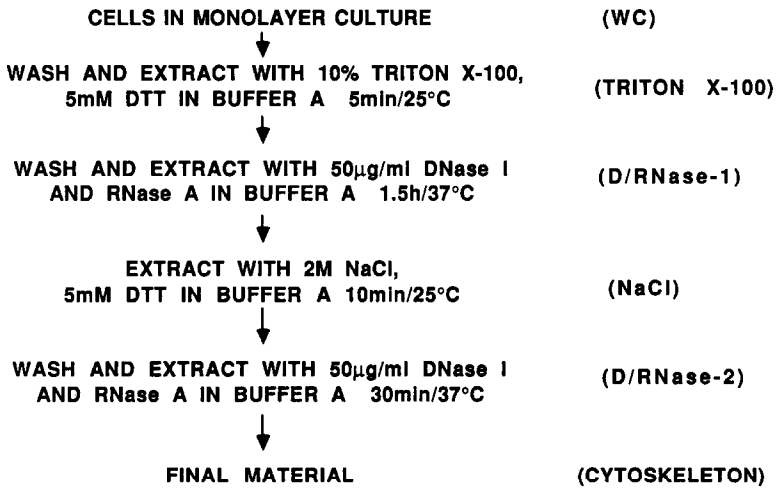

Fig. 1. Biochemical fractionation procedure. WiDr cells were extracted sequentially, and at each fractionation step the solubilized proteins were collected. The collected fractions correspond to the names shown in parentheses at the right of the figure.

0.5 μCi/ml [^{14}C]thymidine (sp. act. 59.3 mCi/mmol, NEN) and RNA was labeled using 0.5 μCi/ml [^{3}H]uridine (sp. act. 1 Ci/mmol, TMM).

Drug accumulation and distribution. In all experiments except drug uptake studies, exponentially growing cells were treated with 50 μM mitoxantrone at 37° for 1 hr. A 1-hr treatment of the WiDr cells with 50 μM mitoxantrone resulted in approximately 1% survival (data not shown), measured by the ability of the cells to form colonies on tissue culture dishes within 2 weeks after treatment. This is in contrast to drug-sensitive and -resistant WiDr cell lines we have used previously whose IC_{50} values were 0.014 and 0.291 μg/ml (0.3 and 0.66 μM respectively) as determined by the ability of the cells to form colonies in soft agar [4]. Although the survival of the cells after drug treatment was low, we do not feel this negates our results since we were studying possible alternative targets for the drug toxicity. For technical reasons, the high drug concentrations (by survival criteria) are necessary to quantitate the amount of radioactive drug within the cellular fractions, especially the cytoskeleton fraction. For drug uptake studies, exponentially growing cells were treated with 50 μM mitoxantrone for 1, 5, or 60 min as described above. If drug efflux was to be allowed, cell monolayers were rinsed twice with fresh medium and returned to 37°. Mitoxantrone (Medical Research Division, American Cyanimide Co.) was stored as 1 mM sterile aliquots in distilled deionized H_2O at −20°. The [^{14}C] ring-labeled mitoxantrone (SRI, sp. act. 8.1 mCi/mmol, chemical purity 96%) was stored similarly in dimethyl sulfoxide (DMSO). Although the ring-labeled compound was chosen to minimize the possibility of metabolic reutilization of the [^{14}C], no mitoxantrone metabolites were detected by HPLC analysis (data not shown). Fifty micromolar treatment solutions were made just prior to use with medium and 50 μl/ml of stock solution.

Biochemical fractionation. Cell monolayers were extracted *in situ* by a modification of the procedure according to Staufenbiel and Deppert [11]. An outline of the method appears in Fig. 1. Triton X-100 was purchased from Sigma Chemical Co. (St Louis, MO), dithiothreitol (DTT) from Calbiochem (La Jolla, CA), DNase I (200 units/ml, RNase free) from BRL, and RNase A (5401 units/mg, phosphate free) from Cooper Biochemical. The final cytoskeletal material was collected by scraping into Buffer A (60 μM PIPES,* 25 μM HEPES, 10 μM EGTA, 2 μM $MgCl_2$, 10 μM NaCl, pH 6.2). Radiolabeled fractions were quantitated after adding 2 ml of extract to 10 ml of liquid scintillation fluid (ICN), using a Tracor Analytic model 6892 liquid scintillation counter. The efficiency of counting [^{14}C] under our conditions was approximately 58%.

Fluorescence microscopy. During the fractionation procedure, as the soluble fractions were removed, an identical set of culture dishes was monitored microscopically to record the morphology of the cellular material after each fractionation step. The cellular structures attached to the dish after the fractionation steps were fixed with 3.7% formaldehyde in PBS (10 mM KH_2PO_4, 150 mM NaCl, pH 7.4) for 10 min at 25°, washed twice with PBS, reacted in the dark with 1 μg/ml Hoechst dye for 10 min at 25°, and rinsed twice with PBS. Unextracted cells were permeabilized with 10% Triton X-100, 5 mM DTT in Buffer A for 5 min prior to staining. The cells within the culture dish were visualized using a Zeiss Photomicroscope II equipped with a Zeiss Epifluorescence condenser IIIRS using 02 barrier filters, a 150 W lamp, and a Plan-Neofluor 63 immersible objective. The images were recorded using Kodak Tmax film (ASA 400).

Flow cytometry measurements. After the appropriate treatment, cells were harvested by trypsinization, and the number of cells was determined using a hemacytometer. The single cell suspensions (1×10^5 cells/ml) were fixed with methanol:acetic acid (3:1) and stored at −20° until the analysis was performed. For analysis, fixed cells were rinsed three times with medium until the pH was neutral, centrifuged at 2000 *g*, resuspended in medium at 1×10^6/

* Abbreviations: PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; and EGTA, ethyleneglycolbis (aminoethylether)tetra-acetate.

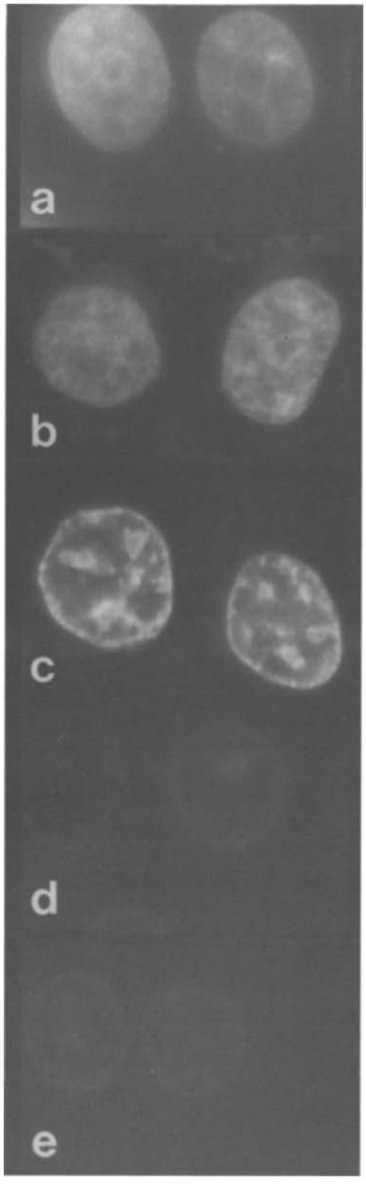

Fig. 2. Morphological appearance of cells during biochemical fractionation. WiDr cells were fractionated according to the sequential procedure outlined in Fig. 1. The residual cellular material remaining attached to the tissue culture dish after the Triton X-100 (panel b), D/RNase-1 (panel c), NaCl (panel d), D/RNase-2 (cytoskeleton) (panel e) as well as intact cells (panel a) was stained with a Hoechst dye (×1000).

ml, and treated with 0.1 mg/ml RNase A (DNase heat inactivated) for 30 min at 25°. Cells were stained with 100 μg/ml propidium iodide (Sigma) and kept at 4° in the dark until analyzed.

Flow cytometric analysis for quantitation of DNA content was performed in a Becton Dickinson FACSTAR analyzer, used at 200 mW, equipped with a water-cooled 5 W argon-ion laser. Excitation wavelength was 488 nm, with red fluorescence detected using a 585 nm filter with bandwidth of 44 nm. The fluorescent signal was read on a 256 channel linear scale. A stream-in-air nozzle tip with a 70 μm orifice diameter was used for cell interrogation. Forward and 90 degree light scatter were used to determine the cell populations. Data analysis was performed on a Hewlett–Packard 9000 series computer (model 310) using the Consort Ver E and DNA Cell-Cycle Analysis software Ver C, polynomial, or sum of broadened rectangles, programs from Becton Dickinson. Ten thousand events per sample were collected, and the percent positive cells and fluorescent intensity were determined. The instrument was standardized daily.

RESULTS

Characterization of cellular fractions. The fractionation procedure used was a modification of a procedure designed to isolate a cellular matrix and is outlined in Fig. 1. The advantage of fractionation *in situ* is that cytoskeletal structures are maintained throughout the fractionation and the extractions are reproducible. During the extraction, the cells remained attached to the monolayer, and the fractions were defined as the material that became soluble during the extraction step. For example, the Triton X-100 fraction refers to the soluble material that was liberated from the cells after the Triton X-100 had been applied and rinsed from the dishes. During the fractionation procedure, the cells were monitored for the attachment of the extracted cells to the culture vessel using fluorescence microscopy (Fig. 2). The cells remained attached to the monolayer as shown in Fig. 2, and a morphology consistent with cells undergoing extraction was observed. Nuclear structures were visualized using a Hoechst DNA fluorochrome during all stages of the fractionation.

The first step in the fractionation procedure involved the solubilization and permeabilization of the plasma membrane and removal of soluble cytosolic proteins by the use of the non-ionic detergent Triton X-100, in the presence of the reducing agent DTT. During this step, 53% of the cellular protein, 6% of the cellular DNA and 19% of the RNA were removed (Table 1).

Extraction with DNase I and RNase A was performed next and the resulting soluble material was designated D/RNase-1 in Fig. 1. This step removed

Table 1. Composition of the biochemical fractions

Fractions	Protein (%)	DNA (%)	RNA (%)
Triton X-100	53.0 ± 2	6.2 ± 1.1	19.4 ± 2.1
D/RNase-1	24.2 ± 1.0	64.4 ± 0.8	68.8 ± 2.0
NaCl	10.9 ± 0.6	27.4 ± 1.2	11.2 ± 0.3
D/RNase-2	1.2 ± 0.1	0.7 ± 1.1	0.4 ± 0.1
Cytoskeleton	10.6 ± 1.3	1.1 ± 0.5	0.3 ± 0.1

The protein, DNA and RNA contents removed from the cells during the fractionation procedure were estimated by [^{35}S]methionine, [^{14}C]thymidine and [^{3}H]uridine incorporation respectively. The incorporation values for protein were approximately 2.4×10^6 cpm/10^6 cells; for DNA, 6.5×10^3 cpm/10^6 cells; and for RNA, 1.1×10^4 cpm/10^6 cells. Each value is the mean ± SE of six determinations.

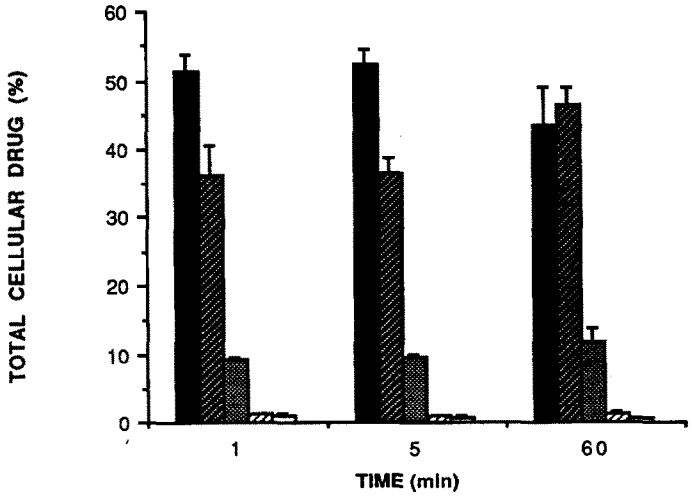

Fig. 3. Intracellular distribution of mitoxantrone during drug treatment. WiDr cells were incubated at 37° in medium containing 50 μM [^{14}C]mitoxantrone for 1, 5, or 60 min and fractionated as described in Fig. 1. The percentages of drug found in each fraction are indicated as follows: (■) Triton X-100, (▨) D/RNase-1, (▩) NaCl, (▨) D/RNase-2, and (□) cytoskeleton. The bars are means ± SE of six determinations, except for those at 60 min, which are means ± SE of twelve determinations. Error bars which are not visible were smaller than the value indicated by the line. The total cpm/dish (100%) of drug for the 1-min exposure was 23,300 ± 2,000, for the 5-min exposure 31,800 ± 1,700, and for the 60-min exposure was 145,600 ± 26,200.

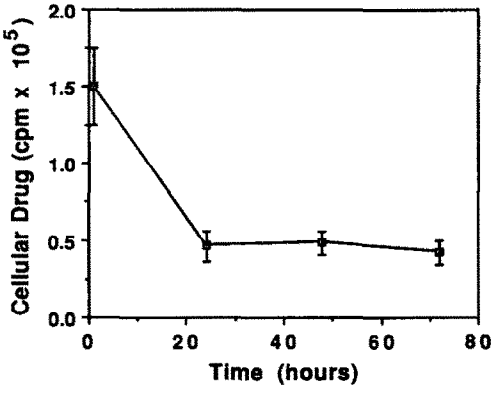

Fig. 4. Intracellular drug concentration during incubation in drug-free medium. WiDr cells were incubated at 37° in 50 μM [^{14}C]mitoxantrone for 1 hr and then placed into drug-free medium. Data points are the means ±SE of six determinations, except for the 0 hr which is the mean ± SE of twelve determinations. The intracellular concentration of the drug after 1 hr of incubation in drug-containing medium was 1.5×10^5 cpm/1.0×10^6 cells which is approximately 60 pmol of mitoxantrone.

64% of the DNA, 69% of the RNA, and 24% of the protein from the cells (Table 1). The majority of the nucleic acid and protein (approximately 77%) was removed from the cells after this step. As expected, the intensity of the DNA staining was diminished after the D/RNase-1, and the residual DNA was concentrated on the periphery of the nucleus and around the nucleoli (Fig. 2, panel c).

The next extraction, with 2 M NaCl, contained 27% of the DNA, 11.2% of the RNA and 10.9% of the cellular protein (Table 1). At this point in the extraction, approximately 98% of the DNA, 99% of the RNA and 89% of the protein had been removed from the cells. Fluorescence microscopy confirmed that very little of the nucleic acid remained in the residual cellular structures (Fig. 2, panel d). Other investigators have reported that a second DNase I and RNase A digestion step is required after the NaCl extraction to remove residual ribonucleoprotein particles which are trapped during the previous steps [12]. The next step, D/RNase-2, was a shorter repeat of the first DNase/RNase step. This step removed approximately 1% of the nucleic acid and 1.2% associated protein (Table 1). Again, the Hoechst DNA staining was minimized (Fig. 2, panel e) in the residual structures.

The final step in the fractionation was the removal of residual material from the tissue culture dish. This cystoskeleton fraction contained 11% of the cellular protein, 1.1% DNA and 0.3% RNA. The predominant proteins present in this fraction were cytokeratin and lamin intermediate filaments as determined by two-dimensional sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (data not shown).

Intracellular distribution of mitoxantrone during drug uptake. Within 1 min of incubation, mitoxantrone was found in all of the biochemical fractions (Fig. 3). The observation of a rapid drug uptake occurring within 0.5 min of incubation has been noted previously for doxorubicin, an anthracycline agent [13]. The majority of the drug (52%) was found associated with the Triton X-100 fraction and approximately 35% of the drug was found in the D/RNase-1 fraction. After a 1-hr incubation of the cells with mitoxantrone, the distribution of the drug among the fractions was altered significantly such that 43 and 46% of drug was found associated with the Triton X-100 and D/RNase-1 fractions respect-

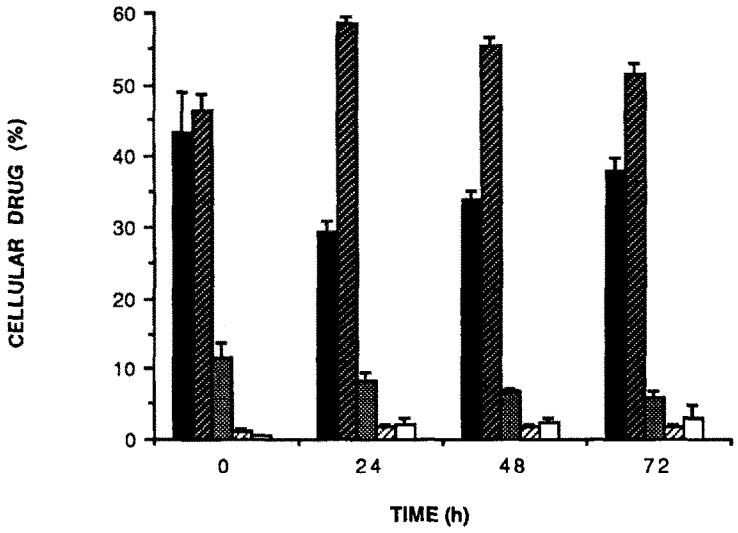

Fig. 5. Intracellular drug distribution during incubation in drug-free medium. WiDr cells were incubated at 37° in 50 μM [^{14}C]mitoxantrone for 1 hr, incubated in drug-free medium, and fractionated as described in Fig. 1. The percentages of the drug found in each fraction are indicated as follows: (■) Triton X-100, (▨) D/RNase-1, (▩) NaCl, (▨) D/RNase-2, and (□) cytoskeleton. The bars are means ± SE of six determinations, except for those at 0 hr, which are the means ± SE of twelve determinations. The total cpm/dish (100%) of drug for the 60-min exposure was 145,600 ± 26,200.

ively. During the initial rapid uptake of the mitoxantrone, more drug was associated with the Triton X-100 fraction, but during the 1-hr incubation period a redistribution of drug occurred so that the greatest percentage of the drug (46%) was present in the D/RNase-1 fraction.

A significant amount of drug (approximately 10%) was found associated with the residual structures (Fig. 1, NaCl, D/RNase-2 and cytoskeleton fractions) after 1, 5 or 60 min of drug incubation. It is interesting that even during the earliest incubation period a portion of the drug was found in the fractions (D/RNase-2 and cytoskeleton) known to contain insoluble proteins. The data are consistent with our earlier report that mitoxantrone binds to the cytokeratins 8, 18 and 19 [14].

Intracellular distribution of mitoxantrone during drug efflux. The WiDr cells were incubated with medium containing 50 μM [^{14}C]mitoxantrone for 1 hr at 37°, and then were placed into drug-free medium to observe the efflux of the drug from the cells. Under these conditions, the cells initially contained approximately 1.5×10^5 cpm of mitoxantrone, corresponding to approximately 60 pmol of drug before the cells were transferred to drug-free medium (Fig. 4). Approximately 8.1×10^3 cpm of drug (3.2 pmol) was bound to the tissue culture dish independent of the presence of cells and was retrievable by solubilization with DMSO (data not shown). After 24 hr of incubation in drug-free medium, the cells retained approximately 31% of the initial amount of drug. A residual amount of drug was observed to be persistent since an incubation period up to 72 hr failed to remove it. Drug-resistant subclones of WiDr that had been maintained in medium containing mitoxantrone had to be incubated for more than 1 week to remove the persistent binding (data not shown).

The distribution of mitoxantrone within cells prior to efflux indicated that 43% of the drug was associated with the Triton X-100 fraction, 46% was present within the D/RNase-1 fraction, 12% in the NaCl fraction, 1.3% in the D/RNase-2 fraction, and 0.6% in the cytoskeleton fraction (Fig. 5). After 24 hr of incubation in drug-free medium, the distribution of mitoxantrone was altered markedly since the amount of drug associated with the Triton X-100 fraction declined to 29% and the drug associated with the D/RNase-1 fraction increased to 58%. Interestingly, the amount of drug associated with the cytoskeleton fraction was observed to increase approximately 3-fold (to 2.2%) during the 24-, 48- and 72-hr incubation period. Taken together, these data suggest that, during prolonged incubation periods in drug-free medium, the majority of drug which leaves the cell does so from the Triton X-100 fraction, as evidenced by the decline of the percent drug present. In contrast, although the drug was lost from all fractions (Fig. 4), drug was retained in the D/RNase-1 and cytoskeleton fractions, perhaps reflecting a diminished rate of drug loss as compared to the Triton X-100 fraction (Fig. 5).

The trend of the increasing percentages of drug in the D/RNase-1 fraction and decreasing percentages in the Triton X-100 fraction, originating in the uptake data, continued through 24 hr of efflux. After 24 hr, the relative percentage of drug in the insoluble cytoskeleton fraction increased, and the percentage in the NaCl fraction decreased during later efflux, suggesting a redistribution of the drug with time. Throughout the drug retention experiments, the drug continued to be present in all fractions, with 22% of the original drug concentration still present in the cells at 72 hr. The persistent drug binding represented binding by the parent compound and not a metabolite as judged by HPLC analysis (data not shown).

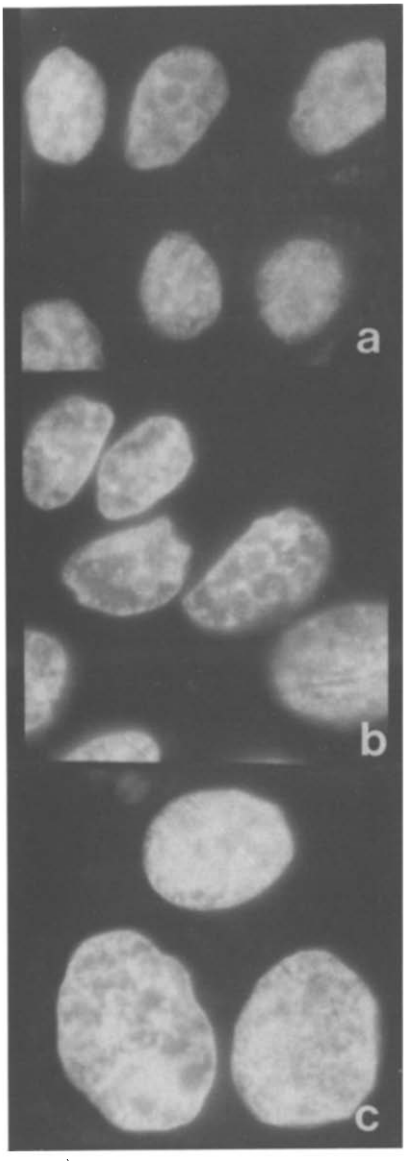

Fig. 6. Morphological appearance of cells during drug retention. WiDr cells were either untreated (panel a), incubated at 37° in 50 μM mitoxantrone for 1 hr (panel b) or treated for 1 hr as in panel b and then incubated in drug-free medium for 72 hr (panel c), stained with a Hoechst dye and photographed (×1000).

Cellular morphology during persistent drug binding. As can be seen from Fig. 6 (panels a and b), cells treated for 1 hr at 37° with 50 μM mitoxantrone were not morphologically different from untreated cells when viewed immediately after the drug treatment. However, if the cells were treated for 1 hr, then allowed to efflux drug for 72 hr, the cells increased in size approximately 2-fold (Fig. 6, panel c). The increase in the size of the cells was not due to osmotic effects of the drug, as observed by others, since the cells did not increase in size within 1 hr of drug treatment, when the intracellular drug concentration was highest, but required a prolonged incubation period (72 hr) after the drug was removed and the intracellular concentration of drug was at its lowest point.

Since the increase in cell size was nuclear as well as cytoplasmic and required prolonged incubation of the cells in drug-free medium, we investigated whether the cell population had been synchronized by the action of the drug. Figure 7 illustrates the cell cycle distributions observed within the different cell populations as determined by DNA content. Exponentially growing WiDr cells had a population doubling time of approximately 24–30 hr (data not shown). During exponential growth, 24.3 and 30.9% of the cells resided in the S and G_2 + M phases, respectively, and the majority of the cells were in G_1 (44.1%) (Fig. 7). After 24 hr of incubation in drug-free medium, corresponding to the approximate doubling time for the population of cells, the percentage of cells within the G_1 phase decreased to 32.7% with corresponding increases in S (37.3%) and the G_2 + M remaining relatively constant at 28.4%. After 72 hr of incubation in drug-free medium, the cells were extensively G_2 blocked, with 5.0% in G_1, 24.9% in S, and 67.9% in G_2 + M. The cell cycle arrest became even more dramatic by 96 hr of incubation in drug-free medium since 84.3% of the cells were in G_2 + M (as compared to 30.9% seen in the untreated cells).

DISCUSSION

In an attempt to discover alternative biological targets for the chemotherapeutic drug mitoxantrone, we investigated the intracellular distribution of the drug within a human carcinoma cell line under conditions of drug uptake and efflux. The association of mitoxantrone within various biochemical fractions of WiDr cells revealed a number of interesting observations. The drug was found in all of the biochemical fractions within 1 min of drug incubation. During the incubation of the cells in drug-free medium, after a 1-hr drug treatment, approximately 20% of the initial intracellular concentration of drug was still associated with the cells after 72 hr. The presence of the persistent drug retention is especially intriguing since it correlates with the increasing size of the cells and a G_2 and S cell cycle arrest. The persistent binding of the drug at 24 hr after the drug had been removed corresponded to a time when the proportion of cells in G_2 began to increase and the cell cycle arrest became readily apparent by 72 hr as over half of the cells were in G_2. We are presently designing more direct experiments to test the strength of this correlation.

During the persistent drug binding, a significant and increasing proportion of the retained drug was found in biochemical fractions which were enriched in cellular structural proteins. We are presently using non-denaturing preparative polyacrylamide gels to identify other protein components of the drug binding. In previous experiments using denaturing two-dimensional SDS–polyacrylamide gel electrophoresis, we have found that mitoxantrone tenaciously binds to cytokeratins 8, 18 and 19 within this cell line [14]. The cytokeratins belong to a family of intermediate filament proteins some of which (the nuclear lamins) are known to be important for cell

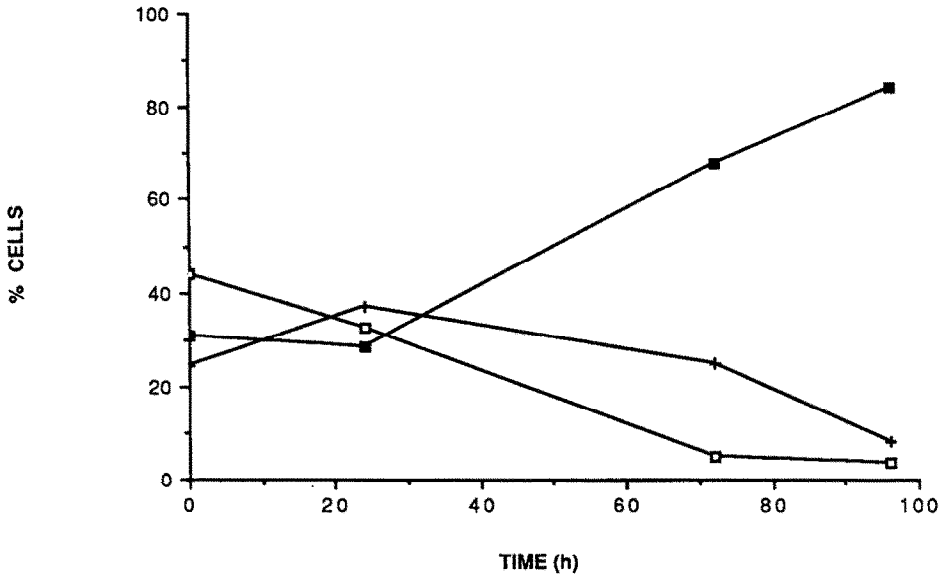

Fig. 7. Cell cycle analysis of cells during persistent drug retention. WiDr cells were incubated at 37° in 50 μM mitoxantrone for 1 hr, incubated in drug-free medium, and analyzed by fluorescence activated cell sorting. The percentages of cells in each cell-cycle population were derived from a gated majority of approximately 6,000 cells/determination, from a population of 10,000 cells at each data value. The percentage of cells found in each population is indicated as follows: (□) G_1 phase, (+) S phase, and (■) G_2 + M phase.

division, [15, 16]. The G_2 arrest associated with the persistent mitoxantrone binding may reflect damage to intracellular proteins such as the intermediate filaments.

The continued retention of significant amounts of chemotherapeutic agents in cells after long incubations (72 hr) in drug-free medium has been noted previously [4], and the existence of a tightly bound non-exchangeable drug pool is also apparent from drug localization studies [7]. An inspection of the distribution of doxorubicin in a sensitive and resistant pair of leukemic ascites tumor cell lines (P388) indicated fast and slow components of drug exchange. The fast (early) drug efflux was identical in the pairs; however, the drug-resistant cell line was more efficient in effluxing the drug from the slow (late) exchange component [17]. Similarly, a human leukemic lymphoblast cell line resistant to Vinca alkaloids was found to contain a decreased proportion of drug within a "non-exchangeable" pool as compared to the drug-sensitive cell line [18]. These data imply that a difference exists in the drug binding between sensitive and resistant cells.

At this time we do not know the biological importance of the persistent drug binding that we observed. However, the prolonged blockage of cells from entering mitosis, perhaps mediated by the persistent drug binding, may lead to cellular toxicity. Using the information reported here, that drug binding does exist in experimentally defined cellular fractions, will allow for the isolation of those fractions specifically to determine the dose dependence of the intracellular persistent drug binding. We are continuing our work to study the drug-sensitive and -resistant subclones derived from the WiDr cell line to determine whether the persistent drug binding, G_2 blockage, and increasing drug binding to cellular structural proteins are related to cytotoxicity.

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