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Cytotoxicity and DNA Damage Associated with Pyrazoloacridine in MCF-7 Breast Cancer Cells

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ABSTRACT. We examined the effects of pyrazoloacridine (PZA), an investigational anticancer agent in clinical trials, on cytotoxicity, DNA synthesis, and DNA damage in MCF-7 human breast carcinoma cells. With PZA concentrations ranging from 0.5 to 50 μM for durations of 3–72 hr, cytotoxicity increased in proportion to the total PZA exposure (concentration × time). Inhibition of DNA and RNA syntheses increased with increasing PZA concentration × time (μM · hr). A 24-hr exposure to 1 and 10 μM PZA reduced DNA synthesis to 62 and 5% of control, respectively, decreased the proportion of cells in S phase with accumulation of cells in G_2 + M phase, and inhibited cell growth at 72 hr by 68 and 100%. Newly synthesized DNA was more susceptible to damage during PZA exposure, with subsequent induction of parental DNA damage. Significant damage to newly synthesized DNA as monitored by alkaline elution was evident after a 3-hr exposure to ≥5 μM PZA. Longer PZA exposures (≥10 μM for 16 hr) were required to elicit damage to parental DNA. Induction of single-strand breaks in parental DNA correlated closely with induction of double-strand breaks and detachment of cells from the monolayer. PZA-mediated DNA fragmentation was not accompanied by the generation of oligonucleosomal laddering in MCF-7 cells, but induction of very high molecular weight DNA fragmentation (0.5 to 1 Mb) was detected by pulsed-field gel electrophoresis. *In vitro* binding of PZA to linear duplex DNA (1 kb DNA ladder) and closed, circular plasmid DNA was demonstrated by a shift in migration during agarose electophoresis. PZA interfered with topoisomerase I- and II-mediated relaxation of plasmid DNA in a cell-free system, but the cytotoxic effects of PZA did not appear to involve a direct interaction with topoisomerase I or II (stabilization of the topoisomerase I- or II-DNA cleavable complex). PZA-mediated cytotoxicity correlated strongly with inhibition of DNA and RNA syntheses, and damage to both nascent and parental DNA. Neither the cytotoxicity of PZA nor induction of double-stranded DNA fragmentation was prevented by aphidicolin, indicating that PZA-mediated lethality occurred in the absence of DNA replication. Since free radical formation was not detected, induction of nascent and parental DNA damage appeared to be a consequence of the avid binding of PZA to DNA, presumably by interfering with the access of replication, repair, and transcription enzyme complexes. BIOCHEM PHARMACOL 51;12:1649–1659, 1996.

KEY WORDS. pyrazoloacridine; DNA intercalation; DNA binding; DNA fragmentation

PZA** is an investigational anticancer agent with a tetracyclic structure containing a 9-methoxy substitution and a potentially reducible 5-nitro group ring substitution [1]. PZA exerted broad cytotoxicity against a panel of 38 human solid tumor cell lines, with mean IC_{50} values for continuous exposure ranging from 0.13 to 0.38 μM [1]. PZA was active against multidrug-resistant murine solid tumor and leukemic cell lines, hypoxic HCT-8 human intestinal adenocarcinoma cells, and epithelial tumor samples in the human tumor stem cell clonogenic assay [1–4]. PZA exhibited similar cytotoxicity against exponentially growing and plateau-phase Chinese hamster ovary cells [1–4]. Based on their structure and ability to displace ethidium from DNA, it is thought that members of this class of compounds intercalate with DNA [1, 5]. The molecular target of amsacrine, another acridine compound, appears to be topoisomerase II [6], and it has been suggested that pyrazoloacridines may also target topoisomerase II [1]. In murine L1210 leukemia cells, inhibition of DNA synthesis by 50% required ~5-fold greater concentrations than were required

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***Abbreviations:* PZA, (9-methoxy-*N*,*N*-dimethyl-5-nitropyrazolo-[3,4,5-*kl*] acridine-2(6*H*)-propanamine, monomethanesulfonate; NSC 366140; PD115934); FBS, fetal bovine serum; PCA, perchloric acid; dH_2O, distilled water; TPAH, tetrapropylammonium hydroxide; and DMPO, 5,5′-dimethylpyrroline-*N*-oxide.

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for RNA synthetic inhibition. Pyrazoloacridine derivatives produced protein-associated single- and double-strand breaks in DNA of L1210 cells, but PZA produced the lowest incidence of DNA breaks among the nine derivatives tested and was 80-fold less potent than the most effective member [1, 5]. PZA was selected among the various derivatives for clinical evaluation because *in vitro* studies suggested selective cytotoxicity against six carcinoma cell lines relative to nine leukemic cell lines (mean IC_{50} values were 0.5-fold lower for carcinoma cell lines) [1]. Since the precise mechanism of action is unclear, we wished to elucidate the determinants of cytotoxicity in MCF-7 breast cancer cells and any possible correlation with damage to nascent and parental DNA.

MATERIALS AND METHODS

Chemicals

PZA and [10-^{14}C]PZA (18 mCi/mmol) were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute (Rockville, MD). PZA was dissolved in dH_2O, and stored in separate aliquots at −70° until used. Etoposide, obtained from the Bristol Myers Co. (Wallingford, CT), and camptothecin sodium (Sigma Chemical Co., St. Louis, MO) were dissolved in dimethyl sulfoxide. PBS, RPMI 1640 culture medium, and FBS were obtained from the Biofluids Co. (Rockville, MD). Moravek Biochemicals (Brea, CA) supplied [^{14}C]thymidine (55 mCi/mmol), [6-^{3}H]uridine (23 Ci/mmol), and [^{3}H]thymidine (25 Ci/mmol). Unless otherwise stated, other chemicals were obtained from Sigma.

Cytotoxicity

MCF-7 cells were grown in RPMI 1640 medium supplemented with 2 mM glutamine and 7% FBS at 37° under a humidified atmosphere containing 5% CO_2. The cytotoxicity of PZA was assessed by effects on cell growth, the sulforhodamine B colorimetric method, and clonogenic capacity. Exponentially growing cells were replicately plated (10,000 cells/well for a 6-well plate, 1,500–2,500 cells/well in 96-well microtiter plates, or 300 cells/well in a 6-well plate for colony formation); after overnight incubation, PZA was added. Following the desired PZA exposure, the medium was aspirated and the cells were gently washed twice with RPMI 1640 medium, and then incubated in drug-free complete medium for a total of 72 hr (cell growth) or 7 days (colony formation). Cell number and volume were determined using a Coulter™ Multisizer II (Coulter Corp., Miami, FL). For the microtiter protein-dye assay, cultures were fixed and stained after a 120-hr PZA exposure as previously described [7]. Colony formation (≥50 cells) was assessed by manual counting after staining with 0.25% methylene blue/50% methanol (w/v). PZA exposure was determined by multiplying the concentration (μM) times the duration of exposure (hr).

Cellular Levels of PZA

Exponentially growing cells were incubated with 1 μM PZA in serum-free tissue culture medium at 37°. At intervals up to 4 hr, the cells were washed three times with 10 mL of ice-cold PBS and lysed with 1.5 mL of 1 N NaOH as previously described [8]. Samples were frozen at −20° until analysis of PZA levels by a previously described HPLC method [9]. Protein content of the cell lysates was determined by the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA).

RNA and DNA Syntheses

Exponentially growing cells were exposed to diluent or PZA at concentrations ranging from 0.1 to 10 μM for 3, 8, and 24 hr (DNA synthesis) or after 24 hr (RNA synthesis). DNA synthesis was estimated by incorporation of [^{14}C]thymidine (3–6 hr) into either purified DNA in conjunction with the alkaline elution assay (described below) or acid-precipitable material. RNA synthesis was determined by [^{3}H]uridine incorporation (1 hr) into acid-precipitable material [10]. To determine endogenous dTTP and UTP pools and the formation of [^{3}H]UTP and [^{14}C]dTTP, control and drug-treated cells were rinsed with ice-cold PBS and extracted with 0.5 N PCA. The soluble fraction was neutralized, lyophilized, and resuspended in dH_2O prior to analysis. UTP, [^{3}H]UTP, and [^{14}C]dTTP formation were quantified by an anion-exchange HPLC method with an in-line scintillation detector [10]. dTTP pools were measured by a DNA polymerase assay using synthetic oligonucleotides as template primers as previously described [11]. The total pool size of either UTP and [^{3}H]UTP or dTTP and [^{14}C]dTTP was then used to correct for radiolabelled precursor incorporation into either RNA or DNA as follows: (total dpm incorporated into RNA or DNA) · (1 μCi/2.2 million dpm) · (total pmol UTP or dTTP/total μCi added per flask). The synthetic rate was defined as the corrected picomoles incorporated into RNA or DNA per million cells per hour.

Cell Cycle Analysis

Following a 24- or 48-hr PZA exposure, MCF-7 cells were harvested and incubated in a solution containing 0.1% sodium citrate (w/v), 0.1% Triton-X (v/v), propidium iodide (50 μg/mL) and 1 μg/mL DNAse-free RNAse (EC 3.1.27.5, Boehringer Mannheim, Indianapolis, IN) overnight at 4° (protected from light). The effect of PZA on distribution in cell cycle phases was determined with a FACSort (Becton Dickinson, San Jose, CA) using "CellFit Cell-Cycle Analysis Software version 2.01.2."

Alkaline Elution of Newly Synthesized DNA

Exponentially growing cells were exposed to PZA at various concentrations for 3, 8, and 24 hr, and pulsed with [^{14}C]thymidine (0.05 μCi/mL, 15 mL/flask) during the fi-

nal 3–6 hr of incubation. Equal numbers of radiolabeled cells were deposited on 25 mm Nuclepore™ filters (1 μm pore size, Costar Corp., Cambridge, MA) held in an alkaline elution funnel (Millipore, Marlborough, MA). The cells were lysed in the dark with lysis buffer 1 as previously described [12, 13]. The DNA was washed subsequently with 20 mM EDTA-disodium salt (pH 10), and then eluted for 15 hr with buffer containing 20 mM EDTA adjusted to pH 12.1 with TPAH at 2.4 mL/hr [12]. The disintegrations per minute in each elution fraction and the filter were corrected for background, and the percentage of total nascent [^{14}C]DNA retained on the filter was calculated. The average cell number loaded per funnel was 310,000 ± 83,000.

Detected of Parental DNA Damage

MCF-7 cells were prelabeled with [^{14}C]thymidine (0.05 μCi/mL, 15 mL/flask) for 24 hr. After a 6-hr chase period, the cells were incubated with 0.1 to 10 μM PZA for 3, 8, or 24 hr. Induction of single-stranded DNA breaks was analyzed by fixed pH alkaline elution as described above with the following exceptions: the lysis buffer containing an equal volume of (i) 50 mM glycine, 40 mM EDTA–disodium salt, and (ii) 4% SDS (lysis buffer two); proteinase K (EC 3.4.21.14) 0.5 mg/mL, was added immediately before cell lysis; and the DNA elution buffer also contained 0.1% SDS. Initially, the elution was carried out over a 15-hr period at a rate of 2.4 mL/hr. Since the majority of the DNA that eluted did so in the initial several hours, the elution period was shortened to 4 hr and the flow rate was increased to 4.8 mL/min. The radioactivities in the initial lysis and wash fraction, the alkaline elution fractions, and the filter were determined. The average cell number loaded per funnel was 430,000 ± 40,000.

A filter binding assay was used to assess double-stranded DNA damage resulting from PZA exposure under non-deproteinizing, non-DNA-denaturing conditions [13–15]. Cells prelabeled with [^{14}C]thymidine were exposed to PZA for 16 hr, deposited on a Metricel™ Membrane (0.8 μm, 25 mm, Gelman Science, Ann Arbor, MI) and processed as previously described [13]. The radioactivities in the lysis/wash fraction, in each subsequent elution fraction, and that retained on the filter were determined. DNA fragmentation was calculated by dividing the disintegrations per minute in the eluting fractions by the total disintegrations per minute.

Detection of Double-Stranded DNA Fragmentation by Agarose Electrophoresis

For conventional agarose electrophoresis, nucleic acids were purified by a salting-out method [16]. The extract was resuspended in 10 mM Tris/1 mM EDTA (pH 8.0) and incubated with DNAse-free RNAse for 60 min at 37°. The samples were extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1; by vol.), and the DNA was precipitated with ammonium acetate and ethanol. The purified DNA was resuspended in 10 mM Tris/1 mM EDTA (pH 8.0). Equal amounts of DNA (2–3 μg) were electrophoresed through a 1.5% agarose gel at 70–90 V for 3–4 hr. The gels were then stained with 0.5 μg/mL ethidium bromide, destained, and photographed under UV illumination.

For pulsed-field electrophoresis, intact cells were embedded in 0.5% low melting point agarose plugs (100,000 cells/plug), digested for 48 hr with 5 vol. of a buffer containing 0.5 M EDTA (pH 9.0), 1% sodium lauroyl sarcosine, and 0.5 mg/mL proteinase K at 50°, then washed and stored at 4° as recommended by the manufacturer (Bio-Rad Laboratories). Individual plugs were placed on the teeth of a gel comb, and a 1.3% chromosomal grade agarose gel (160 mL) was cast. A Chef Mapper™ (Bio-Rad Laboratories) multistate program was employed: state 1 was run at 2 V/cm (−53° and +53°) with an initial switch of 25 min, 18 sec and a final switch of 37 min, 41 sec for a total of 31:56 hr; state 2 was run at 6 V/cm (−60° and +60°) with an initial switch of 9 sec and a final switch of 4 min, 40 sec for 11:10 hr. Tris–borate–EDTA buffer (0.5×, 4 L) was recirculated at 14° during electrophoresis. The gels were then stained as described above.

Assessment of PZA–DNA Binding

PZA (0.5 to 250 μM) was incubated at 37° with 2 μg DNA of the following types (all from Gibco BRL, Grand Island, NY): 1 kb DNA ladder, λ DNA, and pBR322 DNA. After 30 min, 3 μL DNA loading buffer was added; the samples were then electrophoresed through a 1% (pBR322 DNA) or a 1.5% agarose gel (linear duplex DNA) at 90 V for 3 hr in 1× Tris–acetate–EDTA buffer. The gels were then stained as described above.

Interaction of PZA with Topoisomerases I and II

Purified calf thymus, human topoisomerase I (EC 5.99.1.2) and topoisomerase II (EC 5.99.1.3) were obtained from TopoGEN, Inc. (Columbus, OH). The topoisomerase assays were carried out by incubating 0.4 μg plasmid DNA (pBR322 for the topoisomerase I assay; SV40 for the topoisomerase II assay) in a 50 mM Tris–HCl buffer containing 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, 30 μg/mL bovine serum albumin, and 1 unit (relaxation assay) or 5 units (cleavage assay) of either topoisomerase I (no ATP) or topoisomerase II (with 1 mM ATP) at 37° for 60 min [6, 17]. The reaction was terminated by the addition of 10% SDS (1/10 vol.); proteinase K (32.5 μg) was added, and the mixture was incubated at 37° for 30 min. Ficoll-bromophenol blue was then added (final, 3.8%:0.18%, v/v), and the samples were electrophoresed through a 1% agarose gel in 1× Tris–acetate–EDTA buffer; the gels were then stained as described above.

The formation of covalent topoisomerase I– and II–DNA complexes in intact cells was quantitated by the SDS–KCl precipitation assay [6, 17, 18]. Cells were prelabeled with [^{3}H]thymidine (1 μCi/mL), and then exposed to etoposide or PZA (1–100 μM) for 30 or 60 min [6, 17, 18]. The cells

were lysed at 65° as previously described, and chromosomal DNA was sheared by passing the cell lysate through a 22-gauge needle. The sample was transferred to a tube containing 250 μL of 325 mM KCl, and mixed vigourously. The sample was cooled on ice, and then centrifuged for 10 min. The pellet was suspended in 1 mL of a wash solution as previously described and placed at 65° for 10 min with occasional mixing. The suspension was cooled on ice for 10 min, centrifuged, and then washed again before resuspending in 200 μL of dH_2O (preheated to 65°). Radioactive counts were determined after the additon of scintillation fluid.

Cytotoxicity of PZA in Drug-Resistant Cell Lines

A doxorubicin-resistant subline of MCF-7 cells (MCF-7/AD10) was maintained in the presence of 10 μM doxorubicin; this line is cross-resistant to a variety of natural products and overexpresses the P-170 membrane glycoprotein [19]. Doxorubicin-sensitive (HL-60) and -resistant (HL-60/ADR^R) human promyelocytic leukemia cells were grown in RPMI 1640 medium supplemented with 2 mM glutamine, non-essential amino acids, 1 mM sodium pyruvate, 10% FBS, and 20 μg/mL gentamicin [20]. HL-60/ADR^R cells are resistant to doxorubicin and etoposide, do not overexpress P-170 glycoprotein, but have reduced topoisomerase II content [20, 21]. P388/W20 parental and P388/CPT45 topoisomerase I deficient murine leukemia cells were provided by the laboratory of Dr. Randall Johnson (SmithKline Beecham Pharmaceuticals, King of Prussia, PA) [22]. Both parental and camptothecin-resistant P388 cells were grown in RPMI 1640 medium supplemented with 20% FBS, 10 μM 2-mercaptoethanol, 100 μg/mL penicillin, and 100 μg/mL streptomycin. P388/CPT45 cells were maintained in 10 μM camptothecin. All drug-resistant cell lines were grown in drug-free medium for two passages prior to an experiment.

Hydroxyl Radical Formation

ESR studies with a spin-trapping technique using purified DMPO (Aldrich Chemical Co., Milwaukee, WI) were carried out as previously described [23, 24]. Cells were harvested and resuspended in ice-cold PBS at a density of 5×10^6 cells/mL. The amount of DMPO-OH adduct formed provided an index of hydroxyl radical formation. The ESR spectrum was recorded on a Varian 104 spectometer operating at 9.5 GHz.

Statistical Analysis

Sigmaplot™ Software version 5.0 for DOS (Jandel Scientific, San Rafael, CA) was used to graph the data and fit regression curves. The strength of association between two variables was assessed by Pearson Product Moment Correlation (Sigmastat™ Software for DOS version 1.0, Jandel Scientific).

RESULTS

Cytotoxicity of PZA

MCF-7 cells were treated with PZA at various concentrations for durations ranging from 3 to 72 hr. Figure 1 shows the percentage of surviving cells at 72 hr as a function of the PZA exposure (concentration × time). As the duration of PZA exposure increased, the concentration required for a given cytotoxic effect decreased. For example, the IC_{90} values for a 3-, 16-, and 24-hr incubation were 16.1, 4.4, and 1.1 μM, respectively. The mean (±SEM) PZA concentration × time values required for 25, 50, and 90% cytotoxicity were 6.5 ± 2.5, 12.5 ± 4.1, and 51.6 ± 10.6 μM · hr, respectively, and 100% lethality occurred with ⩾200 μM · hr. Near maximum effects were achieved with a 24-hr exposure; extending the exposure to 72 hr did not affect the IC_{50} (24 hr, 0.24 μM; 72 hr, 0.35 μM), suggesting a minimum threshold concentration for PZA cytotoxicity.

PZA-Mediated Inhibition of DNA and RNA Syntheses and Cell Cycle Effects

Cellular accumulation of PZA (1 μM) was rapid and reached a plateau within 1 hr (2.6 ± 0.5 nmol PZA/mg protein). To measure accurately the effects of PZA on RNA and DNA syntheses, the endogenous pools of UTP and dTTP were determined, as were the formation of [^{3}H]UTP and [^{14}C]dTTP during pulse exposure to radiolabeled nucleoside precursors. Indeed, the pool sizes of UTP in-

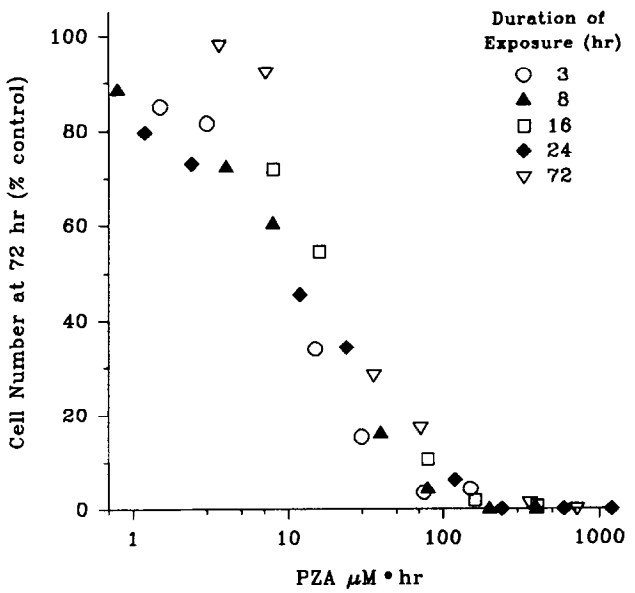

FIG. 1. Cytotoxicity vs PZA concentration × time. MCF-7 cells were treated with diluent or 0.05 to 50 μM PZA for either 3, 8, 16, 24, or 72 hr, followed by a drug-free interval of 69, 64, 56, 48, or 0 hr, respectively. Cell number was enumerated at 72 hr. The data are expressed as the percent of control cell number. The data for each duration of exposure are from two separate experiments, each done in duplicate. The control cell number at 72 hr averaged 198,000 ± 47,000 (N = 10).

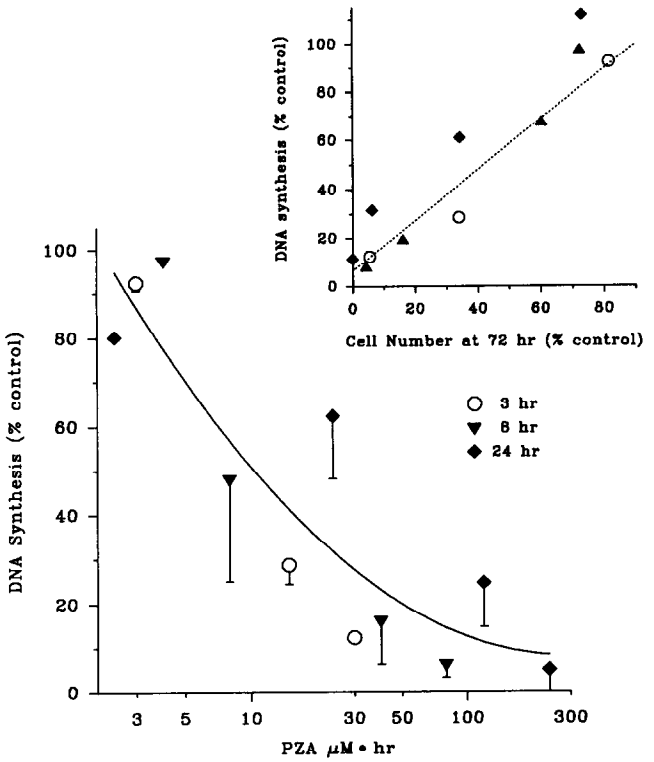

FIG. 2. PZA-mediated DNA synthetic inhibition. MCF-7 cells were treated with diluent or 0.1 to 10 μM PZA for 3, 8, or 24 hr. Cells were incubated with [^{14}C]thymidine (0.05 μCi/mL) for 3–7 hr. Incorporation into acid-precipitable material or purified DNA was determined. The data have been corrected for the total dTTP pool size (both endogenous and [^{14}C]dTTP pools). The DNA synthetic rate in control cells was 8.8 ± 3.4 pmol/10^6 cells/hr (N = 11); the data for drug-treated cells are expressed as percent of control versus PZA μM · hr (r = 0.889). The mean values are shown; the error bars represent the following: 3 hr, ½ range, (N = 2); 8 hr, SD (N = 3); 24 hr, SEM (N = 4–6). The inset shows the correlation between DNA synthetic inhibition during PZA exposure and ultimate cell survival at 72 hr (r = 0.937, P < 0.001).

creased 1.6- to 2.2-fold after a 24-hr exposure to ≥1 μM PZA (mean ± SEM, N = 5): control, 3.1 ± 0.3 nmol/10^6 cells; 1 μM, 5.0 ± 1.0; 5 μM, 5.9 ± 0.2; 10 μM, 6.6 ± 0.6. Similar increases were seen in ATP (1.25- to 1.44-fold), CTP (1.6- to 2.2-fold), and GTP (1.3- to 1.5-fold) pools with 1–10 μM PZA compared with control (data not shown). During a 1-hr incubation with [^{3}H]uridine (1 μCi/flask), [^{3}H]UTP represented 58–68% of the total intracellular [^{3}H]metabolite pool; the absolute amount was about three orders of magnitude lower than the endogenous UTP pools (6.3 to 8.4 pmol/10^6 cells). When the data were corrected for the differences in the endogenous UTP pools, the net RNA synthetic rate in control cells was 312 ± 95 pmol/10^6 cells/hr (mean ± SEM, N = 7). A 24-hr exposure to PZA inhibited RNA synthesis in a concentration-dependent manner (% of control): 0.1 μM, 93 ± 8; 1 μM, 65 ± 4; 5 μM, 50 ± 5; 10 μM, 23 ± 6. The extent of RNA synthetic inhibition after 24 hr correlated with cell survival at 72 hr (r = 0.953, P = 0.047).

dTTP pools in control cells were 172 ± 19 pmol/10^6 cells (mean ± SEM, N = 7). dTTP pools were slightly higher (1.13-fold) than control after a 24-hr exposure to 1 μM PZA. With 5 and 10 μM PZA, in contrast, dTTP pools were 1.5- to 1.6-fold higher than control at 3 and 8 hr, and were 2.6- to 2.7-fold higher after 24 hr. During incubation with [^{14}C]thymidine, [^{14}C]dTTP pools in control cells were 78 ± 10 pmol/10^6 cells (mean ± SEM, N = 7) and represented 93 ± 2% of the total [^{14}C]metabolites. The magnitude of [^{14}C]dTTP pools in PZA-treated cells was similar (data not shown). When [^{14}C]thymidine incorporation into DNA was corrected for the total dTTP pool ([^{14}C]dTTP and endogenous dTTP), the net DNA synthetic rate in control cells was 8.8 ± 3.4 pmol/10^6 cells/hr (mean ± SEM, N = 11). DNA synthetic inhibition increased with increasing PZA exposure (μM · hr) (Fig. 2, r = 0.897). There was a close correlation between DNA synthesis during drug exposures and cell survival at 72 hr (r = 0.937, P < 0.001). The magnitude of RNA and DNA synthetic inhibition after a 24-hr exposure to PZA was similar (r = 0.969, P = 0.031). The expansion of ribonucleotide triphosphate and dTTP pools in PZA-treated cells is therefore most likely due to decreased utilization for RNA and DNA syntheses.

Exposure to 0.1 μM PZA for up to 48 hr had minimal effects on cell cycle distribution (Table 1). A 24-hr exposure to 1 and 10 μM PZA, in contrast, was associated with a decreased proportion of cells in S phase and accumulation in G_2 + M phase. The G_2 + M phase accumulation was more pronounced after 48 hr, although this was accompained by a decreased proportion of cells in G_1 phase.

PZA-Mediated Damage of Nascent and Parental DNA

MCF-7 cells that were labeled with [^{14}C]thymidine during the final hours of PZA exposure were subjected to alkaline elution. A pronounced decrease in the amount of nascent DNA retained was evident after a 3-hr exposure to 5 and 10 μM PZA (Fig. 3), and maximal effects were seen with ≥40 μM · hr (≥5 μM × 8 hr). With 1 μM PZA, a 24-hr exposure was required to produce demonstrable effects on nascent DNA integrity, whereas 0.1 μM PZA was ineffec-

TABLE 1. Effects of Pyrazoloacridine on cell cycle distribution of MCF-7 cells

Drug treatment	G_1 (%)	S (%)	G_2 + M (%)
24 hr			
Control	38	40	22
0.1 μM	39	40	21
1.0 μM	38	33	29
10 μM	42	25	33
48 hr			
Control	40	31	29
0.1 μM	41	27	32
1.0 μM	31	36	33
10 μM	28	26	46

tive. There were strong associations between nascent DNA damage during drug exposure and cell survival at 72 hr (r = 0.825, P = 0.003), and between DNA synthetic inhibition and nascent DNA damage expressed as a percent of control (inset, Fig. 3; r = 0.837, P = 0.003).

MCF-7 cells began to detach from the tissue culture flask with increasing duration of drug exposure beyond 12 hr (Table 2). For the high molecular weight DNA studies, therefore, we collected and quantitated the detached cells, and determined their membrane integrity by trypan blue exclusion. Over 80% of the detached cells excluded trypan blue at both 16 and 24 hr (data not shown). A slight increase in the volume of adherent cells was noted (up to 1.2-fold) with 1–25 μM for 16 hr and 1–10 μM for 24 hr; this may reflect unbalanced cell growth, as suggested by the accumulation of ribonucleotide and dTTP pools. In contrast, the cell volume in adherent cells after a 16-hr exposure to 50 μM PZA was similar to control, but was smaller after 24 hr (0.78-fold). The volumes of the detached cells for all conditions were smaller than that of the control adherent cells; the smallest volumes were observed among those treated with ≥80 μM · hr PZA. The decrease in the volume of detached cells coupled with their membrane integrity are consistent with the changes associated with programmed cell death (apoptosis). Detachment of anchorage-dependent cells portends ultimate cell death [25, 26]. Indeed, when the duration of PZA exposure was extended to 48 hr, the proportion of detached cells that excluded trypan blue ranged from 39% (1 μM) to only 17% (50 μM).

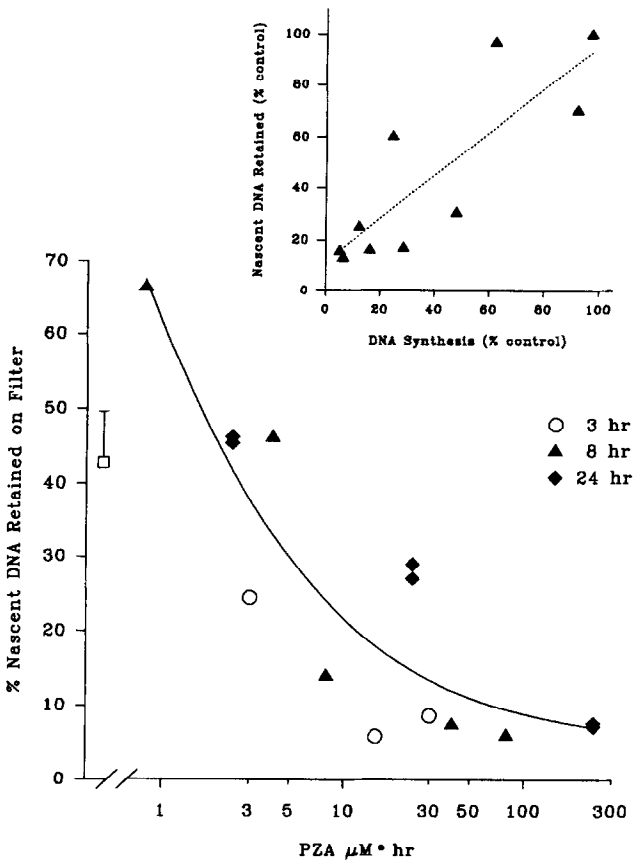

FIG. 3. Interference with nascent DNA integrity by PZA. MCF-7 cells were exposed to diluent or PZA at concentrations ranging from 0.1 to 10 μM for 3, 8, or 24 hr. Cells were incubated with [^{14}C]thymidine for the final 3–6 hr of PZA exposure, lysed under non-deproteinizing conditions, and then subjected to alkaline elution with 20 mM EDTA buffer (pH 12.1) for 15 hr. The data are presented as the percent of the total disintegrations per minute retained on the filter versus PZA μM · hr (r = 0.892), and are from four separate experiments. The mean ± SEM for the control is depicted by the open square to the left of the break; the total nascent DNA for controls was 68,000 ± 44,000 dpm/10^5 cells. The inset shows the relationship between nascent DNA retained (% of control) versus DNA synthesis (% of control) (r = 0.825, P = 0.003).

TABLE 2. Effects of PZA on adherent and detached cell numbers and cell volume

PZA (μM)	Adherent cells: Cell number (% of control)	Adherent cells: Cell volume (fL)	Detached cells: Cell number (% of total)	Detached cells: Cell volume (fL)
×16 hr				
0	100	2380 ± 67	3.0 ± 0.2	1734 ± 342
1	84.8 ± 10.2	2465 ± 78	2.6 ± 0.9	1666 ± 63
5	60.8 ± 13.2	2752 ± 316	8.9 ± 0.3	1210 ± 160
10	54.1 ± 8.9	2630 ± 167	14.9 ± 3.2	1209 ± 179
25	35.5 ± 7.5	2710 ± 181	39.9 ± 8.4	1254 ± 96
50	32.9 ± 12.4	2418 ± 344	61.1 ± 4.5	1350 ± 49
×24 hr				
0	100	2403 ± 95	4.5 ± 0.5	1811 ± 65
1	71.0 ± 4.2	2861 ± 245	4.2 ± 0.4	1752 ± 80
5	45.8 ± 3.2	2617 ± 75	12.5 ± 2.2	1157 ± 88
10	43.1 ± 2.5	2829 ± 229	18.7 ± 3.1	1210 ± 60
25	23.3 ± 3.9	2293 ± 140	62.3 ± 4.5	1227 ± 48
50	11.6 ± 2.6	1882 ± 65	83.2 ± 2.7	1404 ± 67

Exponentially growing cells were treated with diluent or PZA at the indicated concentrations for either 16 or 24 hr. Immediately following drug exposure, the cell number and volume were determined for both the adherent and nonadherent cells. For the adherent cells, cell number was expressed as percent of control. The detached cell number is shown as a percent of the total (the sum of the adherent plus detached cells). The total cell number for the controls averaged 2.1 ± 0.3 million. Although the detached cells excluded trypan blue at 16 and 24 hr, they were destined to die.

We therefore combined the detached and adherent cells for assessment of parental DNA damage (prelabeled with [^{14}C]thymidine) to avoid underestimating drug effects within the initial 24 hr [25, 26]. Compared to PZA-mediated induction of nascent DNA damage, higher PZA exposures were necessary to produce single-strand breaks in parental DNA (Fig. 4). With an 8-hr exposure to 50 μM PZA, a slight increase in the proportion of DNA eluting was evident, while only minor effects occurred with 10 μM (% eluting): control, 10%; 10 μM, 15%; and 50 μM, 29%. As the duration of exposure increased, lower PZA concentrations produced an altered alkaline elution profile. The correlation between parental single-strand breaks at 16 and 24 hr and the percent detached cells is shown in the inset of Fig. 4 (r = 0.933, P < 0.001).

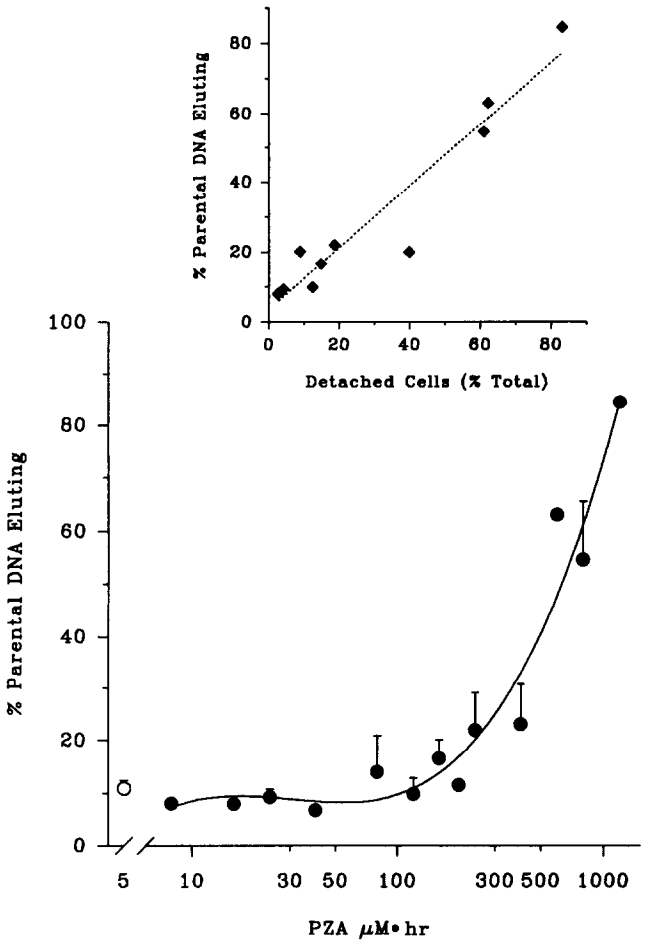

FIG. 4. PZA-mediated induction of parental DNA single-strand breaks. MCF-7 cells prelabeled with [^{14}C]thymidine were exposed to diluent or 1–50 μM PZA for either 8, 16, or 24 hr. Adherent and non-adherent cells were combined and lysed on Nucleopore™ filters under deproteinizing conditions, and eluted with 20 mM EDTA buffer (pH 12.1) containing 0.1% SDS. The data are presented as the percent of the total [^{14}C]dpm eluting versus PZA concentration × time (μM · hr) (r = 0.97). For the control, shown as the open circle to the left of the break, the total dpm/10^5 cells was 19,000 ± 2,300. The data have been compiled from 2–11 separate determinations. The mean values are shown; the error bars represent the following: ± SEM, control (N = 11), 24, 120 and 240 μM · hr (N = 4–5); ± SD, 400 μM · hr (N = 3); ±½ range, 160 and 800 μM · hr; the data for the remaining groups represent the results of a single experiment. The inset shows the percent of parental DNA eluting versus the percent of detached cells after a 16- and 24-hr PZA exposure (r = 0.956, P < 0.001).

Following a 16-hr PZA exposure, double-stranded DNA fragmentation was detected, and it increased with escalating PZA concentration (% [^{14}C]DNA eluting, mean ± range, N = 2): control, 3.0 ± 0.5%; 1 and 5 μM, 3.6 ± 1.6%; 10 μM, 6.4 ± 0.1%; 25 μM, 16.2 ± 3.8%; 50 μM, 34 ± 3.1% (r = 0.996, P < 0.001). A close association was evident between induction of parental single- and double-strand breaks after a 16-hr PZA exposure (r = 0.955, P = 0.003).

DNA Intercalation/Binding

The effect of increasing PZA concentrations on the electrophoretic mobility of several types of DNA in agarose gels was evaluated by preincubating the DNA with PZA for 30 min at 37°. PZA at ≤2.5 μM did not affect appreciably the migration of either a 1 kb DNA ladder or PBR322 plasmid DNA (Fig. 5). With 5–250 μM PZA, however, altered migration of DNA was evident. Only minor effects were seen on ≤1 kb pair fragments (not shown), but decreased migration was noted with ≥1.6 kb DNA (Fig. 5, top panel); the gel shift effect was more pronounced with larger DNA molecules.

Smearing of pBR322 DNA (~ 4.4 kb) was evident with ≥5 μM (Fig. 5, bottom panel). PZA (10–100 μM) also produced smearing of λ DNA (~ 48.5 kb, not shown). The ability of PZA to alter the electrophoretic ability of both linear duplex and circular DNA strongly suggests DNA binding/intercalation.

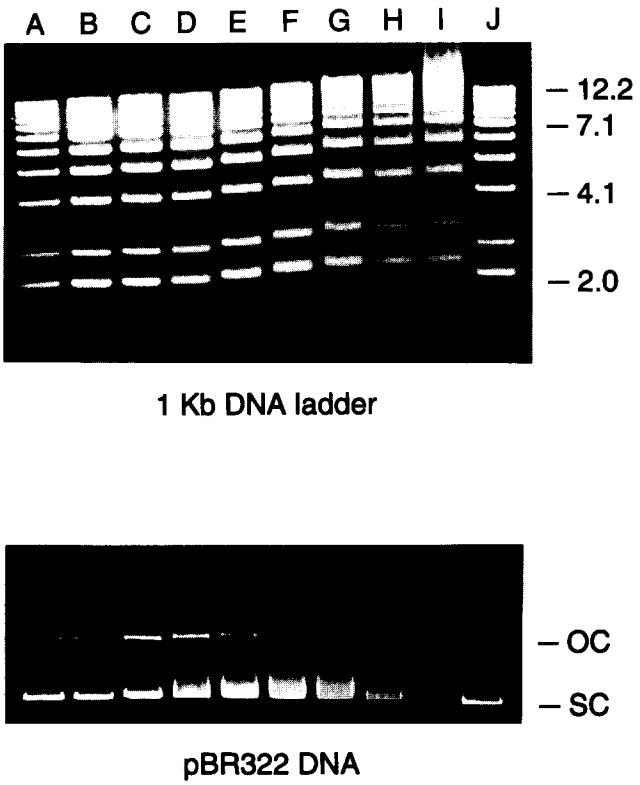

FIG. 5. Effect of PZA on the electrophoretic mobility of linear and supercoiled DNA. One kilobase DNA ladder (2 μg, top panel) and pBR322 DNA (2 μg, bottom panel) were preincubated with various concentrations of PZA for 30 min, following which loading buffer was added and the samples were electrophoresed through a 1.5% (top panel) or 0.9% (bottom panel) agarose (220 mL) at 90 V for 3 hr in 1× Tris–acetate–EDTA buffer at room temperature. Next, the gels were stained with ethidium bromide, destained, and then photographed under UV illumination. The lanes for both panels contained the following concentrations of PZA (μM): A and J, 0; B, 0.5; C, 2.5; D, 5; E, 12.5; F, 25 μM; G, 50; H, 100; and I, 250. Abbreviations: OC, nicked, open circular DNA; SC, supercoiled DNA.

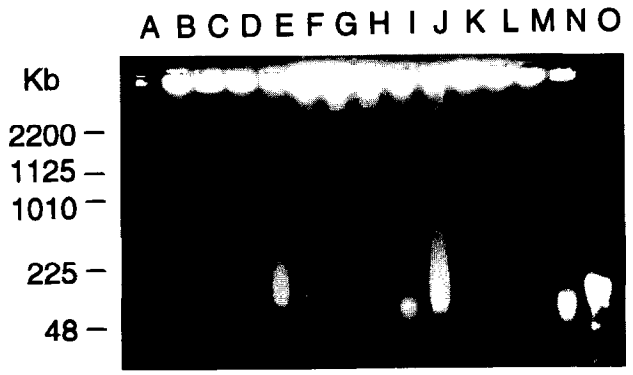

FIG. 6. PZA-mediated induction of double-stranded DNA fragmentation. Control cells and those treated with PZA and/or aphidicolin were embedded in agarose, digested *in situ*, and subjected to pulsed field gel electrophresis. Lane A contains *Saccharomyces cereviseae* DNA and lane O contains 8–48 kb DNA M_r markers. Control cells are in lanes B, F, and K. The duration of drug exposure was 16 hr for lanes B–J and 24 hr for lanes K–N. The concentration of PZA was as follows: L, 5 μM; C, H, and M, 10 μM; D, I, and N, 25 μM; E and J, 50 μM. Lanes G–J represent cells treated with 10 μM aphidicolin alone (G) or aphidicolin plus PZA (H–J).

Using the measured migration of the DNA in the PZA-treated samples, the apparent molecular weight of the retarded DNA was determined. Assuming that the altered migration is due to binding of PZA to the DNA, an apparent dissociation constant of PZA can be estimated by Scatchard analysis: K_D = 61 ± 23 μM. Estimation of the frequency of PZA insertion into the DNA ranged from 1 PZA molecule per 22 bp for 5 μM PZA, 1 per 6 bp for 50 μM, and 1 per 3 bp for 250 μM.

Induction of High-Molecular Weight DNA Fragmentation

Oligonucleosomal DNA laddering using conventional agarose electrophoresis was not seen in DNA purified from MCF-7 cells after a 16- or 24-hr exposure to 5–50 μM PZA (data not shown). In contrast, classic oligonucleosomal laddering was evident in HL-60 leukemic cells treated with 10 and 25 μM PZA for 6 hr (data not shown), at which time viability was 82 and 84% of control, respectively.

Since the DNA purification methods employed result in shearing of DNA, induction of very high molecular weight DNA fragmentation might not be evident using conventional electrophoretic techniques. To avoid fragmentation of chromosomal DNA, we embedded control and PZA-treated cells in agarose plugs followed by enzymatic digestion *in situ* to remove RNA and protein; the purified DNA in the plugs was then analyzed by pulsed-field agarose electrophoresis. A 16-hr exposure to 25 and 50 μM PZA was associated with induction of DNA fragments in the 500–1000 kb size range (Fig. 6, lanes B–E). When the duration of PZA exposure was extended to 24 hr, high-molecular weight DNA fragmentation was detected with lower PZA concentrations (5 and 10 μM), and the extent of DNA damage noted with 25 μM PZA was more prominent (Fig. 5, lanes K–N). These observations suggest that the pattern of PZA-mediated double-stranded DNA fragmentation in MCF-7 cells is distinct from that of classical apoptosis.

We then questioned whether active DNA synthesis was required for PZA-mediated cytotoxicity and induction of megabase DNA fragmentation by evaluating the impact of aphidicolin, which inhibits DNA polymerase α and stops movement of replication forks. In MCF-7 cells, a 1-hr exposure to 10 μM aphidicolin inhibited DNA synthesis by over 96%. A 16-hr exposure to 10 μM aphidicolin reduced cell number at 72 hr to 71% of control. Aphidicolin did not protect MCF-7 cells from PZA-mediated cytotoxicity in either cell growth assays or clonogenic assays (data not shown). Further, aphidicolin did not prevent PZA-mediated high molecular weight DNA fragmentation (Fig. 6, lanes F–J). In contrast to DNA damage mediated by topoisomerase I-targeting agents [27, 28], PZA-mediated cytotoxicity and double-stranded DNA fragmentation occurred in the absence of active DNA synthesis.

Interaction of PZA with Topoisomerases I and II

Since induction of DNA damage may result from either direct interactions with nuclear topoisomerases or indirect effects, we examined the effect of PZA on supercoiled plasmid DNA incubated in the presence or absence of topoisomerases I and II in a cell-free assay. A representative experiment is shown in Fig. 7. Closed, circular pBR322 DNA migrated further in the gel (lane A) than that observed with the addition of topoisomerase I (lane B), due to the induction of nicked, open circular DNA and relaxed DNA (topoisomers). We found that the addition of 0.5 to 50 μM PZA (lanes D–H) interfered with the formation of relaxed DNA compared with topoisomerase I (lane B) alone or 0.1 μM PZA (lane C). The pattern of DNA migration, however, differed depending on the concentration

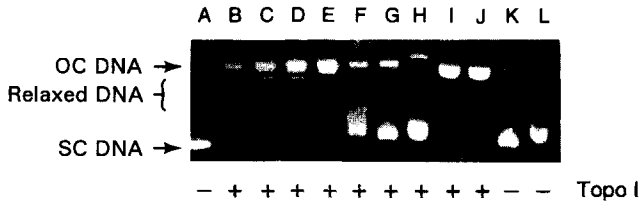

FIG. 7. Interference with topoisomerase I-mediated cleavage of PBR322 DNA. pBR322 plasmid DNA (0.4 μg) was incubated with no drug, PZA, or camptothecin in the presence or absence of 5 units purified calf thymus topoisomerase I at 37° for 60 min. After an additional 30-min incubation in SDS and proteinase K, the samples were subjected to 1% agarose gel electrophoresis. SC = supercoiled DNA; OC = nicked, open circular DNA. The conditions are as follows: plasmid DNA alone (lane A), with topoisomerase I alone (lane B) or enzyme pluse PZA (lanes C–H): 0.1, 0.5, 1, 5, 10, and 50 μM, respectively; pluse camptothecin (lanes I and J): 10 and 25 μM, respectively; PZA without topoisomerase I (lanes K and L): 10 and 50 μM, respectively.

of PZA. With 5, 10, and 50 μM PZA, a gel shift effect was evident (lanes F–H). A similar PZA-mediated shift in electrophoretic mobility of the plasmid DNA was also observed in the absence of topoisomerase I (lanes K and L). In contrast, camptothecin resulted in an increase in open circular DNA and an inhibition of conversion of supercoiled substrate to relaxed DNA (lanes I and J). The effect of PZA is thus distinct from that of camptothecin. In separate experiments, we found that the addition of etoposide (100 μM) interfered with topoisomerase II-mediated relaxation of SV40 DNA (data not shown), whereas PZA (5–50 μM) produced smearing of SV40 DNA in the presence and absence of topoisomerase II (data not shown).

No increase in covalent protein-linked DNA complexes was seen by SDS–KCl precipitation after 30- and 60-min incubations with PZA at concentrations ranging from 1 to 100 μM: e.g. 100 μM PZA, 1.03-fold (±0.12) and 0.84-fold (±0.09) change compared with control cells (mean ± range, N = 2). With 100 μM VP-16, in contrast, a 30- and 60-min incubation produced a 4.05- (±0.38) and 4.65- (±0.26) fold increase in protein-linked DNA complexes over control cells (mean ± SD, N = 3). Taken together, these results suggest that PZA does not directly bind to topoisomerase I or II, but may impede access of topoisomerases I and II to their DNA binding sites through steric hindrance.

Cytotoxicity of PZA against Drug-Resistant Cell Lines

As a further measure of any possible dependence of PZA-mediated cytotoxicity on an interaction with nuclear topoisomerases, we tested the activity of PZA against topoisomerase I- and II-deficient cell lines. The HL-60/ADR^R cell line has a 2- to 3-fold decrease in topoisomerase II protein content and a 40-fold resistance to etoposide-mediated DNA damage compared with parental cells [8, 19, 20]. HL-60/ADR^R cells were 69-fold less sensitive to doxorubicin (120 hr IC_{50}: 24 ± 9 nM vs 1.7 ± 0.1 μM) and are reported to be 280-fold resistant to etoposide [20]. In contrast, the IC_{50} values for PZA in HL-60 parental and HL-60/ADR^R cells were 0.19 ± 0.04 and 0.25 ± 0.05 μM, respectively (120-hr exposure). PZA exerted comparable cytotoxicity in parental and camptothecin-resistant murine P388 leukemia cells (72 hr IC_{50}: 0.43 and 0.31 μM for P388/WT and P388/CPT45). Finally, we tested the activity of PZA against an MCF-7 subline that expresses the classical multidrug resistance phenotype. MCF-7/AD10 cells were 905-fold resistant to doxorubicin (120 hr IC_{50}: 44 ± 13 vs 0.05 ± 0.01 μM) and 131-fold resistant to etoposide (120 hr IC_{50}: 51 vs 0.34 μM). The IC_{50} values for PZA (120 hr), in contrast, were 0.33 ± 0.08 μM (MCF-7) and 0.30 ± 0.07 μM (MCF-7/AD10).

Free Radical Formation

Compounds containing reducible nitro groups may be activated by NADPH–cytochrome P450 reductase to nitro-anion radicals. In the presence of molecular oxygen, these nitro-anion radicals produce reactive superoxide and hydroxyl radicals. Since PZA contains a potentially reducible nitro group, free radical formation might conceivably contribute to DNA damage. We examined free radical formation by both direct ESR and spin-trapping ESR in MCF-7 tumor cells and by purified NADPH–cytochrome P450 reductase. Free radicals (either nitro-anion radical or DMPO-OH) were not detected either in intact cells or by the purified enzyme in the presence of 50–2000 μM PZA.

DISCUSSION

We found that the cytotoxicity of PZA in MCF-7 cells was related to total drug exposure, and did not appear to be schedule dependent. Near-maximal effects (90% cytotoxicity) were seen with an average of 52 μM · hr PZA. However, the similarity of the IC_{50} values with 24- and 72-hr PZA exposures suggests a minimum threshold concentration for cytotoxicity (≤0.1 μM was non-toxic in MCF-7 cells). While perferential inhibition of RNA over DNA synthesis has been reported in murine L1210 leukemia cells [3], we observed comparable degrees of DNA and RNA synthetic inhibition. Single-strand breaks in nascent DNA, detected using non-deproteinizing conditions, were seen after a 3-hr exposure to PZA. There was a strong correlation between the extent of DNA synthetic inhibition and nascent DNA damage during a 24-hr exposure to PZA; these parameters also correlated with the surviving cell number at 72 hr.

Higher PZA exposures were needed for induction of single- and double-strand breaks in parental DNA. A modest effect was noted at 8 hr with 50 μM PZA, while pronounced damage was evident with a 16-hr exposure to 25 and 50 μM PZA, and a 24-hr exposure to 5 and 10 μM PZA. Single-strand breaks were detected in parental DNA under deproteinizing conditions, but double-strand breaks were also observed using non-deproteinizing conditions. A close association was noted between PZA exposure (μM · hr), induction of both parental DNA single- and double-strand breaks, detachment of MCF-7 cells from the monolayer, and ultimate lethality. Induction of parental DNA double-strand breaks increased in concert with single-strand breaks. These observations suggest that induction of nascent single-strand breaks occurs in tandem with DNA synthetic inhibition; induction of single- and double-strand breaks in parental DNA occurs subsequently.

We did not detect oligonucleosomal DNA laddering in MCF-7 cells at times that double-stranded DNA fragmentation was evident. However, PZA produced high molecular weight DNA fragmentation in the range of 50 to 1000 kb. The megabase DNA fragmentation was seen concurrently with detachment of cells from the monolayer; these cells had decreased cell volume but retained membrane integrity, features characteristic of cells undergoing programmed cell death. An increase in cell volume appeared to

precede detachment of cells and the decrease in cell volume; the early changes may reflect cellular consequences of DNA and RNA synthetic inhibition. In HL-60 cells, in contrast, a 6-hr exposure to 10 and 25 μM PZA was sufficient to induce classic oligonucleosomal laddering. Our results suggest that the time–course and the pattern of parental DNA fragmentation produced by PZA in MCF-7 breast cancer cells are distinct from those of HL-60 cells. Exposure of epithelial cancer cell lines to DNA-damaging agents such as fluorodeoxyuridine has been reported to produce high molecular weight DNA fragmentation in the absence of nucleosomal laddering [25, 27, 28]. Internucleosomal fragmentation is thought to result from activation of a calcium- and magnesium-dependent endonuclease. The absence of nucleosomal laddering in the previously reported epithelial malignancies and in the MCF-7 cells in the present report may signify that the cells either do not contain or do not activate that specific endonuclease under the experimental conditions used.

Several investigators have reported that co-exposure of camptothecin, a topoisomerase I-targeting agent, with aphidicolin prevents camptothecin-induced double-strand breaks and cytotoxicity [29, 30]. Ongoing DNA synthesis is required to mediate camptothecin lethality, and collision of the replication fork with the covalent camptothecin–topoisomerase I complex is thought to result in double-strand breaks [29, 30]. Coadministration of aphidicolin with topoisomerase II-targeting agents such as etoposide or amsacrine provides only partial protection against cytotoxicity, and does not affect induction of double-strand DNA fragmentation [29]. Aphidicolin did not protect MCF-7 cells from the cytotoxicity of PZA, nor did it prevent induction of double-stranded DNA fragmentation.

Rowe *et al.* reported that several antitumor acridines, such as amsacrine [4′-(9-acridinylamino)methanesulfon-*m*-anisidide), produce topoisomerase-II-mediated protein-linked DNA strand breaks in cultured mammalian cells [6], but the pyrazoloacridine derivatives were not included in this analysis. A 1-hr exposure to pyrazoloacridine derivatives was associated with protein-linked DNA strand breaks in L1210 cells, although PZA was the least potent of nine members tested [5]. Since differences in the cellular response to genotoxic events such as DNA synthetic inhibition and/or DNA damage are apparent between leukemic and carcinoma cells, we studied the consequences of PZA exposure on DNA damage in MCF-7 cells. The cell-free studies with purified topoisomerase I and II demonstrated that PZA interfered with topoisomerase-mediated relaxation of supercoiled DNA. However, the absence of increased protein-linked DNA complexes by SDS-KCl precipitation coupled with the activity of PZA against the topoisomerase I-deficient P388/CPT45 cells and the topoisomerase II-deficient HL-60 subline argue that a direct, covalent interaction with topoisomerase I or II is not required for PZA-mediated cytotoxicity and DNA damage [20–22]. Our results are in agreement with the observation that PZA retained activity against yeast cells lacking either topoisomerase I or II [31]. PZA-mediated interference with the ability of topoisomerase I and II to reach their binding sites is a likely explanation [31], as has been described for other agents such as doxorubicin. The effect of PZA on the electrophoretic mobility of both linear duplex DNA and closed, circular DNA provides strong evidence of DNA intercalation/binding that, in turn, may alter DNA conformation and interfere with access of replicative, repair, and transcription ezyme complexes. Although PZA has a potentially reducible group, there was no evidence of free radical formation during PZA exposure.

We also confirmed the activity of PZA against a human carcinoma cell line that expresses the classical multidrug resistance phenotype. The cellular uptake and retention of PZA were similar in parental MCF-7 and MCF-AD10 cells, as well as in a human colon carcinoma parental cell line and a doxorubicin-resistant subline with the multidrug-resistant phenotype [32]. Thus, PZA does not appear to be a substrate for the P170 glycoprotein membrane pump.

Based upon its unique properties, PZA appears to be a promising agent for the treatment of epithelial malignancies. In preclinical studies, bolus administration of PZA was associated with acute, dose-limiting neurologic toxicity [33]. A 1-hr infusion of PZA was also associated with acute neurologic toxicity in mice when peak plasma levels exceeded 2 μM; subacute myelosuppression was noted with an AUC of 38 μM · hr [33]. Split dose schedules and longer infusion durations (up to 24 hr), predicted to be associated with lower peak plasma levels, allowed the administration of higher total drug doses [1, 2, 9, 33]. A 1-hr infusion of PZA given every 3 weeks in Phase I clinical trials was associated with a high incidence of neurologic toxicity; increasing the duration of infusion to 3 hr decreased the incidence of neurologic toxicity [34–36]. In dogs and subhuman primates, a single 24-hr infusion that produced steady-state PZA levels of 0.54 to 0.78 μM and 0.2 to 0.45 μM, respectively, was well tolerated [9]. Infusion schedules longer than 3 hr have not been tested in the clinic. The IC_{50} and IC_{90} concentrations in MCF-7 cells for a 3-hr exposure were 2.6 and 16.4 μM, respectively, while concentrations required for comparable toxicity with a 24-hr exposure were 0.24 and 0.94 μM. These latter concentrations are in the same range as those tolerated in animals. If certain clinical toxicities are related to peak plasma levels of PZA, then it is possible that extending the duration of infusion to 24 hr might improve the therapeutic index.

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