Synergistic Cytotoxicity of Pyrazoloacridine with Doxorubicin, Etoposide, and Topotecan in Drug-Resistant Tumor Cells

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ABSTRACT
Pyrazoloacridine (NSC 366140, PD115934, PZA) is a new class of acridine anticancer agents under investigation in Phase II clinical trials in patients with advanced cancers. Although poor responses in patients to the treatment with PZA alone have been observed, this class of agents remains of interest because of its distinct mechanism of action from other topoisomerase poisons. Therefore, the combination of PZA with conventional anticancer agents presents an attractive approach to treat drug-resistant human tumors. In the present study, the cytotoxic effects of PZA combined with doxorubicin, topotecan, and etoposide were determined using paired parental and doxorubicin-resistant human colon carcinoma (SW-620 and SW620/AD-300) and breast cancer cell lines (MCF-7 and MCF-7/TH). Cytotoxicity was measured by soft agar clonogenic assays. Dose effect and combination effects were analyzed by the method of Chou and Talalay. The combination of PZA with doxorubicin, topotecan, and etoposide in fixed ratios demonstrated synergistic cytotoxicity on both SW-620 and SW620/AD-300 cell lines. The combination of PZA with doxorubicin also exhibited synergistic cytotoxicity against both MCF-7 and MCF-7/TH cell lines. The mechanism of synergism appeared independent of topoisomerase I and II inhibition, and interference with protein-DNA complexes. Strategies to define optimal drug combinations are proving to be of significant value when considering potential clinical applications of new and established agents.

INTRODUCTION
Pyrazoloacridine (NSC 366140, PD115934, PZA) is a new class of acridine anticancer agents (1). PZA has selectivity against solid tumors relative to leukemia cells in vitro (2–4) and broad spectrum in vivo activity (5). PZA has nearly equivalent potencies against cycling and quiescent target cells, and against hypoxic and normoxic target cells (2, 3, 4, 6). Furthermore, PZA maintains its activity against several phenotypes of drug-resistant cancer cells including those overexpressing P-glycoprotein, multidrug-resistance protein, and cells deficient in topoisomerase I (Topo I) and II (Topo II; Refs. 6, 7, 8). This unusual activity of PZA is associated with inhibition of DNA and RNA syntheses, damage to both nascent and parental DNA (8), as well as an ability to inhibit Topo I and II in a manner distinct from that of past Topo I- and II-targeting agents (8, 9). A Phase I study completed recently demonstrated that neurotoxicity and thrombocytopenia were not problematic with a weekly 24-h infusion of PZA (10) compared with a 3 h infusion on the once every 3 week schedule (11, 12). However, Phase II studies of PZA using the 3-h infusion schedule in patients with previously treated colorectal cancer, pancreatic cancer, transitional cancer of bladder, germ cell, and renal cell cancer have shown no objective responses (13–17). One of 17 patients with hormone refractory prostate cancer was reported to have a 96% decrease in serum PSA, and this was accompanied by improvements in bone lesions (18). In ovarian cancer, the response rate among 42 patients with platinum-sensitive and 24 patients with platinum-refractory ovarian cancer was 34% and 8%, respectively (19, 20).

Given the unique mechanism of action of PZA, it is worth considering whether its therapeutic activity could be enhanced by combining it with other anticancer agents. From a clinical perspective, it is clear that optimal therapeutic results could be achieved when using drugs in combination, and the rationale for selecting drugs to be used in a particular combination have often been based on toxicity profiles, mechanistic information, and the potential to overcome acquired or innate drug resistance. Perhaps of greater interest, investigators have also tried to identify which drugs to combine by evaluating whether the agents act synergistically in vitro cell-based screening assays (reviewed in Ref. 21). The studies defined in this report evaluate the cytotoxic effects of PZA in combination with doxorubicin, etoposide (VP-16), or topotecan as measured against parental and doxorubicin-resistant human colon carcinoma (SW-620 and SW620/AD-300), and human breast cancer (MCF-7 and MCF-7/TH) cell lines. The effects of the combined drugs were analyzed using the multiple drug equations developed by Chou and Talalay (22). This method is most readily applied to dose titration data collected for individual agents alone, and the combination of drugs added at fixed ratios and over a broad range of effective doses. Evidence obtained in this manner indicate that the therapeutic activity of PZA could be enhance
significantly when combined with the Topo II inhibitor doxorubicin, VP-16, or the Topo I inhibitor topotecan.

MATERIALS AND METHODS

Cell Lines. Human colon adenocarcinoma SW620 and its doxorubicin-resistant subline SW620/AD300 (henceforth referred as AD300) were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 50 units/ml of penicillin, 50 μg/ml of streptomycin, and 2 mM L-glutamine. The AD300 subline was maintained in the presence of 0.52 μM of doxorubicin and was grown in doxorubicin-free medium for 7 days before use for experimentation.

Human breast cancer MCF-7 cell line and its doxorubicin-resistant subline (MCF-7/TH), obtained from Dr. Susan Bates (National Cancer Institute, Bethesda, MD), were cultured in α-MEM supplemented with nucleosides, 12% fetal bovine serum, 100 units/ml of penicillin, 50 μg/ml of streptomycin, and 2 mM L-glutamine. MCF-7/TH cultures were periodically exposed to 0.34 μM of doxorubicin for 7 days and grown in drug-free medium for 7 days before experimentation.

Drugs. Doxorubicin hydrochloride (DOX) was purchased from Adria Laboratories (Columbus, OH). PZA (NSC-366140) [19-methoxy-N,N-dimethyl-5-nitropyrazolo(3,4,5-K) acridine-2 (6H)-propanamine] and topotecan hydrochloride were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD. Etoposide (VP-16) was purchased from Genisia Laboratories, Ltd. (Irvine, CA).

Soft Agar Clonogenic Assays. The effect of PZA alone or in combination with other drugs on cell growth and clonogenicity was determined by soft agar clonogenic assays. Cells exposed to the various drug concentrations at 37°C for 2 h were harvested and washed with cold PBS, resuspended in medium containing 0.3% agar, and layered in multiwell plates on a previously prepared feeder layer of 0.5% agar in complete medium. Plates were incubated at 37°C in an atmosphere of 5% CO₂ and 95% air for 12–15 days. The effect on survival of the clonogenic cells was determined by counting colonies. Each colony had >50 cells. Results presented were derived from three different sequential experiments performed using three wells for each drug concentration tested. IC₅₀ values represent the drug concentration required to inhibit 50% of clonogenicity in tumor cells treated with drug(s) compared with untreated control cells.

Data Analysis. To determine whether effects of the drug combinations on clonogenicity were additive, synergistic, or antagonistic, data from the soft agar assays were analyzed using CalcuSyn software (Biosoft, Ferguson, MO). Fixed ratios of the individual drugs in combination were used for these analyses in SW620 and AD300 cell lines. The fixed ratios of two drugs were selected based on the drug IC₅₀ of each individual drug. The combination results were compared with the cytotoxicity of each drug alone in this software developed on the basis of the median effect analysis of Chou and Talalay (21). Combination index (CI) values of <1 were indicative of drug synergism, >1 indicates drug antagonism, and CI values of ~1 indicate drug additivity. All of the data reflect the mean ± SD from three separate experiments, each done in triplicate. SigmaPlot Software version 5.0 (Jandel Scientific, San Rafael, CA) was used to graph the data.

Crude Nuclear Extracts. Cells from log-phase cultures were collected and washed in ice-cold NB buffer [2 mM K₂HPO₄, 5 mM MgCl₂, 150 mM NaCl, 1 mM EGTA, and 0.1 mM DTT (pH 6.5)]. The washed cells were resuspended in NB buffer supplemented with 0.35% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride. The cell suspension was mixed and incubated for 5 min at 4°C, centrifuged at 1,000 × g for 10 min, and the nuclear pellet was washed in Triton-free NB. Nuclear proteins were extracted by incubation of the suspension for 30 min at 4°C with ice-cold NB containing 0.35 mM NaCl. DNA and nuclear debris were pelleted by centrifugation at 17,000 × g for 10 min, and the protein concentration in the supernatant was determined by the Bio-Rad assay (Bio-Rad Labs, Hercules, CA).

Immunodetection of Topo I and IIα. Nuclear extracts from the SW620-sensitive and the AD300-resistant cells were electrophoresed on a 5.6% or 9% SDS-polyacrylamide gel. Protein from the gel was electrotransferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The blot was incubated for 2 h at room temperature with blocking buffer (5% nonfat dried milk dissolved in 0.05% PBS and Tween 20), followed by incubation with rabbit antihuman Topo I antiserum and mouse monoclonal antibody against human p170 Topo II (Topogen) in fresh blocking buffer (1:1000) overnight at 4°C. The mouse antihuman β-actin monoclonal antibody was purchased from Sigma (St. Louis, MO). The blot was then washed with 5% nonfat dried milk dissolved in 0.05% PBS and Tween 20, and incubated with mouse or rabbit immunoglobulin and horseradish peroxidase-linked second antibody diluted in blocking buffer (1:1500) at room temperature for 1 h. After washing of the membranes, detection with ECL reagents was performed according to the instructions of the manufacturer (Amersham Life Science, Arlington Heights, IL).

Topo I Activity. The Topo I activity in SW620 and AD300 cells was determined by measuring relaxation of supercoiled DNA (pBR322 DNA; Promega). The reaction mixture consisted of 10 mM Tris-HCl (pH 7.9), 1 mM EDTA, 0.15 mM NaCl, 0.1% BSA, 0.1 mM spermidine, 5% glycerol, 0.25 μg pBR322 DNA, and crude nuclear extract from the two cell lines with the indicated amount of protein in a total volume of 20 μl. Inhibition of Topo I activity was analyzed by adding either PZA and/or topotecan at the indicated concentration to the reaction mixture containing 5 units of purified human Topo I (TopoGen, Columbus, OH) and 0.25 μg pBR322 DNA. This sample was incubated for 30 min at 37°C, where the reaction was terminated by addition of 1% SDS. Digestion with proteinase K (50 μg/ml) at 37°C for 30 min was followed by the addition of 2 μl of loading buffer (25% bromphenol blue and 50% glycerol) and 20 μl of chlorofororm:isoamyl alcohol (24:1) and centrifugation in a Microfuge (Biofuge 13; Heraeus Instruments, Langenselbold, Germany). After chlorofororm:isoamyl alcohol extraction, the blue upper layer was loaded onto 1% agarose gels for electrophoresis. Ethidium bromide stained gels were photographed under UV light. The nicked and opened circular DNA forms were quantified by densitometric scanning using HP Desk-Scanner II with 1-D Main Universal software (Advanced American Biotechnology, Fullerton, CA).
Topo II Catalytic Activity. The Topo II activity in SW620 and AD300 cells was studied by an eukaryotic Topo II assay. The assay is based on decatenation of a catenated DNA substrate from Trypanosoma kinetoplasts (kDNA; Topogen), and it is specific for Topo II activity, not Topo I. The reaction mixture contained 50 mM Tris-HCl (pH 8), 120 mM KCl, 10 mM MgCl2, 0.5 mM each of DTT, 30 μg BSA/ml, 0.2 μg/ml kDNA, and crude nuclear extract with the indicated amount of protein in a total volume of 20 μl. The reaction mixture was incubated for 30 min at 37°C and terminated with the addition of 2 μl of stop buffer (5% saccharose, 0.025% bromphenol blue, and 50% glycerol). The samples were then electrophoresed on a 1% agarose gel, stained with ethidium bromide, and photographed under a UV light. The various DNA forms were quantitated by densitometric scanning as described above.

Protein-DNA Complex. Protein-DNA complex formation in intact SW620 and AD300 cell was quantitated using the filter-binding assay previously described by Ellis et al. (23). Briefly, cells were treated with doxorubicin, PZA, VP-16, or topotecan for 2 h at 37°C and centrifuged at 13,000 × g for 1 min. The pellets were immediately lysed with 100 μl of lysis buffer containing 0.5% SDS and 5 mM EGTA (pH 8.0) at 65°C for 10 min. One ml of protein-binding buffer (0.4 M guanidine HCl, 10 mM Tris (pH 8.0), 10 mM EGTA, 0.01% Sarkosyl, 0.3 M NaCl, and 10 mM MgCl2) heated to 65°C was added to each lysate immediately before its application to a nitrocellulose filter using a dot-blot apparatus (Bio-Rad). DNA was fixed to the filter by baking in a vacuum oven for 2 h at 80°C. The amount of DNA fixed to the filter was determined by hybridization to a probe DNA labeled with [32P]dCTP (Amersham) using the Amersham multiprime labeling system. Autoradiography was performed on each blot, and densitometric scanning was used as described above. Results presented were derived from three sequential experiments performed using three wells for each drug concentration tested. Optical absorption density is expressed as the ratio of IC50 values of the resistant AD300 (or MCF-7/TH) cells treated with single or combined drug(s) compared to its parental SW620 (or MCF-7) treated with single drug in soft agar assays.

Table 1 | IC50 (μM) of doxorubicin (DOX), VP-16, topotecan alone, and in combination with pyrazoloacridine (PZA) are determined using SW620 and SW620/AD300, as well as MCF-7 and MCF-7/TH cells.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>SW620</th>
<th>AD300</th>
<th>Resistant fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>PZA</td>
<td>2.03 ± 0.60</td>
<td>2.05 ± 0.05</td>
<td>1.01</td>
</tr>
<tr>
<td>DOX</td>
<td>0.08 ± 0.02</td>
<td>4.60 ± 0.47</td>
<td>57.50</td>
</tr>
<tr>
<td>DOX + PZA</td>
<td>0.02 ± 0.01</td>
<td>0.26 ± 0.09</td>
<td>3.25</td>
</tr>
<tr>
<td>Topotecan</td>
<td>0.49 ± 0.07</td>
<td>0.51 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>Topotecan + PZA</td>
<td>0.025 ± 0.02</td>
<td>0.25 ± 0.06</td>
<td>10.0</td>
</tr>
<tr>
<td>VP-16</td>
<td>0.77 ± 0.31</td>
<td>4.79 ± 0.20</td>
<td>6.22</td>
</tr>
<tr>
<td>VP-16 + PZA</td>
<td>0.51 ± 0.16</td>
<td>0.37 ± 0.07</td>
<td>0.48</td>
</tr>
<tr>
<td>PZA</td>
<td>0.50 ± 0.13</td>
<td>0.48 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>DOX</td>
<td>2.10 ± 0.12</td>
<td>19.8 ± 0.49</td>
<td>9.75</td>
</tr>
<tr>
<td>PZA</td>
<td>4.91 ± 0.25</td>
<td>5.10 ± 0.30</td>
<td>1.04</td>
</tr>
<tr>
<td>Topotecan + PZA</td>
<td>0.75 ± 0.09</td>
<td>2.61 ± 0.15</td>
<td>1.23</td>
</tr>
<tr>
<td>PZA</td>
<td>0.82 ± 0.12</td>
<td>1.25 ± 0.18</td>
<td></td>
</tr>
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</table>

*The concentration of each drug at the point where 50% inhibition of clonogenicity is observed in the parental SW620 (or MCF-7) and resistant AD300 (or MCF-7/TH) cells treated with combined drugs. The IC50 recorded was derived from the median effect analysis of Chou and Talalay using CalcuSyn software as described in “Materials and Methods.”

| Ratio of IC50 values of the resistant AD300 (or MCF-7/TH) cells treated with single or combined drug(s) compared to its parental SW620 (or MCF-7) treated with single drug in soft agar assays. |
|-------|-------|-------|-------|
| PZA   | 2.03 | 1.01 | 1.00 |
| DOX   | 0.13 | 0.03 | 4.00 |
| Topotecan | 2.03 | 0.12 | 17.00 |
| PZA + DOX | 0.82 | 0.25 | 3.31 |
| PZA + Topotecan | 0.82 | 1.25 | 1.56 |
| DOX + Topotecan | 0.82 | 1.25 | 1.56 |
| VP-16 + PZA | 0.51 | 0.48 | 1.06 |
| PZA + VP-16 | 0.51 | 0.48 | 1.06 |
| DOX + Topotecan | 2.10 | 19.8 | 10.90 |
| Topotecan + PZA | 4.91 | 5.10 | 1.04 |
| Topotecan + VP-16 | 4.91 | 5.10 | 1.04 |
| PZA + Topotecan + VP-16 | 4.91 | 5.10 | 1.04 |

Statistical Analyses. Student’s t test was used to measure statistical significance between control and treatment groups. Data were considered significant for P < 0.05.

RESULTS

PZA Circumventing Drug Resistance. The clonogenicity of SW620 and AD300 cells treated with DOX, topotecan, and VP-16, as well as their combination with PZA and PZA alone was determined by soft agar assay. The IC50 values of drugs alone and in combination with PZA have been summarized in Table 1. The data in Table 1 indicate that in SW620 cells the IC50 of PZA, DOX, topotecan, and VP-16 were 2.03, 0.08, 0.025, μM and 0.77 μM, respectively. In AD300 cells, the IC50 of PZA, DOX, topotecan, and VP-16 were 2.05, 4.60 μM, 0.25 μM, and 4.79 μM, respectively. Thus, it can be estimated that the AD 300 cells are 58-, 10-, and 6-fold more resistant to DOX, topotecan, and VP-16 than the parental cells, respectively. The IC50 value of PZA is 2 μM in both SW620 and AD300 cell lines, indicating that PZA does not have the cross-resistance with DOX, topotecan, and VP-16 in the AD300 cell lines.

The combination of DOX with PZA reduced the IC50 values of DOX from 4.6 μM to 0.26 μM. In the presence of PZA, there was a 3-fold residual resistance to DOX compared with its parental cells treated with DOX alone. Moreover, the combination of topotecan with PZA reduced the IC50 values of topotecan from 0.25 μM to 0.03 μM, which is similar to its parental cells treated with topotecan alone, revealing a complete overcome of resistance to topotecan. The combination of VP-16 with PZA completely circumvented the resistance of AD300 to VP-16 and significantly enhanced the cytotoxicity of VP-16 in AD300 cells. These data indicated that PZA could completely or partially circumvent drug resistance induced by DOX, topotecan, and VP-16 in the AD300 cell lines.

The data in Table 1 also indicate that the IC50 of DOX in MCF-7 and MCF-7/TH cells were 2.1 μM and 19.8 μM, respectively. The MCF-7/TH cells are 10-fold more resistant to DOX than the parental cells. The IC50 value of PZA is 5 μM in both MCF-7 and MCF-7/TH cell lines, indicating that PZA does not have the cross-resistance with DOX in MCF-7/TH cell lines. The combination of DOX with PZA reduced the IC50 values of DOX from 2.10 μM to 0.75 μM in MCF-7 cell lines and from 19.8 μM to 2.61 μM in MCF-7/TH cell lines, which is similar to
its parental cells treated with DOX alone. The data indicated that PZA can completely circumvent drug resistance induced by DOX in MCF-7/TH human breast-resistant cells and enhance cytotoxicity of DOX in MCF-7 human breast-sensitive cells. The IC$_{50}$ value of PZA in the presence of DOX was reduced from 4.91 to 0.82 in MCF-7 cell lines and from 5.10 to 1.25 in MCF-7/TH cells. Therefore, the combination treatment significantly reduced the concentration of each drug to achieve 50% inhibition of clonogenicity compared with that of the individual drugs alone.

**Synergistic Cytotoxic Effects of PZA in Combination with Anticancer Drugs.** To determine whether effects of the drug combinations on clonogenicity were additive, synergistic, or antagonistic, data from the soft agar assays were analyzed using CalcuSyn software. Dose effect and CI plots in Fig. 1, A and B, show that the combinations of DOX (0.00625–0.1 μM) with PZA (0.125–2.0 μM) in fixed ratios of 1:20 had modest synergistic effects, with calculated CI values ranging from 0.6 to 0.95 (Fig. 1B) in SW620 cells. Similarly, dose effects versus CI plots in Fig. 1, C and D, indicate that the combinations of DOX (0.125–4.0 μM) and PZA (0.0625–2.0 μM) using a fixed ratio (2:1) had a moderate synergistic effect, with CI values between 0.65 and 0.85 in the AD300 doxorubicin-resistant cell line.

Dose effects versus CI plots in Fig. 2, A and B, indicate that topotecan (0.001–0.04 μM) in combination with PZA (0.1–4 μM) in a ratio of 1:100 had cytotoxic effects ranging from additive to very strong synergy, and CI values decreased from 0.95 to 0.05 with the drug concentrations increasing in SW620 cells. Similarly, dose effects and CI plots in Fig. 2, C and D, suggested that the combinations of topotecan (0.01–0.8 μM) with PZA (0.1–8 μM) in a ratio of 1:10 produced additive activity to very strong synergism in AD300 cells, where the CI decreased from 0.95 to 0.1 in AD300 cells. VP-16 (0.06–2 μM) combined with PZA (0.12–4 μM) using a fixed ratio (1:2) exhibited moderate synergistic effects (Fig. 3, A and B; CI = 0.5–0.85) in SW620 cells. Similarly, the combinations of VP-16 (0.24–2 μM) and PZA (0.12–1 μM) in a fixed ratio of 2:1 exhibited synergistic effects, which increased as the concentrations of the two drugs increased in the AD300 cells as shown in Fig. 3D (CI = 0.3–0.7).

To evaluate whether DOX in combinations with PZA in the MCF-7 and MCF-7/TH cell lines has similar synergistic cytotoxicity as that shown in SW620 and AD300 cell lines, we evaluated the combination at various ratios. Dose effect versus CI plots demonstrated synergistic cytotoxic effects in the MCF-7 and MCF-7/TH cell lines. The calculated CI values ranged from 0.5 to 0.05 with inhibition of 45–95% of colonies (Fig. 4, A and B) in MCF-7 cells, and CI values were between 0.90 and 0.15 with inhibition of 25–75% of colonies in the MCF-7/TH-resistant cells (Fig. 4, C and D).

**Mechanistic Studies of Synergistic Activity.** The amount of DNA Topo I and II in nuclear extracts was measured...
by Western blot analysis. The data in Fig. 5 demonstrated that the Topo I and II protein levels are similar in the SW620 and AD300 cell lines. These results exclude the possibility that doxorubicin resistance in AD300 is due to an increase in the level of these enzymes.

The drug-sensitive SW620 cell line, which exhibits little P-glycoprotein, accumulates higher levels of doxorubicin when compared with the P-glycoprotein overexpressing AD300 cell line. To decide if Topo I activity was involved in the resistance of the AD300 cells to the Topo I poison topotecan and PZA, we determined the catalytic activity of the Topo I in the nuclear extracts of the parental and the resistant cells by measuring relaxation of supercoiled plasmid DNA (see "Materials and Methods"). The results of a representative experiment are shown in Fig. 6A. The amount of supercoiled DNA in the gel (Fig. 6A, Lanes 1 and 7) decreased with the addition of increasing amounts of nuclear extract proteins from SW620 (Fig. 6A, Lanes 2–6) and AD300 (Fig. 6A, Lanes 8–12) cells. This decrease was associated with increases in the amount of detectable nicked (open circular) and relaxed DNA (topoisomers). The relaxation of supercoiled DNA was detectable when the nuclear extract protein concentration was >0.04 μg/assay. The supercoiled DNA was totally catalyzed to relaxed DNA when this protein concentration reached 0.5 μg/assay. These data indicate that the nuclear extracts of the two cell lines, SW620 and AD300, have similar specific activity of Topo I and exclude the possibility that the basis of AD300 resistance is due to changes in Topo I activity.

We examined the inhibitory effect of PZA and/or topotecan on purified human DNA Topo I activity in the presence of plasmid DNA. We found that PZA at concentrations of 2.5–50.0 μM interfered with the formation of relaxed DNA (Fig. 6B, Lanes 3–7) compared with Topo I in absence of drug (Fig. 6B, Lane 2). The different pattern of DNA migration, however, depends on the concentration of PZA. With 25 and 50 μM PZA, a gel shift effect was evident (Fig. 6B, Lanes 6 and 7). In contrast, topotecan resulted in an increase in open circular DNA and inhibition of conversion of supercoiled substrate to relaxed DNA in a concentration-dependent manner (Fig. 6C, Lanes 3–6), reflecting interruption of the phosphodiester backbone when covalent Topo I-DNA complex are formed. The effect of PZA is, thus, distinct from that of topotecan. PZA in combination with 10 μM of topotecan decreased the breakage of supercoiled DNA (Fig. 6D, Lane 3) compared with PZA alone at 2.5 μM (Fig. 6B, Lane 3). However, the amount of supercoiled DNA observed was not greater than that in samples exposed to topotecan alone (Fig. 6C, Lane 3). Quantification of the different DNA forms (Fig. 6, B–D) induced by either PZA or topotecan alone and in combination demonstrated that the combination of

![Fig. 2 Dose effect (A and C) and combination index (B and D) of topotecan (TPT) and/or pyrazoloacridine (PZA) in SW620 cells (A and B) and SW620/AD300 cells (C and D). The concentrations of TPT are used to define drug concentration specified on the X axis for the drug combination studies (A and C); bars, ±SD.](clincancerres.aacrjournals.org)
PZA and topotecan did not provide an activity that was any more than additive.

The catalytic activity of Topo II in nuclear extracts of SW620 and AD300 cells was assayed by decatenation of kDNA and separation by gel electrophoresis of kDNA and monomers, and shown in Fig. 7A. The amount of nicked, opened, and relaxed DNA forms in the gel increased with the addition of increasing amount of nuclear extract proteins from SW620 (Fig. 7A, Lanes 2–7) and AD300 (Fig. 7A, Lanes 8–13) cells compared with kDNA alone in absence of added nuclear extracts (Fig. 7A, Lane 1). The nicked, opened, and relaxed DNA was detectable when the nuclear extract proteins were added at levels of 0.1/µg assay for both the parental and drug-resistant cell lines. These data indicate that the nuclear extracts of the two cell lines, SW620 and AD300, have similar specific activity of Topo II and exclude the possibility that the basis of AD300 resistance is a consequence of differences in Topo II activity.

Nuclear extract proteins (2 µg) from the resistant cells were used to assess drug-mediated changes in the catalytic activity of DNA Topo II (Fig. 7, B–F). Results from the cell-free experiments show that PZA, VP-16, and doxorubicin, at the indicated concentrations, reduced the formation of nicked, opened, and relaxed DNA mediated by Topo II (Fig. 7B, Lanes 2–5; Fig. 7C, Lanes 2–4; and Fig. 7D, Lanes 2–5) as compared with the activity observed in absence of drug (Lane 1 of Fig. 7, B–D). It should be noted that 5 µM PZA was required to achieve 50% inhibition of Topo II activity (Fig. 7B, Lane 3). Similar levels of inhibition could be observed at 150 µM VP-16 (Fig. 7C, Lane 4) and 75 µM doxorubicin (Fig. 7D, Lane 4). The nicked and relaxed DNA forms in the nuclear extract treated with various concentration of PZA combined with 10 µM DOX or 15 µM VP-16 showed no obvious combination effects resulting in enhanced Topo II inhibition (see Fig. 7E, Lanes 2–5 and Fig. 7F, Lanes 2–4) as compared with the cells treated with drug alone.

A filter-binding assay was used to detect Topo I- and II-DNA cross-links complexes stabilized by PZA, DOX, VP-16, or topotecan in SW620 and AD300 cell lines. Cells were treated with the indicated drugs for 2 h at 37°C. As shown in Table 2, DOX at 1.0 µM, VP-16 at 10.0 µM, and topotecan at 0.5 µM produced a 1.8-, 1.8-, and 1.5-fold increase, respectively, in protein-linked DNA complexes over untreated control SW620 cells (P < 0.05). In contrast, PZA at 20 µM caused a 50% reduction in the formation of topoisomerase-DNA complexes compared with control (P < 0.05). These observations suggest that PZA fails to stabilize covalent topoisomerase-DNA complexes in intact SW620 cells. PZA combined with DOX did not change the formation of these complexes compared with DOX alone. However, PZA combined with VP-16 and topotecan reduced topoisomerase-DNA complex formation compared with that of VP-16 or topotecan alone. Similarly, DOX at 50.0 µM, VP-16 at 50.0 µM, and topotecan at 5.0 µM produced 1.7-, 2.5-, and 3.4-fold increases, respectively, in topoisomerase-DNA complex formation over control AD300 cells (P < 0.05). However, PZA at 20 µM caused a 50% reduction in the formation of...
to poiosomerase-DNA complexes compared with control ($P < 0.05$). These results suggest that PZA fails to stabilize covalent topoisomerase-DNA complexes in intact AD300 cells. When PZA was combined with DOX, a reduction of topoisomerase-DNA complexes was observed compared with DOX alone, but this was not observed when PZA was combined with VP-16 and topotecan.

DISCUSSION

In this study, we found that PZA exhibited equivalent cytotoxic effects in drug-sensitive (SW620) and drug-resistant (SW620/AD300) colon carcinoma cells, as well as in drug-sensitive (MCF-7) and -resistant (MCF-7/TH) breast cancer cells. Others have shown that the cellular uptake and retention of PZA, as detected by flow cytometry, are similar when comparing these multidrug resistant to parental cell lines (24). These results suggest that PZA may be an excellent drug choice in treating resistant tumors, or more importantly, in tumors where resistant tumor is likely to emerge. Consistent with this idea, others have reported that PZA exhibited activity against many different types of MDR tumors, such as Adriamycin-resistant sublines of P388 leukemia, B16 melanoma, and 16C mammary adenocarcinoma cells (6), as well as HL-60/ADR human promyelocytic leukemia cells and MCF-7/AD10 human breast cancer cells (8). The results summarized here confirm these previous results and also suggest that PZA can completely circumvent drug resistance in AD 300 cells when used in combination with VP-16 or topotecan, as well as partially restore drugs resistance when combined with DOX. The combination of PZA with DOX, VP-16, and topotecan produced synergistic cytotoxic effects as judged by the median effect analysis of Chou and Talalay on SW620 and SW620/AD300 cells, as well as MCF-7 and MCF-7/TH cells. Synergy has also been observed when PZA is used in combination with cisplatin when tested against A549 human non-small cell lung cancer cells, T98G human glioblastoma

Fig. 4 Dose effect (A and C) and combination index (B and D) of doxorubicin (DOX) and/or pyrazoloacridine (PZA) in MCF-7 cells (A and B) and MCF-7/TH cells (C and D). For the drug combination studies shown in A and C, the concentrations of DOX are used to define drug concentration specified on the X axis. The SD of dose effect and combination index was <15% of their mean values.

Fig. 5 Analysis of topoisomerase I and II protein levels in SW620 and SW620/AD300 cells. The nuclear extract protein (40 μg) isolated from SW620 and SW620/AD300 cells was probed with rabbit antihuman topoisomerase I antisera and mouse monoclonal antibody to human topoisomerase IIa. The mouse antihuman β-actin monoclonal antibody was used as a housekeeper protein.
cells, and HCT8 human intestinal adenocarcinoma cells (25). This evidence of synergy reveals potential advantages of incorporating PZA into combination chemotherapy regimes.

We demonstrated here that the nuclear extracts from SW620 and AD300 cells show comparable levels of Topo I and Topo II expression and activity. PZA inhibits activity of Topo I and Topo II in cell-free assays in a concentration-dependent manner. Unlike doxorubicin, topotecan, and VP-16, it appears that PZA destabilizes topo-DNA complexes. As indicated in Table 2, PZA inhibited topotecan-induced stabilization of the Topo I-DNA complexes as well as doxorubicin- and VP-16-induced stabilization of the Topo II-DNA complexes. Grem et al. (8) suggested that PZA-mediated cytotoxicity correlated strongly with inhibition of DNA and RNA synthesis, and the associated damage to both nascent and parental DNA. Because the cytotoxicity of topotecan is highly dependent on inhibition of DNA and RNA synthesis, which may alter the subnuclei distribution of Topo I (26), deregulation of cyclins or phosphatases may also influence the cytotoxicity of Topo I inhibitors (27). Doxorubicin inhibits nucleic acid synthesis and deregulates cyclins or phosphatases, as well as induces free radicals (27, 28). Etoposide-induced cytotoxicity is also associated with inhibition of RNA and protein synthesis (29). These studies would suggest that synergistic cytotoxicity of PZA and doxorubicin, topotecan, or VP-16 may be due to their combined effects on DNA and RNA synthesis and/or multilevel therapeutic targets. It is important to recognize that others have shown that PZA retained similar activity against the Topo I-deficient P88/CPT45 and the Topo II-deficient HL-60 sublines when compared with the parental cell lines (8, 30, 31) and yeast cells lacking either Topo I or II (9). These results argue that cytotoxicity of PZA does not relate directly with topoisomerase activity. In fact, the effect of PZA on the electrophoretic mobility of both closed and opened circular DNA (Fig. 6B) provides strong evidence of DNA interaction/binding that, in turn, may alter DNA conformation and interfere with access of replicative, repair, and transcription enzymes complexes. Taken together, these results suggest that PZA does not directly bind to Topo I and II, but may impede access of Topo I and II to their DNA binding sites through steric hindrance.

PZA can induce classic apoptosis with oligonucleosomal DNA ladders in HL-60 leukemia cells and high molecular weight DNA fragmentation (500–1,000 kb) without nucleosomal laddering in MCF-7 breast cancer cells (8). However, in SW620 and AD300 cells treated with PZA at concentrations ranging from 2 to 20 μM for 2–24 h, there were no detectable oligonucleosomal DNA ladders and no significant amount of high molecular weight DNA fragmentation (500–1,000 kb; data not shown). Therefore, the apoptosis induced by PZA seems dependent on the type of cell lines. Intracellular nucleosomal fragmentation is thought to result from activation of a calcium- and magnesium-dependent endonuclease. The absence of nucleosomal ladders reported previously in the MCF-7 cells (8), and in the SW620 and AD300 cells in our present report may signify that the cells either do not contain or do not activate that specific endonuclease under the experimental condition used.

A 24-h exposure to 1 and 10 μM PZA was associated with a decreased proportion of cells in S phase, and accumulation in G2 + M phase accumulation was more pronounced after 48 h, although this was accompanied by a decreased proportion of cells in G1 phase (8). However, in our study, SW620 and AD300 cells were exposed to 1 and 10 μM PZA for 2 h, and there were little effects observed on cell cycle distribution up to 24 h.

The in vitro data presented in this report clearly suggest that the therapeutic activity of PZA could be enhanced through strategies that combine this agent with agents such as doxorubicin, topotecan, and VP-16. In vitro drug synergy assays, however, provide little information as to how the potential synergy between two or more agents can be attained in vivo. We believe that this problem will be overcome through strategies that control pharmacokinetic behavior of the combined agents. Typically, in vitro data are used to define drug concentrations known to be effective against cell populations of interest. Ideally when the drug is administered in vivo a pharmacokinetic goal is to define a dosing strategy that will insure that the plasma concentrations of drug achieved are equal to or greater than the concentration required to achieve in vitro cytotoxicity. In this context both peak plasma drug concentration and steady state concentrations need to be considered. The pharmacokinetics of PZA in patients given a well-

Fig. 6 The DNA relaxation activity of topoisomerase I in SW620 and SW620/AD300 cells (A). Supercoiled plasmid DNA (A, Lanes 1 and 7) was incubated with various amounts of nuclear extracts isolated from SW620 cells (Lanes 2–6) and SW620/AD300 cells (Lanes 8–12), respectively. Inhibition of topoisomerase I activity by drug alone and in combinations (B–D). Supercoiled plasmid DNA (B–D, Lane 1) was incubated with purified topoisomerase I enzyme (B–D, Lane 2) and various concentration of pyrazoloacridine (PZA; B, Lanes 3–7), topotecan (C, Lanes 3–6) and PZA +20 μM topotecan (D, Lanes 3–6), respectively. O.C., Rel., and S.C. represent opened circular DNA, relaxed DNA, and supercoiled DNA, respectively.
tolerated dose of 281 mg/m² as a 24 h i.v. infusion weekly for 3 of 4 weeks have suggested that peak plasma drug concentrations of 1.6 μM can be achieved (10). These studies suggested that the terminal half-life of PZA was 27 h, and the average plasma concentration achieved during one cycle was 1.1 μM (10). On the basis of previous study, this concentration can induce 90% inhibition of MCF-7 breast cancer cell growth as judged by a 24-h in vitro cytotoxicity assay (8). And PZA-induced parental DNA fragmentation occurred in MCF-7 cells in a concentration-dependent manner and correlated with loss of viability (8). Furthermore, high molecular weight DNA fragmentation was seen in 38% of post-therapy bone marrow mononuclear cells taken from patient treated with 281 mg/m² (10). In addition, it was demonstrated by Adjei et al. (32) that when K562 cells were incubated for 1 h with 1–10 μM extracellular PZA, intracellular PZA content, which was determined by high-performance liquid chromatography, was ~250-fold higher. On the basis of these observations, an extracellular PZA concentration of 1 μM would be expected to result in an average intracellular PZA concentration of 200 μM. The concentration is well within the range shown to inhibit Topo I and Topo II in a cell-free system. In our study, the IC₅₀ of PZA in combination with doxorubicin, VP-16, or topotecan (Table 1) in SW620 and SW620/AD300 cells, as well as MCF-7 cells exposed to the drugs for as little as 2 h were ~1.3 μM. These data suggest that the concentrations of PZA required to engender a cytotoxic response are well within the range achievable in patients.

When considering these pharmacokinetic parameters, particularly in view of designing clinical trials for defined synergistic drug combinations, the plasma concentrations of all of the drugs must be assessed. Results from in vitro synergy assays, such as the approach defined in this report, indicate that optimal drug synergy can only be achieved at defined effective doses (the Fa value defined by the Chou and Talalay median effect analysis) and at specific drug ratios. The latter factor is of

![Fig. 7 The topoisomerase II activity in nuclear extracts from SW620 and SW620/AD300 cells (A). Kinetochores (kDNA) and decatenated kDNA were detected in absence of nuclear extract (A, Lane 1); in the presence of indicated amount of nuclear extract protein from SW620 cells (A, Lanes 2–7), and SW620/AD300 cells (A, Lanes 8–13). Inhibition of topoisomerase II activity by pyrazoloacridine (PZA; B), VP-16 (C), doxorubicin (DOX; D), PZA + VP-16 (E), and PZA + Dox (F), respectively. kDNA and decatenated kDNA were detected with kDNA and 2μg of nuclear extracts from SW620/AD300 cells in absence of drugs (B–F, Lane 1); and in presence of indicated concentrations of drugs (B–F, Lanes 2 and above). Decatenated kDNA products include nicked, opened, circular, and relaxed DNA.](image)

### Table 2 DNA-protein complexes (absorbance values) in SW620 and SW620/AD300 cells treated with drugs

<table>
<thead>
<tr>
<th>Drug (μM)</th>
<th>Absorbance SW620</th>
<th>Drug (μM)</th>
<th>Absorbance SW620/AD300</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>103.5 ± 10.1</td>
<td>Control</td>
<td>63.0 ± 11.8</td>
</tr>
<tr>
<td>DOX 1.0</td>
<td>181.0 ± 8.1</td>
<td>DOX 50.0</td>
<td>108.1 ± 10.0</td>
</tr>
<tr>
<td>PZA 20.0</td>
<td>56.3 ± 4.8</td>
<td>PZA 20.0</td>
<td>34.3 ± 10.9</td>
</tr>
<tr>
<td>VP-16 10.0</td>
<td>177.2 ± 4.3</td>
<td>VP-16 50.0</td>
<td>162.1 ± 24.3</td>
</tr>
<tr>
<td>TPT 0.5</td>
<td>149.3 ± 12.0</td>
<td>TPT 5.0</td>
<td>212.1 ± 7.2</td>
</tr>
<tr>
<td>DOX 1.0 + PZA 20.0</td>
<td>155.8 ± 13.0</td>
<td>DOX 50.0 + PZA 20.0</td>
<td>79.1 ± 7.9</td>
</tr>
<tr>
<td>VP-16 10.0 + PZA 20.0</td>
<td>137.1 ± 20.8</td>
<td>VP-16 50.0 + PZA 20.0</td>
<td>127.7 ± 13.6</td>
</tr>
<tr>
<td>TPT 0.5 + PZA 20.0</td>
<td>132.5 ± 24.8</td>
<td>TPT 5.0 + PZA 20.0</td>
<td>170.2 ± 7.9</td>
</tr>
</tbody>
</table>

*DOX, doxorubicin; PZA, pyrazoloacridine; TPT, topotecan.
*P < 0.05, **P < 0.001 vs. control (Student’s t-test).
particular interest, because it presently is very difficult to control the pharmacokinetic behavior of two therapeutic agents such that their delivery to a target cell population occurs at defined ratios as well as optimal concentrations.

In view of our observations that PZA has synergistic activity with several conventional anticancer drugs and the fact that cytotoxic concentrations are achievable in patients plasma using a weekly 24-h infusion schedule in a Phase I clinical study (10), this drug may be a good candidate for combination studies that cytotoxic concentrations are achievable in patients plasma activity with several conventional anticancer drugs and the fact particular interest, because it presently is very difficult to control anticancer agents with selectivity against solid tumors.

REFERENCES

Synergistic Cytotoxicity of Pyrazoloacridine with Doxorubicin, Etoposide, and Topotecan in Drug-Resistant Tumor Cells

YanPing Hu, Awtar Krishan, WeiJia Nie, et al.


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