Pyrazoloacridine Is Active in Multidrug-resistant Neuroblastoma Cell Lines with Nonfunctional p53[1]

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ABSTRACT

Purpose: The purpose of this study was to determine the activity of pyrazoloacridine (PZA) in neuroblastomas that have acquired high-level resistance to multiple drugs (not associated with multidrug resistance-associated protein or P-glycoprotein) during therapy, including those with loss of p53 function.

Experimental Design: We determined the activity of PZA in 12 drug-sensitive and 10 multidrug-resistant (MDR) neuroblastoma cell lines. Six of the MDR cell lines lacked functional p53. Drug cytotoxicity was measured using the DIMSCAN fluorescence/digital imaging microscopy assay with a 4-log dynamic range.

Results: LC_{90} (*i.e.*, the drug concentration that was lethal for 90% of the cell population) values ranged from 0.01 to 1.1 μM for the drug-sensitive cell lines, from 0.8 to 2.4 μM for the MDR cell lines with functional p53, and from 0.9 to 2.1 μM for the MDR cell lines that lacked functional p53. To confirm that PZA cytotoxicity is independent of p53 function, we compared two neuroblastoma cell lines in which p53 function was abrogated via human papilloma virus-16 E6 transduction (which mediates increased degradation of p53) to the mock-transduced (LXSN) controls. LC_{90} values for human papilloma virus-16 E6 clones (abrogated p53) ranged from 0.2 to 2.04 μM, whereas LC_{90} values for LXSN controls (functional p53) were 0.1 and 0.5 μM. PZA was active with 72-h *in vitro* exposures against p53-nonfunctional MDR cells at drug levels (2–3 μM) obtained for shorter periods (1–3 h) in Phase I and II clinical trials. Washout experiments showed that 3 μM PZA achieved 0.5–1 log of cell kill with 1–3-h exposures *versus* 3 logs at 24 h.

Conclusions: These data suggest that expanding the time of PZA systemic exposure, which may be clinically tolerable with hematopoietic stem cell support, should be tested in clinical trials. Prolonged systemic exposure to PZA with hematopoietic stem cell support may be effective against recurrent neuroblastomas that have failed conventional chemotherapeutic regimens, including those neuroblastomas with loss of p53 function.

INTRODUCTION

Aggressive chemoradiotherapy followed by autologous BMT[3] and 13-*cis*-retinoic acid improved survival of high-risk neuroblastoma patients; however, progressive disease is still fatal for over 50% of such patients (1). Drug resistance acquired during the course of treatment is one of the major reasons for treatment failure (2), with p53 loss of function being identified as one of the major mechanisms responsible for neuroblastoma drug resistance (3). Therefore, identifying drugs that are p53 independent may provide agents active against recurrent, drug-refractory neuroblastomas.

PZA (NSC366140, PD115934), a novel DNA intercalator, is a rationally synthesized acridine derivative that has been shown (*a*) to have a broad-spectrum activity against tumor cells *in vitro* and *in vivo* (4–6), (*b*) to be equally potent in normoxic and hypoxic cells (5, 6), (*c*) to be equally effective in cycling and noncycling cells (5, 6), (*d*) to be active in cells resistant to agents due to overexpression of Pgp (7) or MRP (8), and (*e*) to be toxic to cells with selective loss of topoisomerase I or II (9).

Phase I (10–13) and II (14–26) clinical trials of PZA showed that major toxicities were myelosuppression in pediatric patients (12) and, in adults, neurotoxicity (prominent with 1-h PZA infusion times) as well as myelosuppression (13). However, only modest tumor responses were seen at the administered schedules (15, 17).

We found that PZA effectively induced cytotoxicity in MDR cell lines, and this prompted us to determine whether PZA could be effective in drug-resistant p53-nonfunctional neuroblastoma cell lines. Here we demonstrate that PZA showed a dose-dependent cytotoxicity for a range of neuroblastoma cell lines in a p53-independent manner, even under hypoxic conditions, but only with PZA exposure times that exceeded those tested in previous clinical trials.

[3] The abbreviations used are: BMT, bone marrow transplantation; PZA, pyrazoloacridine; L-PAM, melphalan; CBDCA, carboplatin; ETOP, etoposide; FDA, fluorescein diaceate; HPV, human papilloma virus; MDR, multidrug-resistant (not MRP- or Pgp-associated); MRP, multidrug resistance-associated protein; Pgp, P-glycoprotein; AUC, area under the curve; PPL, peak plasma level; FBS, fetal bovine serum; CI, confidence interval; MTD, maximum tolerated dose.

Table 1 p53 status and PZA efficacy in neuroblastoma cells lines

	Cell lines	p53 function	PZA (μM)		AUC_{90}^{c} (μM·h)
			LC_{90}^{a}	LC_{99}^{b}	
Drug-sensitive	SK-N-BE(1)	$+^{d}$	0.5	3.7	38.9
	SMS-SAN	+	<0.01	0.1	0.7
	CHLA-15	+	0.2	0.6	14.4
	SMS-KAN	+	0.9	4.4	64.8
	SMS-KCN	+	1.1	5.9	79.2
	SMS-LHN	+	0.1	0.5	9.4
	SMS-KANR	+	0.6	2.8	41.0
	SMS-KCNR	+	0.2	1.0	12.2
	CHLA-20	+	0.4	1.6	26.6
	CHLA-42	+	0.7	1.3	46.8
	CHLA-8	+	0.6	1.5	43.9
	CHLA-51	+	0.4	1.8	29.5
Mean^{e}			0.41	1.75	29.19
95% CI^{e}			(0.24–0.62)	(1.05–2.64)	(16.75–45.06)
MDR, p53-functional	CHLA-140	+	0.9	2.8	62.6
	LA-N-6	+	0.8	2.5	55.4
	CHLA-79	+	2.4	17.4	169.2
	CHLA136	+	1.2	5.0	83.5
Mean			1.26	3.35	87.89
95% CI			(0.73–1.93)	(1.46–6.01)	(50.50–135.58)
MDR, p53-nonfunctional	SK-N-BE(2)	$-^{f}$	1.0	4.7	69.1
	CHLA-119	−	1.1	2.5	77.8
	CHLA-171	−	0.9	2.6	62.6
	CHLA-90	−	1.5	3.9	108.7
	CHLA-134^{g}	−	1.3	4.3	91.4
	CHLA-172	−	2.1	3.0	151.2
Mean			1.29	3.45	91.29
95% CI			(0.84–1.83)	(2.01–5.27)	(59.33–130.12)

[a] LC_{90}, PZA concentration lethal for 90% of treated cells.

[b] LC_{99}, PZA concentration lethal for 99% of treated cells.

[c] AUC_{90} calculated as $LC_{90} \times 72$ h.

[d] +, p53 function intact was determined as low basal level of p53 and induction of p21 and/or MDM2 in response to 16-h exposure to 6 μg/ml melphalan by Western blotting.

[e] Means and associated CIs were based on the square root transformed data. LC_{99} value of CHLA-79 was excluded from analysis as an outlier.

[f] −, loss of p53 function determined as high basal level p53 and failure to induce both p21 and MDM2 in response to 16-h exposure of 6 μg/ml melphalan by Western blotting.

[g] Cell line with loss of p53 function associated with *MDM2* genomic amplification.

MATERIALS AND METHODS

Cell Lines. We used a panel of 22 neuroblastoma cell lines (Table 1) obtained from patients at various phases of therapy: 6 at diagnosis before any therapy [SMS-SAN, SK-N-BE(1), SMS-KAN, SMS-KCN, CHLA-15, and CHLA-42]; 3 at progressive disease during dual-agent induction therapy (SMS-LHN, SMS-KCNR, and SMS-KANR); 6 at progressive disease during intensive multiagent chemotherapy [SK-N-BE(2), LA-N-6, CHLA-20, CHLA-119, CHLA-140, and CHLA-171]; and 7 derived at relapse after myeloablative therapy and BMT (CHLA-8, CHLA-51, CHLA-79, CHLA-90, CHLA-134, CHLA-136, CHLA-172). Neuroblastoma origin of these cell lines has been confirmed previously (2, 3, 27, 28).

None of the tumors that gave rise to cell lines used in this study had been treated with PZA. As we have shown previously, the cell line panel displays a spectrum of resistance (acquired during the therapy) to L-PAM, CBDCA, ETOP, cisplatin, doxorubicin, SN-38, and topotecan (2, 3, 27). Cell lines with LC_{90} (*i.e.*, the drug concentration that was lethal for 90% of the cell population) values greater than clinically achievable drug levels

for at least two of three agents (ETOP, L-PAM, and CBDCA) were considered MDR. Clinically achievable reference drug concentrations were 10 μg/ml for L-PAM, 3 μg/ml for CBDCA, and 5 μg/ml for ETOP (2). Thus, 12 drug-sensitive and 10 MDR cell lines were used in this study (Table 1). SK-N-BE(1), SMS-SAN, SMS-LHN, LA-N-6, CHLA-171, CHLA-79, CHLA-134, CHLA-136 (3), and SMS-KAN (29) have been shown to carry wild-type *TP53*, whereas SK-N-BE(2), CHLA-119, CHLA-90, and CHLA-172 (3) carry mutant *TP53*.

SMS-KAN, SMS-KANR, SMS-KCN, SMS-KCNR, SK-N-BE(1), SK-N-BE(2), SMS-SAN, SMS-LHN, and LA-N-6 were cultured in complete medium made from RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 10% heat-inactivated FBS (Gemini Bioproducts, Inc., Calabasas, CA). CHLA-15, CHLA-20, CHLA-42, CHLA-119, CHLA-140, CHLA-171, CHLA-51, CHLA-8, CHLA-79, CHLA-90, CHLA-134, CHLA-136, and CHLA-172 were cultured in complete medium made from Iscove's modified Dulbecco's medium (BioWhittaker, Walkersville, MD) supplemented with ≈3 mM L-glutamine (Gemini Bioproducts), 5 μg/ml insulin, 5 μg/ml
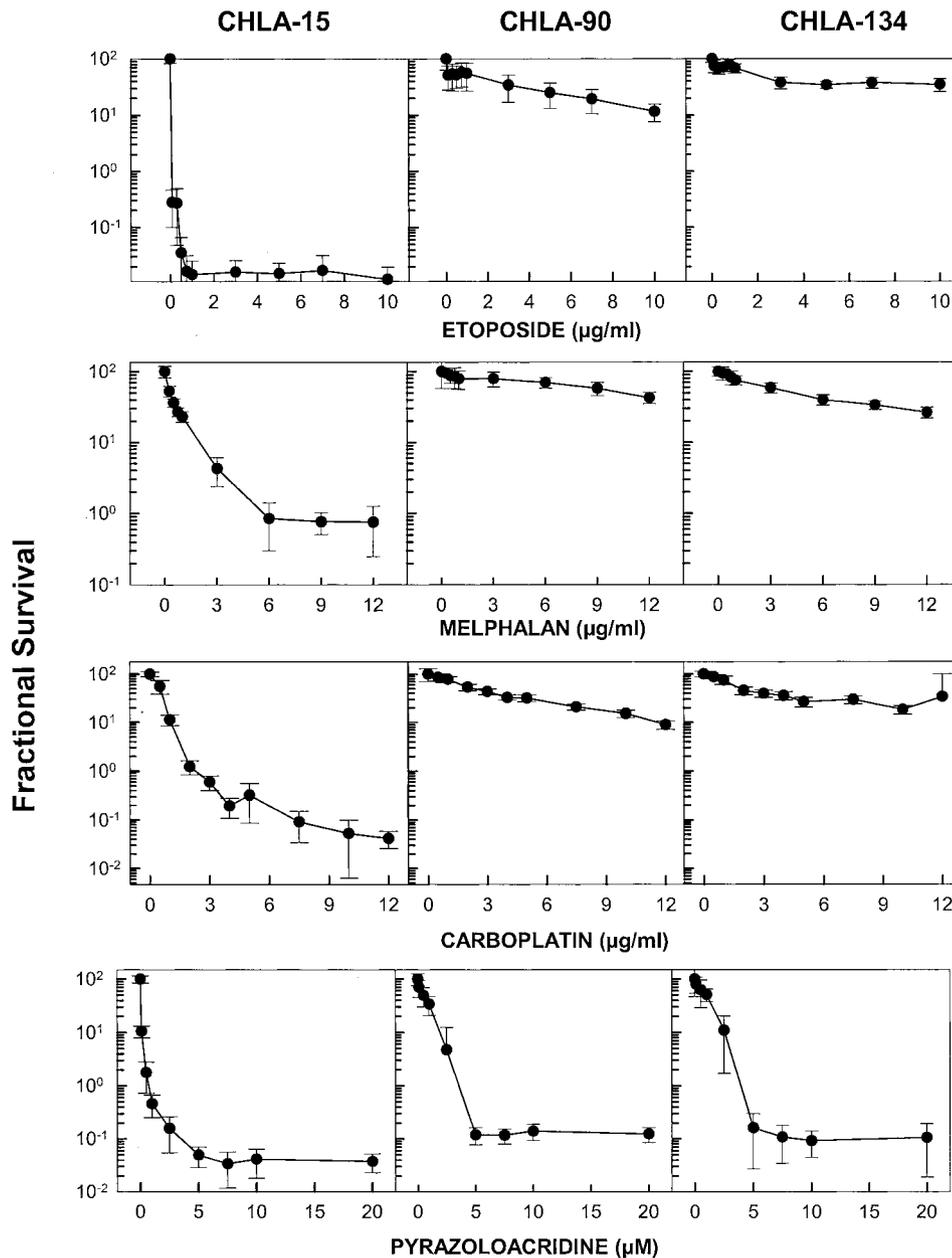
Fig. 1 Representative dose-response curves obtained by DIMSCAN assay. CHLA-15, a drug-sensitive cell line with functional p53, was established from a patient at diagnosis before treatment. CHLA-90 and CHLA-134, MDR cell lines with nonfunctional p53, were obtained from patients after myeloablative chemoradiotherapy followed by BMT. Cell lines established after BMT are highly resistant to ETOP, L-PAM, and CBDCA but are sensitive to PZA.

transferrin, and 5 ng/ml selenous acid (ITS Culture Supplement; Collaborative Biomedical Products, Bedford, MA) and 20% heat-inactivated FBS. All neuroblastoma cell lines used in the study were under passage 30. Non-neuroblastoma cell lines were maintained in complete medium made from RPMI 1640 supplemented with 10% heat-inactivated FBS. The cell lines were cultured at 37°C in a humidified incubator containing 95% air + 5% CO_2 atmosphere without antibiotics. All cell lines tested negative for *Mycoplasma*. Cell lines were not selected for drug resistance *in vitro*.

SMS-SAN (*MYCN* gene amplified) and SMS-LHN (*MYCN* gene not amplified; both are drug-sensitive cell lines) were transduced with HPV-16 E6, which encodes a protein that promotes degradation of p53 and renders cells p53 nonfunctional (30), or with the LXSN retrovirus empty vector as a control; G418-resistant clones were selected from each. HPV-16 E6-transduced clones were designated as SAN/E6 D4, SAN/E6 B3, LHN/E6 2–6 and LHN/E6 5-B5. LXSN-transduced clones were designated as SAN/LXSN and LHN/LXSN. Reduced p53 activity in HPV-16 E6 clones was confirmed by the lack of p53 expression and the lack of induction of p53, p21, and MDM2 in L-PAM-challenged samples using Western blotting and also using a p53 transactivation assay (3). Using this system, we have shown previously that transduction of HPV-16 E6 conferred high-level multidrug resistance for L-PAM, CBDCA, and ETOP to both SMS-SAN and SMS-LHN cell lines (3).
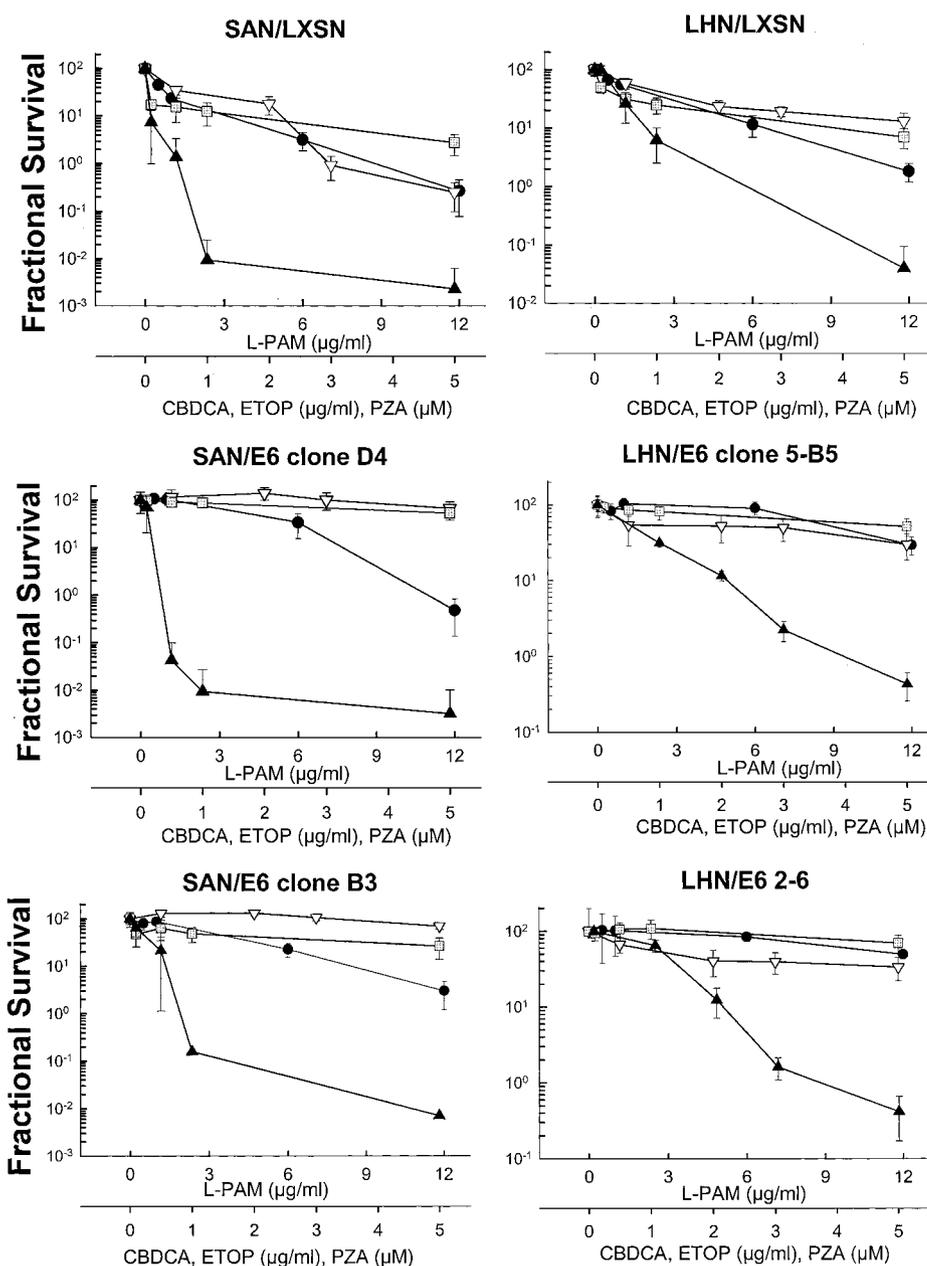
SAN/LXSN

LHN/LXSN

SAN/E6 clone D4

LHN/E6 clone 5-B5

SAN/E6 clone B3

LHN/E6 2-6

Fig. 2 DIMSCAN assay comparing the cytotoxicity of melphalan (L-PAM), carboplatin (CBDCA), etoposide (ETOP), and pyrazolo-acridine (PZA) in p53-functional and p53-nonfunctional cells lines. Clones of the p53 functional cell lines SMS-SAN (*MYCN* amplified) and SMS-LHN (*MYCN* nonamplified) were rendered p53 nonfunctional by HPV 16 E6-transduction (SAN/E6 and LHN/E6). The same cell lines transduced with the LXSN "empty vector" (SAN/LXSN and LHN/LXSN) retained p53 function and were tested as controls. The cell lines with loss of p53 function demonstrated increased drug resistance to L-PAM (●), CBDCA (▽), and ETOP (□), but not to PZA (▲).

Drugs and Chemicals. PZA, L-PAM, CBDCA, and ETOP were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD). FDA was from Eastman Kodak Co. (Rochester, NY), and eosin Y was from Sigma Chemical Co. (St. Louis, MO).

Cytotoxicity Assay. The cytotoxicity of chemotherapeutic agents for cell lines was determined with the DIMSCAN assay system (2, 3, 27, 31). DIMSCAN uses digital imaging microscopy to quantify viable cells, which selectively accumulate FDA. DIMSCAN is capable of measuring cytotoxicity over a 4–5-log dynamic range by quantifying total fluorescence/well (which is proportional to viable,

clonogenic cells) after eliminating background fluorescence with digital thresholding and eosin Y quenching (2). Cell lines were seeded at 10,000–15,000 cells in 150 μl of complete medium per well into 96-well plates. After overnight incubation, various concentrations of PZA in 100 μl of complete medium were added to each well. The final drug concentrations ranged from 0 to 20 μM. Each condition was tested in 12 replicates. After incubation of cell lines with PZA for 3 days, 150 μl of medium were removed from each well, FDA in 50 μl of medium (final concentration of FDA 10 μg/ml) was added, and plates were incubated for an additional 25 min at 37°C before adding 30 μl of 0.5% eosin Y to each well. Total fluorescence was then measured using

digital image microscopy, and results were expressed as surviving fractions of treated cells compared with control cells.

Using four cell lines (SMS-KANR, SMS-KCNR, LA-N-6, and CHLA-90), PZA activity was tested in atmospheric (20.7% oxygen, 5% CO_2, and 74% N_2) and hypoxic (5% or 2% oxygen, 5% CO_2, and the balance N_2) conditions. The stated hypoxic conditions were created and maintained by flushing respective gas mixtures into the hermetic chambers containing 96-well plates as described previously (32). The chambers were incubated at 37°C for 3 (SMS-KANR, LA-N-6, and CHLA-90) and 4 (SMS-KCNR) days, and the plates were then assayed for cytotoxicity by DIMSCAN as described above.

Drug Washout Experiments. CHLA-90, and CHLA-15 cell lines were treated with PZA (1, 2, 3, and 5 μM) in 96-well plates. After incubation for 1, 3, 6, and 24 h with PZA, 150 μl of medium were removed from each well and replaced with an equal amount of fresh complete medium without disturbing the cell monolayer. This procedure was repeated immediately twice. Plates were then incubated for 3 days and subjected to DIM-SCAN analysis as described above.

AUC 30, 60, 90, and 120 Assays. CHLA-90 was assayed with a range of PZA concentrations and times (for 1–72 h) in such a manner that a PZA concentration × time (here designated AUC) of 30, 60, 90, and 120 was achieved. We have adopted the term AUC (AUC was calculated as μM PZA·h) from clinical pharmacokinetics to refer to PZA cytotoxicity as a function of drug exposure. Cell lines seeded in 96-well plates were exposed to concentrations of PZA for 1, 2, 3, 6, 8, 16, 24, and 72 h, such that for each point on a graph, the indicated AUC was obtained. Plates were then washed three times with fresh medium (except for the 72-h condition), incubated for 3 days, and subjected to DIMSCAN analysis as described above.

Data Analysis. The LC_{90} and LC_{99} (the drug concentration that was lethal for 90% or 99% of the cell population) values were calculated using the software "Dose-Effect Analysis with Microcomputers" (33). A cell line with a LC_{90} value greater than or equal to the clinically achievable concentration was considered resistant to PZA. The reported PPL for PZA varies with administration schedule (10, 12, 13) from 2.2 to 7 μM. We chose 2 μM as a clinically achievable reference value for PZA. The area under the time-concentration curve that results in cytotoxicity for 90% of treated cells (AUC_{90}) was calculated as (LC_{90} × exposure time to the drug).

Statistical Analysis. Before analysis, the data were examined to evaluate compatibility with the assumptions of the normal distribution and homoscedasticity that would permit the use of standard parametric methods of analysis, in particular, the ANOVA. For the effect of hypoxia on PZA cytotoxicity, the logarithm transformation was used, and for the comparison analysis of LC_{90} and LC_{99} values for drug-sensitive *versus* drug-resistant cell lines and for p53-functional *versus* p53-nonfunctional cell lines, the square root transformation was used. These transformations rendered the data compatible with the assumptions required for ANOVA.

To adjust for the dependence of the assay-to-assay variability on dose, the observations were weighted by the inverse of PZA dose + 0.1. Each of the cell lines was analyzed separately using a factorial design ANOVA employing the
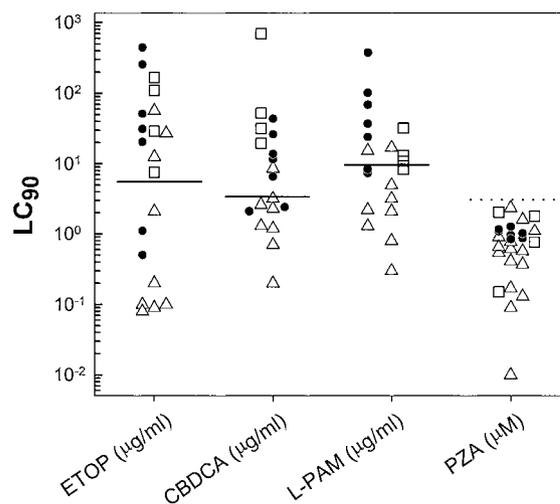

Fig. 3 LC_{90} values for ETOP, CBDCA, L-PAM, and PZA measured by DIMSCAN assay in neuroblastoma 22 cell lines are shown. △, neuroblastoma cell lines with functional p53 ($n = 15$). ●, neuroblastoma cell lines with nonfunctional p53 ($n = 7$). □, HPV-16 E6-transduced clones ($n = 4$). The *bar* indicates the drug levels that are clinically achievable in the setting of myeloablative therapy. The clinically achieved level of PZA over a 72-h exposure (which is the time of exposure used to derive the LC_{90} values) remains to be determined. A *dashed bar* indicates the reference level (2 μM) reported in Phase I clinical trials using short exposure times.

generalized linear models procedure. When comparing LC_{90} and LC_{99} values for drug-sensitive *versus* drug-resistant cell lines and for p53-functional *versus* p53-nonfunctional cell lines, a one-way ANOVA was used. The overall P was based on the ANOVA F test. If the F test P was <0.05, the least significant difference method (34) was used for multiple comparisons. The means and the associated 95% CIs were calculated to summarize the data.
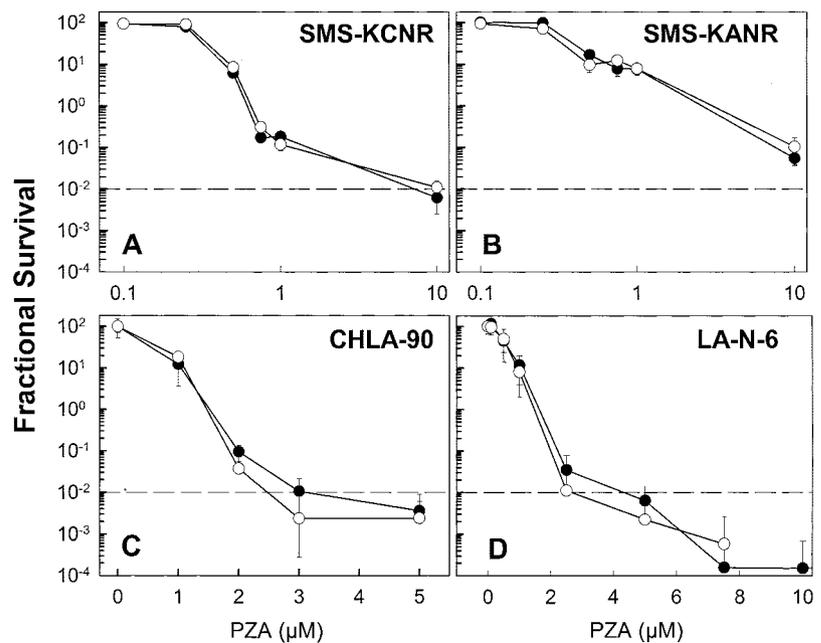
To perform statistical analysis, SAS Software, Version 8.2 was used (SAS Institute, Inc., Cary, NC).

RESULTS

Neuroblastoma Cell Line Panel. The cell line panel used in this study has been characterized previously for the profile of drug sensitivity to commonly used agents in neuroblastoma: L-PAM; CBDCA; and ETOP (2, 3, 27). Cell lines with LC_{90} values greater than clinically achievable drug levels for at least two of the three agents were considered drug resistant. This panel included 12 drug-sensitive and 10 drug-resistant cell lines (Table 1). Representative dose-response curves for L-PAM, ETOP, CBDCA, and PZA are shown in Fig. 1 for a drug-sensitive neuroblastoma cell line established at diagnosis (CHLA-15) and two MDR cell lines established at relapse after myeloablative therapy and BMT (CHLA-90 and CHLA-134). The LC_{90} and LC_{99} values for PZA and the AUC_{90} calculated from the LC_{90} values are listed in Table 1. The LC_{90} and LC_{99} of treated cells were calculated from dose-response curves for each drug tested using the DIMSCAN assay.

PZA showed dose-dependent cytotoxicity against all cell lines tested. The LC_{90} values of drug-sensitive cell lines ranged

from 0.003 to 1.1 μM, and the LC_{99} values ranged from 0.07 to 5.9 μM. The LC_{90} values of MDR cell lines ranged from 0.8 to 2.4 μM, and the LC_{99} values ranged from 2.2 to 17.4 μM. PZA was more effective against the drug-sensitive cell lines than against MDR cell lines (LC_{90} values $P < 0.001$, LC_{99} values $P = 0.028$), but PZA was highly cytotoxic at ~5 μM levels (72-h exposure) for most cell lines with the MDR phenotype.

PZA Activity against p53-nonfunctional Cell Lines. PZA LC_{90} values of cell lines with loss of p53 function ranged from 0.9 to 2.1 μM, and the LC_{99} values ranged from 2.2 to 4.7 μM (Figs. 2 and 3; Table 1). PZA LC_{90} values for p53-funtional/drug-resistant cell lines ranged from 0.8 to 2.4 μM, and the LC_{99} values ranged from 2.5 to 5 μM. There was no statistical difference between PZA activity in drug-resistant/p53-functional cell lines *versus* MDR/p53-nonfunctional cell lines ($P > 0.05$).

To confirm that PZA is a p53-independent agent, PZA efficacy was tested in neuroblastoma cell lines in which p53 function was selectively abrogated by HPV-16 E6 transduction (3). We compared dose-response curves for PZA (Fig. 2) with other drugs (L-PAM, CBDCA, and ETOP) in the p53-nonfunctional cell lines.

SAN/E6 and LHN/E6 clones have been studied previously for their sensitivities to L-PAM, ETOP, and CBDCA (3). The LC_{90} values of SAN/E6 and LHN/E6 clones were generally higher than drug concentrations achieved in patients treated with myeloablative doses, whereas those values in LXSN controls were in the range of clinically achievable levels. The LC_{90} values of SAN/E6 clones were 8.3 and 10.8 μg/ml for L-PAM, 19.6 and 32 μg/ml for CBDCA, 29 and 110 μg/ml for ETOP, whereas the LC_{90} values of SAN/LXSN controls were 1.8 μg/ml for L-PAM, 1.3 μg/ml for CBDCA, and 0.7 μg/ml for ETOP. Similarly, the LC_{90} values of LHN/E6 clones were 13 and 32 μg/ml for L-PAM, 53 and 694 μg/ml for CBDCA, and 7.6 and 166 μg/ml for ETOP. The LC_{90} values of LHN/LXSN

controls were 4.7 μg/ml for L-PAM, 6.4 μg/ml for CBDCA, and 3.7 μg/ml for ETOP.

The PZA LC_{90} values of SAN/E6 clones were 0.15 and 0.76 μM, whereas the LC_{90} value of the SAN/LXSN control was 0.1 μM. Similarly, LC_{90} values of LHN/E6 clones were 1.8 and 2.04 μM, whereas the LC_{90} value of LHN/LXSN cells was 0.5 μM. Although LC_{90} values of SAN/E6 (clones B3 and D4) and LHN/E6 (clones 5-B5 and 2-6) were 1.5–7.6 times higher than those of SAN/LXSN and LHN/LXSN controls, they were within the range of the clinically achievable levels of PZA, except for the LHN/E6 2-6 clone, whose LC_{90} value (2.04 μM) reached the upper limit.

We compared the LC_{90} values obtained when testing p53-functional ($n = 16$), p53-nonfunctional ($n = 6$), and p53 nonfunctional via HPV-16 E6 transduction ($n = 4$) neuroblastoma cell lines against ETOP, CBDCA, L-PAM, and PZA (Fig. 3). Most p53-nonfunctional cell lines were highly resistant to ETOP, CBDCA, and L-PAM ($LC_{90} >$ clinically achievable concentrations in the myeloablative setting), whereas PZA did not show any relationship between p53 functionality and drug sensitivity.

PZA is Equally Active in Atmospheric and Reduced Oxygen Conditions. We have tested PZA in drug-sensitive (SMS-KANR and SMS-KCNR) and MDR [LA-N-6 and CHLA-90 (the latter is p53 nonfunctional)] cell lines at atmospheric and reduced oxygen conditions, and we found that this agent was equally effective in standard culture conditions and hypoxia (Fig. 4). A 3-day exposure to PZA resulted in equivalent cell kills for LA-N-6 (drug-resistant but p53 functional) and CHLA-90 (MDR, p53 nonfunctional) cell lines in atmospheric (20.7% O_2, 5% CO_2, and 74% N_2) and hypoxic (2% O_2, 5% CO_2, and 93% N_2) conditions ($P > 0.05$). PZA dose-response curves obtained in 5% O_2, 5% CO_2, and 90% N_2 after 3-day exposure in SMS-KANR and after 4-day exposure in SMS-

Table 2 Peak plasma PZA concentrations achieved at MTD as reported in Phase I clinical trials

Dose (mg/m^2)	Schedule	Peak plasma level (μM)	AUC (μM·h)	Ref. no.
600	1-h i.v. every 3 wks	2.17 ± 1.05	30 ± 25	10
750	3-h i.v. every 3 wks	2.3 (range, 1.4–4.5)		12
150	1-h i.v. × 5 days every 3 wks	Day 1 2.5 (range, 0.7–11.3) Days 2–5 5.6 (range, 2.6–9.5)		12
281	24-h i.v. every 3 or 4 wks	1.6	56	11

Table 3 Summary of Phase II clinical trials of PZA

Tumor type	No. of patients	Dose schedule	Responses	Ref. no.
Cervical squamous carcinoma	21	750 mg/m^2 3-h i.v.; every 3 wks	None	18
Metastatic colorectal cancer	14	750 mg/m^2 3-h i.v.; every 3 wks	None	20
Cisplatin-refractory germ cell tumors		600 mg/m^2 3-h i.v.; every 3 wks	None	21
Unresectable or metastatic transitional cell carcinoma	14	750 mg/m^2 3-h i.v.; every 3 wks	None	22
Advanced renal cell carcinoma	12	750 mg/m^2 3-h i.v.; every 3 wks	None	23
Hormone-refractory prostate cancer	17	750 mg/m^2 3-h i.v.; every 3 wks	1	24
Advanced pancreatic carcinoma	15	750 mg/m^2 3-h i.v.; every 3 wks	None	25
Recurrent platinum-sensitive ovarian cancer	42	750 mg/m^2 3-h i.v.; every 3 wks	1 CR[a]; 9 PR	17
Recurrent platinum-resistant ovarian cancer	24	750 mg/m^2 3-h i.v.; every 3 wks	1 CR; 1 PR	15
Persistent or recurrent endometrial carcinoma	23	750 mg/m^2 3-h i.v.; every 3 wks	1 PR	16
Advanced colorectal carcinoma	15	750 mg/m^2 3-h i.v.; every 3 wks	None	26
Pediatric solid tumors[b]	47	640 mg/m^2 3-h i.v.; every 3 wks	None	19

[a] CR, complete response; PR, partial response.

[b] This included children with rhabdomyosarcomas ($n = 8$), Ewing's Family Tumors ($n = 10$), neuroblastomas ($n = 7$), osteogenic sarcomas ($n = 8$), Wilms' tumor ($n = 2$), and other solid tumors ($n = 12$) [except brain tumors].

KCNR cell lines were comparable with those obtained in atmospheric (20.7% O_2, 5% CO_2, and 74% N_2) conditions (Ps >0.05; data not shown).

PZA Washout Assays. In the clinical setting, PZA has been given as a 3-h infusion (Tables 2 and 3). To model the activity of PZA at various delivery schedules, we tested the dose-response to PZA after 1-, 3-, 6-, 24- and 72-h exposures in the CHLA-15 and CHLA-90 cell lines (Fig. 5). Exposure to PZA of 6 h or greater appeared to increase activity relative to shorter exposures.

AUC 30, 60, 90, and 120 Assays. We tested the MDR, p53-nonfunctional cell line CHLA-90 in the presence of various PZA concentrations for 1, 2, 3, 6, 8, 16, 24, and 72 h (Fig. 6), such that for each concentration tested, the cells were exposed to a time-concentration for PZA of 30, 60, 90, or 120 μM·h, here designated as AUC. These *in vitro* assays were designed to model the responses to varying exposure times to PZA, which may occur during continuous infusion clinical trials. The highest PZA cytotoxicity (~2.5 logs and greater of cell killing) was achieved with brief exposures to >20 μM PZA, but such high concentrations are clinically unachievable. However, exposure to 5 μM PZA for 6–24 h, which may be clinically achievable with stem cell support, was cytotoxic for the CHLA-90 cell line. Increasing the time of exposure (*i.e.*, increasing the AUC without increasing the drug concentration) for 5 μM PZA led to a multilog increase in the degree of cell kill achieved: 0.7 log at 3 h; 1.0 log at 6 h; 1.4 logs at 12 h; 2.1 logs at 18 h; and 3.2 logs at 24 h.

DISCUSSION

We have shown that neuroblastomas manifest sustained resistance to multiple drugs (non-Pgp- and non-MRP-associated multidrug resistance) *in vitro* and that the resistance correlates with the intensity of chemotherapy used in patients (2, 35, 36). We have also observed that all drug-sensitive neuroblastoma cell lines established before chemotherapy had functional p53, whereas in MDR cell lines, p53 was frequently nonfunctional due to mutations or nonmutational events [such as MDM2 genomic amplification (3, 37)]. Moreover, abrogation of p53

Fig. 5 PZA cytotoxicity increased with longer exposure. CHLA-15 (*A* and *B*), and CHLA-90 (*C* and *D*) cell lines were incubated with PZA for 1 (○), 6 (▽), and 72 h (●) [*A* and *C*] and for 24 (□) and 3 h (▼) [*B* and *D*]. After incubation for 1, 3, 6, and 24 h, 150 μl of media were removed from each well and replaced with an equal amount of fresh media. This procedure was repeated immediately once more. Plates were then incubated for 3 days and subjected to DIMSCAN analysis, as described in "Materials and Methods." LC_{90} values decreased with longer drug exposure.
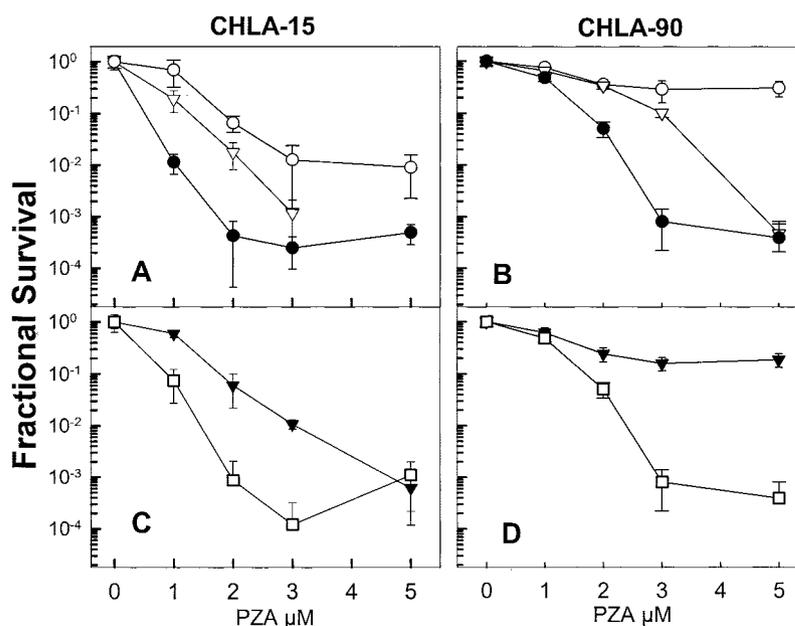
function in drug-sensitive neuroblastoma cell lines conferred the MDR phenotype (3), even at drug levels obtained during myeloablative therapy. Drugs that are independent of p53 may be non-cross-resistant with those drugs in current use for neuroblastoma and could have activity in patients who develop chemotherapy-refractory disease.

PZA is a novel drug that has shown impressive activity *in vitro* and in preclinical studies conducted in mice (4, 5). Previous *in vitro* studies showed that cells resistant to drugs due to Pgp (7) or MRP (8) overexpression or due to altered topoisomerases I or II were invariably affected by PZA (9). Although responses (2 complete responses, 12 partial responses, and 5 minor responses) were seen in clinical trials (13, 15–17), PZA antitumor activity in Phase II trials was disappointing (Table 3). The present study was undertaken to investigate the effect of PZA on drug-resistant neuroblastoma in general and to determine whether PZA had activity against neuroblastomas manifesting MDR due to loss of p53 function (3).

It has been well documented that p53, a regulatory nuclear protein, is activated in response to a variety of DNA-damaging agents, resulting in cell cycle arrest (38) and/or apoptosis (39). PZA-mediated cytotoxicity has been shown to correlate with DNA damage and inhibition of DNA synthesis (40), yet we found that PZA was cytotoxic for neuroblastoma cell lines that lacked p53 function and were resistant to other drugs that cause DNA damage (ETOP, CBDCA, and L-PAM). In the panel of cell lines we tested, there were four cell lines with a loss of p53 function due to *TP53* mutations and two additional cell lines with loss of p53 function due to nonmutational events (in one instance, associated with MDM2 genomic amplification). All of these cell lines were highly resistant to various drugs (2, 3). We also tested four HPV-16 E6-transduced clones derived from two different drug-sensitive/p53-functional cell lines, which, due to abrogation of p53 function, showed high-level resistance to ETOP, CBDCA, and L-PAM (3). In this study we showed that,

in general, PZA was more active in cell lines lacking the MDR phenotype than in MDR cell lines (*P* < 0.05; Table 1). However, this trend was due to reasons other than a lack of p53 function because PZA was equally toxic in resistant cell lines with nonfunctional p53 (LC_{90} values ranged from 1.0 to 2.1 μM; LC_{99} values ranged from 2.5 to 4.7 μM) and those with functional p53 (LC_{90} values ranged from 0.9 to 2.4 μM; LC_{99} values ranged from 2.5 to 5 μM; *P* > 0.05; Table 1 and Fig. 3). The LC_{90} values of HPV-16 E6-transduced clones were 0.2–2.04 μM (*i.e.*, within the range of clinically achievable levels).

Phase I clinical trials of PZA have tested several dose schedules [Table 2 (10, 12, 13)]. With an administration schedule using a 1-h infusion every 21 days, the reported MTD was 600 mg/m² with a PPL of 7.1 μM (13), and neurotoxicity was dose-limiting (13). However, prolongation of infusion duration from 1 to 3 h resulted in a reduction in the incidence of neurotoxicity and permitted further dose escalation to 750 mg/m² (PPL = 2.3 μM), during which neutropenia emerged as the principal dose-limiting toxicity. The total dose of 750 mg/m² administered as a single dose or as 150 mg/m²/day for 5 consecutive days was the recommended dose for adults (13). In another Phase I clinical trial, a 1-h PZA infusion at the recommended 600 mg/m² dose resulted in an AUC of 30 ± 25 μM·h and peak drug levels of 2.2 ± 1.05 μM (10). In a Phase I clinical trial conducted in adults as a weekly 24-h infusion, 281 mg/m² was reported as the MTD, and the maximum plasma level was 1.6 μM with a maximum AUC of 56 μM·h. This schedule was well tolerated, and neurotoxicity was not problematic (11). This Phase I clinical trial of PZA given as a 24-h continuous infusion was designed to minimize neurological toxicity and to achieve steady-state plasma concentrations that were reported to be associated with *in vitro* cytotoxicity [IC_{50} levels ranging from 0.03 to 0.88 μM as determined by colony-forming assay in over 40 cell lines of various tumor types (5, 6)]. Further dose escalation was precluded due to myelosuppression and one case of
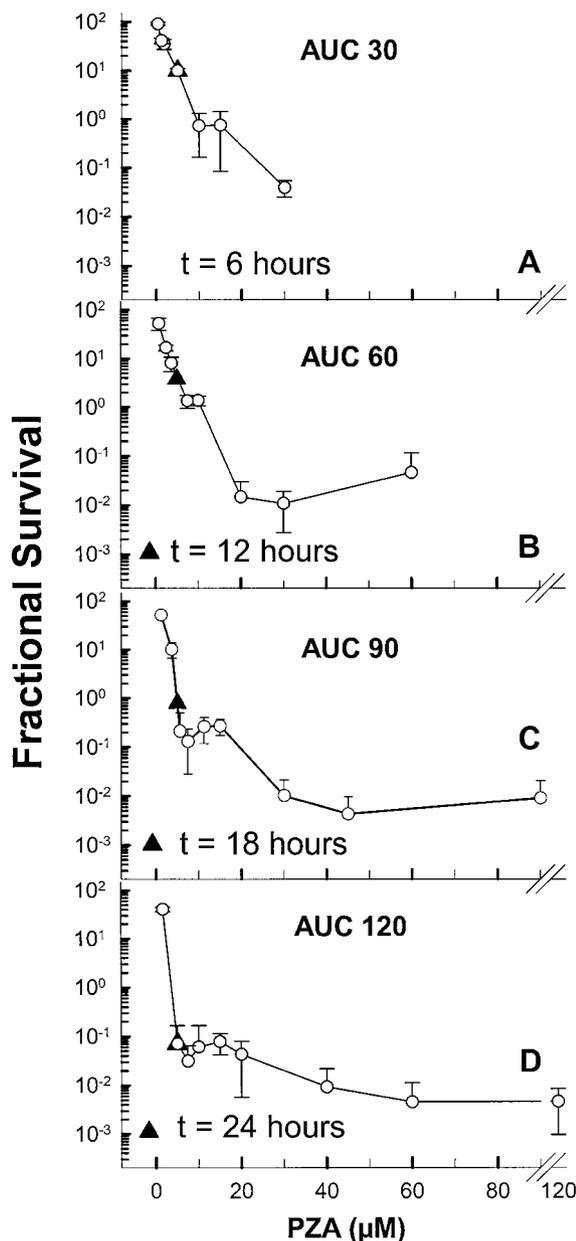
Fig. 6 Model for time and dose dependency of PZA cytotoxicity *in vitro*. Using the DIMSCAN assay, AUC 30 (AUC = PZA μM·h), 60, 90, and 120 were determined in the CHLA-90 cell line. Cells were exposed to various PZA concentrations (○) for 1, 2, 3, 6, 8, 12, 16, 24, or 72 h, such that total drug exposure at each point shown on the graphs would be (*A*) 30, (*B*) 60, (*C*) 90, and (*D*) 120 μM·h. Cytotoxicity was assayed after 71, 70, 69, 66, 64, 60, 56, 48, or 0 h of drug-free interval. The cytotoxicity of 5 μM PZA achieved in (*A*) 6, (*B*) 12, (*C*) 18, and (*D*) 24 h is shown on each curve (▲). Administration of 5 μM PZA for 6–24 h achieved 1–3 logs of tumor reduction, and a 24-h exposure to 5 μM PZA achieved cytotoxicity comparable with that of higher drug levels (>20 μM) given for shorter periods of time.

grade 3 mucositis; however, the authors suggested that dose escalation could have been feasible with colony-stimulating factor support.

In a study conducted in children and young adults, 640

mg/m² PZA administered as a 1- or 24-h infusion was reported as the recommended dose (12). The predictive AUC_{50} (*i.e.*, the AUC that produces 50% of the maximum effect) was reported as 45 μM·h for leukopenia and 34 μM·h for neutropenia. Phase I pharmacokinetic studies have shown that the PZA half-life $t_{1/2}\alpha$ was 1–85 min, and the $t_{1/2}\beta$ ranged from 2.7 to 158 h (10, 12). Actual PZA plasma concentrations, measured in two patients at the end of a 24-h infusion, were 7 and 2.3 μM. In this age group, myelosuppression was reported as the dose-limiting toxicity; in contrast to the adult population, neurotoxicity was not prominent (12).

Multiple Phase II clinical studies used a total dose of PZA ranging from 600 to 750 mg/m² PZA administered as a 3-h infusion every 21 days, which produced only limited clinical activity (Table 3). Our *in vitro* data demonstrate that PZA cytotoxicity is dose and time dependent. This observation was in agreement with a study of Grem *et al.* (40), which showed that with an increased PZA exposure time, the concentration required for a given cytotoxic effect in MCF-7 breast cancer cells was decreased. We determined dose-response curves for CHLA-15 (drug-sensitive) and CHLA-90 (MDR due to loss of p53 function) cell lines for 1, 3, 6, 24, and 72 h with PZA. We found that longer exposures resulted in higher cell kills at a given concentration in these cell lines. In the CHLA-90 cell line, 1-, 3-, and 6-h PZA exposures resulted in LC_{90} values 7, 4, and 1.5 times greater than that of a 72-h exposure, respectively. After a 24-h exposure, the LC_{90} value was identical to that obtained after a 72-h PZA exposure. In CHLA-15, the LC_{90} values after 1-, 3-, 6-, and 24-h exposures were 10, 8.8, 6.7, and 2.7 times higher, respectively, relative to a 72-h exposure. We have also determined the (concentration × time) relationship to cytotoxicity for PZA in the CHLA-90 *TP53*-mutated, MDR neuroblastoma cell line, such that PZA exposures of 30, 60, 90, or 120 μM·h were achieved. Over a 3-log cell kill was achieved with a 1–3-h exposure to 20 μM PZA, but a similar effect was seen with 12–24-h exposures to 5–10 μM PZA (Fig. 6).

The AUC_{90} for all 10 MDR neuroblastomas and for 3 of the 12 non-MDR cell lines (Table 1) exceeded that reported in clinical trials. These data suggest that neuroblastoma patients with drug-resistant tumors receiving PZA at a schedule used in Phase II studies would be unlikely to respond (according to *in vitro* data, cell kills <1 log), consistent with the lack of response seen in the seven neuroblastoma patients treated in the Phase II study (19). However, our *in vitro* data suggest that if a concentration of 5–10 μM PZA was sustained for 8–24 h (equal to an AUC of 80–120 μM·h), PZA could be active against recurrent neuroblastomas. Due to the significant hematopoietic toxicity of PZA, such prolonged exposure would require hematopoietic stem cell support, precluding a study of such a dose schedule against xenografts in immunocompromised mice. Most Phase I clinical trials reported a PZA C_{max} of 1.6–2.3 μM. Only two pediatric patients received PZA in a 24-h infusion, and the 7 μM PZA level measured in one patient at the end of 24-h infusion (12) suggests that plasma concentrations over a time >8h of 5–10 μM PZA may be achievable in pediatric patients, especially if given with hematopoietic stem cell support. Our data suggest that achieving >5 μM PZA for >8 h may be effective against recurrent neuroblastoma, but such a schedule has yet to be tested against neuroblastoma in a clinical trial.

Preclinical investigation of PZA in solid tumors in mice concluded that PZA was a schedule category III agent, *i.e.*, a schedule-independent agent (4). Due to the variety of tumor types used in that study, distinct drug sensitivity profiles of tumor cells, and drug administration schedules, it is difficult to interpret the results. In general, treatment schedules delivering higher total drug doses showed better activity, but such high levels tended to be lethal for treated mice. Therefore, the data in mice do not adequately address the possibility that a higher systemic exposure of PZA could be more effective and might be achievable using hematopoietic stem cell support.

(*a*) Our observation that PZA activity increased with a more prolonged drug exposure, (*b*) pharmacokinetic data showing a relatively short $t_{1/2}\alpha$ for PZA, (*c*) peak levels being limited by neurotoxicity, and (*d*) myelosuppression being the dose-limiting toxicity in pediatric patients, taken together, suggest that dose escalation to achieve a high PZA AUC using prolonged (6–24 h) infusions supported by hematopoietic stem cell infusion should be tested. Increasing the AUC of PZA may be tolerable with stem cell support and may prove effective in recurrent neuroblastoma patients (and potentially other tumor types), despite discouraging results seen in Phase II clinical trials using shorter infusions of PZA. A Phase I trial testing this hypothesis (N2002-01) is ongoing in the New Approaches to Neuroblastoma Therapy consortium.[4]

ACKNOWLEDGMENTS

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Pyrazoloacridine Is Active in Multidrug-resistant Neuroblastoma Cell Lines with Nonfunctional p53

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