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Schedule dependence, activity against natural metastases, and cross-resistance of pyrazine diazohydroxide (sodium salt, NSC 361456) in preclinical models in vivo

Steadman D. Harrison, Jr., Jacqueline Plowman,* Donald J. Dykes, William R. Waud, and Daniel P. Griswold, Jr.

Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, AL 35255-5305 and *Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD 20892, USA

Summary. Pyrazine diazohydroxide (sodium salt, NSC 361456; PZDH) is a new antitumor drug with relatively broad activity in initial evaluations against murine leukemias, solid tumors, and two human tumor xenografts *in vivo*. The present studies were designed to address questions about PZDH activity on different treatment schedules, its activity against metastases, and the extent of its cross-resistance with established drugs. Human LOX amelanotic melanoma xenografts in athymic mice were used to explore schedule dependence and activity against natural metastases, and a series of drug-resistant murine leukemias provided an *in vivo* cross-resistance profile. Single-dose treatment and prolonged treatment provided equivalent therapeutic responses to PZDH by both the i.p. and i.v. routes in the i.p. LOX model. A s.c. LOX model resulting in spontaneous pulmonary metastases was adapted for bioassay and quantitation of the numbers of LOX cells killed by PZDH among both primary and metastatic cell populations. It was demonstrated that PZDH afforded about 2-$\log_{10}$ orders of magnitude greater cell kill among pulmonary metastases than against primary s.c. LOX tumors in the same mouse. Murine leukemias resistant to doxorubicin (ADR), vincristine (VCR), cisplatin (DDPt), methotrexate (MTX), *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU), and cyclophosphamide (CPA) were not cross-resistant to PZDH. However, both P388 and L1210 leukemia sublines resistant to melphalan (L-PAM) were cross-resistant to PZDH, suggesting that patients previously treated with L-PAM might have less likelihood of response to PZDH than those who had had no opportunity to develop L-PAM resistance. Although these observations should not be applied to clinical studies without due caution, they support clinical evaluation of PZDH as well as continued investigation of its molecular pharmacology.

Introduction

PZDH is an analog of pyridine-2-diazohydroxide that comprises a symmetric dinitrogen heterocyclic ring monosubstituted with a diazohydroxide moiety by means of nitrous acid deamination [1]. It was developed [1, 13] to provide improved chemical stability in a clinical formulation. PZDH has exhibited promising antitumor activity after i.p. administration in *in vivo* screening models including i.p. P388, i.p. L1210, and s.c. L1210 leukemias; i.p. B16 melanoma; i.p. M5076 sarcoma; i.p. human LOX melanoma xenografts; and MX-1 human mammary carcinoma in the subrenal capsule assay in athymic mice [1, 13]. The availability of [2-^{14}C]-PZDH has facilitated studies of the drug's distribution, metabolism, and pharmacokinetics in mice and dogs [12]. These studies have indicated that [2-^{14}C]-PZDH is rapidly cleared from plasma in both species and is virtually totally converted to metabolites. The possibility was explored that monooxygenase-dependent metabolism to a reactive intermediate might be required for PZDH activity. Cultured tumor cells were coincubated with microsomal fractions of rat-liver homogenate and, although the presence of microsomes enhanced PZDH cytotoxicity, the effect was not monooxygenase-dependent and was not related to metabolite production [2]. PZDH seemed to exhibit enhanced cytotoxicity under acidic and hypoxic conditions *in vitro*, suggesting the possibility of selectivity for solid tumors *in vivo* [2].

The purpose of the present study was to explore the possible dependence of therapeutic activity on treatment schedule. We also determined the activity of PZDH in a metastatic model of LOX melanoma and describe the cross-resistance profile of PZDH determined from its activity against specific drug-resistant murine leukemias. A preliminary account of this research has previously been presented [9].

Material and methods

Animals, tumors, and drugs. Mice were young adult females of an appropriate strain, either BALB/c × DBA/2 F_1 (otherwise designated $CD2F_1$) for the murine leukemias or athymic NCr-nu for the LOX melanoma. These mice were obtained from various suppliers under contract with the Biological Testing Branch, Developmental Therapeutics Program (DTP), Division of Cancer Treatment (DCT), National Cancer Institute (NCI). Each strain was housed in a separate barrier facility. $CD2F_1$ mice were caged in stainless-steel cages with hardwood bedding (Beta Chip, Northeastern Products Corp.); their diet consisted of Wayne Rodent Blox (Continental Grain Co.) and tap water *ad libitum*. Using aseptic techniques, athymic mice were transferred to sterile, polycarbonate, filter-capped

Offprint requests to: S. D. Harrison, Jr., Southern Research Institute, P. O. Box 55305, Birmingham, AL 35255-5305, USA

Microisolator cages (Lab Products, Inc.) containing sterile hardwood bedding. Athymic mice were fed Wayne Sterilizable Rodent Blox and filter-sterilized water *ad libitum*. All manipulations of these mice were conducted in laminar-flow biosafety hoods. Temperature in the barrier facilities was maintained at 74° ± 2° F; relative humidity was 50% ± 10%. Lighting was operated automatically on 12-h light/dark cycles.

The human amelanotic melanoma designated LOX was established and characterized by Fodstad et al. [7]; its usefulness as a preclinical model for antitumor drug evaluation has been reported by Shoemaker et al. [16]. For the present studies, the LOX tumor line was obtained from the Biological Testing Branch (NCI). LOX grows i.p. as a combination of solid tumor and monodispersed cells in ascitic fluid. Isolated, counted cells (10^6) were inoculated i.p. into recipient mice for tumor propagation. Murine leukemias P388/0 and L1210/0 were obtained from the same source and propagated according to standard NCI protocols [3]. Drug-resistant lines of P388 and L1210 (e.g., P388/ADR, the ADR-resistant subline of P388/0) were developed at Southern Research Institute [15].

PZDH was supplied by the Pharmaceutical Resources Branch (DTP, DCT, NCI). For injection into mice, PZDH was prepared fresh daily as a solution in distilled water or aqueous phosphate buffer. All doses were given to groups of mice on the basis of their average body weight; treatment routes and schedules are indicated in the tables. Mice were observed daily for survival. Other observations (e.g., tumor measurements) were recorded as indicated in the text.

Each experiment reported included multiple doses of PZDH ranging from nontoxic to frankly toxic. For the purpose of this report, results reflect optimal, nontoxic doses ($\leq LD_{10}$) only. Typically, each dose group consisted of ten mice. All experiments were internally controlled with appropriate groups of tumor-bearing mice. Tumor-bearing control groups were generally not treated with diluent. All experiments were repeated at least once for confirmation unless otherwise noted.

Schedule dependence. Different treatment schedules (and routes; see text) were compared simultaneously in single, internally controlled experiments in accordance with the principles and methods described by Schabel et al. [14]. To avoid the pitfall of comparing different schedules on the basis of increases in life span (ILS), comparisons were based on estimates of net tumor-cell kill for each schedule. To assess tumor-cell kill at the end of treatment, the difference in duration of survival between treated and control groups was adjusted to account for the regrowth of tumor-cell populations that may occur between individual treatments. This adjustment consisted of subtracting the treatment duration from the life span increase, i.e. $(T\text{-}C)\text{-}(t_n\text{-}t_1)$, where t_n = the time (in days) of the nth treatment [11].

LOX metastasis model. This model has previously been described [5]. It enables the simultaneous assessment of drug activity in primary and metastatic tumor. Hemacytometer-counted LOX cells (10^6) from *in vivo* passage (ascites) were implanted s.c. in athymic nude mice. These tumors were shown in previous studies to metastasize to the lungs by day 12 postimplantation. Accordingly, mice were treated with i.p. PZDH on days 12, 16, and 20 (single doses of 44.5 – 150 mg/kg). At 24 h after the last treatment, the mice were euthanized with ether and their lungs were removed, minced, and transplanted s.c. to recipient athymic mice. Primary tumors were transplanted similarly. These bioassay groups were observed for tumor growth. When growth became evident, tumors were measured (two perpendicular diameters by caliper) twice weekly until they reached 1 g. From these data, T-C values were calculated. This value is indicative of the number of viable cells implanted (i.e., at the time of transplant for bioassay) and is used, along with the volume-doubling time, to calculate cell kill by the method of Lloyd [11]. The volume-doubling time was determined by s.c. implantation of $\log_{10}$ dilutions (10^7–10^3) of LOX amelanotic melanoma cells.

Cross-resistance in vivo. An *in vivo* cross-resistance profile was developed by comparing the activity of PZDH against a parent ("wild-type") sensitive leukemia and against a subline developed for resistance to a specific drug [15]. The rationale and experimental design for this approach have been described in detail [8]. Each experiment included a range of PZDH doses, and all comparisons were based on therapeutically optimal, nontoxic doses only. Dying mice were necropsied, and if death was judged to be non-leukemic based on the absence of ascites or splenomegaly, it was considered likely to have been drug-induced.

In each experiment, additional tumor-bearing groups were treated with a range of doses of the appropriate drug to confirm the resistance of the drug-resistant subline used. Moreover, in each experiment a resistant leukemia was directly compared with the drug-sensitive parent leukemia from which resistant sublines were derived, and the parallel groups of mice were treated identically with a single drug preparation. With two exceptions, experiments were repeated at least once for confirmation. Each of these internally controlled experiments included six groups of mice bearing initial tumor burdens ranging from 10^7 to 10^2 cells (by serial dilution). These "titration" groups provided the basis for an assessment of therapeutic response as described by Schabel et al. [14]. Therapeutic response was based on the median day of death of dying mice only. Long-term (45- to 60-day) survivors were excluded from calculations of the percentage of ILS and from estimates of changes in tumor burden as a result of treatment [14]. If an optimal dose of PZDH effected a net cell kill that was $\geq 2\text{-}\log_{10}$ units lower in a drug-resistant subline than the parallel cell kill in the sensitive leukemia, PZDH was judged to be cross-resistant with that drug.

Results

Schedule dependence

Presented in Table 1 are data from two experiments with LOX melanoma, one each exploring the effect of i.p. and i.v. routes of PZDH administration. PZDH seemed to be more potent by the i.v. route, but its therapeutic effect was not route-dependent. The treatment schedule used was not remarkably important for therapeutic outcome in either experiment, although more total PZDH was tolerated on the repeated-dose schedules. The effect of two i.p. injections separated by 8 days suggests that with careful dose adjustment to avoid toxicity, this repeated-course regimen might provide a therapeutic advantage.

Table 1. Effects of treatment schedule on the antitumor activity of PZDH against i. p. implanted LOX melanoma xenografts

Treatment schedule	Route	Dose range (mg/kg)	Optimal dose (mg/kg)	Total dose (mg/kg)	ILS[a] (%)	Day 60 survivors/ total	Approximate $\log_{10}$ change in tumor burden at end of treatment[a]	Day 60 survivors/ total (non-tumor-bearing controls)
Single dose, day 5	i. p.	288–57	128	128	70	0/10	−1.5	6/6
Q3h × 8, day 5		57– 7.5	25	200	59	0/10	−1.1	6/6
Q1d, days 5–13		57–11	57	513	120	0/10	−1.7	0/6
			38[b]	342	80	2/10	−0.8 (−6.3)	6/6
Q4d, days 5, 9, 13		192–38	85	255	77	1/10	−0.8 (−6.2)	6/6
Q8d, days 5, 13		288–38	192	384	−35	5/7	— (−6.2)	4/6
			128[b]	256	80	0/10	−0.8	6/6
Single dose, day 5	i. v.	288–57	192	192	35	0/10	−1.4	2/8
			85[b]	85	20	0/10	−0.8	7/8
Q1d, days 5–9		128–17	57	285	91	0/10	−2.7	1/8
			38[b]	190	53	0/10	−1.3	7/8
Q4d, days 5, 9, 13		192–38	85	255	59	0/10	−0.7	4/8
			38[b]	114	23	0/10	+0.7	8/8

[a] Calculations based on dying mice only; values in parentheses represent changes in tumor burden for day-60 survivors
[b] Highest nontoxic dose, which resulted in no more than one death in groups of six or eight non-tumor-bearing mice
NCr-nu athymic mice were inoculated i. p. with 10^6 LOX ascites cells on day 0. Treatment with PZDH was initiated on day 5; solutions were injected within 15 min of preparation. The dose range included the highest and lowest doses indicated in the table as well as intermediate doses determined by multiplying each dose in turn by 0.67, starting with the highest dose. A parallel group of non-tumor-bearing mice received drug injections identical to these. The median survival of the treated mice (day-60 survivors excluded) was compared with that of the untreated controls and expressed as the percentage of increase in life span (ILS). In addition, the study contained internal tumor titrations to establish tumor-doubling time and to enable the estimation of changes in tumor burden with treatment

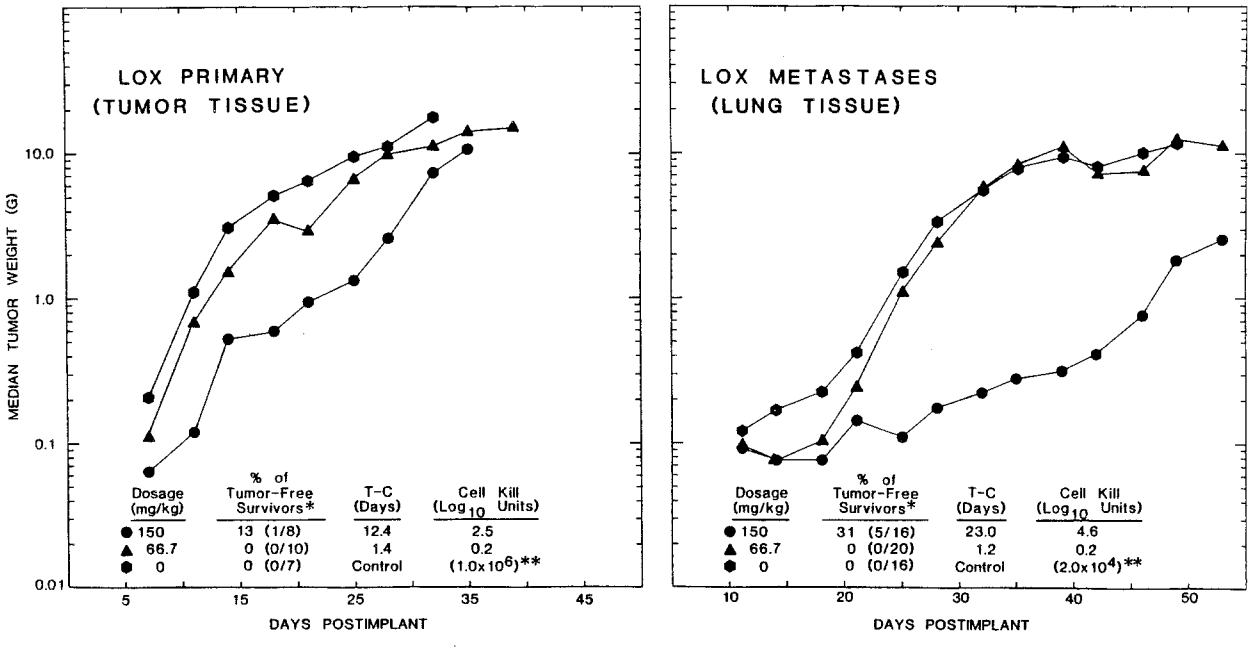

Fig. 1. Activity of PZDH against primary and metastatic LOX tumor burdens assessed by *in vivo* bioassay. NCr-nu female athymic mice were implanted s. c. with 10^6 LOX cells on day 0. PZDH was injected i. p. on days 12, 16, and 20. On day 21, the control and treated animals were killed, and their primary tumors and lungs were removed and bioassayed by s. c. implantation into recipient animals. Antitumor activity was reflected by the number of bioassay survivors that exhibited no tumor growth, by tumor growth delay, and by $\log_{10}$ cell kill. Growth delay (*T-C*) was determined from the time for bioassay growth to reach a median size of 1 g. * Tumor-free survivors provide an additional indication of therapeutic activity. ** Established number of LOX cells in the primary tumor or lungs on day 21, based on a historic doubling time of 1.5 days

Table 2. Cross-resistance profile of PZDH derived from drug-resistant leukemias in vivo

Resistant leukemia	Dose, i.p., Q1d×9 (mg/kg):		Sensitive leukemia:		Resistant leukemia:		Cross-resistance
	Range	Optimal[a]	ILS (%)	Approximate $\log_{10}$ change in tumor burden at end of treatment[b]	ILS (%)	Approximate $\log_{10}$ change in tumor burden at end of treatment[b]	
P388/ADR	11–56	38	145	−4	177	−7	No
	11–56	38	171	−6	213	−7	
P388/VCR	17–56	38	179	−7	225	−7	No
	17–56	38	191[c]	−7	248[c]	−7	
P388/DDPt	17–56	38	201[c]	−7	119	−7	No
	17–56	38	168	−6	132	−7	
P388/MTX[d]	17–56	38	248[c]	−7	210[c]	−7	No
P388/BCNU	11–38	38	100	−2	108	−4	[e]
	11–38	25[f]	72	0	87	−2	
P388/CPA[d]	11–56	38	124	−4	195	−7	[e]
P388/L-PAM	17–56	38	184[c]	−7	63	−1	Yes
	17–56	38	164[c]	−7	45	+1	
L1210/L-PAM	11–56	38	121	−2	67	+2	Yes
	11–56	38	123	−2	–	–	
		25[f]			34	+3	

[a] $\leq LD_{10}$

[b] $\log_{10}$ change in viable tumor stem cell population at the end of treatment compared with that at the start of treatment, based on the median day of death among the animals that died, survivors excluded. This calculation accounts for different volume-doubling times among tumor lines, a variable that influences ILS values and their interpretation

[c] 1/10 long-term survivors

[d] Single experiment

[e] Possible collateral sensitivity

[f] 38 mg/kg was $> LD_{10}$ in one or both arms of this experiment

In each experiment, a resistant leukemia was compared directly to the drug-sensitive parent leukemia from which the resistant lines were derived, and the parallel groups of mice were treated identically with a single drug preparation. Duplicate experiments were conducted, each including a range of at least four PZDH doses. Dose ranges, optimal doses, and effects of the latter are shown. Experiments included internal tumor titrations to establish tumor-doubling time and calculate cell kill as described in Materials and methods

Activity against metastases

One site of natural metastasis of s.c. LOX in athymic nude mice is the lung. The process of metastasis required about 2 weeks, depending on the size of the initial s.c. implant. We have found [5] that late treatment of mice bearing primary LOX tumors affords an opportunity to evaluate simultaneously the activity of a drug against primary and metastatic disease. Shown in Fig. 1 are results of an evaluation of PZDH in this model. It bears reemphasizing that the two sets of growth curves presented in Fig. 1 reflect the growth after treatment of transplanted primary tumor and lung tissue in recipient (bioassay) mice. This growth, in turn, is a reflection of the viable tumor burden that survived treatment in the original donor mice. Therefore, the measured growth delays (T-C, Fig. 1) enable the calculation [11] of the number of LOX cells killed by treatment ($\log_{10}$ units). PZDH exhibited noteworthy activity, particularly against the burden of metastatic LOX cells in the lungs of the original mice. These results were readily confirmed [9; data not shown].

Cross-resistance profile

Multiple experiments (Table 2) were conducted, each of which comprised a simultaneous comparison of PZDH activity in a drug-sensitive and a drug-resistant murine leukemia over a range of doses. If PZDH effected a cell kill $\geq$2-$\log_{10}$ units lower in the drug-resistant line than in the drug-sensitive line, the drug-resistant line was interpreted to be cross-resistant to PZDH. This was the result obtained and confirmed with P388/L-PAM and L1210/L-PAM. Tumor lines reflecting resistance to an anthracycline, a *Vinca* alkaloid, a DNA cross-linking agent, and an antimetabolite were sensitive (i.e., were not cross-resistant) to PZDH. Tumor lines reflecting resistance to two alkylating agents other than L-PAM, namely, BCNU and CPA, were collaterally sensitive (i.e., exhibited at least a 2-$\log_{10}$ unit greater cell kill than the parent sensitive line) to PZDH.

Discussion

The observations reported provide information potentially important to the design of strategies for the optimal clinical use of PZDH. These results suggest that clinical treatment schedules may be chosen with a view toward minimizing PZDH toxicity but without initial concern that the choice of schedule may significantly affect therapeutic responses. PZDH may be evaluated in advanced-stage malignancies with the reasonable expectation that control of metastases will equal or exceed objective responses in primary disease. PZDH may be evaluated in patients who have received prior chemotherapy with ADR, VCR, DDPt, MTX, and even selected alkylating agents with no more than the usual *a priori* concern that cross-resistance will be encountered. However, failure to observe objective

responses in patients previously treated with L-PAM should be viewed as a possible manifestation of cross-resistance to PZDH. As always, none of these approaches may be applied clinically without caution and concern for the recognized gap between preclinical prediction and clinical validation.

The dependency of therapeutic response on treatment schedule has most recently been interpreted in light of the implications of dose intensity [4, 10, 17, 18]. In the present study, dose intensities [i. e., average dose intensity, calculated by dividing the total dose (in mg/kg) by the total treatment duration (in days)] for the first four schedules listed in Table 1 were 128, 200, 38, and 28 mg/kg per day, respectively. Although these dose intensities almost spanned a 10-fold range, there was no dose-intensity-related effect on therapeutic responses, strengthening the conclusion that PZDH activity was independent of treatment schedule.

The implications of controlling cancer metastasis for success in cancer therapy are well recognized [6]. In general, preclinical metastasis models are based on the use of either experimental or natural metastases. The LOX melanoma model used in the present study is a natural metastasis model in that it depends on spontaneous translocation of cells from the primary tumor to the lungs of nude mice rather than on i.v. injection of cells and subsequent colonization of the lungs. It enabled us to analyze concurrently PZDH effects against primary and metastatic lesions *in vivo*, and these effects are expressed quantitatively [14] in terms of the numbers of residual, viable LOX stem cells remaining in primary and metastatic lesions after PZDH treatment.

The agreement between P388/L-PAM and L1210/L-PAM with regard to their cross-resistance to PZDH is noteworthy. Previous results [8] suggest that the resistance of these lines may be related to elevated total intracellular glutathione. Of interest is the fact that both leukemias exhibit about the same ratio of glutathione in resistant vs sensitive cells (2.4 and 2.9 for P388 and L1210 lines, respectively). Thus, if glutathione played a role in cross-resistance, one would expect the response of two resistant lines to PZDH to agree, as they did. An important question to be addressed by future studies is: what is the correlation between cross-resistance profiles, such as the current one reported for PZDH, and clinical experience with resistance and cross-resistance?

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