Antitumor Activity and Toxicity of Novel Nitroheterocyclic Phosphoramidates

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Received August 17, 2000

A series of novel nitroheterocyclic phosphoramidates has been evaluated for antitumor activity in murine and xenograft tumor models and for toxicity in mice. Significant increases in lifespan and long-term survivors were noted in L1210 leukemia and B16 melanoma models, and both complete and partial tumor regressions were observed in the MX-1 breast cancer xenograft model. All compounds exhibited some degree of toxicity to granulocyte/macrophage progenitors in the bone marrow of mice. Two drugs were selected for further toxicologic, histopathologic, and pharmacokinetic evaluations. Toxicity of potential clinical significance was observed only in the bone marrow at the highest drug dose; otherwise no significant abnormalities in blood chemistries or organ histopathology were noted. The bone marrow lesions consisted of reduced numbers of progenitor cells in the myeloid and erythroid series; platelets were not affected. The compounds were eliminated rapidly by first-order kinetics, with half-lives in the 4−12 min range. The best of these compounds exhibits excellent antitumor activity and minimal toxicity at therapeutically effective doses in mice.

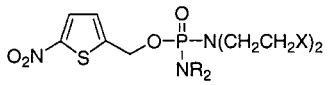
Introduction

The presence of a reducing environment in solid tumors is well-established. This reducing environment represents an attractive target for drug development, and there is considerable interest in the design of antitumor prodrugs that undergo activation under bioreductive conditions.[1] Although many drug types have been investigated, prodrugs that generate an alkylating agent upon activation are the most widely exploited.[2] Compounds containing a nitroheterocyclic group are among the most versatile because their reduction potentials are favorable and because reduction converts a strongly electron-withdrawing group to an electron-donating group.[3] Reduction of some nitroheterocyclic compounds leads directly to the formation of cytotoxic intermediates, whereas in other cases reduction serves to activate latent alkylating groups. We have developed a number of nitroaromatic compounds that are designed to deliver a cytotoxic phosphoramide mustard following reductive activation.[4] More recently we described a series of nitroheterocyclic phosphoramidates that are highly potent in vitro under both aerobic and hypoxic conditions and also show modest hypoxic selectivity.[5] The antitumor activity, toxicity, and pharmacokinetics of selected analogues are the subjects of this report.

Results and Discussion

Antitumor Activity. Nitrothienyl and nitrofuryl phosphoramidates **1a**−**c** and **2a**−**c** (Chart 1) were synthesized as described previously.[5] Preliminary experiments showed that at the maximum tolerated dose (MTD) these compounds had greater efficacy using a divided-dose schedule compared to a single-dose schedule, so a daily ×5 schedule was used in all further antitumor experiments. The antitumor activity of these compounds was evaluated against ip implanted B16 melanoma; the results are summarized in Table 1. Two parameters were used to assess efficacy: increased life span (% T/C) and 60-day survivors. All compounds showed a significant increase in life span compared to untreated controls in this model, and the efficacies were generally comparable to that of cyclophosphamide. In contrast to cyclophosphamide, however, long-term survivors were observed in several of the treatment groups. Compound **2a** appeared to be the most potent in this model, giving a 2.3-fold increase in life span and 4/10 long-term survivors at a dose of 16 mg/kg given daily ×5.

Chart 1



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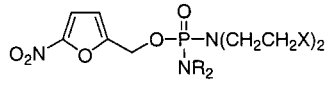

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a, R = H, X = Br
b, R = CH_2CH_2Br, X = Br
c, R = CH_2CH_2Cl, X = Cl

The nitrothienyl and nitrofuryl phosphoramidates did not exhibit cross-resistance to cyclophosphamide-resistant MCF-7 cells in our previous in vitro studies.[5] To assess whether cross-resistance would occur in vivo, the antitumor activity of **1a** and **2a** was evaluated against cyclophosphamide-resistant L1210 leukemia using a daily ×5 dosing schedule (Table 1). Both

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10.1021/jm000359y CCC: $20.00
Published on Web 12/01/2000

Table 1. Antitumor Activity of Nitroheterocyclic Phosphoramidates Against Murine Tumors[a]

compd	X	R	dose,[b] mg/kg	wt chg[c]	T/C,[d] %	% LTS[e]
			B16 Melanoma			
1a	Br	H	12.5	+2	186	30
			25	−17	180	20
1b	Br	CH_2CH_2Br	50	−10	185	0
1c	Cl	CH_2CH_2Cl	50	−8	166	0
			75	−10	248	20
2a	Br	H	8	+4	199	0
			16	−19	228	40
2b	Br	CH_2CH_2Br	50	−11	202	0
2c	Cl	CH_2CH_2Cl	100	−6	172	10
cyclophosphamide			125	−10	207	0
			L1210/WT[f]			
1a	Br	H	12.5	+2	139	10
			25	−13	155	70
2a	Br	H	8	+5	131	0
			16	−8	150	60
cyclophosphamide[g]			300	−6	>300	100
			L1210/CP[f]			
1a	Br	H	12.5	+5	131	0
			25	−14	160	10
2a	Br	H	8	+4	120	10
			16	−11	150	20
cyclophosphamide[g]			300	−6	146	10

[a] See Experimental Section for details; 10 mice/group. [b] Drug given daily ×5 by the ip route unless specified otherwise. [c] Percent weight change on day 8. [d] T/C = 100% × (mean survival time$_{treated}$/mean survival time$_{control}$). [e] 60-Day survivors for B16; 30-day survivors for L1210. [f] WT= wild-type; CP = cyclophosphamide-resistant. [g] Single dose.

compounds showed significant antitumor activity against wild-type cells at the higher dose, although neither compound was as potent as single-dose cyclophosphamide in this model. The increases in life span in response to **1a** and **2a** against the cyclophosphamide-resistant cell line were comparable to those seen in the wild-type cell line, but a significant decrease in long-term survivors was observed in the resistant cell line. Although the primary mechanism of resistance in this cell line is believed to be the overexpression of aldehyde dehydrogenase,[6] this would not account for the cross-resistance observed for phosphoramidates **1a** and **2a**, because activation of these compounds does not progress through an aldehyde intermediate.[6]

The compounds were then evaluated against sc implanted human MX-1 breast cancer cells in a xenograft model using a daily ×5 ip dosing schedule; the results are presented in Table 2. The MX-1 tumor is highly sensitive to these drugs; all of the compounds produced dramatic growth inhibition, and tumor regression was observed in all mice following treatment with **1a**, **2a**, and **2b**. Compounds **1a** and **2a** were also administered by gavage (po in Table 2). Although no attempt was made to optimize the dose by this route, significant growth inhibition and some tumor regressions were observed.

Toxicity. Cyclophosphamide and its activated analogues are known to be less toxic to bone marrow progenitors than other alkylating agents, presumably because these cells overexpress aldehyde dehydrogenase.[6,7] Although compounds **1** and **2** ultimately generate cytotoxic phosphoramide mustards related to that of cyclophosphamide, activation of these compounds does not proceed through an intermediate that would be inactivated by aldehyde dehydrogenase. Thus it was of interest to assess the toxicity of these compounds to

Table 2. Antitumor Activity of Nitroheterocyclic Phosphoramidates in the MX-1 Xenograft Model[a]

compd	Y	R	dose,[b] mg/kg	wt chg[c]	% GI[d]	no. total[e]	no. partial[f]
1a	Br	H	25	−8	>99	6	4
			12.5	+3	98	0	4
			40 po[g]	+7	87	0	2
1c	Cl	CH_2CH_2Cl	50	+1	>99	0	10
2a	Br	H	16	−4	>99	6	4
			8	0	93	0	4
			40 po[g]	+3	98	1	5
2b	Br	CH_2CH_2Br	50	−11	>99	4	6
2c	Cl	CH_2CH_2Cl	50	+2	99	0	7
cyclophosphamide			125	−9	>99	4	6

[a] See Experimental Section for details; 10 mice/group. [b] Drug given daily ×5 by the ip route unless specified otherwise. [c] Percent weight change on day 8. [d] Growth inhibition = 100% × (1 − tumor weight$_{treated}$/tumor weight$_{control}$). [e] Number of mice with complete tumor regression. [f] Number of mice with partial tumor regression. [g] Drug given by gavage.

Table 3. Toxicity of Nitroheterocyclic Phosphoramidates to Bone Marrow Granulocyte/Macrophage Progenitors[a]

compd	X	R	dose/schedule[b]	G/M (% of control)[c]
1a	Br	H	30 × 1	87 ± 6
			100 × 1	45 ± 4
			25 × 5	14 ± 6
1b	Br	CH_2CH_2Br	50 × 1	93 ± 5
			100 × 1	64 ± 7
1c	Cl	CH_2CH_2Cl	150 × 1	106 ± 7
			200 × 1	81 ± 8
2a	Br	H	25 × 1	79 ± 9
			50 × 1	52 ± 6
			16 × 5	28 ± 4
2b	Br	CH_2CH_2Br	50 × 1	102 ± 4
			100 × 1	32 ± 7
			150 × 1	23 ± 9
2c	Cl	CH_2CH_2Cl	100 × 1	100 ± 9
			150 × 1	104 ± 13
			200 × 1	26 ± 6

[a] See Experimental Section for details; 5 mice/group. [b] Dose (mg/kg) administered ip daily × number of doses. [c] Granulocyte/macrophage progenitors per femur, 100% × treated/control; mean ± SEM.

bone marrow granulocyte/macrophage progenitors. Mice were treated with drug or vehicle ip using a single dose or daily ×5 schedule. Twenty-four hours after the last dose, bone marrow cells were harvested from the femur and the number of granulocyte/macrophage colony-forming cells (GM-CFC) was determined. The results are presented in Table 3. All compounds showed some degree of dose-dependent toxicity when administered as a single dose. The myelotoxicity of compounds **1a** and **2a** was also evaluated using the optimum therapeutic dose and schedule (daily ×5) for each drug. Multiple dosing resulted in increased toxicity for both compounds, and the degree of myelosuppression was more profound for **1a** than for **2a**.

To assess whether myelosuppression was likely to be the dose-limiting toxicity, toxicologic profiles were generated for compounds **1a** and **2a** in female B6D2F_1 mice using an iv daily ×5 schedule and doses of 0, 0.1, 0.5, and 1.0 × MTD. On day 8 the mice were euthanized and hematology, blood chemistries, and necropsies were completed. The results are summarized in Table 4. Toxicity to the granulocyte/macrophage progenitors is reflected in the decreased white blood cell counts at the highest dose for both compounds. In contrast to many other alkylating agents, however, compounds **1a** and **2a** appear to be platelet-sparing. The other hematologic and

Table 4. Toxicity of Phosphoramidates **1a** and **2a** in Mice[a]

compd	dose, mg/kg[b]	WBC × 10^{-3}, μL^{-1}	platelets × 10^{-3}, μL^{-1}	RBC × 10^{-6}, μL^{-1}	ALT,[c] U/L	alk phos,[d] U/L	BUN, mg/dL	spleen wt, mg	thymus wt, mg
1a	0	6.7 ± 2.5	890 ± 371	10.1 ± 1.1	22 ± 5	143 ± 22	23.4 ± 2.5	64 ± 25	41 ± 8
	2.5	5.6 ± 2.8	737 ± 217	7.5 ± 2.2	34 ± 10	144 ± 5	18.4 ± 4.3	91 ± 10	62 ± 17
	12.5	3.9 ± 1.1	1208 ± 112	8.6 ± 0.8	27 ± 6	133 ± 19	19.2 ± 2.9	67 ± 19	32 ± 17
	25	2.1 ± 0.5	523 ± 177	9.7 ± 0.3	43 ± 23	63 ± 3	17.0 ± 1.0	23 ± 10	7 ± 4
2a	0	5.6 ± 1.3	846 ± 265	8.5 ± 0.5	40 ± 15	144 ± 26	30.6 ± 3.5	68 ± 4	41 ± 8
	1.6	4.5 ± 1.4	970 ± 378	8.7 ± 1.2	43 ± 20	134 ± 15	20 ± 1.7	88 ± 11	57 ± 7
	8	2.5 ± 1.4	1191 ± 198	7.9 ± 0.9	37 ± 12	123 ± 8	23.6 ± 3.0	48 ± 16	33 ± 13
	16	1.9 ± 1.0	689 ± 135	7.7 ± 0.8	45 ± 21	89 ± 14	23.0 ± 1.2	45 ± 14	17 ± 11
normal range		2.6−10.7	600−2900	6.4−9.4	24−140	45−222	9−28		

[a] See Experimental Section for details; 5 mice/group. [b] Drug given daily ×5 by the ip route. [c] Alanine aminotransferase. [d] Alkaline phosphatase.

blood chemistry values are within normal limits at all drug doses tested. Organ weights for spleen and thymus are significantly reduced at the highest dose of each drug; presumably this results from toxicity to hematologic progenitors. Other organ weights (heart, liver, kidney, brain) were within normal limits. Finally, histopathology was carried out on all organs. The only significant lesions were found in the bone marrow of mice receiving **1a** at the highest dose. The lesions were characterized by an overall diminution in the number of cells of the immature myeloid and erythroid series, consistent with toxicity to bone marrow progenitors discussed above. The megakaryocytes were relatively unaffected, which is consistent with the platelet sparing properties of these drugs. The bone marrow lesions were not observed in the mice receiving the highest dose of **2a**. The remaining tissues examined were devoid of significant qualitative morphologic lesions in all groups. In summary, **1a** and **2a** are toxic to myeloid progenitors at optimum therapeutic doses but exhibit no other toxicity likely to be clinically significant.

Pharmacokinetics. The pharmacokinetics of compounds **1a** and **2a** were investigated in B6D2F_1 mice. Drugs were administered as a single ip dose of 50 mg/kg, and drug levels in plasma were monitored for 60 min after drug administration using HPLC analysis. Representative pharmacokinetics profiles for **1a** and **2a** are shown in Figure 1. Both drugs were eliminated by first-order kinetics, but the elimination rates were significantly different for the two compounds: $t_{1/2} = 4.3 \pm 0.8$ min for compound **1a** compared to $t_{1/2} = 11.6 \pm 2.5$ min for compound **2a**. This difference in half-life may account for the difference in effective dose and maximum tolerated dose for the two compounds. Each compound was converted to a more polar metabolite, and the peak intensity for the metabolite was ca. 3−4-fold greater than the peak intensity for the parent drug. The metabolites are presumed to be the hydroxylamine reduction products; LC/ESMS analysis of the **1a** metabolite showed $(M + H)^+$ at *m/z* 438 with an isotope composition characteristic of a compound containing two bromine atoms. The metabolites from **1a** and **2a** disappeared with half-lives of 14.2 ± 1.3 and 14.8 ± 3.0 min, respectively.

Conclusions

A series of reductively activated cytotoxic phosphoramidates has demonstrated antitumor activity in murine and xenograft tumor models. The bis(bromoethyl) compounds appear to be the most potent; although the tetrakis(haloethyl) compounds show activity, they are less potent and are less effective at the maximum tolerated dose. Compounds **1a** and **2a** are remarkably free of toxicity compared to other alkylating agents, exhibiting only moderate toxicity to bone marrow progenitors at the maximum tolerated dose. Pharmacokinetics studies show that both **1a** and **2a** are rapidly metabolized, but the nitrofuryl compound has a somewhat longer plasma half-life. We conclude that the nitrofuryl phosphoramidate **2a** is the most promising of these compounds based on its efficacy in the murine and xenograft models, its moderate degree of myelosuppression, and its pharmacokinetic profile.

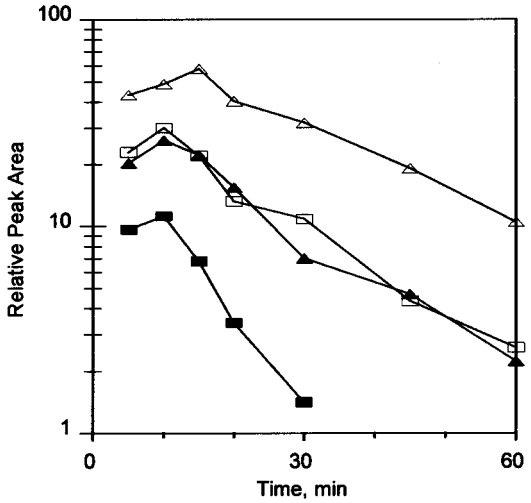

Figure 1. Pharmacokinetics of **1a** and **2a** in the B6D2F_1 mouse. Drug (50 mg/kg) was administered ip, and plasma samples were collected at the designated times. Plasma samples were deproteinized and analyzed by HPLC; see Experimental Section for details: (■) **1a**; (□) **1a** metabolite; (▲) **2a**; (△) **2a** metabolite.

Experimental Section

Drug Synthesis and Administration. The drugs evaluated in this study were synthesized as described previously.[5] Drugs were prepared in a vehicle consisting of 1:2:7 Cremophor:ethanol:saline, and all drug solutions were prepared fresh before each administration.

B16 Melanoma. Female B6D2F_1 mice were inoculated intraperitoneally (ip) with 0.2 mL of tumor brei prepared fresh from B16 tumors growing subcutaneously (sc) in the same mouse strain (day 0). The experimental mice were then randomized in groups of 10, and on days 1−5 the mice were treated ip with the appropriate dose of drug dissolved in 0.2 mL of vehicle. Mice were monitored daily and weighed twice per week. The mean survival times (MST) of all groups were calculated and the results expressed as 100% × ($MST_{treated}$/

$MST_{control}$). Mice that survived for 60 days were considered long-term survivors in the B16 model and were excluded from the calculation of mean survival time.

Wild-Type and Cyclophosphamide-Resistant L1210. L1210/WT and L1210/CP cells were maintained by ip inoculation in female $B6D2F_1$ mice. For the drug evaluation experiments, mice were inoculated ip with 1×10^5 leukemia cells obtained by removing ascites fluid from tumor-bearing mice, separating the cells by centrifugation, and resuspending the cells in saline. The mice were then randomized in groups of 10, and on days 1−5 the mice were treated ip with the appropriate dose of drug dissolved in 0.2 mL of vehicle. Mice were monitored daily and weighed twice per week, and the mean survival times were calculated as described above. Mice that survived for 30 days were considered long-term survivors and were excluded from the calculation of mean survival time.

MX-1 Human Breast Tumor Xenograft. Tumor fragments (1−2 mm^3) harvested from MX-1 tumors growing sc were harvested and implanted sc by trocar in the right foreflank of nude mice. When tumors were ca. 5 × 5 mm in size (~10 days), the animals were pair-matched into treatment and control groups of 10 mice each and individually ear-tagged (day 1). On days 1−5 the mice were treated ip with the appropriate dose of drug dissolved in 0.2 mL of vehicle. Twice weekly the mice were weighed, and tumor measurements were taken using calipers. Tumor weights were estimated from the tumor measurements using the formula $W = S^2 \times L/2$, where S and L are the short and long tumor dimensions, respectively. The experiment was terminated when the control tumors exceeded an estimated weight of 2 g. Upon termination, all mice were weighed and sacrificed, and the tumors were excised and weighed. Mean tumor weight was calculated, and tumor growth inhibition was determined from the formula $GI = 100\% \times (1 - WGT_{treated}/WGT_{control})$. If the final weight of a given tumor was smaller than its weight at the start of treatment on day 1, this was considered partial tumor shrinkage. If the tumor completely disappeared, this was considered complete tumor shrinkage. The number of mice with partial or complete tumor shrinkage was recorded.

Granulocyte/Macrophage Progenitor Cell (GM-CFC) Assay. This assay was carried out as previously described.[8] Male $B6D2F_1$ mice (5/group) were treated ip with drug, and bone marrow cells were harvested 24 h after the last drug dose. The cells were suspended at a density of 2×10^4 viable nucleated cells/mL in α-MEM supplemented with 0.8% methylcellulose, 20% fetal bovine serum, 10% pokeweed mitogen-stimulated spleen cell conditioned medium, and 50 μg/mL gentamicin. Aliquots of the cell suspension were plated and cultured at 37 °C (5% CO_2) for 7 days. Granulocyte/macrophage colonies (>50 cells/colony) were counted using a dissecting microscope. The mean number of colonies counted from each treatment group was divided by the mean number of colonies from the control group to give the percent control value.

Toxicology. For each drug, 20 female $B6D2F_1$ mice were randomly divided into four groups (5/group) and treated iv with drug in Cremophor:ethanol:saline using a day 1−5 schedule, with the groups receiving 0, 0.1, 0.5 and 1.0 × maximum tolerated dose (MTD). On day 8 the mice were anesthetized with carbon dioxide, and a terminal bleed was carried out for hematology and blood chemistries. Necropsies and histopathology were completed on all mice and were carried out by Dr. Raymond Baggs. Hematology and blood chemistries were carried out by AniLytics, Inc., Gaithersburg, MD.

Pharmacokinetics. For each drug, five female $B6D2F_1$ mice were treated with drug (50 mg/kg) administered ip in 0.2 mL of vehicle. Blood samples (80 μL) were collected by retroorbital puncture using heparinized 40-μL pipets at 5, 15, 30, 45 and 60 min after drug administration. Samples were spun for 5 min, and the plasma was separated, transferred to a Microcon filter, and centrifuged for 20 min at 14*g*. 20 μL of the plasma ultrafiltrate was analyzed by HPLC (Analtech C18 column, flow rate 1.0 mL/min, 60:40 $CH_3CN:H_2O$, UV detector at 315 nm). Retention times for **1a** and **2a** were 4.7 and 4.1 min, respectively. The drugs were quantified for each mouse by measurement of peak area, and the elimination constants were obtained by linear regression of the log values of peak area vs time during the elimination phase of the drug.

Acknowledgment. Financial support from Grant CA-34619 provided by the National Cancer Institute, DHHS, and from MGI Pharma, Inc., is gratefully acknowledged. We thank Marcy Hernick, Caren Freel Meyers, Deb Miles, and Karl Wood for assistance with the plasma metabolite analysis and John MacDonald, MGI Pharma, for helpful discussions and review of the manuscript.

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JM000359Y