Synthesis and Evaluation of 1-Acyl-1,2-bis(methylsulfonyl)-2-(2-chloroethyl)hydrazines as Antineoplastic Agents

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A series of 1-acyl-1,2-bis(methylsulfonyl)-2-(2-chloroethyl)hydrazines, conceived as more potent analogs of 1-(2-chloroethyl)-1,2,2-tris(methylsulfonyl)hydrazine, were synthesized and evaluated for antineoplastic activity against the L1210 leukemia in mice. Of these, 1-acetyl-1,2-bis-(methylsulfonyl)-2-(2-chloroethyl)hydrazine produced "cures" of mice bearing the L1210 leukemia at dosage levels that were considerably less than those at which the tris(sulfonyl) analog produced its antineoplastic effects. This compound was also found to have pronounced activity against the P388 leukemia and against several solid tumors, including the B16F10 melanoma, the M5076 reticulum cell sarcoma, and the M109 lung carcinoma. Furthermore, the acyl derivatives were in general considerably more resistant to hydrolysis in aqueous media and more prone to protease- and thiol-mediated activation than the tris(sulfonyl) analog. The former property is important to formulation, while the latter properties may result in some degree of drug targeting and enhancement of the therapeutic indices of these agents.

Introduction

Recent studies in our laboratory have identified a number of 1-methyl- and 1-(2-chloroethyl)-1,2,2-tris-(sulfonyl)hydrazines with antineoplastic activity. The most active compound of this class to emerge from this study, 1-(2-chloroethyl)-1,2,2-tris(methylsulfonyl)hydrazine (1), displayed potent antineoplastic activity in experimental systems, producing several 60-day "cures" of mice bearing leukemia L1210, leukemia P388, or Sarcoma 180,[1] and delaying by 5 days the growth of 10-day established (100 mm^3 at initiation of therapy) intradermally implanted B16 melanoma.[2] The *N*-methyl analog (2) was highly active as a trypanocide.[1] Although compound 1 is an exceedingly effective anticancer agent, with an excellent therapeutic index in murine tumor systems, it suffers from two drawbacks: (a) instability in aqueous media and (b) low water solubility. Although this compound can be formulated in dimethylacetamide for use in humans, it would be desirable to have an analog that is more easily formulatable.

$CH_3SO_2N(R)N(SO_2CH_3)_2$

1: R = CH_2CH_2Cl

2: R = CH_3

The 1-alkyl-1,2,2-tris(sulfonyl)hydrazines have been hypothesized to undergo spontaneous hydrolysis at N-2 in aqueous solution at physiological pH and temperature to generate 1-alkyl-1,2-bis(sulfonyl)hydrazines, which in turn are capable of generating an alkylating species via base-catalyzed elimination, as previously described.[3]

Recently, we compared the antitrypanosomal activity of the newly synthesized compound, 1-methyl-1,2,2-tris(methylsulfonyl)hydrazine (2) with that of the corresponding bis-analog, 1,2-bis(methylsulfonyl)-1-methylhydrazine (3).[1] Although compound 2 was considerably less potent than compound 3, it was more efficacious because its markedly lower toxicity to the murine host allowed the administration of much larger doses. Thus, when given at the same dose of 0.2 mmol/kg, compound 2 increased the survival time of mice bearing *T. brucei rhodesiense* by 7.7 days, whereas compound 3 produced an increase of 12 days. Similarly, although both compounds 2 and 3 displayed approximately the same level of activity against the L1210 leukemia, compound 2 was much less potent. Thus, compound 2 had to be administered at a dosage level of 150 mg/kg per day for 6 consecutive days to achieve the same antitumor activity as that produced by 40 mg/kg × 6 of compound 3. A similar drop in potency was also observed in the chloroethyl series when the N-2 proton in 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine (4) was replaced by a methanesulfonyl group, although such a modification resulted in a dramatic decrease in host toxicity, which produced an increase in the therapeutic index.[1] Thus, compound 1 produced "cures" of mice bearing the L1210 leukemia when administered as six daily intraperitoneal doses of 60 mg/kg of body weight.

$CH_3SO_2N(R)NHSO_2CH_3$

3: R = CH_3

4: R = CH_2CH_2Cl

The relatively low potency observed with the tris-compounds 1 and 2 could, at least in part, be due to a decrease in the yield of the alkylating species generated *in vivo* from these agents.[1] This may be the result of three inactivating decomposition pathways: (a) hydrolysis at N-1 to form 1-alkyl-2,2-bis(methylsulfonyl)hydrazine, a product incapable of generating an alkylating species by the postulated mechanism;[1] (b) cleavage of the N–N bond resulting from the relatively good leaving group ability of the *N,N*-disulfonimide;[4] and/or (c) metabolic *N*-dealkylation. A very slow rate of hydrolysis of the masking group

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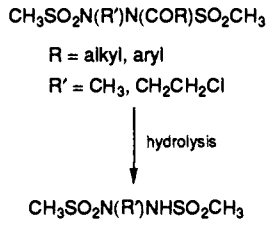
Scheme I



may indirectly promote the latter two pathways. It is, therefore, probable that an agent that would generate the alkylating species at a rate intermediate between compound 4 ($t_{1/2}$ = *ca.* 30 s) and compound 1 ($t_{1/2}$ = *ca.* 1 h) may not only enhance potency but also result in the retention of the relatively low host toxicity associated with compound 1.

The *N*-acyl bond is prone to enzyme-mediated hydrolytic cleavage and therefore may be a particularly appropriate masking group. Thus, for example, the *N'*-acetyl derivative of sulfisoxazole, a taste-masking prodrug of sulfisoxazole, undergoes selective removal of the acetyl group *in vivo* to generate the parent compound.[5] Hence, the hydrolytic removal of the acyl group from 1-acyl-1,2-bis(methylsulfonyl)-2-methyl- or -(2-chloroethyl)hydrazines (Scheme I) may also result in a higher yield of the alkylating species *in vivo* than is obtainable with compound 1. We reasoned that such activation should result in an increase in potency and, thereby, in a lower net amount of drug being required for administration compared to the tris(sulfonyl) analog. The *N*-acyl bond also resembles a peptide linkage and should be prone to protease-mediated cleavage, as well as to cleavage by nucleophilic attack. These compounds, therefore, might in addition exhibit selective toxicity to tumors through two mechanisms: (a) protease-mediated cleavage, since metastatic tumors contain high levels of proteolytic enzymes[6] and (b) glutathione- and glutathione S-transferase-mediated activation, since many tumors resistant to alkylating agents have elevated levels of glutathione- and glutathione S-transferase intrinsically or as a consequence of the attainment of resistance.[7] The design of these compounds, therefore, can exploit these properties of malignant cells, thereby providing selectivity in their cytodestructive action.

This paper reports the synthesis of 1-acyl-1,2-bis(methylsulfonyl)-2-(2-chloroethyl)hydrazines and their evaluation as antineoplastic agents in mice bearing various transplanted murine tumors. Their susceptibility to activation in aqueous media by spontaneous hydrolysis, various nucleophiles, serum, and proteinase K is also described.

Chemistry

1-Acyl-1,2-bis(methylsulfonyl)-2-(2-chloroethyl)hydrazines were prepared by reacting 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine with the appropriate acyl chloride or acid anhydride as shown in Scheme II.

Results and Discussion

The antineoplastic activity of compounds 5–10 was initially assessed by measuring their effects on the survival time of mice bearing the L1210 leukemia; the results obtained are summarized in Table I. All of the compounds tested were capable of producing "cures" of mice bearing the L1210 leukemia, as measured by animals surviving for >60 days. Furthermore, the most hydrophilic analogs of this class, i.e., compounds **5**, **6**, and **9**, produced their curative effects at dosage levels that were much lower than those at which compound 1 effected "cures" (i.e., 60 mg/kg × 6). Thus, the acetyl derivative (**5**) was highly active against the L1210 leukemia, with 80% of L1210-bearing mice being free of tumor when treated with 7.5 mg/kg of compound **5** for 6 consecutive days. The propionyl analog (**6**) in this series appeared to be the most potent agent, producing some "cures" of mice bearing the L1210 leukemia at dosage levels as low as 5 mg/kg per day for 6 consecutive days. This compound also produced 80–90% "cures" at 10 and 15 mg/kg per day × 6. The methoxyacetyl analog (**9**) was capable of curing 100% of tumor-bearing mice at 10 mg/kg per day × 6, but was lethal at the higher dose levels examined. Compounds **5**–**10** were all less toxic than compound **4**, which caused lethality at daily doses greater than 5 mg/kg.

Scheme II

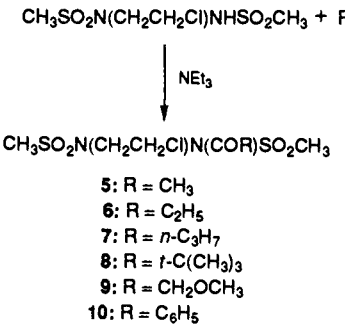


Table I. Effects of 1-Acyl-1,2-bis(methylsulfonyl)-2-(2-chloroethyl)hydrazines on the Survival Time of Mice Bearing the L1210 Leukemia

compd	optimum effective daily dose, mg/kg[a,b]	av Δwt, %[c]	% T/C[d]	% cures
1	60	−7.2		100
5	7.5	+1.0	341	80
6	10	−6.4	217	90
7	10	0.0	207	20
8	20	0.0	302	60
9	10	−8.4		100
10	20	−7.7	160	20

[a] Administered once daily for 6 consecutive days, beginning 24 h after tumor implantation, with 5–10 mice being used per group. [b] Average day of death for untreated tumor-bearing mice was 8.4. [c] Average percent change in body weight from onset to termination of therapy. Average percent change in body weight for vehicle-treated controls was +2.0. [d] % T/C = average survival time of treated/control mice × 100; cures (60-day survivors) are listed separately and are not included in this calculation.

The antitumor activity of a representative agent of the 1-acyl-1,2-bis(methylsulfonyl)-2-(2-chloroethyl)hydrazine class, compound **5**, was evaluated and found to possess significant activity against several other transplanted mouse tumors (Table II). In addition to producing 50% "cures" of mice bearing the P388 leukemia, this compound displayed activity against intraperitoneally (ip) implanted M109 lung carcinoma. In this latter system, at the highest dose of **5** examined (60 mg/kg per injection), a maximum T/C of 231% was achieved when the drug was administered intraperitoneally every third day for four total doses.

Compound **5** was evaluated in three separate experiments against the M109 lung carcinoma implanted subcutaneously (sc). In the initial test using this model, a dose of 30 mg/kg per injection of compound **5** was administered intravenously in 10% dimethyl sulfoxide (DMSO) in saline every third day for a total of four doses;

Table II. Summary of Optimal Antitumor Effects of 1-Acetyl-1,2-bis(methylsulfonyl)-2-(2-chloroethyl)hydrazine (5) on Several Preclinical Tumor Models

tumor, site	treatment schedule, route	optimum effective dose, mg/kg/injection	%T/C (cures/total) and/or [T - C, days]
P388, ip	qd1→5; ip	200[a]	(3/6)
B16, id	single dose; d.10;[b] ip	100[c]	[12]
M109, ip	q3dx4; d.1;[b] ip	60[d]	231
M109, sc	q3dx4; d.1;[b] iv	30	(a)[e] 128 [10.5]
		60	(b) 133 [3.0]
		32 [48][f]	(c) 136 [14.5]
M5076, sc	q2dx5; d.1;[b] iv	20 [40][f]	(a) 156 [18.3]
		36[c,d]	(b) 122 [20.8]

[a] Administered in suspension. [b] Day treatment initiated. [c] Administered in 100% DMSO. [d] Highest dose tested. [e] Each letter signifies a different experiment. [f] Dose in brackets producing the maximum T - C obtained.

this regimen produced a maximum T/C of 128% accompanied by a delay in tumor growth (T - C) of 10.5 days. Mitomycin C, included as a reference drug, produced a maximum T/C of 128% and a T - C of 14.0 days. In the second experiment, using the same treatment schedule, a dose of 60 mg/kg per injection of compound 5 was tolerated and produced a maximum T/C of 133% but only a 3.0 day delay in tumor growth. Mitomycin C, used again for comparison, failed to cause a significant increase in life span, but produced a growth delay of 11.0 days. Due to the inconsistency of compound 5 in delaying tumor growth, a third experiment was conducted. At a dose level of 32 mg/kg per injection, compound 5 achieved a maximum T/C of 136%, and at a slightly higher dose of 48 mg/kg per injection, it produced a maximum growth delay of 14.5 days. Cyclophosphamide and mitomycin C were both included as positive controls in this last experiment; the former drug produced a maximum T/C of 143% accompanied by a T - C value of 8.8 days, while mitomycin C produced a maximum T/C of 134% and a growth delay of 9.3 days.

In addition, compound 5 was evaluated twice against subcutaneously implanted M5076 reticulum cell sarcoma. In the first experiment, 20 mg/kg per injection of compound 5, administered intravenously in 10% DMSO in saline, produced a maximum T/C of 156% and a growth delay of 17.8 days. When the dose level was doubled to 40 mg/kg per injection, a T/C of 137% and a growth delay of 18.3 days were achieved. In the second experiment, a dose of 36 mg/kg per injection of compound 5, administered intravenously in 100% DMSO on an every other day treatment schedule for 5 days, produced a T/C of only 122% but effected a delay in tumor growth of 20.8 days.

In addition, in a preliminary study, established (50–100 mm^3 at the start of therapy) intradermally (id) implanted B16F10 melanoma tumors in mice treated with a single intraperitoneal dose of 100 mg/kg of compound 5 in 100% DMSO took 12 days longer than control (untreated) tumors to reach 10-times the initial volume.

1-Acyl-1,2-bis(methylsulfonyl)-2-(2-chloroethyl)hydrazines have been hypothesized to undergo selective hydrolysis at N-1 to generate 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine (4). Since these compounds were conceived as more stable analogs of 1-(2-chloroethyl)-1,2,2-tris(methylsulfonyl)hydrazine (1), their ability to undergo spontaneous decomposition in aqueous media was measured in phosphate buffer at pH 7.4 and 37 °C using the spectroscopic assay described in the Experimental Section to monitor acidification. As shown in Table III, compounds 5–10 were considerably more stable under these conditions than compound 1. The aim of synthesizing an agent that was more potent than compound 1, but also one that was considerably more stable than compound 1 in aqueous media, appeared to be satisfied with the development of compounds 5–10.

Table III. Initial Rates of Decomposition of 1 mM Solutions of 1-Acyl-1,2-bis(methylsulfonyl)-2-(2-chloroethyl)hydrazines at pH 7.4 and 37 °C in 1 mM Potassium Phosphate Buffer

compd	rate of decomposition, nmol of drug/mL/min ± SD	relative rate of decomposition[a,b]
1	9.58 ± 0.40	1.00
5	0.24 ± 0.04	0.03
6	≤0.1	
7	≤0.1	
8	≤0.1	
9	2.90 ± 0.15	0.30
10	1.02 ± 0.05	0.11

[a] Compounds 6, 7, and 8 decompose at rates close to or less than the limit of detection; therefore, relative rates of decomposition cannot be expressed for these compounds. [b] Relate rate of decomposition = rate of decomposition of acyl compound/rate of decomposition of compound 1.

Table IV. Effects of 2% CD-1 Mouse Serum on the Rate of Hydrolysis of 1-Acyl-1,2-bis(methylsulfonyl)-2-(2-chloroethyl)-hydrazines at pH 7.4 and 37 °C

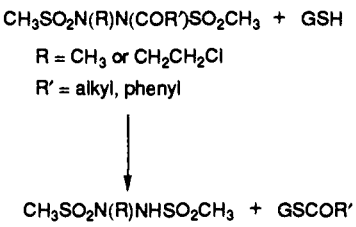
compd	rate of decomposition, nmol of drug/mL/min ± SD	relative rate of decomposition[a,b]
5	0.89 ± 0.06	3.7
6	0.45 ± 0.02	
7	0.34 ± 0.07	
8	0.78 ± 0.07	
9	8.57 ± 0.67	3.0
10	7.86 ± 1.14	7.7

[a] Compounds 6, 7, and 8 decompose at rates close to or less than the limit of detection; therefore, relative rates of decomposition cannot be expressed for these compounds. [b] Relate rate of decomposition = rate of decomposition in the presence of 2% serum/rate of hydrolysis in buffer alone.

The spontaneous hydrolysis of compound 5 at pH 7.4 and 37 °C in 1 mM phosphate buffer occurs, with essentially first-order kinetics, at approximately 2.5% of the rate of compound 1 under the same conditions (calculated $T_{1/2}$ values are 48 and 1.2 h, respectively). The rate of hydrolysis was highly sensitive to changes in temperature and pH. The rate was found to approximately double for every 5 °C rise in temperature and increased approximately 40% for every 0.2 unit rise in pH in the 7.0–7.8 range. At pH 7.4 and 37 °C, a 1.0 mM solution of compound 5 hydrolyzed at an initial rate of approximately 1.44% per hour. This rate could be appreciably decreased by slight acidification and/or by storage at low temperature. Therefore, this agent could be readily formulated as a stable aqueous solution. The other analogs that were synthesized (compounds 6–10) were also hydrolyzed at much slower rates than compound 1 (Table III).

Studies on the kinetics of hydrolysis of these compounds in the presence of 2% mouse serum indicated that serum was capable of readily catalyzing this process (Table IV). The addition of 2% CD-1 mouse serum increased the rate of hydrolysis of compound 10 approximately 8-fold. Furthermore, the rate of hydrolysis was found to be approximately proportional to the concentration of serum. Therefore, in 100% mouse serum, the rates of hydrolysis of compounds 5–10 can be calculated to be elevated 100–340-fold over buffer alone. In 100% mouse serum, a 1 mM solution of compound 10, the most labile compound

Scheme III



under these conditions, would hydrolyze at a calculated initial rate of approximately 35% per minute. Both bovine and human serum were also capable of activating these agents (data not shown). Heating dilute solutions of bovine serum in boiling water for 10 min decreased hydrolysis of compound **5** by >75%, suggesting that serum proteins, possibly esterases and proteases, were important for this activity. The residual activity could be due to "non-denaturable" thiols or other nucleophiles present in the serum. In support of enzyme-mediated decomposition, proteinase K, a protease of low specificity, was found to catalyze the decomposition of these agents. Thus, the addition of 1 mg/mL of proteinase K increased the rates of hydrolysis of 1.0 mM solutions of compounds **5** and **10** by approximately 100- and 30-fold, respectively. As anticipated, 10% mouse serum was capable of increasing the alkylation of the model nucleophile, 4-(4'-nitrobenzyl)pyridine,[8] by 1-acetyl-1,2-bis(methylsulfonyl)-2-(2-chloroethyl)hydrazine (**5**) more than 6-fold over buffer alone, providing direct evidence for serum-enhanced generation of alkylating species from these prodrugs. The molar yields ± SDs of 2-chloroethanol from compounds **1** and **10** were 0.58 ± 0.06 and 0.80 ± 0.06, respectively, supporting the idea that enzymatic cleavage of compound **10** produces a slightly higher yield of the alkylating species than is generated by the spontaneous decomposition of compound **1**. The differences in the yields of the alkylating species between the acyl derivatives, as exemplified by compound **10**, and compound **1** may be much more dramatic *in vivo* where, in contrast to compound **1**, the rapid activation of compounds **5–10** by serum and other mechanisms would leave relatively little time for excretion and metabolic inactivation.

Resistance to a large number of alkylating agents has been shown to be correlated with increased levels of glutathione (GSH) and glutathione S-transferase (GST) enzymes,[7] suggesting that resistance is due to a direct interaction between the sulfhydryl group and the electrophilic alkylating species. Buthionine sulfoximine has been shown to reduce cellular GSH levels, to reverse resistance, and to increase *in vitro* cytotoxicity and *in vivo* anticancer activity of a variety of alkylating agents, such as cyclophosphamide, melphalan, and the nitrosoureas.[7] Furthermore, some neoplastic cell lines have intrinsically high levels of GSH, without having been subjected to drug selection.[9] The resistance of these cell lines to various therapeutic strategies has been attributed to their high non-protein thiol and GST contents. The elevated thiol levels of these tumor cell lines can theoretically be exploited by the selective targeting of thiol-activated prodrugs.

We have reasoned that the removal of the acyl group from 2-acyl-1-alkyl-1,2-bis(methylsulfonyl)hydrazines could proceed not only by hydrolytic cleavage of the *N*-acyl bond, but also via nucleophilic attack of the carbonyl carbon by a sulfur-containing nucleophile (Scheme **III**). Since the envisioned activation process involves consumption of free thiols, the protective effect afforded tumor cells by non-protein sulfhydryls would also be minimized.

Table V. Rates of Acidification of a 1 mM Solution of 1-Benzoyl-1,2-bis(methylsulfonyl)-2-(2-chloroethyl)hydrazine (**10**) at pH 7.4 and 37 °C in the Presence and Absence of Various Nucleophiles

conditions/nucleophile	rate of acidification, nmol of H^+/mL/min ± SD
buffer alone	3.2 ± 0.2
+ 10 mM potassium chloride	3.1 ± 0.2
+ 10 mM lithium chloride	3.2 ± 0.3
+ 10 mM potassium iodide	3.0 ± 0.2
+ 10 mM sodium azide	79.0 ± 6.3
+ 10 mM sodium cyanide	36.7 ± 2.4
+ 10 mM sodium acetate	2.9 ± 0.4
+ 10 mM ammonium acetate	16.4 ± 0.7
+ 10 mM glycine	24.2 ± 0.9
+ 10 mM histidine	20.3 ± 2.3
+ 10 mM thioglycerol	170 ± 6.6
+ 10 mM GSH	461 ± 21

Table VI. Decomposition Rates of 1-Acyl-1,2-bis(methylsulfonyl)-2-(2-chloroethyl)hydrazines in the Presence of 10 mM Glutathione at pH 7.4 and 37 °C

compd	rate of decomposition in the presence of 10 mM GSH, nmol of drug/mL/min ± SD	relative rate of decomposition[a]
5	14.5 ± 0.9	60.3
6	12.8 ± 0.4	
7	10.3 ± 0.6	
8	11.0 ± 0.3	
9	147 ± 4.6	50.6
10	227 ± 10.5	223

[a] Relative rate = rate of decomposition in the presence of 10 mM GSH/rate of decomposition in buffer alone. Compounds **6**, **7**, and **8** decompose at rates close to or less than the limit of detection in the absence of glutathione; therefore, relative rates cannot be expressed for these compounds.

We have examined the effects of various nucleophiles on the rate of activation of compound **10**. That GSH is a potent activator of this prodrug and compounds containing amino groups also accelerate the decomposition of this agent to a significant extent is shown in Table V. We also examined the relative susceptibilities of compounds **5–10** to undergo activation by glutathione, and these findings are presented in Table VI. As expected, the greater the electron-withdrawing inductive effect of the substituent attached to the carbonyl group, the more susceptible the agent is to thiol-mediated activation. The reactions of compounds **5** and **9** with GSH were examined in more detail and found to be approximately first order with respect to both GSH and drug concentration.

To confirm that the increase in decomposition rates measured in the acidification assay in the presence of GSH represents an increased rate of generation of the alkylating species and not of an unrelated reaction which results in acidification, a solution of 1-benzoyl-1,2-bis(methylsulfonyl)-2-methylhydrazine (**11**) was incubated in phosphate buffer at pH 7.4 and 37 °C in the presence and absence of GSH. The amount of methanol generated in 10 min in the presence of 1 mM GSH was approximately 10-fold greater than that produced in phosphate buffer alone, implying that the rate of generation of the alkylating species from compound **11** is much faster than the rate of reaction of the alkylating species with GSH; subsequent experiments have demonstrated that the rate of activation of the prodrug was directly proportional to the concentration of GSH (data not shown). Similar results were

obtained with the 1-acetyl analog. In the case of the 1-(2-chloroethyl) derivative (10), precise quantitation was difficult due to the limitations of the assay; however, 1 mM compound 10 gave a 6–7-fold greater amount of alkylated product when reacted with the nucleophile 4-(4′-nitrobenzyl)pyridine in the presence of 1 mM GSH for 30 min at 37 °C than in its absence. It is conceivable, therefore, that tumors resistant to conventional chloroethylating agents because of the protective effect conferred by high levels of intracellular thiols may be particularly sensitive to 2-acyl-1,2-bis(methylsulfonyl)-1-methyl(or 2-chloroethyl)hydrazines. Furthermore, we have found that the GSH activation of the prodrug derivative 11 can be catalyzed by GST (13.5 units/mL of equine liver enzyme as determined by the 1-chloro-2,4-dinitrobenzene assay; Sigma Chemical Co.), producing a doubling of the activation rate. Thus, although elevations in GST activity have been associated with increased resistance to a variety of electrophilic alkylating agents, such enzymatic activity should increase sensitivity to the 2-acyl-1,2-bis(methylsulfonyl)-1-methyl(or 2-chloroethyl)hydrazine thiol-activated prodrugs.[7,10]

In conclusion, 1-acyl-1,2-bis(methylsulfonyl)-2-(2-chloroethyl)hydrazines have the following advantages over the tris(sulfonyl)hydrazine (1): (a) they are more resistant to spontaneous hydrolysis, and the lower homologs are also 5–10-fold more potent and more water soluble, making them easier to formulate than compound 1; (b) a wide range of serum half-lives can be obtained by altering the acyl group; and (c) their capacity to undergo protease and/or thiol-mediated activation may result in selective tumor cell targeting and enhanced therapeutic indices. Furthermore, a representative agent of this class, compound 5, showed broad-spectrum activity against several different tumors, including stringent distal site tumor models. The levels of activity observed in the M109 lung carcinoma model were comparable to those obtained with clinically active alkylating anticancer drugs. These properties make compound 5 a potential new candidate for clinical evaluation.

Experimental Section

Synthesis. Melting points were determined with a Thomas-Hoover capillary melting point apparatus and are uncorrected. Proton magnetic resonance spectra were recorded on a Varian EM-390 spectrometer with tetramethylsilane as an internal standard. Elemental analyses were performed by the Baron Consulting Co. (Orange, CT), and the data were within ±0.4% of the theoretical values for the 1-acyl-1,2-bis(methylsulfonyl)-2-(2-chloroethyl)hydrazines reported.

1-Acetyl-1,2-bis(methylsulfonyl)-2-(2-chloroethyl)hydrazine (5). To a stirred solution of 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine (4; 1.5 g, 0.006 mol) and acetic anhydride (4.3 g, 0.042 mol) in dry acetone (50 mL) was added dropwise triethylamine (3.6 g, 0.035 mol) at room temperature. After an additional 16 h, the reaction mixture was evaporated to dryness. The residue was taken up in ethyl acetate (50 mL) and washed with dilute hydrochloric acid (3 × 10 mL), followed by water (3 × 10 mL). The ethyl acetate layer was dried over anhydrous magnesium sulfate, treated with charcoal, and filtered. Upon evaporation of the solvent, a solid was obtained which, upon recrystallization from ethanol, gave 1.1 g (62.8%) of the title compound: mp 148–149 °C; ^{1}H NMR (acetone-d_6) δ 3.8–4.2 (m, 4H, CH_2CH_2), 3.5 and 3.3 (2 s, 6 H, $2\,CH_3SO_2$), 2.4 (s, 3 H, CH_3CO). Anal. ($C_6H_{13}ClN_2O_5S_2$) C, H, N.

1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-propionylhydrazine (6). To a stirred solution of 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine (4; 1.25 g, 0.005 mol) and propionyl chloride (3.20 g, 0.035 mol) in dry acetone (20 mL) was added dropwise triethylamine (0.014 mol) at room temperature. A precipitate was formed immediately. After an additional 20 h, the reaction mixture was filtered and the filtrate was evaporated to dryness *in vacuo*. The residue was taken up in ethyl acetate (100 mL) and washed with dilute hydrochloric acid (3 × 15 mL), followed by water (2 × 10 mL). The ethyl acetate layer was dried over anhydrous magnesium sulfate and filtered, and the filtrate was evaporated to dryness *in vacuo*. The residue was triturated with petroleum ether to give a solid which, upon recrystallization from absolute ethanol, afforded 0.71 g (46.4%) of the title compound: mp 97–98 °C; ^{1}H NMR (acetone-d_6) δ 3.8–4.2 (m, 4H, CH_2CH_2), 3.5 and 3.3 (2 s, 6 H, $2\,CH_3SO_2$), 2.8 (q, 2 H, $COCH_2$), 1.1 (t, 3 H, $COCCH_3$). Anal. ($C_7H_{15}ClN_2O_5S_2$) C, H, N.

The following compounds were synthesized using procedures similar to that described for compound 6.

1,2-Bis(methylsulfonyl)-1-(*n*-butyryl)-2-(2-chloroethyl)hydrazine (7). Compound 7 was recrystallized from ethanol–petroleum ether: yield 59.4%; mp 68–69 °C; ^{1}H NMR (acetone-d_6) δ 3.8–4.2 (m, 4 H, CH_2CH_2Cl), 3.5 and 3.3 (2 s, 6 H, $2\,CH_3SO_2$), 2.8 (t, 2 H, $COCH_2$), 1.6 (m, 2 H, CCH_2C), 1.0 (t, 3 H, CCH_3). Anal. ($C_8H_{17}ClN_2O_5S_2$) C, H, N.

1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-pivaloylhydrazine (8). Compound 8 was recrystallized from ethanol: yield 4.6%; mp 115–116 °C; ^{1}H NMR (acetone-d_6) δ 3.9–4.2 (m, 4 H, CH_2CH_2Cl), 3.5 and 3.4 (2 s, 6 H, $2\,CH_3SO_2$), 1.4 [s, 9 H, $C(CH_3)_3$]. Anal. ($C_9H_{19}ClN_2O_5S_2$) C, H, N.

1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-(methoxyacetyl)hydrazine (9). Compound 9 was recrystallized from ethanol: yield 77.7%; mp 99–100 °C; ^{1}H NMR (acetone-d_6) δ 4.5 (d, 2 H, $COCH_2$), 3.8–4.3 (m, 4 H, CH_2CH_2), 3.6 (s, 3 H, OCH_3), 3.5 and 3.4 (2 s, 6 H, $2\,CH_3SO_2$). Anal. ($C_7H_{15}ClN_2O_6S_2$) C, H, N.

1-Benzoyl-1,2-bis(methylsulfonyl)-2-(2-chloroethyl)hydrazine (10). Compound 10 was recrystallized from ethanol: yield 53.5%; mp 106–107 °C; ^{1}H NMR (acetone-d_6) δ 7.5–7.9 (m, 5 H, aromatic H), 4.0 and 3.7 (2 t, 4 H, CH_2CH_2), 3.6 and 3.2 (2 s, 6 H, $2\,CH_3$). Anal. ($C_{11}H_{15}ClN_2O_5S_2$) C, H, N.

1-Benzoyl-1,2-bis(methylsulfonyl)-2-methylhydrazine (11). Compound 11 was synthesized by reacting compound 3 with benzoyl chloride using a method similar to the one described for the preparation of compound 6. It was recrystallized from ethanol: yield 57.3%; mp 111–112 °C; ^{1}H NMR (acetone-d_6) δ 7.3–7.8 (m, 5 H, aromatic H), 3.6, 3.4, and 2.9 (3 s, 9 H, $3\,CH_3$). Anal. ($C_{10}H_{14}N_2O_5S_2$) C, H, N.

Antitumor Activity. The ascites cell form of leukemia L1210 was obtained from the Frederick Cancer Research Facility, Division of Cancer Treatment Tumor Repository of the National Cancer Institute, and was maintained by serial passage in tissue culture. Every 8 weeks, tumor cells were injected intraperitoneally into five donor CD_2F_1 mice 8–10 weeks of age and were allowed to grow for 7 days. The peritoneal fluid was withdrawn, and the suspension was centrifuged for 5 min at 1600*g*. The supernatant was decanted, and 10^5 cells/mL were seeded into 10 mL of RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% L-glutamine and once again maintained in culture. To assay for antineoplastic activity, 0.1 mL of the cell suspension containing 10^5 leukemia cells was injected into each recipient mouse. Test compounds were administered over a wide range of dosage levels (5–20 mg/kg), beginning 24 h after tumor implantation, and continued once daily for 6 consecutive days. All drugs were administered intraperitoneally as a solution in 100% DMSO, in a volume not exceeding 0.025 mL. In each experiment, animals were distributed into groups of five mice of comparable weight and maintained throughout the course of the experiment on Purina Laboratory Chow pellets and water ad libitum. Control tumor-bearing animals given comparable volumes of vehicle were included in each experiment. Mice were weighed during the course of the experiments, and the percentage change in body weight from onset to termination of therapy was used as an indication of drug toxicity. Determination of the sensitivity of neoplasms to these agents was based upon the prolongation of survival time afforded by the drug treatments.

Cultures of B16F10 melanoma were grown *in vitro* as monolayers in minimum essential medium with Hank's salts supplemented with 10% fetal bovine serum and 1% L-glutamine. Solid tumors were produced in C57BL6 female mice 12–14 weeks of

age by the intradermal injection in the right flank of each mouse of 0.1 mL of a cell suspension containing 10^6 cells/mL from freshly trypsinized cultures. After 10 days, animals bearing 50–100 mm^3 tumors were treated with a single intraperitoneal dose of compound **5** dissolved in 100% DMSO, and tumor volumes were measured on alternate days until reaching 10 times their initial volume.

The P388 leukemia was propagated weekly by intraperitoneal transfer into DBA/2 mice. The M5076 sarcoma was passaged biweekly by subcutaneous transfer of tumor fragments into C57BL/6 mice, and the M109 lung carcinoma was similarly passaged in Balb/c mice. In these systems, compound **5** was dissolved in (a) 100% DMSO and administered by intravenous injection in a fixed volume of 10 μL; (b) DMSO and diluted with saline to a final concentration of solvent of 10% and administered intravenously in a volume of 0.01 mL/g of body weight; or (c) 10% DMSO in carboxymethylcellulose containing a drop of Tween 80 for intraperitoneal administrations as a fine suspension in an injection volume of 0.5 mL. These different modes of formulation resulted in differences in the optimum effective dose found in the various tumor systems. Mitomycin C and cyclophosphamide were dissolved and administered in saline.

Four to six mice per group were employed in experiments with the P388 leukemia, six to eight mice per group with the B16F10 melanoma, and eight mice per group with the M5076 sarcoma and the M109 carcinoma. A minimum of three dose levels per compound were included in each evaluation, and drug therapy was initiated 24 h after tumor implantation for P388 leukemia, M5076 sarcoma, and M109 carcinoma. Therapeutic results are presented in terms of (a) increases in life span reflected by the relative median survival time (MST) of treated versus control groups (i.e., %T/C values), and by long-term survivors, and (b) primary tumor growth inhibition determined by calculating the relative median times for treated (T) and control untreated (C) mice to grow tumors of a 1 g size (i.e., $T - C$ values). Tumor weights were interchangeable with tumor size on the basis of 1 mm^3 = 1 mg. Treated mice dying prior to either day 5 in the P388 experiment, day 10 in the ip M109 experiment, or having their tumors achieve 1 g in size, were considered to have died from drug toxicity. Groups of mice with more than one death due to drug toxicity were not used in the evaluation of antitumor efficacy.

Decomposition Studies. The decomposition of various compounds was studied by following the acidification of weakly buffered (1 mM potassium phosphate, pH 7.4) solutions of phenol red (20 mg/L) spectrophotometrically at 560 nm. Since the carboxylic and sulfinic acids that are produced are essentially 100% ionized at near neutral pH values, stoichiometries used to calculate the decomposition rates of the drugs were as follows: 2 mol of protons released/mol of drug during the decomposition of 1-alkyl-1,2-bis(sulfonyl)hydrazines, 3 mol of protons released during the decomposition of the 1-acyl-2-alkyl-1,2-bis(sulfonyl)-hydrazines and 2 mol of protons generated during the GSH-initiated decomposition of 1-acyl-2-alkyl-1,2-bis(sulfonyl)hydrazines as shown below.

$$CH_3SO_2N(R)NHSO_2CH_3 + H_2O \rightarrow 2CH_3SO_2^- + N_2 + ROH + 2H^+$$

$$CH_3SO_2N(R)N(COR')SO_2CH_3 + 2H_2O \rightarrow 2CH_3SO_2^- + ROH + R'COO^- + N_2 + 3H^+$$

$$CH_3SO_2N(R)N(COR')SO_2CH_3 + GSH + H_2O \rightarrow 2CH_3SO_2^- + GSCOR' + N_2 + ROH + 2H^+$$

All mixtures were adjusted to pH 7.4 prior to drug addition, and the assay was calibrated using HCl standards. Calibrations were carried out for all of the reaction mixtures tested to allow for changes in sensitivity resulting from differences in buffering capacity that were due to other additives (i.e., serum/GSH). The assay was approximately linear over a range of H^+ additions of 0–100 μmol/L. All determinations were conducted at 37 °C and at pH 7.4 unless otherwise stated. Drugs were added in 10 μL volumes of 100 mM solutions in DMSO to 0.99 mL of assay buffer with rapid mixing to prevent precipitation. The initial rate of decomposition was also measured for compounds **5** and **9** over 0.1–2.0 mM drug and 0–16 mM GSH concentration ranges to determine the order of these reactions with respect to the concentration of drug and GSH.

Alkylation of 4-(4′-Nitrobenzyl)pyridine. The ability to alkylate the model nucleophile 4-(4′-nitrobenzyl)pyridine (NBP) was determined, using a modification of the method of Wheeler and Chumley.[8] To 0.5 mL of 100 mM sodium phosphate buffer (pH 7.4) was added 10 μL of NBP (80 mg/mL) in acetone. The alkylating agent to be tested (compound **5**) was added to this mixture as a concentrated solution dissolved in 1 μL of DMSO to give a final concentration of 2 mM. The mixture was incubated at 37 °C for either zero or 2 h. The experiment was also repeated with 50 μL of buffer being replaced by mouse serum. The extent of alkylation of NBP was determined spectroscopically by the addition of 1 mL of 1-octanol followed by 0.2 mL of 10 M NaOH to the reaction mixture. The mixture was shaken immediately and centrifuged at 10000g for 1 min. The absorbancy of the upper octanol layer was then recorded at 545 nm. The generated blue color is unstable in aqueous solution and must be extracted promptly into octanol, in which it is stable, for reproducible results. The absorbancy measured at zero time was subtracted from that measured at 2 h to account for any activation of drug by alkaline hydrolytic cleavage of the acyl group during the procedure. This amount represented approximately 6% of the absorbance measured in the presence of serum after the 2-h incubation period.

Determination of Chloroethanol. 2-Chloroethanol was assayed using bakers' yeast alcohol dehydrogenase (EC 1.1.1.1). This enzyme was found to use 2-chloroethanol as a substrate, although the rate of oxidation of this alcohol was much slower than that of the authentic substrate, ethanol. The assay conditions were as follows: 100 mM Tris-HCl buffer (pH 8.8, 37 °C) containing 2 mM NAD^+ to which known amounts of 2-chloroethanol were added as a standard at concentrations between 0 and 1 mM. The initial rate of reduction of NAD^+ was measured spectroscopically at 340 nm after the addition of 120 units/mL of alcohol dehydrogenase. The initial rate of NAD^+ reduction was found to be proportional to the concentration of 2-chloroethanol. The yields of chloroethanol resulting from the decomposition of various chloroethylating agents were determined by the addition of 1–2 μL of a concentrated solution of drug in DMSO to Tris-HCl buffer (pH 8.8) at 37 °C to give a final concentration of 1 mM. Proteinase K (1 mg/mL) was also added to this mixture when chloroethanol yields from acyl derivatives were determined; this mixture was incubated for 24 h and then assayed for chloroethanol as described above following the addition of NAD^+/alcohol dehydrogenase. The addition of 1 μL/mL of DMSO or 1 mg/mL of proteinase K after 24 h of incubation had no measurable effect on the determination of chloroethanol.

Determination of Methanol. The amount of methanol generated in 10 min was measured using alcohol oxidase by methodology previously described by our laboratory using a Gilson Oxygraph, *Pichia pastoris* alcohol oxidase, and methanol standards.[11]

Preparation of Mouse Serum. Female CD-1 mice anesthetized using ethyl ether were bled by cardiac puncture, and the pooled blood from three mice was incubated at 37 °C for 30 min. The serum was then separated by centrifugation at 10000g for 2 min and stored on ice for no more than 6 h prior to use.

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