Antitumor 2-(Aminocarbonyl)-1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-hydrazines

Krishnamurthy Shyam,[†] Philip G. Penketh,[†] Regina H. Loomis,[†] William C. Rose,[‡] and Alan C. Sartorelli*,[†]

Department of Pharmacology and Experimental Therapeutics Program, Cancer Center, Yale University School of Medicine, New Haven, Connecticut 06520, and Department of Experimental Therapeutics, Pharmaceutical Research Institute, Bristol-Myers Squibb Co., Princeton, New Jersey 08543

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Several 2-(aminocarbonyl)-1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazines were synthesized and primarily evaluated for antitumor activity against the murine L1210 leukemia. All of the compounds tested were capable of producing "cures" of mice bearing this tumor. One of the most active agents of this class, 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[(2-chloroethyl)-amino]carbonyl]hydrazine, was further evaluated against a spectrum of transplanted murine and human solid tumors. Pronounced activity was found against all of the tumors including the murine B16F10 melanoma, M109 lung carcinoma, M5076 reticulum cell sarcoma, and the human LX-1 lung carcinoma. The activities observed compared favorably with those of the established antitumor drugs, cyclophosphamide, mitomycin C, and the nitrosoureas, evaluated concomitantly.

Several *N*-(2-chloroethyl)-*N*-nitrosoureas (CNUs) have been evaluated clinically and have been shown to possess significant antineoplastic activity against brain tumors, colon cancer, and lymphomas.[1,2] Characterization of the decomposition products of the clinically used CNUs, such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), has resulted in the identification of several reactive products, including chloroethylating,[3,4] carbamoylating,[3−5] and hydroxyethylating species.[6] The antitumor activity of the CNUs has been suggested to result from chloroethylation and subsequent cross-linking of DNA.[7] In support of this view is the observation that many chloroethylating agents with no carbamoylating activity (e.g., clomesome[8]) possess excellent antineoplastic activity. Furthermore, replacement of the chloro group in the CNUs by a hydroxyl group resulted in a considerable drop in antineoplastic activity.[9] There is also some evidence that hydroxyethylation of DNA is a carcinogenic and/or mutagenic event.[10,11]

While hydroxyethylation seems to have no salutary effect on the antineoplastic activity of the CNUs, there appears to be some uncertainty regarding the role played by the carbamoylating species (i.e., the isocyanate). The isocyanate generated from the CNUs reacts with thiol and amine functionalities on proteins and inhibits DNA polymerase,[12] the repair of DNA strand breaks,[13] and RNA synthesis and processing.[14] It is conceivable that the inhibition of DNA repair may potentiate the cytotoxicity of the DNA lesions caused by the chloroethylating species. In addition, BCNU has been shown to inhibit glutathione reductase, ribonucleotide reductase, and thioredoxin reductase.[15] Although it is believed by many that some of these properties contribute to the toxic side effects of the CNUs,[16−18] it is entirely possible, as speculated by Gibson and Hickman[19] in their study of the effects of BCNU on the TLX tumor in mice, that intracellular release of isocyanates plays a role in modulating the biological activity of the CNUs against some specific tumor types. Thus, caracemide, an investigational antitumor agent developed by the Dow Chemical Company,[20,21] is thought to act as a latent form of methyl isocyanate. This agent was shown to be active in a number of National Cancer Institute tumor models, including the mammary MX-1 and colon CX-1 human tumor xenografts implanted in the subrenal capsule of athymic mice.[22] Therefore, a case can be made for the synthesis of agents that retain chloroethylating and carbamoylating properties, but are devoid of hydroxyethylating activity.

The hydroxyethylating species generated from the CNUs, 2-hydroxyethyl diazohydroxide, is thought to be formed from 4,5-dihydro-1,2,3-oxadiazole which, in turn, has been hypothesized to result from an internal cyclization reaction involving the *N*-nitroso group.[6] The *N*-nitroso group is also involved in the enzymatic inactivation of the CNUs. For example, BCNU can be inactivated by denitrosation by liver microsomal enzymes in an NADPH-dependent reaction, with the formation of 1,3-bis(2-chloroethyl)urea.[23,24] The denitrosation reaction is catalyzed by NADPH:cytochrome P-450 reductase in the case of CCNU.[25,26] BCNU has also been shown to undergo glutathione-dependent denitrosation catalyzed by rat[27] and human[28] glutathione S-transferase μ isoenzymes. Since tumor cell-catalyzed denitrosation can conceivably be a potential mechanism of resistance to the CNUs, our aim was to synthesize a series of compounds that (a) were capable of generating a chloroethylating species, (b) were capable of forming a carbamoylating species, (c) were

[†] Yale University School of Medicine.
[‡] Bristol-Myers Squibb Co.
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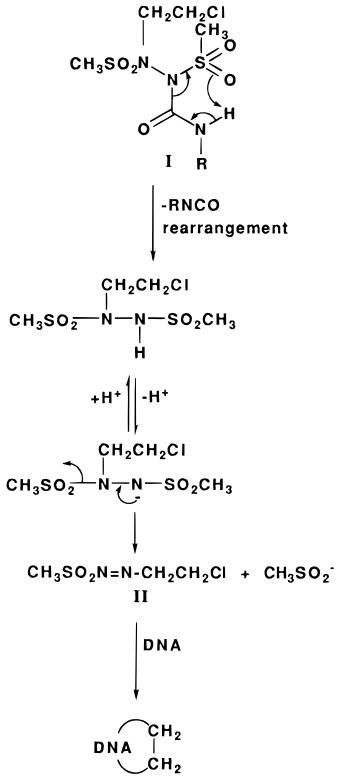
devoid of hydroxyethylating activity, and (d) were free from structural features that would make them highly prone to metabolic inactivation. We concluded that 2-(aminocarbonyl)-1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazines (**I**) would satisfy the above conditions for the following reasons: (a) In vivo decomposition of compounds **I** would result in the formation of a chloroethylating species and a carbamoylating agent as shown below. The loss of the alkyl isocyanate from **I** may occur via a six-membered cyclic transition state intermediate resulting in the formation of 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine. Base-catalyzed elimination of the latter will give the putative alkylating species **II**.[29]

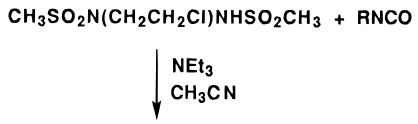


(b) At least three classes of prodrugs of species **II**, i.e., 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine,[30] 1-(2-chloroethyl)-1,2,2-tris(methylsulfonyl)hydrazine,[30] and 1-acyl-1,2-bis(methylsulfonyl)-2-(2-chloroethyl)hydrazine,[31] with potent antitumor activity have been identified. (c) The formation of a 4,5-dihydro-1,2,3-oxadiazole intermediate would be prevented by the absence of an *N*-nitroso moiety. This, in turn, would be expected to prevent the formation of a 2-hydroxyethylating agent. The absence of an *N*-nitroso group would also make compounds **I** less prone to metabolic inactivation.

This paper reports the synthesis of 2-(aminocarbonyl)-1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazines (**I**) and their evaluation for antineoplastic activity against the L1210 leukemia in mice. A representative agent of this class has also been screened against several other transplanted murine and human tumors.

Chemistry

2-(Aminocarbonyl)-1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazines (**I**) were synthesized by reacting 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine with the appropriate isocyanate in dry acetonitrile in the presence of triethylamine as shown below.



I

III. R = $-CH_2CH_2Cl$
IV. R = $-CH_3$
V. R = $-CH_2CH{=}CH_2$
VI. R = $-CH_2CH_2CH_2Cl$
VII. R = $-CH_2COOC_2H_5$
VIII. R = $-CH(CH_3)COOC_2H_5$
IX. R = $-CH(CH_2C_6H_5)COOC_2H_5$

Antitumor Activity

The tumor-inhibitory properties of compounds **III**−**IX** were determined in initial tests by measuring their effects on the survival time of mice bearing the intraperitoneally (ip) implanted L1210 leukemia; the results of these tests are summarized in Table 1. With the exception of compound **VI**, all of the agents synthesized produced "cures" (defined as symptom-free 60 days post-tumor implant) in 100% of mice bearing the L1210 leukemia at one or more of the dosage levels examined following ip administration. It is conceivable that compound **VI** failed to do so only because it was not evaluated at daily dosage levels greater than 15 mg/kg given for 6 consecutive days. Compound **VI** did, however, produce a partial cure rate of tumor-bearing mice at the highest dosage level examined. Compounds **III** and **IV** appeared to have much better therapeutic potential than the amino acid ester derivatives, i.e., compounds **VII**, **VIII**, and **IX**. Thus, the methylurea derivative (**IV**) produced a 40% cure rate of tumor-bearing mice at 5 mg/kg administered for 6 consecutive days with no body weight loss. This agent also cured 100% of mice bearing the L1210 leukemia at 10 and 15 mg/kg × 6 with less than 6% weight loss. The (2-chloroethyl)urea derivative (**III**), which can be regarded as a structural analog of BCNU, cured 80−100% of leukemic mice at 10−20 mg/kg × 6, although at the highest dose examined, i.e., 20 mg/kg, it appeared to be somewhat toxic, as evidenced by a 10.4% loss in body weight. The allylurea derivative (**V**) was also highly efficacious against this tumor, curing 100% of mice receiving a daily dosage of 15 mg/kg given for 6 consecutive days. The amino acid ester derivatives **VII**−**IX**, in general, appeared to be considerably less potent than compounds **III**−**V** requiring daily dosage levels in the range of 25−100 mg/kg to achieve optimum cure rates, and early deaths of treated mice occurred at higher doses in each case.

One of the most active and potent compounds in the series, compound **III**, was also evaluated against several other transplanted tumors (Table 2). When administered at the highest dose examined, i.e., three ip doses of 50 mg/kg given at 4 day intervals in the ip-implanted M109 lung carcinoma model, this compound produced

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

compd	daily dose, mg/kg[a]	av Δwt, %[b]	%T/C[c]	% 60-day survivors
III	10	−4.7		100
	15	−4.0	216	80
	20	−10.4	239	80
IV	5	+9.9	234	40
	10	−5.6		100
	15	−2.1		100
V	5	−1.6	184	20
	10	−2.1	394	40
	15	−2.9		100
VI	5	−2.8	111	
	10	−5.6	187	
	15	−8.8	192	20
VII	5	−1.5	151	
	10	−1.4	202	20
	15	−0.5	202	
	20	−3.7	191	20
	25	−0.5		100
	50	−2.5	138	80
	75	−1.5	119	60
	100	−4.0	115	
VIII	5	−2.0	170	
	10	−1.4	178	20
	15	−0.5	185	
	20	−1.5	227	
	25	−2.5	239	60
	50	−3.3		100
	75	−2.5	125	80
	100	−2.5	118	
IX	5	−2.5	147	
	10	−0.5	165	
	15	−1.9	160	
	20	−1.9	174	
	25	−0.9	177	
	50	−2.0	225	60
	100	−2.0		100
	150	−5.0	169	20

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

a %T/C of 267. In the same system, but in a different experiment, the acetyl derivative (**X**) produced a comparable %T/C of 231 at the highest dosage level examined (60 mg/kg per injection), when the drug was administered ip using the same schedule.[31]

$CH_3SO_2N(CH_2CH_2Cl)N(COCH_3)SO_2CH_3$

X

Compound **III** was also evaluated against the M109 lung carcinoma implanted subcutaneously (sc). In the initial test using this model, a dose of 50 mg/kg per injection of this compound was administered intravenously (iv) in 10% dimethyl sulfoxide (DMSO) in saline every fourth day for a total of three injections. While the maximum %T/C achieved (115) was not considered to be an active result, a meaningful delay in tumor growth ($T - C$) of 8.3 days was observed under these conditions. Mitomycin C, used as a reference drug, produced a maximum %T/C of 103 and a delay in tumor growth of 10 days. A subsequent evaluation of compound **III** was performed using four different doses on a slightly different schedule, i.e., 24, 32, 48, and 64 mg/kg administered every third day for four total injections, and two vehicles, 10% DMSO in saline and 100% DMSO. When administered in 10% DMSO in saline, compound **III** produced a maximum %T/C of 143 and a maximum delay in tumor growth of 9.3 days at 24 and 32 mg/kg per injection, respectively; the next higher dose evaluated, 48 mg/kg per injection, was excessively lethal. At the highest level evaluated, 64 mg/kg per injection, made possible by the use of 100% DMSO as the vehicle, compound **III** achieved a maximum %T/C of 145 and a delay in tumor growth of 17.8 days, without causing any treatment-associated lethalities. The latter antitumor effect was statistically superior ($p < 0.01$) to the best $T - C$ value achieved with this compound in 10% DMSO in saline. Cyclophosphamide and mitomycin C were included as reference drugs in the last experiment. The former compound produced a maximum %T/C of 143 and a delay in tumor growth of 8.8 days, while mitomycin C produced a maximum %T/C of 134 and a $T - C$ value of 9.3 days. As reported earlier, compound **X** achieved a maximum %T/C of 136 and a maximum $T - C$ value of 14.5 days against this tumor.[31]

Table 2. Summary of Optimal Antitumor Effects of 1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[(2-chloroethyl)-amino]carbonyl]hydrazine (**III**) on M109, M5076, and LX-1 Tumors

tumor, site	treatment schedule, route	optimum effective dose, mg/kg per injection	antitumor activity
M109, ip	q4d×3; d.1;[a] ip	50[b,c]	267[d]
M109, sc	q4d×3; d.1;[a] iv	50[c]	(a)[e] 115[d] [8.3][f]
	q3d×4; d.1;[a] iv	24[32][c,g]	(b)[e] 143[d] [9.3][f]
		64[h]	(b)[e] 145[d] [17.8][f]
M5076, sc	q2d×5; d.1;[a] iv	48[h]	>157[d] (6/8)[i]
LX-1, sc	q2d×5; d.6;[a] iv	40[h]	[14.5][f]

[a] Day treatment initiated. [b] Highest dose tested. [c] Administered in 10% DMSO in saline. [d] %T/C = median survival time of treated/control mice × 100. [e] Each letter (a, b) signifies a different experiment. [f] Primary tumor growth inhibition ($T - C$ value) determined by calculating the relative median times for treated (T) and control untreated (C) mice to grow tumors of a 0.5 g size for the LX-1 carcinoma or a 1 g size for the M109 carcinoma. [g] Dose in brackets producing the maximum $T - C$ obtained. [h] Administered in 100% DMSO. [i] Number of cures/number of treated animals.

Compound **III** was also evaluated against the M5076 reticulum cell sarcoma implanted sc. When administered iv at a level of 48 mg/kg per injection in 100% DMSO every other day for five days, compound **III** cured six out of eight mice and consequently, no median time ($T - C$ value) to reach 1 g size tumors was expressed for this group. Tumor growth in mice receiving only 100% DMSO was indistinguishable from that of untreated control animals. 1-(2-Chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (MeCCNU) and BCNU were included in this experiment for comparison. The former achieved a maximum %T/C of 128 and a delay in tumor growth of 33.5 days at 16 mg/kg per injection administered iv every fourth day for three total injections, while BCNU, administered iv on the same treatment schedule, produced a %T/C in excess of 157, with two out of eight cures, and a $T - C$ of >62 days. Since compound **X**, a chloroethylating agent with no carbamoylating activity, produced a growth delay of only 20.8 days with no cures against this tumor,[31] it is possible that the generation of an isocyanate intermediate contributes to the antineoplastic properties of chloroethylating agents against the M5076 sarcoma.

The human lung tumor, LX-1, xenografted sc in athymic mice, was also used to examine the antineo-

Table 3. Antitumor Activity of 1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[(2-chloroethyl)amino]carbonyl]hydrazine (**III**) and 1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl]hydrazine (**IV**) against sc B16F10 Melanoma

compound	treatment schedule, route	optimum effective dose, mg/kg per injection	[$T - C$, days]
III	qd×6; d.10;[a] ip	20[b]	(a)[c] 11.0
	qd×6; d.12;[a] ip	20[b]	(b)[c] 5.5
		30[b]	(b)[c] 13.5
IV	qd×6; d.10;[a] ip	10[b]	(a)[c] 5.0
		20[b]	(a)[c] 15.5
	qd×6; d.12;[a] ip	20[b]	(b)[c] 10.0
		30[b]	(b)[c] 25.5

[a] Day treatment initiated. [b] Administered in 100% DMSO. [c] Each letter (a, b) signifies a different experiment.

plastic potential of compound **III**. Treatment was initiated on day 6 postimplant when the median weight of the tumors was approximately 100 mg. A dose of 40 mg/kg per injection of compound **III** administered iv in 100% DMSO on an every other day schedule for a total of five injections was optimal; this regimen produced a median delay of 14.5 days in the growth of this tumor to a target size of 0.5 g. This level of activity, 1.6 $\log_{10}$ cell kill (LCK), compared favorably with that obtained with BCNU in the same experiment, which produced a $T - C$ of 11.8 days (1.3 LCK) at the optimum dosage of 20 mg/kg per injection when administered iv every fourth day for a total of three injections.

In addition, both compound **III**, which was conceived as a structural analog of BCNU, and compound **IV**, which emerged as one of the best agents in terms of activity and therapeutic index in the L1210 screen, were evaluated in 100% DMSO against the B16F10 melanoma implanted intradermally (id) in mice (Table 3). In an initial experiment, compound **IV** produced a $T - C$ of 15.5 days at a daily dosage level of 20 mg/kg administered once daily for six consecutive days beginning on day 10 postimplant. In the same experiment, using the same treatment schedule, a growth delay of 11 days was obtained with compound **III**. In the second experiment, when the daily dose of compound **IV** was increased to 30 mg/kg, a more substantial growth delay of 25.5 days was achieved, whereas compound **III** at the same daily dose of 30 mg/kg was less active, with the $T - C$ value obtained being 13.5 days.

In summary, 2-(aminocarbonyl)-1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazines were highly active against the L1210 leukemia in mice. A representative agent of this class, compound **III**, was found to have substantial activity in several more stringent distal site tumor models that was better than or equal to some of the clinically active alkylating agents used for comparison in these assays. Furthermore, a comparison of compounds **III** and **IV** against the B16F10 melanoma demonstrated that the aminocarbonyl substituent influenced the degree of antineoplastic activity attainable. These observations make compounds **III** and **IV** and other agents of this class potential candidates for clinical development. Experiments are underway to determine the mechanism(s) of activation of these compounds, with a view to arriving at meaningful correlations between structure and activity and/or toxicity.

Experimental Section

Synthesis. Melting points were determined in capillary tubes on a Thomas-Hoover melting point apparatus and are uncorrected. ^{1}H NMR 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.

1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[(2-chloroethyl)amino]carbonyl]hydrazine (III). To a stirred solution of 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine[30] (2.5 g, 0.010 mol) and 2-chloroethyl isocyanate (1.2 g, 0.011 mol) in dry acetonitrile (100 mL) was added triethylamine (1.1 g, 0.011 mol) at room temperature. After an additional 10 min, the reaction mixture was evaporated to dryness in vacuo. The residue was triturated twice with 15 mL quantities of petroleum ether, and the petroleum ether layer was discarded each time. The residue was then taken up in ethyl acetate (150 mL) and washed with dilute hydrochloric acid (3 × 15 mL). The ethyl acetate layer was dried over anhydrous magnesium sulfate and filtered. Upon evaporation of the solvent, a semisolid residue was obtained which, upon trituration with absolute ethanol, gave a white solid. Recrystallization from ethanol afforded 1.5 g (42.2%) of the title compound: mp 96−97.5 °C; ^{1}H NMR (acetone-d_6) δ 7.0 (br, 1 H, N*H*), 3.7−4.2 (m, 4 H, $SO_2NCH_2CH_2Cl$), 3.5−3.7 (m, 4 H, $CONHCH_2CH_2Cl$), 3.5 and 3.3 (2 s, 6 H, 2 CH_3). Anal. ($C_7H_{15}Cl_2N_3O_5S_2$) C, H, N.

The following compounds were prepared using procedures similar to the one described for compound **III**.

1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl]hydrazine (IV). Compound **IV** was recrystallized from ethanol: yield 42.4%; mp 146−147.5 °C; ^{1}H NMR (acetone-d_6) δ 6.7 (br, 1 H, N*H*), 3.7−4.2 (m, 4 H, CH_2CH_2Cl), 3.5 and 3.3 (2 s, 6 H, 2 CH_3), 2.9 (d, 3 H, NCH_3). Anal. ($C_6H_{14}ClN_3O_5S_2$) C, H, N.

2-[(Allylamino)carbonyl]-1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine (V). Compound **V** was recrystallized from ethanol: yield 42.2%; mp 105−106 °C; ^{1}H NMR (acetone-d_6) δ 6.9 (br, 1 H, N*H*), 5.6−6.1 (m, 1 H, CH=C), 5.4, 5.2 and 5.1 (3 d, 2 H, C=CH_2), 3.7−4.2 (m, 6 H, $NHCH_2$ and CH_2CH_2-Cl), 3.5 and 3.3 (2 s, 6 H, 2 CH_3). Anal. ($C_8H_{16}ClN_3O_5S_2$) C, H, N.

1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[(3-chloropropyl)amino]carbonyl]hydrazine (VI). Compound **VI** was recrystallized from ethanol: yield 35.2%; mp 85−86 °C; ^{1}H NMR (acetone-d_6) δ 6.8 (br, 1 H, N*H*), 3.7−4.2 (m, 4H, $SO_2NCH_2CH_2Cl$), 3.4−3.8 (m, 6 H, $CH_2CH_2CH_2Cl$), 3.5 and 3.3 (2 s, 6 H, 2 CH_3). Anal. ($C_8H_{17}Cl_2N_3O_5S_2$) C, H, N.

1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[[(ethoxycarbonyl)methyl]amino]carbonyl]hydrazine (VII). Compound **VII** was recrystallized from ethanol: yield 42.2%; mp 121−122 °C; ^{1}H NMR (acetone-d_6) δ 7.1 (br, 1 H, N*H*), 3.7−4.4 (m, 8 H, OCH_2, $NHCH_2$, and CH_2CH_2Cl), 3.5 and 3.3 (2 s, 6 H, 2 CH_3), 1.2 (t, 3 H, CCH_3). Anal. ($C_9H_{18}ClN_3O_7S_2$) C, H, N.

1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[[1-(ethoxycarbonyl)ethyl]amino]carbonyl]hydrazine (VIII). Compound **VIII** was recrystallized from ethanol: yield 28.0%; mp 111−112 °C; ^{1}H NMR (acetone-d_6) δ 6.9 (br, 1 H, N*H*), 3.7−4.6 (m, 7 H, OCH_2, $NHCH$, and CH_2CH_2Cl), 3.5 and 3.3 (2 s, 6 H, 2 CH_3), 1.4 (d, 3 H, $CHCH_3$), 1.2 (t, 3 H, CH_2CH_3). Anal. ($C_{10}H_{20}ClN_3O_7S_2$) C, H, N.

1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[[1-(ethoxycarbonyl)phenylethyl]amino]carbonyl]hydrazine (IX). Compound **IX** was recrystallized from ethanol−petroleum ether: yield 12.8%; mp 106−107 °C; ^{1}H NMR (acetone-d_6) δ 7.1−7.3 (m, 5 H, C_6H_5), 6.8 (br, 1 H, N*H*), 4.6 (m, 1 H, $NHCH$), 3.6−4.3 (m, 6 H, OCH_2 and CH_2CH_2Cl), 3.5 (s, 3 H, CH_3SO_2), 3.0−3.3 (s, m, 5 H, $CH_2C_6H_5$, CH_3), 1.2 (t, 3 H, CH_2CH_3). Anal. ($C_{16}H_{24}ClN_3O_7S_2$) C, H; N: calcd, 8.94; found, 8.10.

Antitumor Activity. Leukemia L1210 cells were obtained from the Frederick Cancer Research Facility, Division of Cancer Treatment Tumor Repository of the National Cancer Institute, and were 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 1600g. 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% 200 mM L-glutamine and once again maintained in culture. To assay for antineoplastic activity, 0.1 mL of the cell suspension containing 10^5 L1210 leukemia cells was injected ip into each recipient mouse. Test compounds were administered over a wide range of dosage levels, beginning 24 h after tumor implantation, and continued once daily for 6 consecutive days. All drugs were administered ip 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.

B16F10 melanoma cells were grown in vitro as monolayers in minimum essential medium with Hank's salts supplemented with 10% fetal bovine serum and 1% 200 mM L-glutamine. Solid tumors were produced in C57BL/6 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 B16F10 cells/mL from freshly trypsinized cultures. After 10−12 days, animals bearing approximately 100 mm^3 tumors were treated ip with compound **III** or **IV** dissolved in 100% DMSO for 6 consecutive days, and tumor volumes were measured on alternate days until reaching 1000 mm^3.

The M5076 reticulum cell sarcoma was passaged biweekly by sc transfer of tumor fragments into C57BL/6 mice, and the M109 lung carcinoma was similarly passaged in BALB/c mice. The LX-1 human lung carcinoma was passaged sc every 2−3 weeks in BALB/c background athymic (nu/nu) mice. In these systems, compound **III** was dissolved in (a) 100% DMSO and administered by iv injection in a fixed volume of 10 μL or (b) DMSO diluted with saline to a final concentration of 10% DMSO and administered iv in a volume of 0.01 mL/g of body weight. 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; BCNU and MeCCNU were dissolved in ethanol and diluted 1:9 (v/v) with water prior to administration.

Five mice per group were employed in experiments with the B16F10 melanoma and eight mice per group with the M5076 sarcoma, the M109 carcinoma, and the LX-1 carcinoma. A minimum of two dose levels per compound were included in each evaluation, and drug therapy was initiated 24 h after tumor implantation for M5076 sarcoma and M109 carcinoma. In the LX-1 experiment, tumor-bearing mice were selected and sorted into treatment and control groups on day 6 post-tumor implant such that all tumor weights ranged from 50−100 mg, and median tumor weights per group were reasonably similar. Therapeutic results are presented in terms of (a) increases in lifespan 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 (i.e., $T - C$ values) determined by calculating the relative median times for treated (T) and control untreated (C) mice to grow tumors of a 0.5 g size for the LX-1 carcinoma or a 1 g size for the murine neoplasms. Tumor weights were interchangeable with tumor size on the basis of 1 $mm^3 = 1$ mg. The activity criterion for increased lifespan was a T/C of ≥125%. The activity criterion for tumor inhibition was a delay in tumor growth consistent with one LCK. The absolute $T - C$ value needed to attain this level of efficacy varied from experiment to experiment and depended upon the tumor volume doubling time of the control mice in each study. Treated mice dying prior to day 10 in the ip M109 experiment, or dying before their tumors achieved 0.5 g for the LX-1 carcinoma or 1 g in size for all other sc tumor models, 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. Statistical evaluations of data were performed using Gehan's generalized Wilcoxan test.[32]

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