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Antitumor activity and cross-resistance of carmethizole hydrochloride in preclinical models in mice*

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Summary. Carmethizole hydrochloride [1-methyl-2-methylthio-4,5-bis(hydroxymethyl)imidazole-4′, 5′-bis(*N*-methylcarbamate)hydrochloride, NSC 602668; hereafter called carmethizole] is a new antitumor drug that has shown relatively broad activity in initial evaluations against several murine tumors and human tumor xenografts in vivo. The present studies were designed to address questions about carmethizole's activity against established disease, its activity on different treatment schedules, and the extent of its cross-resistance with established drugs. Human MX-1 mammary carcinoma, human NCI-H82 small-cell lung carcinoma, and human LOX amelanotic melanoma xenografts in athymic mice were used to determine the drug's activity against established disease; the NCI-H82 lung-tumor xenograft in athymic mice was used to explore its schedule dependence; and a series of drug-resistant murine leukemias provided an in vivo cross-resistance profile. When injected i.p., carmethizole exhibited antitumor activity against advanced-stage s.c. MX-1 mammary, s.c. NCI-H82 lung, and i.p. LOX melanoma xenografts and was as effective against established disease (MX-1 and LOX) as it was against early-stage disease (no data are available for early-stage NCI-H82). The therapeutic effect of carmethizole was not route-dependent, as was evidenced by the similar delays observed in tumor growth following i.p. and i.v. administration. The use of a split-dose schedule on a single day instead of one bolus injection yielded an increase in the total dose delivered, resulting in an increased delay in tumor growth. Murine leukemias resistant to vincristine (VCR), amsacrine (AMSA), or methotrexate (MTX) were not cross-resistant to carmethizole. However, murine leukemias resistant to doxorubicin (ADR), melphalan (L-PAM), cisplatin (DDPt), 1-β-D-arabinofuranosylcytosine (ara-C), and 5-fluorouracil (5-FU) were cross-resistant to carmethizole, suggesting that patients who have previously been treated with any of these agents might be less likely to respond to carmethizole than those who have had no opportunity to develop resistance to any of these compounds. We anticipate that the information derived from these studies may be useful in the design of clinical trials of carmethizole and may stimulate additional basic research on the mechanism of action of this new agent.

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Introduction

Carmethizole hydrochloride [1-methyl-2-methylthio-4,5-bis(hydroxymethyl)imidazole-4′,5′-bis(*N*-methylcarbamate) hydrochloride, NSC 602668; hereafter called carmethizole], a congener of pyrrolizine biscarbamate (NSC 278214), was developed to provide a compound with greater aqueous solubility and stability than pyrrolizine biscarbamate [2]. Carmethizole has exhibited antitumor activity after i.p. administration in preclinical models in vivo, including i.p. P388 and i.p. L1210 leukemias, i.p. M5076 sarcoma, i.p. human LOX melanoma xenografts, and MX-1 human mammary carcinoma in the subrenal capsule assay in athymic mice [2]. In these efficacy studies, treatment was started at 1 day after tumor implantation. Studies of carmethizole in more advanced-stage, solid tumor models have recently been reported [10].

The chemical breakdown of carmethizole in vitro and its pharmacokinetics in the mouse and the beagle dog have been examined by Brodfuehrer et al. [4]. Carmethizole degraded within 24 h at 37° C to carmethizole diol in 0.9% sodium chloride or phosphate buffer. Its half-life in whole blood and plasma was about 1 h at 37° C in vitro. Protein binding was equivalent in human and mouse plasma. The compound was rapidly eliminated from the plasma of mice in vivo after i.v. bolus administration, but it was more slowly eliminated by dogs (elimination half-lives, about 12 and 40 min, respectively). Carmethizole diol was the major

product formed in vivo, possibly by spontaneous degradation.

The purpose of the present study was to address questions raised by the results cited above. It was important that the activity of carmethizole be explored against advanced-stage, solid tumors. Concurrently, it was possible to confirm and extend previously reported results. The schedule dependence of carmethizole treatment was evaluated in light of its rapid elimination in mice. Finally, a cross-resistance profile was developed using specific drug-resistant murine leukemias in vivo. Preliminary accounts of these findings have been reported elsewhere [1, 20].

Materials and methods

Drugs. Carmethizole was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program (DTP), Division of Cancer Treatment (DCT), National Cancer Institute (NCI). During the studies, carmethizole was prepared in either distilled water or saline just prior to its use and was injected within 30 min. The antitumor drugs used in the cross-resistance studies were also provided by the Drug Synthesis and Chemistry Branch.

Mice and tumors. Mice obtained from various commercial suppliers were housed either in open-top, stainless-steel cages (immunocompetent mice) or in Microisolator cages (Lab Products, Inc., Maywood, N.J.; athymic mice). Immunocompetent and athymic mice were housed in separate barrier facilities. Mice were allowed access to commercial mouse food and water ad libitum. Blood samples obtained from randomly selected (non-tumor-bearing) mice were tested every 3 months for antibodies to selected viruses.

Tumor lines (murine P388/0 and L1210/0 leukemias, human MX-1 mammary carcinoma, human LOX melanoma, and human NCI-H82 small-cell lung carcinoma) were obtained from the DTP Tumor Repository, DCT, NCI. The human amelanotic melanoma designated LOX has been used as a preclinical model for antitumor drug evaluation [18]. LOX grows i.p. as a combination of solid tumor attached to abdominal organs and peritoneum and monodispersed cells in ascitic fluid. Isolated, counted cells (10^6) were inoculated i.p. into recipient mice for tumor propagation. The human small-cell lung tumor designated NCI-H82 was established and characterized by Carney et al. [5]. The NCI-H82 tumor was inoculated s.c. (30- to 60-mg fragments) into recipient mice for tumor propagation. Murine leukemias P388/0 and L1210/0 and human MX-1 mammary carcinoma were propagated according to standard NCI protocols [9]. Drug-resistant lines of P388 and L1210 were developed at Southern Research Institute [17]. Only tumor lines that tested negative for antibodies to selected viruses were used.

Evaluation of antitumor activity. For studies using the murine leukemias, BALB/c × DBA/2 F_1 (hereafter called $CD2F_1$) mice were implanted i.p. with either 10^6 P388 cells or 10^5 L1210 cells. For studies using the human tumor xenografts, NCr-*nu* athymic mice were implanted either i.p. with 10^6 LOX melanoma cells or s.c. with tumor fragments (30–60 mg) of MX-1 mammary carcinoma or NCI-H82 small-cell lung-carcinoma xenografts. The date of tumor implantation was designated day 0. Carmethizole was given according to the schedules and routes listed in the tables and figures. In each experiment, carmethizole was evaluated at several dose levels (ranging from toxic to nontoxic), with each dose being given to 6–10 mice. All doses were given to mice on the basis of individual body weight except for the cross-resistance studies, in which all doses were given to groups of mice on the basis of their average body weight. Tumor-bearing control mice (20/experiment) were not treated with diluent. Mice were observed for life span and/or tumor growth.

The antitumor activity of carmethizole was evaluated in mice bearing advanced-stage MX-1 mammary carcinoma, LOX melanoma, or NCI-H82 small-cell lung-carcinoma xenografts. For the studies using LOX melanoma xenografts, an internal tumor titration, which consisted of 5 groups of mice (10/group) bearing initial tumor burdens ranging from 10^7 to 10^3 cells (by serial dilution), was included to permit an assessment of therapeutic response.

The effects of schedule and route of administration on the antitumor activity of carmethizole were studied in mice bearing s.c. implanted, advanced-stage NCI-H82 lung xenografts. For the i.p. and i.v. studies, treatment with carmethizole was initiated on day 14 and day 15, respectively. For each schedule, carmethizole was given over a range of doses (ratio of consecutive doses, 0.67). The injection volume per mouse was 0.1 ml/10 g body weight for both i.p. and i.v. treatments.

Evaluation of drug-resistant P388 and L1210 sublines for cross-resistance to carmethizole was conducted as previously described by Harrison et al. [11]. In each experiment, groups of tumor-bearing mice were treated with a range of doses of the appropriate drug to confirm the resistance of the tumor; in addition, a drug-resistant leukemia was compared directly with the drug-sensitive parental leukemia from which the resistant line was derived, and the parallel groups of mice were identically treated with a single drug preparation. Each of these internally controlled experiments included 6 groups of mice (10/group) bearing initial tumor burdens ranging from 10^7 to 10^2 cells (by serial dilution).

Quantitation of antitumor activity. For L1210, P388, and LOX tumors, antitumor activity was assessed on the basis of the median percentage of increase in life span (%ILS) and the net $\log_{10}$ cell kill. Calculations of net $\log_{10}$ cell kill were made from the tumor-doubling time that was determined from an internal tumor titration consisting of implants from serial 10-fold dilutions [16]. Each experiment contained a titration. Long-term (45- to 60-day) survivors were excluded from calculations of %ILS and tumor cell kill. To assess the tumor-cell kill at the end of treatment, the difference in survival duration between treated and control groups was adjusted to account for possible regrowth of tumor-cell populations between individual treatments [14]. The net $\log_{10}$ cell kill was calculated as follows:

$$\text{Net } \log_{10} \text{ cell kill} = \frac{(T - C) - (\text{duration of treatment in days})}{3.32 \times T_d},$$

where $(T - C)$ represents the difference in the median day of death between the treated (T) and the control (C) groups and T_d represents the mean tumor-doubling time (days) calculated from a log-linear least squares fit of the implant sizes and the median days of death of the titration groups.

For the MX-1 mammary and NCI-H82 lung-carcinoma xenografts, antitumor activity was assessed on the basis of net $\log_{10}$ cell kill and delay in tumor growth (T – C). The delay in tumor growth represents the unweighted average of the differences in the median times (days) postimplantation for the treated (T) and control (C) groups to attain each of two evaluation sizes (1000 and 1500 mg). Drug deaths, tumor-free survivors, and animals whose tumors failed to attain the evaluation sizes were excluded. Tumors were measured (two perpendicular diameters) with calipers twice weekly.

Results

MX-1 mammary carcinoma xenografts

Previous studies have shown that carmethizole given at 1 day after tumor implantation is active against MX-1 mammary carcinoma in the subrenal capsule assay in athymic mice [2]. A study was conducted to determine whether the compound would also be effective against established disease. Treatment of the s.c. implanted tumor staged at approximately 500 mg with an optimal ($\leq LD_{10}$) dose of carmethizole (115 mg/kg) given i.p. on days 11, 15, and 19 resulted in 100% regressions as determined on day 35 postimplantation, with no tumor recurrence being observed on day 63 (Fig. 1). Elliott and co-workers [10] recently reported similar results. Treatment of the s.c. im-

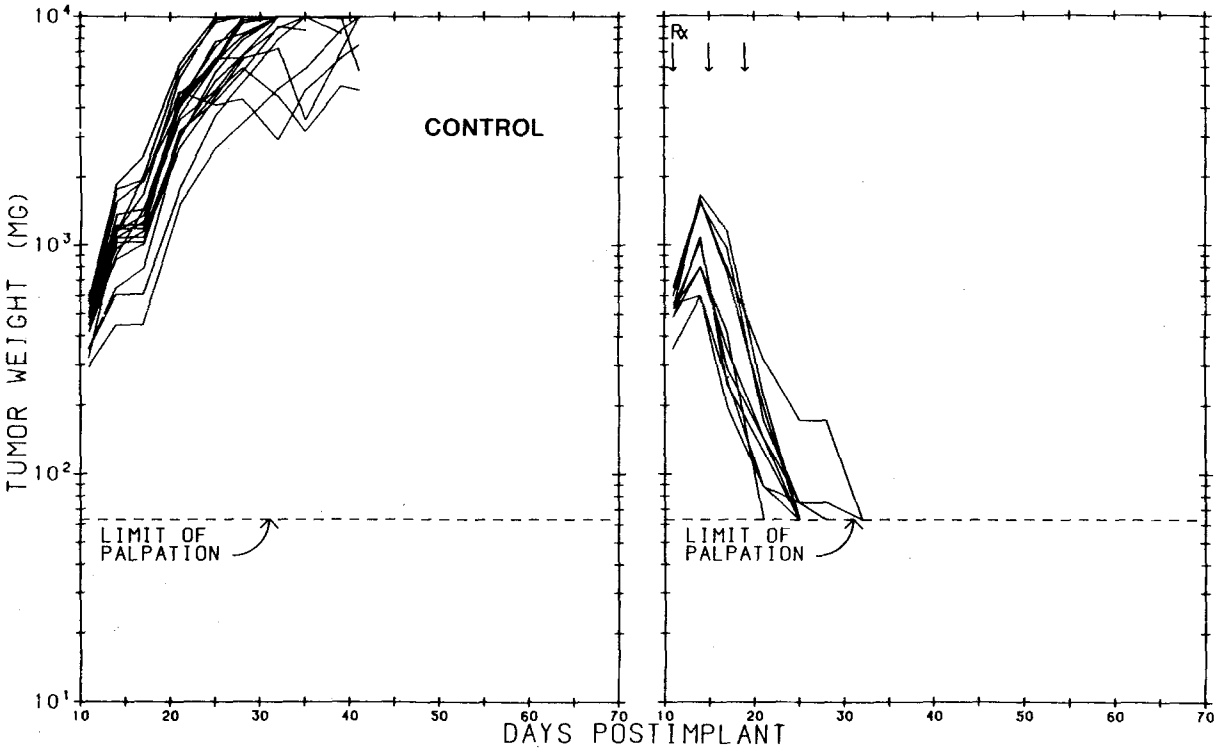

Fig. 1. Response of advanced-stage human MX-1 mammary carcinoma xenografts to treatment with carmethizole. NCr-*nu* mice were implanted s.c. with fragments of MX-1 tumor on day 0. Carmethizole was injected i.p. at an optimal dose of 115 mg/kg on days 11, 15, and 19

Table 1. Antitumor activity of carmethizole against advanced-stage, i.p. implanted LOX melanoma xenografts

Dose (mg/kg)	Median % ILS	Approx. $\log_{10}$ change in tumor burden[a]	60-Day survivors/ total
172	–28	Toxic	1/6
115	~	– (–7.6)	6/6
76	+129	–3.4	0/6
51	+69	–1.2 (–6.9)	2/6
34	+45	–0.3	0/6

NCr-*nu* mice were implanted i.p. with 10^6 LOX cells on day 0. Drug was injected i.p. on days 5, 9, and 13

[a] $\log_{10}$ change in the viable tumor-cell population at the end of therapy as compared with that at the start of therapy, based on the median day of death among the animals that died. Figures in parentheses are based on the percentage of survivors

planted MX-1 tumor with 160 mg/kg carmethizole given i.p. on days 13, 17, and 21 resulted in 7/10 tumor-free survivors on day 60.

LOX human amelanotic melanoma xenografts

Previous studies have shown that carmethizole given at 1 day after tumor implantation is active against i.p. implanted LOX melanoma [2]. A study was conducted to determine whether the compound would also be effective against established disease with its accompanying metastases. Treatment of the i.p. implanted tumor at a burden of approximately 8.0×10^6 cells with an optimal dose of carmethizole (115 mg/kg) given i.p. on days 5, 9, and 13 resulted in 6/6 tumor-free survivors on day 60 (Table 1).

NCI-H82 human lung-carcinoma xenografts

The antitumor activity of carmethizole was also evaluated in mice bearing advanced-stage NCI-H82 lung-carcinoma xenografts. Treatment of the s.c. implanted tumor staged at approximately 330 mg with an optimal dose of carmethizole (115 mg/kg) given i.p. on days 15, 19, and 23 resulted in a delay in tumor growth of 30.5 days and in 2/10 tumor-free survivors on day 64 (Fig. 2).

Since NCI-H82 lung carcinoma was less sensitive to treatment with carmethizole than was MX-1 mammary carcinoma, the effects of schedule and route of administration on the antitumor activity of the drug were studied in mice bearing s.c. implanted, advanced-stage NCI-H82 lung-carcinoma xenografts. Presented in Table 2 are data from two experiments on NCI-H82 lung-tumor xenografts exploring the effect of i.p. and i.v. carmethizole administration, respectively. Both single i.p. and single i.v. injections of the drug at an optimal dose effected a delay in tumor growth of approximately 10 days, corresponding to a reduction in tumor burden of 1 $\log_{10}$ unit. The use of a split-dose schedule on a single day instead of one bolus injection yielded an increase in the total dose delivered, resulting in an increased delay in tumor growth (and in net $\log_{10}$ cell kill). This finding is consistent with the rapid

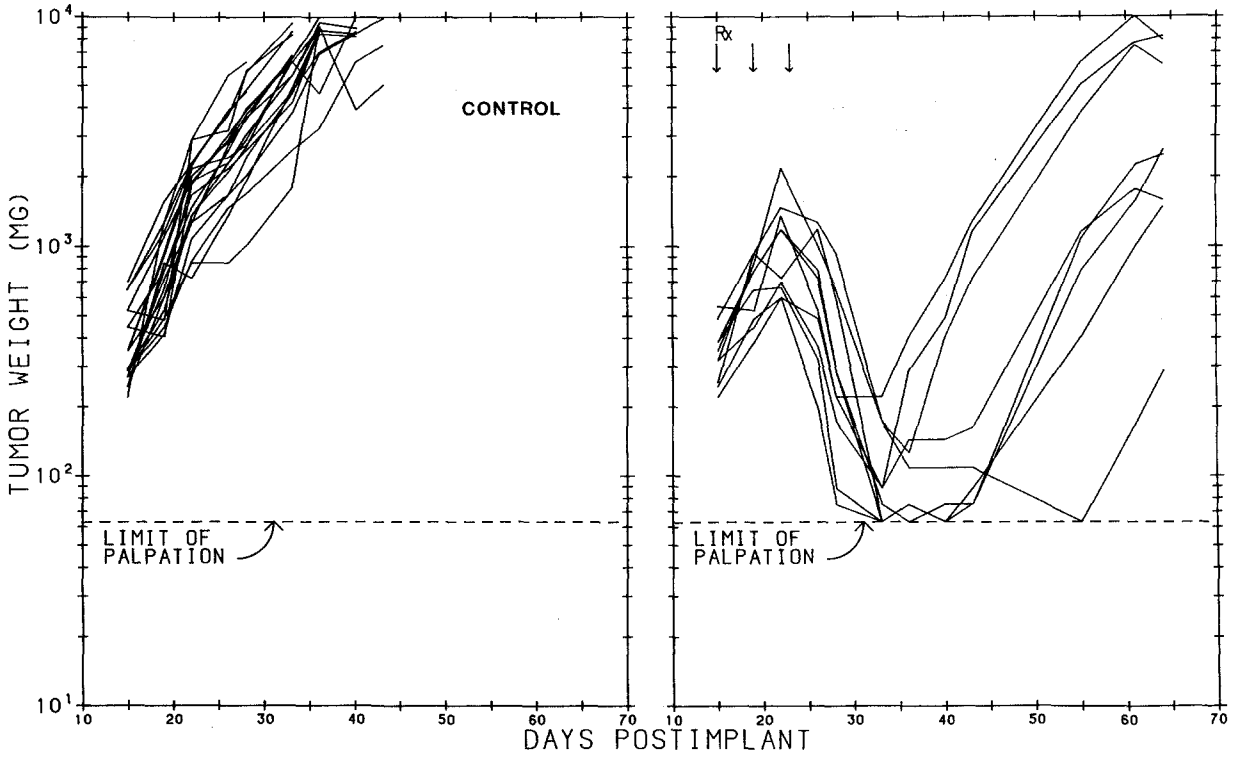

Fig. 2. Response of advanced-stage human NCI-H82 small-cell lung-carcinoma xenografts to treatment with carmethizole. NCr-*nu* mice were implanted s.c. with fragments of NCI-H82 tumor on day 0. Carmethizole was injected i.p. at an optimal dose of 115 mg/kg on days 15, 19, and 23

elimination of the drug from the plasma of mice in vivo after i.v. bolus administration [4]. Three courses of single-bolus treatment given at 4-day intervals increased the antitumor activity observed for i.p. but not i.v. administration. Single i.p. injections on days 14, 18, and 22 effected a reduction of 2.3 $\log_{10}$ units in the tumor burden and resulted in 4/10 tumor-free survivors on day 62. The effect observed for i.p. administration was related to an increased total dose of the drug being tolerated by the mice.

In vivo cross-resistance of drug-resistant murine leukemias

Multiple experiments (Table 3) were conducted, each of which comprised a simultaneous comparison of carmethizole activity in a drug-sensitive and a drug-resistant murine leukemia over a range of doses. If carmethizole effected a cell kill in the drug-resistant line that was >2 $\log_{10}$ units lower than that induced in the drug-sensitive line, the drug-

Table 2. Effects of schedule and route of administration on the antitumor activity of carmethizole against s.c. implanted NCI-H82 lung-tumor xenografts

Schedule[a]	Route	Dose range (mg/kg)	Optimal dose ($\leq LD_{10}$, mg/kg)	Total dose (mg/kg)	T-C[b] (days)	Approx. $\log_{10}$ change in tumor burden[c]	Tumor-free survivors/ total
Day 14 only	i.p.	450 –89	134	134	9.1	–0.9	0/10[d]
q3h×8, Day 14 only		56.3–16.7	25	200	16.0	–1.6	0/10
q4d, Days 14, 18, 22		150 –44.5	100	300	31.2	–2.3	4/10
Day 15 only	i.v.	200 –59.4	89	89	10.4	–1.0	0/9[e]
q3h×4, Day 15 only		75 –22.3	33.4	134	24.5	–2.2	0/10
qd, Days 15–19		90 –26.7	40	200	21.9	–1.6	0/9
q4d, Days 15, 19, 23		150 –44.5	66.7	200	3.8	+0.4	0/9

NCr-*nu* athymic mice that had been inoculated s.c. with fragments of NCI-H82 lung tumor on day 0 were treated with bulk drug dissolved in distilled water. For the i.p. study, tumor size was approximately 250 mg on day 14, whereas for the iv study, tumor size was approximately 290 mg on day 15

[a] qd, daily; q3h×8, every 3 h for a total 8 injections/day

[b] Unweighted average of the differences in the median times postimplantation for the treated (T) and control (C) groups to attain each of two evaluation sizes (1000 and 1500 mg). Drug deaths, tumor-free survivors, and animals whose tumors failed to attain the evaluation size were excluded

[c] $\log_{10}$ change in the viable tumor-cell population at the end of therapy as compared with that at the start of therapy, based on the delay in tumor growth in the treated group relative to the control group. The volume-doubling times in the controls were estimated to be 3.0 and 3.3 days for the i.p. and i.v. studies, respectively

[d] Day 62

[e] Day 72

Table 3. Cross-resistance profile of carmethizole derived from drug-resistant leukemias in vivo

Resistant leukemia	Dose (mg/kg)		Sensitive leukemia		Resistant leukemia[a]		Cross-resistance?
	Range	Optimal ($\leq LD_{10}$)	Median % ILS	Approx. $\log_{10}$ change in tumor burden[b]	Median % ILS	Approx. $\log_{10}$ change in tumor burden[b]	
P388/ADR	172–34	51	+96	–4.0	+63	–1.4	Yes
	80–30	65	+100	–4.8	+55	–1.3	
P388/VCR	80–40	65	+93	–4.5	+158	–6.7	No
	65–40	65	+135	–6.4	+105	–6.9	
P388/AMSA	80–40	50	+82	–3.1	+125	–6.8	No
	65–40	50	+143	–6.6	+110	–6.3 (–6.3)[c]	
P388/L-PAM	115–34	51	+100	–4.3	+11	+1.3	Yes
	80–30	65	+117	–5.2	+13	+1.3	
P388/DDPt	115–34	51	+86	–3.9	+26	+0.2	Yes
	80–30	50	+103	–3.9	+36	–0.6	
P388/MTX	80–40	65	+119	–6.5 (–6.3)[c]	+134	–6.2	No
	65–40	50	+121	–4.6	+95	–2.9 (–6.0)[c]	
P388/ara-C	80–40	50	+87	–4.3	+32	+0.1	Yes
	65–40	50	+171	–6.7	+32	–0.1	
L1210/5-FU	80–40	65	+75	–2.1	+41	–0.2	Marginal
	65–40	65 (LD_{30})	+57	–0.8	+20	+1.7	
		50	+35	+0.6	+27	+1.1	

For the P388 studies, $CD2F^1$ mice were implanted i.p. with 10^6 P388/0 or P388/drug-resistant cells on day 0. For the L1210 studies, $CD2F^1$ mice were implanted i.p. with 10^5 L1210/0 or L1210/5-FU cells on day 0. Carmethizole was administered i.p., days 1–5.

[a] In these studies, the degree of resistance of a drug-resistant subline to the drug used to select the subline in comparison with its parental line was as follows: ADR, 5–6 $\log_{10}$ units; VCR, 6 $\log_{10}$ units; AMSA, 4–6 $\log_{10}$ units; L-PAM, 6 $\log_{10}$ units; DDPt, 6–7 $\log_{10}$ units; MTX, 3 $\log_{10}$ units; ara-C, 6–7 $\log_{10}$ units; 5-FU, 4 $\log_{10}$ units

[b] $\log_{10}$ change in the viable tumor-cell population at the end of therapy as compared with that at the start of therapy, based on the median day of death among the animals that died. Figures in parentheses are based on the percentage of survivors. 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

resistant line was interpreted to be cross-resistant to carmethizole. This result was obtained and confirmed in four drug-resistant P388 leukemias [doxorubicin-resistant (P388/ADR), melphalan-resistant (P388/L-PAM), cisplatin-resistant (P388/DDPt), and arabinofuranosyl cytosine-resistant (P388/ara-C)]. Marginal cross-resistance (whereby the cell kill in the drug-resistant line was approx. 2 $\log_{10}$ units lower than that in the drug-sensitive line) was observed for the 5-fluorouracil-resistant L1210 leukemia (L1210/5-FU). Tumor lines selected for resistance to vincristine (VCR), amsacrine (AMSA), or methotrexate (MTX) were sensitive (i.e., were not cross-resistant) to carmethizole.

Discussion

Structure-activity relationships reported by Anderson et al. [2] have emphasized the importance of substitution that enhances the alkylating characteristics of the imidazole biscarbamates. Agents more active against P388 leukemia have been those that are more amenable to ready displacement of the carbamate groups. Carmethizole was selected from a series of analogs for drug development because of both its superior reactivity for carbamate displacement and its initial antileukemic activity in the P388 screen. The subsequent observations reported by Brodfuehrer et al. [4] on its chemical pharmacodynamics in biological systems are consistent with a reactive alkylating agent, particularly the reactivity of carmethizole with glutathione (GSH).

The observations reported herein provide information that is potentially important to the design of strategies for the optimal clinical use of carmethizole. Although phase I clinical pharmacokinetic data are needed, both the present findings and those of previous pharmacokinetic studies [4] suggest that clinical treatment schedules should use repeated dosing. In the present investigations, carmethizole was as effective against established disease (MX-1 and LOX) as it was against early-stage disease. The activity exhibited by carmethizole against MX-1 and NCI-H82 tumors was comparable to that observed for the alkylating agent L-PAM, whereas the activity of carmethizole against the LOX tumor was superior to that found for L-PAM. The alkylating agents cyclophosphamide and carmustine are generally less effective than carmethizole against these three human tumors (unpublished data) [10]. The in vivo cross-resistance profile reported in the present study suggests that carmethizole may be evaluated in patients who have received prior chemotherapy with AMSA, MTX, and VCR with no more than the usual a priori concern that cross-resistance might be encountered. However, a failure to observe objective responses in patients previously treated with ADR, ara-C, L-PAM, DDPT, or, possibly,

5-FU should be viewed as a possible manifestation of cross-resistance to carmethizole. As always, none of these approaches may be applied clinically without the exercise of caution and concern for the recognized gap between preclinical prediction and clinical validation.

For the four drug-resistant leukemias that exhibited cross-resistance to carmethizole, numerous reports have been published concerning the mechanisms of resistance [3, 6–8, 11–13, 15, 19]. Nevertheless, it remains unclear as to which mechanism(s) was operative in the observed cross-resistance to carmethizole. P388/ADR, P388/DDPt, and P388/L-PAM exhibited increases in intracellular GSH or GSH transferase activity. Because carmethizole is an alkylating agent that has been shown to react with GSH, an increase in intracellular GSH and/or GSH-metabolizing enzymes could confer resistance to carmethizole. We have found that our P388/ara-C leukemia is cross-resistant to a number of other alkylating agents: DDPt, cyclophosphamide, mitomycin C, spirohydantoin mustard, and tetraplatin (unpublished data). Possibly P388/ara-C also possesses an alteration in GSH metabolism that could confer resistance to carmethizole.

Other mechanisms of resistance appear less likely to be responsible for the observed cross-resistance to carmethizole. Whereas a decreased accumulation of drug was common to three of the four drug-resistant leukemias that exhibited cross-resistance to carmethizole, the underlying mechanisms differ. Altered repair of drug-induced damage to DNA may be involved in cross-resistance to carmethizole; however, the mechanisms of repair that are operative in P388/ADR and P388/DDPt appear to be different. The relationship between the alterations in various enzyme activities that lead to ara-C resistance and cross-resistance to carmethizole is unclear. Possibly other mechanisms of resistance are operative in P388/ara-C that are responsible for the observed cross-resistance to carmethizole. Clearly, more information about the mechanisms of resistance of the four drug-resistant leukemias, especially P388/L-PAM and P388/ara-C, would aid in the elucidation of both the cross-resistance to carmethizole and the mechanism(s) of action of the drug.

In summary, the present study provides information concerning the activity of carmethizole against advanced-stage, solid tumors, the effect of the treatment schedule on the therapeutic activity of the drug, and the cross-resistance profile of the drug. We anticipate that this information may be useful in the design of clinical trials of carmethizole and may stimulate additional basic research on the mechanism of action of this new agent.

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