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SENSITIVITY OF ALDEHYDE DEHYDROGENASES IN MURINE TUMOR AND HEMATOPOIETIC PROGENITOR CELLS TO INHIBITION BY CHLORAL HYDRATE AS DETERMINED BY THE ABILITY OF CHLORAL HYDRATE TO POTENTIATE THE CYTOTOXIC ACTION OF MAFOSFAMIDE*

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Abstract—Several murine aldehyde dehydrogenases, most notably AHD-2, are known to catalyze the detoxification of cyclophosphamide, mafosfamide, and other oxazaphosphorines. Thus, cellular sensitivity to these agents decreases as the relevant aldehyde dehydrogenase activity increases, and vice versa. Chloral hydrate is a sedative/hypnotic agent that is sometimes administered to patients being treated with cyclophosphamide. It is known to inhibit some, but not all, aldehyde dehydrogenases. Murine (CFU-S, CFU-GEMM and CFU-Mk) and human (CFU-Mix, CFU-GM, BFU-E and CFU-Mk) hematopoietic progenitor cells, as well as murine oxazaphosphorine-resistant (L1210/OAP and P388/CLA) tumor cells, are known to contain the relevant aldehyde dehydrogenase activity but the identity of the specific enzyme present in the normal cells is unknown and may be different than that, namely AHD-2, present in neoplastic cells. In that event, the potential exists to inhibit the detoxification of the oxazaphosphorines in tumor cells without inhibiting this event in normal cells; the net effect of such a selective inhibition would be to increase the margin of safety of the oxazaphosphorines. In *ex vivo* experiments, chloral hydrate markedly potentiated the antitumor activity of mafosfamide against oxazaphosphorine-resistant L1210/OAP and P388/CLA cells. It did not potentiate the cytotoxic action of mafosfamide against any of the murine or human hematopoietic cells tested, even at concentrations which fully restored the sensitivity of the resistant tumor cell lines to this agent. One explanation for these observations is that hematopoietic progenitor, and the resistant tumor, cells express different relevant aldehyde dehydrogenases and that these aldehyde dehydrogenases differ in their sensitivity to inhibition by chloral hydrate. Consistent with this notion were the observations that AHD-2 was exquisitely sensitive to inhibition by chloral hydrate, whereas two other aldehyde dehydrogenases that also catalyze the detoxification of aldophosphamide, namely AHD-12a,b and AHD-13, were relatively unaffected.

Cyclophosphamide is a prodrug requiring metabolic activation to exert its pharmacological effect [reviewed in Ref. 1]. The parent compound is converted to the primary circulating metabolite, 4-hydroxycyclophosphamide, by the hepatic mixed-function oxidases. 4-Hydroxycyclophosphamide exists in equilibrium with its ring-opened tautomer, aldophosphamide; each, *per se*, is without cytotoxic activity. Aldophosphamide can undergo β-elimination of acrolein thus giving rise to phosphoramide mustard, a cytotoxic metabolite. Alternatively, aldophosphamide can be converted to carboxyphosphamide, a noncytotoxic metabolite that does not give rise to a cytotoxic one. The latter reaction is catalyzed by NAD(P)-linked aldehyde dehydrogenases.

Aldehyde dehydrogenases are virtually ubiquitous in nature. Several are usually found in any given species with each exhibiting a unique substrate preference/specificity, tissue distribution, and sensitivity to inhibitors [2, 3]. Ten different aldehyde dehydrogenases capable of catalyzing the detoxification of aldophosphamide have been identified in mouse tissues [3–5]. The efficiency with which these enzymes catalyze the oxidation of aldophosphamide varies greatly; a cytosolic enzyme, viz. AHD-2,§ was identified as the aldehyde dehydrogenase making the greatest contribution to the hepatic detoxification of aldophosphamide in mice.

Greater expression of the relevant aldehyde dehydrogenase activity in critical normal tissues versus that in some tumor tissues has been suggested as the basis for the relatively favorable margin of safety sometimes exhibited by the oxazaphosphorines

* A description of parts of this investigation has appeared in abstract form [Maki PA and Sladek NE, *Proc Am Assoc Cancer Res* **27**: 391, 1986].

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§ Abbreviations: AHD, mouse aldehyde dehydrogenase; and LC_{50} and LC_{99}, concentrations of drug required to render 50 and 99% of the cells, respectively, incapable of further indefinite proliferation.

[reviewed in Ref. 1]. Thus, the relevant enzyme activity is present in murine and human multipotent and committed hematopoietic progenitor cells [6–10], and in murine intestinal crypt and villus cells [11], but not in a number of neoplastic murine and human cell lines [7, 12]; consequently, the former are, relative to the latter, insensitive to cyclophosphamide and other oxazaphosphorine nitrogen mustards, e.g. mafosfamide and 4-hydroperoxycyclophosphamide, that give rise to 4-hydroxycyclophosphamide and then undergo an identical detoxification reaction [1].

Some murine tumor cells that ordinarily do not express the relevant aldehyde dehydrogenase activity and that are quite sensitive to the oxazaphosphorines, e.g. L1210/0 and P388/0 cells, become insensitive to these agents when they are pressured (by exposure to increasing amounts of an oxazaphosphorine) into expressing the relevant enzyme activity [7, 12–15]. The oxazaphosphorine-resistant counterparts of the aforementioned oxazaphosphorine-sensitive cell lines are known as L1210/OAP and P388/CLA cells, respectively. ADH-2 has been identified as the relevant enzyme in each of these cell lines [11, 12, 16–18].

A number of clinically useful drugs are reported to produce a "disulfiram-like" effect, characterized by nausea, vasodilation, headache, etc., when alcohol is ingested following their administration [3]. In at least some cases, these symptoms are thought to result from the accumulation of acetaldehyde, i.e. the inhibition of aldehyde dehydrogenase-catalyzed oxidation of acetaldehyde to acetic acid. Some of these drugs, e.g. chloramphenicol and moxalactam, are likely to be given to cancer patients being treated with cyclophosphamide. Not known is what the effect of concurrent administration of an oxazaphosphorine and one of the drugs that produce a "disulfiram-like" effect would be on the margin of safety of the oxazaphosphorine but, clearly, the outcome will depend on the relative amount, and sensitivity to the putative inhibitor, of any relevant aldehyde dehydrogenases that may be present in the uneconomic (neoplastic cells) and/or economic (critical normal cells) species. Thus, a decrease in the margin of safety work would be expected if only relevant aldehyde dehydrogenases present in critical normal cells were inhibited. An increase in the margin of safety would be expected if only relevant aldehyde dehydrogenases present in tumor cells were inhibited. No change in the margin of safety would be expected if aldehyde dehydrogenases present in tumor cells and critical normal cells were present in approximately equal amounts and were equally inhibited, although a downward adjustment in dose might be required.

Another drug that is reported to produce a "disulfiram-like" effect is chloral hydrate [19, 20], a sedative/hypnotic first introduced into medicine in 1869 [21]. Chloral hydrate is an analog of acetaldehyde that is not susceptible to oxidation catalyzed by aldehyde dehydrogenases [22, 23] but is a good inhibitor of some, though not all, of them [24–27]. Although chloral hydrate has, to some extent, been replaced by newer sedative/hypnotics, it is still extensively used, and is considered by some to be the hypnotic of choice for pediatric patients [28]. Reported herein are experiments designed to examine the effect of chloral hydrate on the toxicity of the oxazaphosphorines to hematopoietic progenitor cells and to tumor cells known to contain the relevant aldehyde dehydrogenase activity.

MATERIALS AND METHODS

Mafosfamide (2-[bis-(2-chloroethyl)-amino]-4-(2-sulfoethylthio) - tetrahydro - 2*H* - 1, 3, 2 - oxazaphosphorine-2-oxide · cyclohexylamine) and 4-hydroperoxycyclophosphamide were provided by Dr. P. Hilgard (Asta–Werke AG, Bielefeld, Federal Republic of Germany). Phosphoramide mustard · cyclohexylamine and conditioned medium from phytohemagglutinin-stimulated human peripheral blood leukocytes were supplied by Mr. L. H. Kedda (Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD), and Dr. F. Uckun (Department of Therapeutic Radiology, University of Minnesota, Minneapolis, MN), respectively. Chloral hydrate was obtained from the University of Minnesota Hospital Pharmacy, Minneapolis, MN.

All drugs were dissolved in triple-distilled water or drug-exposure medium and were sterilized by passage through 0.22-μm Millipore filters; all were used within 1 hr of preparation and were kept on ice until used. Drug-exposure medium was a phosphate-buffered, saline-based solution, pH 7.4, prepared as previously described [14].

(Semi)purified aldehyde dehydrogenases, viz. AHD-2, AHD-12a,b and AHD-13, were provided by Dr. C. L. Manthey. A detailed description of the preparative procedure is available [5, 12]. Selected physical and kinetic characteristics of these enzymes have been published previously [4, 5, 12].

Male, BALB/c mice, 12- to 16-weeks-old, were used as bone marrow donors for the murine CFU-S, CFU-GEMM and CFU-Mk experiments. They were also used as marrow recipients in the CFU-S experiments. These animals were obtained from the University of Minnesota Mouse Colony or Taconic, Germantown, NY. Animals were housed in plastic cages fitted with filtered lids and were given standard laboratory food and water *ad lib.* A strict 12-hr photoperiod was maintained.

Human bone marrow cells were obtained from a healthy adult volunteer.

Cultured mouse L1210 and P388 leukemia cells, sensitive (L1210/0, P388/0) and resistant to oxazaphosphorines specifically (L1210/OAP) or to cross-linking agents in general (P388/CLA) [14], were obtained from the Southern Research Institute, Birmingham, AL, through the courtesy of Drs. R. F. Struck and L. J. Wilkoff. Culture and growth conditions were as previously described [14].

Cultured human HPB-Null, MOLT-4, and Raji cells were gifts from Dr. T. W. LeBien (Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN). K-562 and KG-1 cells were gifts from Dr. D. A. Vallera (Department of Therapeutic Radiology, University of Minnesota, Minneapolis, MN) and Dr. B. C. Bostrom (Department of Pediatrics, University of Minnesota, Minneapolis, MN), respectively. U-937 cells were

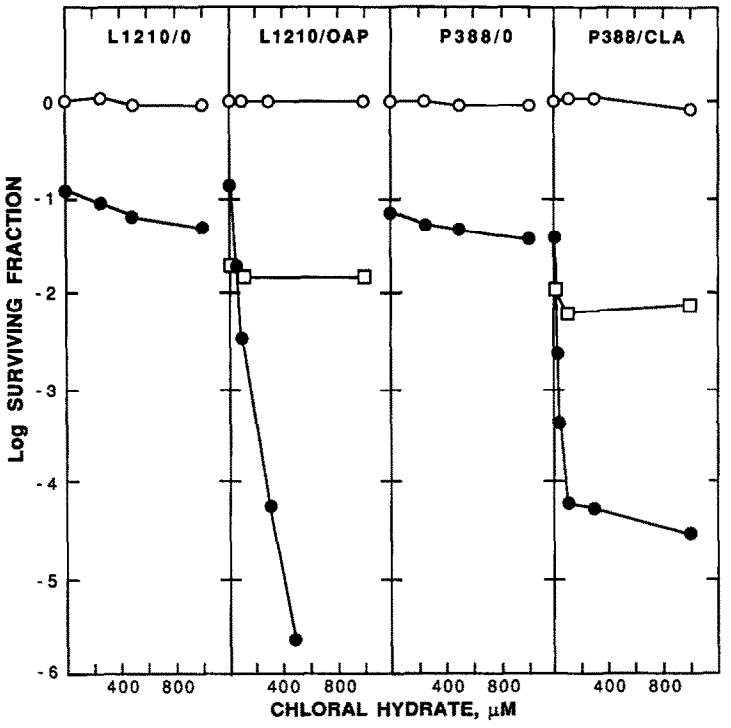

Fig. 1. Sensitivity of cultured murine tumor cells to mafosfamide and phosphoramide mustard in the presence of chloral hydrate. Tumor cells were incubated with vehicle or chloral hydrate for 60 min at 37°. Mafosfamide (●), phosphoramide mustard (□) or vehicle (○) was added, and incubation was continued at 37° for an additional 30 min. Concentrations of cytotoxic agents were 8.3 (L1210/0), 75 (L1210/OAP), 3.5 (P388/0), and 28 (P388/CLA) μM mafosfamide, and 90 (L1210/OAP), and 150 (P388/CLA) μM phosphoramide mustard. Cells were harvested at the end of the incubation period and subsequently resuspended and grown in drug-free medium. Surviving fractions were determined by the back-extrapolation method as described in Materials and Methods.

purchased from the American Type Culture Collection, Rockville, MD. All cell lines were grown in static suspension culture at 37° in RPMI 1640 culture medium supplemented with 10% horse serum, and a humidified atmosphere of 5% CO_2 in air; all grew exponentially [7].

The back-extrapolation method of Alexander and Mikulski [29] was used as described previously [14] to determine the sensitivity of cultured tumor cells to drugs except that the total drug exposure period was 90 min. Thus, cells were incubated with chloral hydrate or vehicle for 60 min; mafosfamide, phosphoramide mustard or vehicle was then added, and incubation was continued for an additional 30 min.

The sensitivity of murine CFU-S to mafosfamide ± chloral hydrate was determined by the method of Till and McCulloch [30] as modified by Kohn and Sladek [6, 9]. Preparation of murine bone marrow cell suspensions for this assay was as previously described [6, 9]. The sensitivity of murine CFU-GEMM and CFU-Mk to drug treatment was determined as previously described [8]. Preparation of bone marrow cell suspensions for these assays was also as before [8]. Control values ranged from 16 to 33 colonies formed per 8×10^4 nucleated cells injected (CFU-S), 6 to 16 colonies per 1×10^5 nucleated cells plated (CFU-GEMM), and 25 to 41 colonies per 1×10^5 nucleated cells plated (CFU-Mk).

The sensitivity of human hematopoietic stem cells to mafosfamide ± chloral hydrate was determined as previously described [7]. Preparation of these cells was as previously described [7].

Initial rates of aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide were quantified as described elsewhere [5, 31].

Wilkinson weighted linear regression analysis [32] was used to fit lines to the double-reciprocal plot (Lineweaver–Burk kinetic analysis) values. Computer-assisted unweighted regression analysis was carried out using the StatView™ [BrainPower, Inc., Calabasas, CA] statistical program to generate all other straight-line functions. The method of Bliss [33] was used to generate LC_{99} and LC_{50} values and 90% confidence intervals.

RESULTS

Effect of chloral hydrate on the sensitivity of murine tumor cell lines to mafosfamide. The effect of chloral hydrate on the sensitivity of L1210 and P388 cells, known to be sensitive (L1210/0, P388/0) and resistant (L1210/OAP, P388/CLA) to mafosfamide,

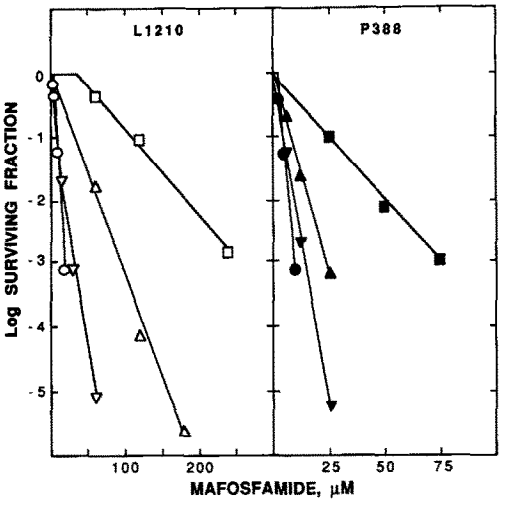

Fig. 2. Sensitivity of cultured murine tumor cells to mafosfamide in the presence of chloral hydrate. L1210/OAP (□, △, ▽), or P388/CLA (■, ▲, ▼), cells were incubated with vehicle (□, ■) or 100 (△, ▲) or 1000 (▽, ▼) μM chloral hydrate for 60 min at 37°. Mafosfamide was added, and the incubation was continued at 37° for an additional 30 min. Cells were harvested at the end of the incubation period and subsequently resuspended and grown in drug-free medium. Surviving fractions were determined by the back-extrapolation method as described in Materials and Methods. The responses of cultured murine L1210/0 (○) and P388/0 (●) cells to mafosfamide are included for comparison.

was determined in initial experiments (Fig. 1). Chloral hydrate alone was without cytotoxic activity. It markedly potentiated the antitumor activity of mafosfamide against the oxazaphosphorine-resistant L1210/OAP and P388/CLA cells. This is consistent with the fact that these cells contain an aldehyde dehydrogenase that catalyzes the detoxification of aldophosphamide, and the notion that chloral hydrate inhibits this enzyme. Chloral hydrate did not potentiate the cytotoxic action of mafosfamide against oxazaphosphorine-sensitive L1210/0 and P388/0 cells. This observation is consistent with the fact that these cells do not contain the relevant aldehyde dehydrogenase activity. Chloral hydrate did not potentiate the cytotoxic action of phosphoramide mustard against oxazaphosphorine-resistant L1210/OAP and P388/CLA cells. Phosphoramide mustard is a metabolite of cyclophosphamide that is not susceptible to aldehyde dehydrogenase-catalyzed oxidation (detoxification); therefore, potentiation by chloral hydrate would not be expected. To quantify the effect of chloral hydrate on the cytotoxic action of mafosfamide, L1210/OAP and P388/CLA cells were exposed to increasing concentrations of mafosfamide in the presence of 100 and 1000 μM chloral hydrate (Fig. 2). LC_{99} values were calculated from these data. LC_{99} values for mafosfamide alone were 180 and 49 μM when L1210/OAP and P388/CLA, respectively, were the target. These values were reduced to 65 and 16 μM, respectively, when 100 μM chloral hydrate was included in the exposure medium, and were further decreased to 15 and 10 μM, respectively, when 1000 μM chloral hydrate was included in the exposure medium. LC_{99} values for mafosfamide were 14 and 7 μM, respectively, when L1210/0 and P388/0 cells were the target. Thus, the decrease in LC_{99} values that resulted from inclusion of 1000 μM chloral hydrate in the drug-exposure medium reflects a nearly full restoration of the sensitivity of L1210/OAP and P388/CLA cells to mafosfamide.

Effect of chloral hydrate on the sensitivity of human tumor cell lines to mafosfamide. Potentiation by

Table 1. Sensitivity of cultured human tumor cells to mafosfamide ± chloral hydrate*

Cell line†	Classification‡	Differentiation stage‡	LC_{99} (μM) H_2O§	CH (100 μM)	CH (1000 μM)
HPB-Null	ALL	Pre-B blast	7.5	6.9	4.5
MOLT-4	ALL	T-Blast II	20	19	18
Raji	Burkitt's lymphoma	B-Blast I	35	33	29
U-937	Histocytic lymphoma	Monoblast	85	61	38
KG-1	AML	Pre-myeloid blast	77	42	30
K-562	CML	Pre-erythroid blast	60	45	38

* Tumor cells were incubated with vehicle (H_2O) or chloral hydrate (CH, 100 or 1000 μM) for 60 min at 37°. Mafosfamide or vehicle was added and incubation was continued at 37° for an additional 30 min. Immediately following drug exposure, cells were harvested and the number of surviving cells was determined via the back-extrapolation assay as described in Materials and Methods. Chloral hydrate itself was not cytotoxic at the concentrations used.

† There was no detectable aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide (70 μM) by soluble or particulate fractions obtained from any of these cell lines [7].

‡ Human tumor cell classification and differentiation stage according to Minowada *et al.* [34]. Abbreviations: ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; and CML, chronic myelogenous leukemia.

§ From Kohn *et al.* [7].

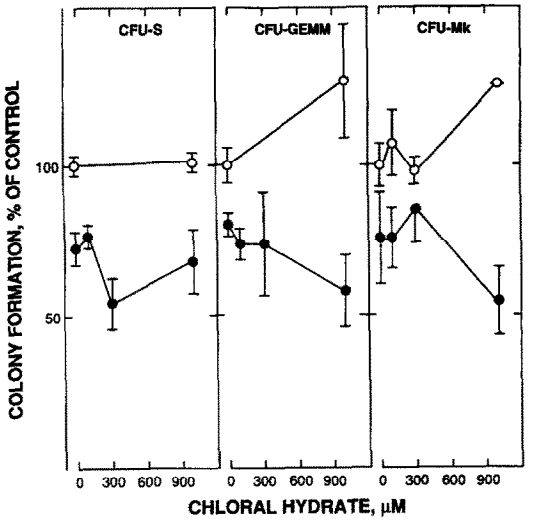

Fig. 3. Sensitivity of murine CFU-S, CFU-GEMM, and CFU-Mk to a fixed concentration of mafosfamide in the presence of increasing concentrations of chloral hydrate. Bone marrow cells obtained from 3 to 4 BALB/c donor mice were pooled and exposed to vehicle or chloral hydrate for 60 min at 37°. Vehicle (○) or mafosfamide (●) was added, and incubation was continued at 37° for an additional 30 min. Cells were harvested at the end of the incubation period, resuspended in drug-free medium, and assayed for CFU-S, CFU-GEMM or CFU-Mk as described in Materials and Methods. Concentrations of mafosfamide were 40 (CFU-S), 50 (CFU-GEMM), and 33 (CFU-Mk) μM. Points are mean ± SEM of observations made in 3–4 recipient mice (day-12 CFU-S), mean ± SEM of 5 plates (CFU-GEMM), and mean and range of duplicate plates (CFU-Mk). Results are expressed as percent of control (exposed to vehicle only).

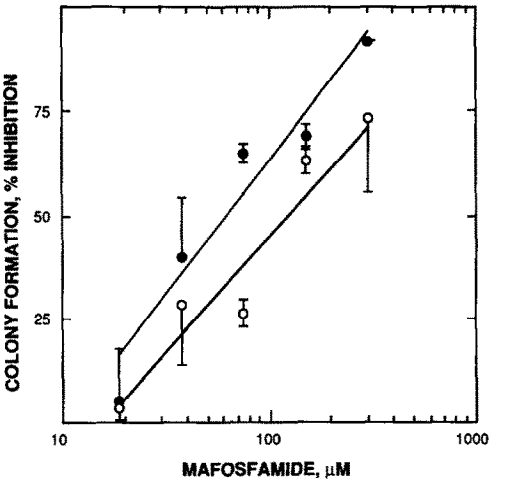

Fig. 4. Sensitivity of murine CFU-S to mafosfamide in the absence and presence of a fixed concentration of chloral hydrate. Bone marrow cells obtained from 4 donor BALB/c mice were pooled and incubated with vehicle (○) or 1000 μM chloral hydrate (●) for 60 min at 37°. Vehicle or mafosfamide was then added, and incubation was continued at 37° for an additional 30 min. Following incubation, cells were harvested, resuspended in drug-free medium, and assayed for day-12 CFU-S as described in Materials and Methods. Chloral hydrate alone was without cytotoxic activity. Results are expressed as percent inhibition of the relevant control. Points are the mean and range of values obtained in 2 recipient mice.

chloral hydrate of the antitumor activity of mafosfamide against six different cultured human tumor cells lines, viz. HPB-Null, MOLT-4, Raji, U-937, KG-1 and K-562, was minimal (Table 1). This is consistent with the observation that these cell lines do not contain an aldehyde dehydrogenase capable of catalyzing the detoxification of aldophosphamide [7].

Effect of chloral hydrate on the sensitivity of murine hematopoietic progenitor cells to mafosfamide. Certain murine bone marrow hematopoietic progenitor cells, viz. CFU-S, CFU-GEMM and CFU-Mk, are known to contain aldehyde dehydrogenase(s) capable of catalyzing the oxidation (detoxification) of aldophosphamide [6, 8, 9]. Chloral hydrate, at concentrations as high as 1000 μM, had little or no effect on the sensitivity of these cells to mafosfamide (Fig. 3), indicating that the relevant aldehyde dehydrogenase activity in these cells is not, or is only minimally, inhibited by this agent. LC_{50} values were determined in experiments in which bone marrow cells were exposed to increasing concentrations of mafosfamide in the absence and presence of 1000 μM chloral hydrate (Figs. 4 and 5, and Table 2); again, the results are consistent with the notion that the relevant aldehyde dehydrogenase activity in these cells is, at best, only minimally inhibited by chloral hydrate. In contrast, disulfiram, cyanamide, diethyldithiocarbamate, ethylphenyl (2-formylethyl)phosphinate and *N*-isopropyl-*p*-formylbenzamide clearly potentiate the cytotoxic action of mafosfamide and 4-hydroperoxycyclophosphamide against these cells [6, 8, 9, 35]. Disulfiram and cyanamide are demonstrated inhibitors of a number of aldehyde dehydrogenases and are used clinically for this purpose, i.e. as alcohol deterrents. Diethyldithiocarbamate is a metabolite of disulfiram and is also a demonstrated inhibitor of several aldehyde dehydrogenases. Ethylphenyl (2-formylethyl)phosphinate is a suspected inhibitor of aldehyde dehydrogenases. *N*-Isopropyl-*p*-formylbenzamide is a metabolite of procarbazine and is a demonstrated substrate for mouse AHD-2 [35]; procarbazine is known to produce a "disulfiram-like" effect when alcohol is ingested following its administration, suggesting that *N*-isopropyl-*p*-formylbenzamide is a substrate for at least one additional aldehyde dehydrogenase, namely, the hepatic mitochondrial aldehyde dehydrogenase that is primarily responsible for catalyzing the oxidation of acetaldehyde to acetic acid *in vivo*.

LC_{99} values defining the sensitivity of tumor cells to mafosfamide ± 1000 μM chloral hydrate are presented in Table 2 for comparison. The data presented in this table give quantitative support to the conclusion that, while the cytotoxic activity of mafosfamide against L1210/OAP and P388/CLA

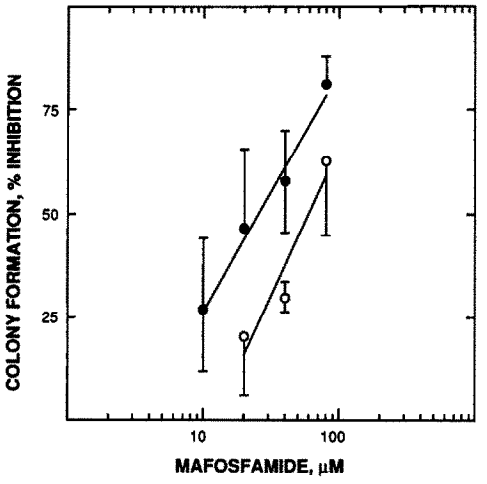

Fig. 5. Sensitivity of murine CFU-GEMM to mafosfamide in the absence and presence of a fixed concentration of chloral hydrate. Bone marrow cells obtained from 3 to 4 donor BALB/c mice were pooled and incubated with vehicle (○) or 1000 μM chloral hydrate (●) for 60 min at 37°. Vehicle or mafosfamide was then added, and incubation was continued at 37° for an additional 30 min. Following incubation, cells were harvested, resuspended in drug-free medium, and assayed for CFU-GEMM as described in Materials and Methods. Chloral hydrate alone was without cytotoxic activity. Results are expressed as percent inhibition of the relevant control. Points are the means ± SEM of values obtained in 3 separate experiments; each of the values in a given experiment was the mean value of duplicate or triplicate plates.

Table 2. *Ex vivo* sensitivity of murine tumor cells and hematopoietic progenitor cells to mafosfamide in the absence and presence of 1000 μM chloral hydrate

Cell	LC_{99}* (μM)	
	Control	Chloral hydrate
L1210/OAP†	180 (153–207)‡	15 (1–31)
L1210/0§	14 (13–15)	
P388/CLA	49 (42–56)	10 (8–12)
P388/0	7 (6–8)	
	LC_{50}‖ (μM)	
CFU-S	117 (70–194)	62 (49–79)
CFU-GEMM	59 (34–104)	26 (15–44)

* Values were obtained from data presented in Fig. 2.

† Aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide (70 μM) to carboxyphosphamide occurred at a rate of 31 nmol/min/mg protein when a 105,000 *g* soluble fraction obtained from these cells was evaluated; there was no activity in a 105,000 *g* particulate fraction [7].

‡ 90% Confidence intervals.

§ Subcellular fractions obtained from these cells were unable to catalyze NAD-dependent oxidation of aldophosphamide to carboxyphosphamide [7].

‖ Values were obtained from data presented in Figs. 4 and 5.

tumor cells was potentiated dramatically by chloral hydrate, the cytotoxic activity of mafosfamide against normal hematopoietic progenitor cells was not.

Effect of chloral hydrate on the sensitivity of human hematopoietic progenitor cells to mafosfamide. To determine whether chloral hydrate potentiates the cytotoxic action of mafosfamide against human hematopoietic progenitor cells, bone marrow cells were exposed to mafosfamide in the presence of 1000 μM chloral hydrate and assayed for CFU-Mix, CFU-Mk, CFU-GM and BFU-E (Fig. 6). Each of these progenitor cells is known to contain at least one aldehyde dehydrogenase that catalyzes the detoxification of aldophosphamide [7, 10]. In no case was potentiation observed. In contrast, disulfiram, cyanamide and *N*-isopropyl-*p*-formylbenzamide markedly potentiate the cytotoxic action of mafosfamide and 4-hydroperoxycyclophosphamide against these cells [7, 35].

Sensitivity of some (semi)purified aldehyde dehydrogenases to inhibition by chloral hydrate. The relevant aldehyde dehydrogenase present in mouse L1210/OAP cells has been identified as AHD-2, a cytosolic enzyme, *vide supra.* The unavailability of adequate methods to isolate sufficient numbers of normal murine bone marrow hematopoietic progenitor cells in acceptably pure form makes it impossible, at this time, to determine the identity of the relevant aldehyde dehydrogenase(s) in these cells. However, one or more of the ten aldehyde dehydrogenases (six cytosolic and four particulate) known to catalyze the detoxification of aldophosphamide in other mouse tissues [3–5] is(are) likely to be the operative enzyme(s).

Our data suggest that (1) oxidation (detoxification) of aldophosphamide in normal bone marrow hematopoietic progenitor cells is catalyzed by (an) aldehyde dehydrogenase(s) other than AHD-2, and (2) that the former, as compared to AHD-2, is(are) relatively insensitive to inhibition by chloral hydrate. Aldehyde dehydrogenases with differing sensitivities to inhibition by chloral hydrate have been reported previously; thus, one cytosolic aldehyde dehydrogenase was highly sensitive to inhibition by chloral hydrate (substrate was propionaldehyde), whereas another cytosolic and several particulate aldehyde dehydrogenases were not [26].

To determine if aldehyde dehydrogenases known to catalyze the oxidation of aldophosphamide are differentially sensitive to inhibition by chloral hydrate, the sensitivity of three of the more active hepatic aldehyde dehydrogenases, viz. AHD-2, AHD-12a,b and AHD-13, to inhibition by chloral hydrate was tested. Chloral hydrate proved to be a very potent (mixed) inhibitor of AHD-2-catalyzed oxidation of aldophosphamide; K_i and αK_i values were 3 and 13.8 μM, respectively (Table 3). This observation is in agreement with the report that chloral hydrate is a potent (noncompetitive) inhibitor ($K_i = 13$ μM) of ALDH-1-catalyzed oxidation of aldophosphamide [37]. ALDH-1 is the human homolog of mouse AHD-2. In contrast, chloral hydrate was a very poor (competitive) inhibitor of AHD-12a,b- and AHD-13-catalyzed oxidation of aldophosphamide; K_i values were 6,000 and 22,000 μM, respectively (Table 3). Similarly, chloral

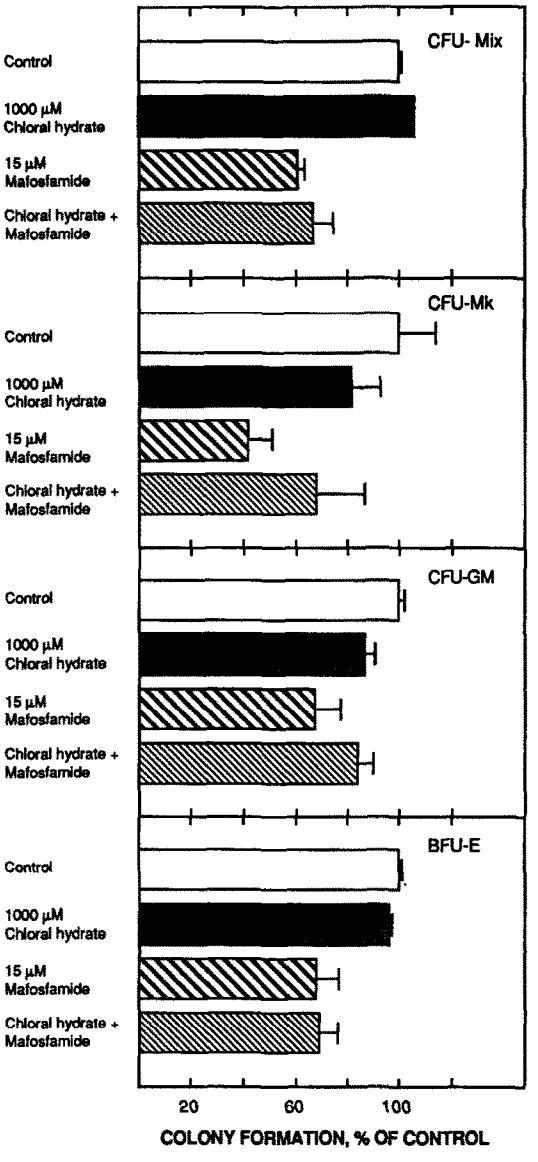

Fig. 6. Sensitivity of human progenitor cells to a fixed concentration of mafosfamide in the presence of chloral hydrate. Mononuclear bone marrow cells were incubated with vehicle or chloral hydrate for 60 min at 37°. Vehicle or mafosfamide was added and incubation was continued for an additional 30 min at 37°. Cells were harvested at the end of the incubation period, resuspended in drug-free medium, and assayed for CFU-Mix, CFU-Mk, CFU-GM and BFU-E. Control colony numbers were 38.5 (CFU-Mix), 32.5 (CFU-Mk), 293 (CFU-GM), and 202 (BFU-E). Bars are means and range of duplicate measurements on one sample. Results are expressed as percent of the relevant control, viz. vehicle only (no mafosfamide or chloral hydrate) for chloral hydrate and mafosfamide, and chloral hydrate for mafosfamide plus chloral hydrate.

hydrate only very weakly inhibits aldophosphamide oxidation catalyzed by the human homolog of AHD-12 [37].

DISCUSSION

The results of the present and previous [3, 6–9, 15, 35] investigations demonstrate that a number of clinically used drugs which produce a "disulfiram-like" effect when given in conjunction with ethanol ingestion potentiate the cytotoxic action of cyclophosphamide and other oxazaphosphorines against tumor and/or critical normal cells when these cells contain an aldehyde dehydrogenase that (1) catalyzes the detoxification of the oxazaphosphorines, and (2) is sensitive to inhibition by the agent that produces the "disulfiram-like" effect. Whether these drug interactions will be therapeutically beneficial, neutral, or harmful will depend on the relative aldehyde dehydrogenase status of the economic and uneconomic species. In the murine model presented herein, the neoplastic, and bone marrow hematopoietic progenitor, cells each contained an aldehyde dehydrogenase that catalyzed the detoxification of oxazaphosphorines but only the aldehyde dehydrogenase present in the former was inhibited by chloral hydrate. Considering only these two cell populations then, a beneficial drug interaction is obtained. However, at least one other murine critical normal cell population, viz. intestinal crypt cells, does contain an aldehyde dehydrogenase, viz. AHD-2, that is highly sensitive to inhibition by chloral hydrate [11]. Thus, *in vivo*, the drug interaction under consideration may not be therapeutically beneficial or only marginally so.

To what extent the mouse model used in the present experiments reflects the human situation is uncertain. The relevant aldehyde dehydrogenase activity is not present in the panel of cultured human tumor lines examined in the present study (Table 1) nor was it found in over 100 human primary leukemias and lymphomas obtained from oxazaphosphorine-naive patients (Sladek NE and Uckun FE, unpublished observations). On the other hand, Colvin and Hilton [38] did detect aldehyde dehydrogenase activity in human nonlymphocytic leukemia cells although it is not clear as to whether the activity was relevant since the substrate used for these studies was not identified. Measurable amounts of the relevant aldehyde dehydrogenase activity were

Table 3. Sensitivity of several mouse aldehyde dehydrogenases that catalyze the oxidation of aldophosphamide to inhibition by chloral hydrate*

AHD-	K_i (μM)
2	3†
12a,b	6,000
13	22,000

* Initial rates of aldehyde dehydrogenase-catalyzed oxidation were determined as described in Materials and Methods. Wilkinson weighted statistical regression analysis [32] was used to fit straight lines to Lineweaver–Burk plots of the data. The relationship $K_i = [I]/((V_{max}/K_m)(\text{slope}) - 1)$ was used to calculate K_i values for AHD-12a,b and AHD-13. K_i and αK_i values for AHD-2 were calculated from plots of the slope and y-intercept values, respectively, obtained from the Lineweaver–Burk plots, against the chloral hydrate concentration [36].

† The αK_i value was 13.8 μM.

identified in four Wilm's tumors and a testicular tumor (Manthey CL and Sladek NE, unpublished observations). The identity of the aldehyde dehydrogenase(s) present in these tumors is not known nor is it known whether this enzyme(s) is sensitive to inhibition by chloral hydrate, or for that matter, disulfiram, cyanamide, and other inhibitors of aldehyde dehydrogenases. It is known that at least three human aldehyde dehydrogenases catalyze the detoxification of oxazaphosphorines [37, 39].

High levels of the relevant aldehyde dehydrogenase are likely to be found in tumor populations that become relatively insensitive to the oxazaphosphorines (but not to other antitumor agents) after repeated exposure to them. At present, there is no evidence that this occurs clinically, but recent reports demonstrating the development of resistance specifically to oxazaphosphorines on the part of human mammary and melanoma cell lines cultured in medium containing progressively increasing concentrations of an oxazaphosphorine [40, 41] underscore the possibility.

Human hematopoietic progenitor cells, like their murine counterparts, apparently contain an aldehyde dehydrogenase that catalyzes the detoxification of oxazaphosphorines and is sensitive to inhibition by several agents, *vide supra*, but not by chloral hydrate. Thus, it would seem not to be ALDH-1, the human homolog of mouse AHD-2, although the experiments of Kastan and coworkers [10] argue that it is. In any case, it is likely that, as in the mouse, certain other human critical normal cell populations, e.g. gut epithelial stem cells, contain a relevant aldehyde dehydrogenase that is sensitive to inhibition by chloral hydrate, as well as to inhibition by other inhibitors of aldehyde dehydrogenases. If that is the case, the strategy of using chloral hydrate to selectively sensitize candidate tumor cells to oxazaphosphorines would be considerably less viable unless the relevant aldehyde dehydrogenase activity in the tumor cell population greatly exceeded that in the critical normal cell population(s).

The strategy of sensitizing tumor cells to oxazaphosphorines by co-administering chloral hydrate has an additional limitation. Concentrations of chloral hydrate that approach 1000 μM were required to effect the full restoration of tumor cell sensitivity to the oxazaphosphorines in our murine model. Therapeutic plasma concentrations of chloral hydrate when used as a hypnotic/sedative range from 40 to 120 μM [42]. However, these values include the contribution of trichloroethanol, the metabolite of chloral hydrate which exerts the hypnotic/sedative action. Trichloroethanol does not inhibit aldehyde dehydrogenases. The conversion of chloral hydrate to trichloroethanol is extremely rapid ($T_{1/2}$ = 8 min); thus, chloral hydrate itself is present only transiently in the circulation.

The pharmacokinetic and certain other problems discussed above would not be factors if oxazaphosphorines and chloral hydrate were used to purge bone marrow, intended for autologous transplantation, of tumor cells including any that contain a relevant aldehyde dehydrogenase. Similarly, they would not be factors if chloral hydrate was used to inhibit ALDH-1 present in the erythrocytes that, unless removed, populate bone marrow preparations intended for autologous transplantation, and, apparently because ALDH-1 catalyzes the inactivation of the oxazaphosphorines and the erythrocyte concentration varies with each preparation, account for the variability in 4-hydroperoxycyclophosphamide and mafosfamide potency when these agents are used to purge marrow of residual tumor cells [43].

The strategy of selectivity inhibiting erythrocyte ALDH-1 during the bone marrow purging procedure thus minimizing variability in oxazaphosphorine potency without having to remove erythrocytes seems to be worthy of further attention, especially since this strategy would also sensitize otherwise relatively insensitive (because they contain ALDH-1) tumor cells to the oxazaphosphorines. Despite the problems outlined above, the strategy of selectively sensitizing candidate tumor cells to oxazaphosphorines *in vivo* by co-administering a suitable aldehyde dehydrogenase inhibitor would also seem to merit further attention.

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