Notes

Chemically Stable *N*-Methyl-4-(alkylthio)cyclophosphamide Derivatives as Prodrugs of 4-Hydroxycyclophosphamide

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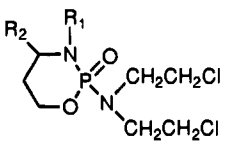
Two prototype *N*-methyl-4-thio-substituted cyclophosphamide (CP) derivatives (**5** and **6**), prodrugs of 4-hydroxycyclophosphamide (4-HO-CP), were designed to undergo oxidative N-demethylation to release the active alkylating agent. These prodrugs were chemically stable until oxidatively N-demethylated in the presence of hepatic microsomal P-450 enzymes. While the metabolism of **5** was enhanced in the presence of phenobarbital-induced microsomes, **6** was unaffected. Compound **6** was more active than **5** against L1210 leukemia cells grown in mice and exhibited statistically significant activity against the small cell lung cancer panel in the National Cancer Institute anticancer drug screen. Compound **5**, like CP (**1**), was inactive in this screen. Thus, placement of a dithioester at the 4-position of *N*-methyl-HO-CP as in **6** markedly changes its spectrum of activity and has resulted in a new type of CP-based prodrug with antitumor activity against small cell lung cancer as well as leukemia cells in vitro as shown by their ability to inhibit tumor cell growth at concentrations as low as 10^{-6} M.

Introduction

Numerous structural modifications of cyclophosphamide (CP, **1**, NSC-26271) have been made to elucidate its mechanism of action and enhance its efficacy as an antitumor agent.[1-5] The antitumor activity of the latter is dependent on its conversion in vivo to 4-hydroxycyclophosphamide (4-HO-CP), an intermediate that is converted to the ultimate alkylating species. Among the more promising analogues studied, mafosfamide (NSC-345842), a chemically "stabilized" derivative of 4-HO-CP, bears a thiol substituent (2-mercaptoethanesulfonate) at the C_4-position of the oxazaphosphorine ring. Its stability under physiological conditions is pH dependent.[6] The kinetics of this decomposition reaction are complex[7,8] but involve the base-catalyzed release of the thiol group to form a transient imino-CP intermediate followed by immediate hydration to 4-HO-CP. Preclinical evaluation of mafosfamide indicates that its spectrum of antitumor activity is similar to that of CP (**1**)[9,10] while its myelo and urotoxic effects are lower than those of CP.[11] However, phase I clinical studies indicate that mafosamide causes severe local toxicity due to its rapid hydrolysis to 4-HO-CP.[12,13] Thus, premature hydrolysis of 4-(alkylthio)-CP derivatives severely limits their potential use in cancer chemotherapy.

While mafosfamide and related compounds undergo facile base-catalyzed decomposition to 4-HO-CP under physiological conditions, N^3-protected 4-thio-substituted CP derivatives, e.g., 4-[(2-sulfamoylethyl)thio]ifosfamide, are chemically stable to hydrolysis in aqueous buffer and plasma under physiological conditions.[14] This suggests that the presence of an alkyl substitutent at the N^3-position of the oxazaphosphorine ring must stabilize 4-(alkylthio)cyclophosphamides and prevent premature hydrolysis of the latter to 4-HO-CP. Since N-demethylation is a common oxidative pathway for the metabolism of many xenobiotic substances, we have placed a methyl substitutent at the N^3-position of the oxazaphosphorine ring to stabilize the molecule and thus avert premature hydrolysis. N-Demethylation of structurally similar cyclic amide- or imide-containing compounds, e.g., diazepam [15] and trimethadione,[16] has been reported in both rats and humans. This suggests that a similar N-dealkylation of these *N*-methyl-4-(alkylthio)-CP derivatives may occur in vivo to release 4-HO-CP in the presence of hepatic microsomal mixed function oxidases (MFO).

Chart 1



no.	R_1	R_2
1	H	H
4	CH_3	OOH
5	CH_3	$SCH_2CH_2CH_3$
6	CH_3	$S-\overset{\overset{S}{\|}}{C}-N(CH_2CH_3)_2$

We now report the synthesis of N^3-methyl-4-(*n*-propylthio)-CP (**5**, *N*-Me-4-PrS-CP) and N^3-methyl-4-(diethyldithiocarbamoyl)-CP (**6**, *N*-Me-4-DDTC-CP) (Chart 1) as chemically stabilized prodrugs of 4-HO-CP and the pharmacological evaluation of their cytotoxic activity (Chart 1).

Chemistry

N-Methyl-4-hydroperoxycyclophosphamide (**4**, *N*-Me-4-HOO-CP) was synthesized essentially as described previously[14] in 21.5% yield. The *N*-methyl-4-thio-CP derivatives were prepared by reaction of *N*-methyl-4-hydroxy-CP (*N*-Me-4-HO-CP), obtained from the reduc-

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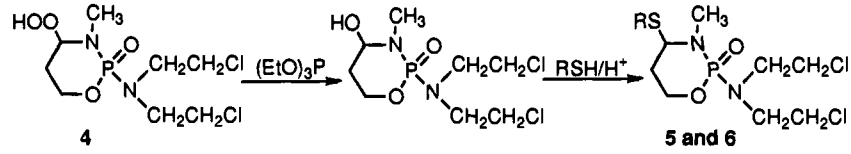
Scheme 1



tion of **4** with triethyl phosphite, with the corresponding thiols (2–3 equiv) and a catalytic amount of trifluoroacetic acid (Scheme 1). N^3-Methyl-4-(*n*-propylthio)-CP (**5**) was designated as the *cis* isomer on the basis of the large H_4–P coupling constant ($J_{C_4\text{-}H,P}$ = 22.2 Hz) and a small H–H coupling constant ($J_{C_4\text{-}H,\ C_5\text{-}H}$ < 2.5 Hz), indicating the equatorial relationship of the two protons to one another. The designations, *cis* and *trans*, refer to the relative stereochemistry of the phosphoryl oxygen and the substituent at the C_4-position in the oxazaphosphorine ring.[17–19] *N*-Methyl-4-(diethyldithiocarbamoyl)-CP (**6**) was prepared by reaction of the ozonolysis product of *O*-3-butenyl *N*,*N*-bis(2-chloroethyl)-*N'*-methylphosphordiamidate (**3**) with 2 equiv of diethyldithiocarbamic acid, sodium salt trihydrate in aqueous acetone. A key step in the synthesis of **6** was the lowering of the pH from 12.8 to 5.2 by addition of aqueous HCl 1 h after the reaction was initiated. This pH change facilitates the intramolecular cyclization of the hemithioacetal intermediate giving a single isomer. The H_4–P coupling constant was 24.5 Hz, indicating that the C_4-substituent is in the axial position. The chemical shift of H_4 in compound **6** was shifted downfield (5.68 ppm) compared to that for compound **5** (4.25 ppm). This is consistent with the greater deshielding effect due to the presence of the dithiocarbamoyl moiety.

Biological Results and Discussion

Compounds **5** and **6** were stable in aqueous phosphate buffer (pH 7.4) at 37 °C for 24 h. No significant alkylating activity was detectable when assayed for an alkylating species by monitoring the reaction with NBP [4-(4-nitrobenzyl)pyridine].[20] On the addition of rat liver microsomes and a NADPH-generating system, an alkylating species was detectable in the presence of **5** or **6** at rates of 0.11 ± 0.03 and 0.18 ± 0.03 equiv of mechlorethamine/g of wet weight liver/h, respectively, compared to **1** (0.16 ± 0.02 equiv of mechlorethamine/g wet weight liver/h). N-Demethylation of **5** and **6** was followed by monitoring formaldehyde release with time. Formaldehyde was formed in a time-dependent manner for at least 40 min (data not shown). Omission of the NADPH-generating system did not result in formaldehyde generation, indicating that the hepatic microsomal enzymes were involved. Incubation of compound **5** with phenobarbital (PB)-induced rat liver microsomal enzymes doubled the rate of formation of the alkylating species and resulted in a 2.2-fold increase in formaldehyde formation. This supports our hypothesis that N-dealkylation by the hepatic mixed function oxidases was the mechanism of 4-HO-CP release. In sharp contrast, no significant enhancement of metabolite formation occurred when compound **6** was incubated with PB-induced microsomes, indicating that **6** may not be metabolized in the same manner as **5**.

Since these prodrugs require metabolic biotransformation to exhibit their biological effects in vivo, **5** and **6** were initially screened in a cell line known to possess mixed function oxidase activity. The cytotoxicity of **5** and **6** was compared with CP (**1**) in a mouse embryo Balb/c 3T3 cell line.[21] The LC_{50} value for compound **5** (21.8 μM) indicated that it was only slightly more active than **1** (37.6 μM). Compound **6** was considerably more cytotoxic than either **1** or **5** with a LC_{50} value of 0.6 μM (Table 1). The cytotoxicity of DDTC, a metabolite of **6**, may be a contributory factor since the latter is highly cytotoxic to 3T3 cells (LC_{50} <0.5 μM).

Table 1. Cytotoxicity of **5** and **6** Against Balb/c 3T3 Cells in Vitro and Against 10^5 L1210 Leukemia Cells in Mice

compd	Balb/c 3T3 IC_{50} (μM ± SEM)[a]	% ILS[b] (1.08 mmol/kg dosage)
1	37.6 ± 4.8	>187 (3/4)
4	1.7 ± 0.2	toxic
5	21.8 ± 2.0	42
6	0.6 ± 0.1	>92 (1/4)

[a] Mean ± SEM of three experiments using five different drug concentrations. [b] Where long term survivors (>30 days) occurred, their number is shown in parentheses. % ILS (increased life span) = (treated/control - 1) × 100. Mean survival times for mice receiving L1210 and vehicle was 9.5–10.6 days.

The ability of a single dose (1.08 mmol/kg) of compound **5** or **6**, administered 24 h after inoculation with L1210 leukemia cells in mice, to increase the lifespan (ILS) of these animals over untreated controls is shown in Table 1. It is interesting to note that while both compounds were equally stable to hydrolysis, compound **5** exhibited only modest activity against L1210 leukemia in mice as shown by the 42% increase in lifespan. Compound **6**, however, was highly active, increasing the lifespan of these animals by >92% and yielding 1/4 survivors, while *N*-CH_3-HOOCP (**4**) was toxic at this dose and CP (**1**) gave a >187% increase in life span and 3/4 survivors. The observance of a survivor for **6** indicates activity closely comparable to that of **1** and a likely difference in cell kill of only ca. 1–10 cells. The reason for the difference in activity between **5** and **6** is not clear. It is possible that there may be alternate mechanism(s) for activation of **6** and that oxidative N-demethylation may not be the major contributing factor.

The in vitro cytotoxicity of **5** and **6** was evaluated against a battery of 60 human tumor cell lines representing eight different types of cancer over a range of five concentrations (10^{-4}–10^{-8} M). Sensitivity of the tumor panel to the drug is indicated on a log scale by the magnitude of the difference from the mean value (MG MID). Compound **5** was only weakly cytoxic in this protocol expressing minimal activity toward the leukemia panel at the highest concentration used (10^{-4} M) (Table 2). This observation is in agreement with the known inactivity of anticancer agents that require bioactivation in this screen. Thus, compound **5** and CP (**1**) exhibited a similar spectrum of cytotoxic activity as indicated by comparison of their mean graphs (data not shown). In contrast, the spectrum of activity exhibited by compound **6** was sharply different from that of **5**. While the leukemia panel was most sensitive to **5**, the

Table 2. Comparison of the Cytotoxicity of **5** (NSC-638242) and **6** (NSC-638241) Against Leukemia and Small Cell Lung Cancer Subpanels

	$\log_{10}$ GI_{50}[a]	
	5[b]	**6**[c]
leukemia subpanel	*	
CCRF-CEM	>−4.00	−7.50, −7.40
HL-60(TB)	−4.75	−7.50, −6.54
K562	−4.31	−7.28, −7.06
MOLT-4	>−4.00	−7.31, −7.16
RPMI-8226	−4.10	−6.48, −6.40
SR	−4.32	−7.43, −6.41
small cell lung cancer subpanel		*
DMS 114	−4.36	−6.77, −6.68
DMS 273	>-4.00	−6.92, −6.42
MG MID GI_{50}[d]	−4.06	−5.78, −5.26
MGD_H[e]	54.49	69.72, 87.78

[a] $\log_{10}$ GI_{50} (or IC_{50}) represents the log of the prodrug concentration (M) required to achieve 50% inhibition of tumor cell growth. [b] One experiment. [c] Two separate experiments. [d] MG MID GI_{50} represents the calculated mean for all panels. [e] MGD_H represents the selectivity or relative sensitivity of the tumor cell panel to the drug. An asterisk (*) indicates that the sensitivity of the panel to the drug is statistically significant when compared to the others.

small cell lung cancer panel was the most sensitive to the cytotoxic effects of compound **6**. This effect was statistically significant on the basis of the D_H value calculated from parameters representing every tumor panel [D_H values for **6** were 75.00 and 84.21 ($\times 10^{-6}$M), respectively]. D_H values ≥ 75 are statistically significant. The IG_{50} (IC_{50}) values indicate that **6** is 100–1000-fold more active than **5** in the leukemia panel and 100-fold more active than **5** in the small cell lung cancer panel. These results suggest that release of the active alkylating agent may occur via pathways already present in these tumor cell lines. Thus, placement of a dithioester at the 4-position (**6**) has resulted in a new type of CP-based prodrug that has antitumor activity against small cell lung cancer as well as leukemia.

In conclusion, two prototype *N*-methyl-4-thio-substituted CP derivatives (**5** and **6**) were designed to undergo oxidative N-demethylation to generate 4-HO-CP. These N-methylated prodrugs were chemically stable until activated in vivo. Compound **6** was active in vivo (L1210 leukemia in mice) and in vitro [small cell lung cancer and leukemia in the National Cancer Institute (NCI) anticancer drug screen]. Replacement of a thioether with a dithioester group in the 4-position of an N-methylated 4-HO-CP drastically changed its spectrum of activity in the NCI human tumor panel by enhancing its potency and changing the tumor panel target. Further studies are in progress to elucidate the mechanism by which **6** is converted to an alkylating species.

Experimental Section

Chemistry. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Elemental analyses were performed by Atlantic Microlab Inc., Norcross, GA. ^{1}H NMR spectra were recorded on a Nicolet modified NT-360 (360 MHz) instrument, and chemical shifts are reported as δ values (ppm) downfield from tetramethylsilane (internal standard). Analytical and preparative thin layer chromatography were performed on silica gel GF 254 (Analtech Inc., Newark, DE). Silica gel, 40 μm (J. T. Baker Inc., Phillipsburg, NJ), was used for flash chromatography. A Milton Roy Spectronic 301 spectrophotometer was used for spectral analysis. Ozonolysis was carried out with an ozone generator (Welsbach Corp.) at an oxygen pressure of 10 psi, 120 V, and 0.07 scfm air flow (5 psi). Tetrahydrofuran (THF) was dried by refluxing with sodium hydride under a nitrogen atmosphere followed by distillation. Cyclophosphamide was purchased (Sigma Chemical Co., St Louis, MO). All chemicals were reagent grade. *N,N*-Bis(2-chloroethyl)phosphoramidic dichloride was prepared essentially as described, mp 53.5–55 °C (lit.[22] mp 54–56 °C). *N,N*-Bis(2-chloroethyl)phosphorodiamidic acid (**2**) was prepared as the cyclohexylamine salt, mp 104–105 °C (lit.[23] mp 107–108 °C). *N*-Methyl-4-hydroperoxycyclophosphamide (**4**) was synthesized essentially as described previously.[14]

***N*-Methyl-*cis*-4-(*n*-propylthio)cyclophosphamide (5).** *N*-Methyl-*cis*-4-hydroperoxycyclophosphamide (**4**) (491 mg, 1.6 mmol) was dissolved in 20 mL of methylene chloride and deoxygenated with triethyl phosphite (267 mg, 0.28 mL, 1.6 mmol) at 4 °C for 10 min. 1-Propanethiol (366 mg, 0.44 mL, 4.8 mmol) was added to the reaction mixture followed by 0.1 mL of trifluoroacetic acid. The reaction was allowed to proceed at 4 °C for 2 h and the mixture brought to room temperature and stirred overnight. The reaction solution was concentrated and directly applied to a silica gel column (40 μm, 3 × 15 cm), drypacked, equilibrated with solvent (ethyl acetate/hexane, 1:1), and eluted with ethyl acetate under positive pressure (15 psi). Fractions were collected (50 × 8 mL). Fractions (16–29) were pooled, and the solvent was removed in vacuo to give **5** as a yellow oil (yield, 42.9%): TLC $R_f = 0.88$ in CH_2Cl_2/MeOH (9:1); ^{1}H NMR ($CDCl_3$) δ 4.78–4.80 (1H, m, H_{6e}), 4.25 (1H, ddd, $J_{C_4\text{-}H,P} = 22.2$ Hz, H_4), 4.07–4.25 (1H, m, H_{6a}), 3.60 (4H, t, CH_2Cl), 3.38 (4H, m, CH_2N), 2.72 (3H, d, $J_{H,P} = 10.1$ Hz, NCH_3), 2.40–2.52 (2H, m, CH_2S), 0.95 (3H, m, CH_2CH_3). Anal. ($C_{11}H_{23}Cl_2N_2O_2PS$) C, H, N, Cl.

***N*-Methyl-*cis*-4-(diethyldithiocarbamoyl)cyclophosphamide (6).** *O*-3-Butenyl *N,N*-bis(2-chloroethyl)-*N'*-methylphosphordiamidate (**3**) (4.01 g, 15.5 mmol)[14] was dissolved in acetone/water (2:1, 150 mL) and ozonized at 4 °C for 40 min. Acetone was added periodically to maintain the original solvent level during the ozonolysis. Diethyldithiocarbamic acid, sodium salt trihydrate (6.98 g, 31.0 mmol) was added. The reaction was allowed to proceed at 4 °C for 1 h. The pH of the mixture was lowered from 12.8 to 5.2 by the addition of 5% HCl. The reaction was continued at 4 °C for an additional 2.5 h. Evaporation of the solvent gave a greenish aqueous mixture which was extracted with ethyl acetate (3 × 50 mL). The organic layer was dried over Na_2SO_4 and filtered and the filtrate evaporated to a dark yellow liquid (5.98 g). The liquid was applied directly to a silica gel column (40 μm, 5 × 15 cm), drypacked, equilibrated with solvent (ethyl acetate/hexane, 1:1), and eluted with ethyl acetate under positive pressure (15 psi). Fractions (36 × 20 mL) were collected. Fractions (12–22) were pooled and evaporated in vacuo to give **6** as a semisolid which solidified on cooling. The solid was recrystallized from anhydrous ether to give 1.37 g of **6** as a white solid (20.9% yield): mp 101–102 °C; TLC $R_f = 0.6$ in ethyl acetate; ^{1}H NMR ($CHCl_3$) δ 5.68 (1H, ddd, $J_{C_4\text{-}H,P} = 24.5$ Hz, H_4), 4.72–4.82 (1H, m, H_{6a}), 4.20–4.35 (1H, m, H_{6e}), 4.00 and 2.45–2.60 (2H, m, H_5), 1.20–1.40 (6H, m, CH2CH3). Anal. ($C_{13}H_{26}Cl_2N_3O_2PS_2$) C, H, N, Cl, S.

Stability Test Under Physiological Conditions. Chemical stability of the compounds under physiological conditions was determined by assaying for the release of an alkylating species. Alkylating activity was determined as previously described using the NBP assay,[20] except that the heating process of test compounds and mechlorethamine was performed at 60 °C for 20 min.

Microsomal Incubations. Microsomes were prepared as previously described[20] from male Sprague–Dawley rats (201–225 g) (Taconic, Germantown, NY) maintained on water ad libitum or on aqueous 0.1% sodium phenobarbital solution in place of their drinking water for 5 days. On day 6, all animals received regular water and were fasted overnight before sacrifice. All steps were carried out at 4 °C. The microsomal pellet was suspended in 0.25 M sodium phosphate buffer, pH 7.4, to a final concentration of 270 mg of wet weight liver/mL and used immediately or stored at −80 °C. Incubation mixtures contained microsomes (105 000 g fraction) from PB-

treated or naive rats corresponding to 270 mg of liver, test drug (1 mM in 0.05 mL of EtOH), NADPH-generating system (consisting of 5 mM glucose-6-phosphate, 5 mM NADP, 0.8 mM $MgCl_2$) in 0.92 mL of 0.025 M sodium phosphate buffer (pH 7.4), and 5 units of glucose-6-phosphate dehydrogenase [500 units/3 mL of 0.005 M citrate buffer (pH 7.5)] in a total volume of 2 mL. Incubations were carried out initially in a shaking water bath for varying times periods (10, 20, and 40 min) at 37 °C. After preincubation for 2 min, incubations wereinitiated by the addition of drug and terminated by the addition of 2 mL of cold absolute EtOH. The precipitated proteins were removed by centrifugation at 3000 rpm for 10 min. The supernatant was filtered through a syringe filter (0.45 μM, PTFG membrane) and the filtrate used to test for alkylating activity. Microsomes were heated at 100 °C for 5 min before use in control incubations. Alkylating activity and formaldehyde were determined as previously described.[20]

In Vitro Cytotoxicity in Balb/c 3T3 Fibroblasts. The cytotoxicity of **5** and **6** was determined using mouse embryo Balb/c 3T3 fibroblasts (American Tissue Culture Collection, Rockville, MD; ATCC #163).[20] Poly(styrene) tissue culture petri dishes (100 mm; Costar, Cambridge, MA) were seeded with 500 cells in 10 mL of Dulbecco's minimum essential media (Gibco, Grand Island, NY) in the presence or absence of drugs or solvent in varying concentrations. Drugs were dissolved prior to use in EtOH/H_2O (1:1). Cultures were incubated in a 95% air, 5% CO_2 humidified atmosphere under standard conditions, undisturbed for 10–14 days. At this time, cultures were rinsed twice with Hank's balanced salt solution (HBSS), fixed with 95% EtOH, and stained with 0.4% toluidine blue. Colonial growth was assessed by macroscopically counting the number of surviving colonies per dish. LC_{50} values were calculated semilogarithmically by plotting the drug concentration versus cell viability as determined by the number of colonies surviving treatments.

Anticancer Drug Screening Program (National Cancer Institute). The cytotoxicity of **5** and **6** was evaluated in 60 human tumor cell lines representing eight different types of cancer.[24] Briefly, compounds **5** and **6** were dissolved in DMSO and tested over a range of five concentrations (10^{-4}–10^{-8} M). The effect of the drug on cell growth was monitored over time with the results expressed as percent growth (PG) with the cell population at zero time equal to zero. Three response parameters, GI_{50} (PG > 0), TGI (PG = 0), and LC_{50} (PG < 0), were calculated for each cell line at each concentration tested, and the results are displayed as a mean graph of the most significant response and indicate the tumor subpanel(s) most sensitive to the drug. The tumor subpanels most sensitive to **5** and **6** are shown in Table 2.

In Vivo Antitumor Evaluation. Preliminary antitumor activity of **5** and **6** was determined in CDF_1 mice (17–18 g) against 10^5 lymphoid leukemia L1210 cells according to NIH protocol 1.100 for testing anticancer drugs as described previously.[20] L1210 cells were kindly provided by Dr. Norman E. Sladek, Department of Pharmacology, University of Minnesota, Minneapolis, MN.

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