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Inhibition of Drug-Naive and -Resistant Leukemia Cell Proliferation by Low Molecular Weight Thiols

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ABSTRACT. The aim of these studies was to investigate the ability of cysteamine and its congeners to arrest the proliferation of leukemic cells and to determine the physico-chemical properties responsible for this ability. Fifteen low molecular weight thiol-bearing compounds were shown to arrest the proliferation of CCRF-CEM cells and a methotrexate-resistant subline, with IC_{50} values between 10^{-5} and 10^{-4} M. Cysteamine arrested proliferation by slowing the passage of cells through S phase. These cells subsequently resumed cycling, although a proportion went on to die by apoptosis. The antiproliferative action of cysteamine was shown to depend, in part, on H_2O_2 production. This ability to generate peroxide is shared by many thiol compounds, and molecular modeling indicated that thiol groups were required for the antiproliferative actions of the congeners of cysteamine. Molecular modeling also revealed that the most efficacious antiproliferative agents were those that had their amino acid and thiol moieties separated by an intramolecular distance of 3.17 to 5.9 Å, as exemplified by WR 1065 and the aminothiophenols. These findings indicate that thiol-bearing compounds may have some efficacy in the treatment of drug-naive and -resistant leukemia cells. BIOCHEM PHARMACOL **55**;6:793–802, 1998.
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KEY WORDS. aminothiol; cysteamine; leukemia; neoplasia; cell cycle; drug resistance

The aminothiol CySH[‖] (β-mercaptoethanolamine) is an effective radioprotectant and also the parent compound for the WR series of radioprotective agents, including WR 1065 and WR 2721 [1]. These compounds all bear a free thiol group or one that can be exposed by metabolism, and it is the electrophilic nature of this group that is responsible for the ability of these compounds to reduce radiation-induced oxidants. This same electrophilic nature is also responsible for the ability of CySH [2, 3], WR 2721 [4], and the related compound penicillamine [5, 6] to act as detoxifying agents and accounts, in part, for their clinical use. Investigations into the radioprotective effects of CySH have revealed that while some of its actions undoubtedly involve free radical scavenging, this agent does not accumulate at sufficiently high intracellular concentrations to scavenge all of the hydroxyl radicals produced by X-irradiation, suggesting other modes of action [7]. Brown [8] hypothesized that CySH might also act to inhibit proliferation and thereby protect the cells by limiting the propagation of DNA damage and increasing the fidelity of DNA repair. This hypothesis is supported by numerous studies detailing the antiproliferative effects of CySH [9–15]. Of even greater interest are observations suggesting that CySH preferentially arrests the proliferation of neoplastic cells [16–20].

Previously, we hypothesized that CySH inhibited the proliferation of neoplastic neural cells by generating an oxidant stress according to the following reactions [15]:

$$RSH + M^n \rightarrow \tfrac{1}{2}\,RSSR + M^{n-1} + H^+ \quad (1)$$

$$O_2 + M^{n-1} \rightarrow O_2^{\cdot-} + M^n \quad (2)$$

$$O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 \quad (3)$$

Reaction 1 describes the reduction of transition metals (M^n, with n referring to the oxidation state) by a thiol (RSH), which is concomitantly oxidized to its corresponding disulfide (RSSR). The reduced metal then donates the electron to oxygen to produce $O_2^{\cdot-}$ (reaction 2), which in turn, dismutates to H_2O_2 (reaction 3). It was hypothesized that H_2O_2 would go on to react with serum and cell-associated transition metals to produce a number of reactive metabolites that would act to block cellular proliferation [15]. The ability of micromolar concentrations of CySH to generate H_2O_2 is shared by many low molecular weight

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‖ *Abbreviations:* CySH, cysteamine; FBS, fetal bovine serum; IC_{50}, concentration that produces 50% inhibition of proliferation; and SOD, superoxide dismutase.

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thiol-bearing compounds [21–26]. Thus, the aim of these studies was to investigate the ability of congeners of CySH to inhibit the proliferation of leukemia cell lines and to determine the chemical properties responsible for this ability.

MATERIALS AND METHODS

Materials

Catalase (20,000 units/mg), SOD (25,000 units/mg), dithiocarbamate, ceruloplasmin (7,020 units/mg), CySH, cysteamine-*S*-phosphate (WR 638), cystamine, *N*-acetylcysteine, *N*-acetylcysteamine, L-penicillamine, D-penicillamine, D-penicillamine methyl ester, ethanolamine, and 2-, 3- and 4-aminothiophenol were all purchased from the Sigma Chemical Co. Chelex 100 resin was purchased from BioRad. WR 1065 and WR 2721 were supplied by Dr. N. R. Lomax, formerly of the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. Similarly, WR 2822 and WR 2823 were supplied by Dr. R. R. Engle, Department of Medicinal Chemistry, Division of Experimental Therapeutics, Walter Reed Army Institute of Research. 2,3-Dimercapto-1-propane sulfonic acid, and D- and L-lipoic acid were the gifts of Drs. H. Ullrich and K. Wessel (Asta Pharma AG). All of the aminothiols either were prepared fresh or were from 100 mM diluted stocks stored at −80°, just prior to their use in the assays. The aminothiols were dissolved either in Chelex-treated 75 mM potassium phosphate buffer, pH 7.0, or triple-distilled H_2O. Ethanolamine was prepared fresh in H_2O.

Cell Culture of Leukemic Cell Lines

The human leukemia cell lines CCRF-CEM, B4D [27], Namalwa, and VB 1000 [28] were maintained in RPMI-1640 medium (ICN Biomedicals) supplemented with 10% FBS, 100 units/mL penicillin, 10 mg/mL streptomycin, and 2 mM glutamine, at 37°, in 5% CO_2:95% air. Under these conditions, CCRF-CEM, B4D, VB 1000, and Namalwa cells completed one division cycle by 17, 21, 23, and 23 hr, respectively. The resistance of B4D cells to methotrexate was confirmed using the experimental design described by Taylor *et al.* [27] and the viability assay described by Hida *et al.* [29].

Measures of Cell Proliferation and Viability in Leukemia Cell Lines

Proliferation was assessed by measurement of DNA synthesis and cell number. CCRF-CEM cells were diluted from log-phase cultures to 2.5×10^5 cells/mL, were placed in 200 μL/well on 96 flat-bottom multiwell tissue culture plates in the presence or absence of the test compounds, and were incubated for 24 hr at 37° and 5% CO_2:95% air. [^{3}H]Thymidine at 0.4 μCi/well was applied for the final 2.5 hr of the culture period. Labeled DNA was precipitated onto glass fiber discs after hypotonic lysis of the cells, using a PHD harvester (Cambridge Technology Inc.), and the discs were suspended in 2 mL of Optifluor-O liquid scintillant (Canberra Packard) to allow counting of the β-emissions.

The number of CCRF-CEM cells was counted on a Neubauer hemocytometer, and their viability was judged by the exclusion of eosin, present at a final concentration of 0.025% (w/v). Between 100 and 400 live cells or dead cells were counted per sample and averaged from quadruplicate wells. Percent control values for the cell number measured were calculated according to the following expression, % Control = [Drug-treated cell number $- 2.5 \times 10^5$]/[Control cell number $- 2.5 \times 10^5$] $\times$ 100, to account for the differences seen in the proliferation over the 24-hr test period, rather than overall changes in the cell number *per se*.

Biochemical Measurements

Oxygen consumption was measured using a Clark electrode oxygen monitor [30] (Yellow Springs Instrument Co.) fitted with a Perkin–Elmer recorder. For these experiments, RPMI was buffered with 10 mM HEPES and 4.17 mM sodium bicarbonate to pH 7.4 for use in air [31] and stirred constantly during the incubations. The medium was equilibrated with the atmosphere at 37° for at least 6 hr prior to the start of the experiments. The number of thiol groups in a 100 μM CySH solution prepared in Chelex-treated 100 mM potassium phosphate buffer, pH 7.2, with or without 20 μg/mL catalase, was measured after 24 hr at 37° in air according to protocol of Collier [32].

Molecular Modeling

A molecular modeling study was carried out on the fifteen thiol derivative compounds to ascertain if there were any physico-chemical determinants of differences in activity. The structures of the compounds were, if available, derived from each compound's respective X-ray crystal data. These matrices were then entered into Chem-X (Chemical Design Ltd., 1995) and optimized using functions within Chem-X. The optimized structures were then submitted to a Molecular Orbital Package, version 6.0 [33], and optimized using reduced Hartree–Fock, Austin model 1 theory. The resultant optimized data were then submitted to the Molecular Orbital Package to obtain single point charges for each atom. This was achieved using minimal neglect of differential overlap, electrostatic potential theory with a net charge on each compound equal to zero.

DNA Flow Cytometry and Hoechst 33342 Cell Staining

CCRF-CEM cells at 2.5×10^5 cells/mL were treated with CySH and incubated at 37° and 5% CO_2:95% air and then processed for DNA flow cytometry as described previously [34]. Analysis of the frequency of cells in the cell cycle phases was performed using Cellfit software (Becton–Dick-

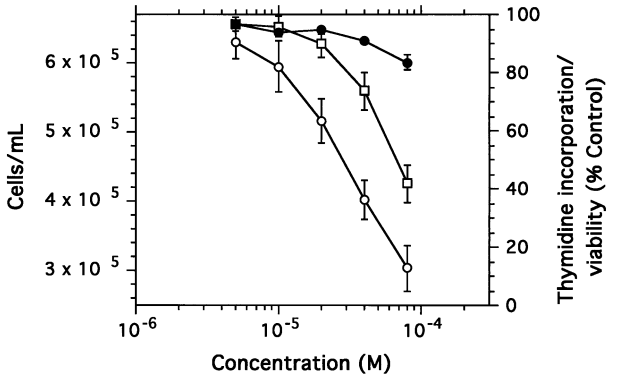

FIG. 1. Inhibition of the proliferation of CCRF-CEM cells by CySH. CCRF-CEM cells at 2.5 × 10^5/mL were treated with CySH at the indicated concentrations for 24 hr. The effects on cell density (●), viability (○), and thymidine incorporation (□) were recorded for six experiments, for which the mean and SEM are shown. After 24 hr, the untreated cells had increased to a density of 6.45 × 10^5/mL and incorporated 1.17 × 10^5 dpm of [^{3}H]thymidine/min. The Y1 axis has been scaled such that the high point corresponds to density of the untreated control cells after the 24-hr experimental period (6.45 × 10^5/mL) and the 100% level on the Y2 axis. Similarly, the low point on the Y1 axis is equivalent to both the seeding density (2.5 × 10^5 cells/mL) and 0% on the Y2 axis. Thus, the percent changes in cell density can be read from the Y2 axis.

inson). During acquisition, the data were gated by the manufacturer's method to exclude doublets. Staining with Hoechst 33342 was performed as described previously [35].

Statistics

The differences between the means of treated and untreated groups were examined by analysis of variance. In the text, the mean and SEM are reported with the number of experiments given in parentheses.

RESULTS

Inhibition of the Proliferation of CCRF-CEM Cells by CySH

Previous studies have shown that CySH has profound effects on the cell cycle progression of human peripheral blood lymphocytes [13, 14] and neoplastic neural cell lines [15]. Therefore, to ensure that we observed early perturbations in the cell cycle and affected all the CySH-sensitive cells within one division cycle, it was decided to examine the antiproliferative action of aminothiols within 24 hr, which approximates one cell cycle period of the leukemia cell lines under investigation. Treatment with CySH for 24 hr resulted in a concentration-dependent inhibition of proliferation, as measured in terms of cell number (Fig. 1). At 80 μM, CySH prevented any significant increase in cell number above the seeding density, clearly indicating arrested proliferation. CySH at the IC_{90} concentration of 80 μM also induced a modest increase in toxicity (~10%) over that seen in the control populations (Fig. 1). Higher

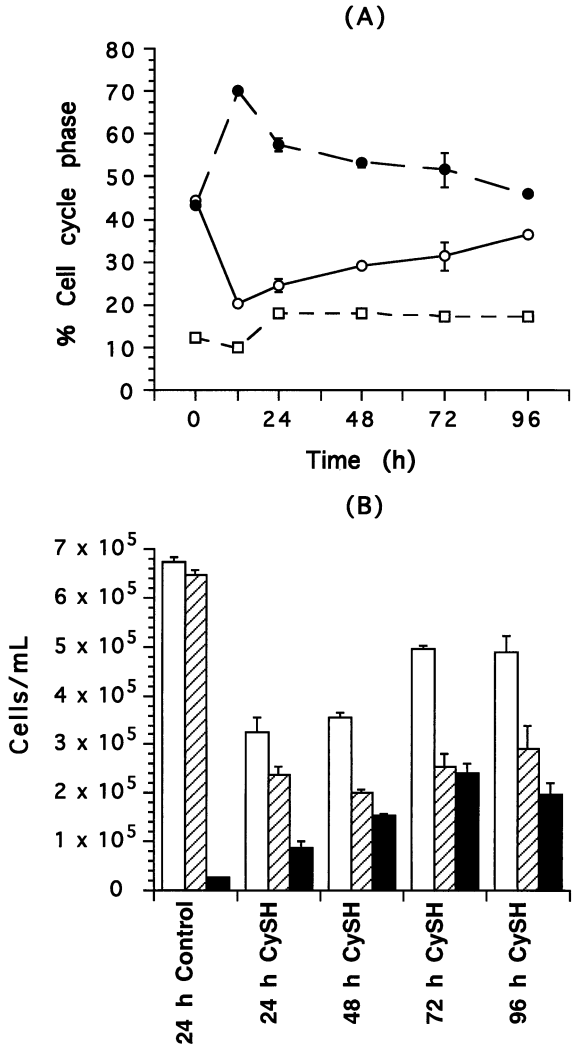

FIG. 2. Effect of CySH on the cell cycle progression of CCRF-CEM cells. CCRF-CEM cells at 2.5 × 10^5/mL were treated with 80 μM CySH and the effect on the proportion of cells in the G_1 (○), S (●), and G_2/M (□) phases of the cell cycle was measured by flow cytometry (A). Shown is the mean and SEM of three experiments. The effect of CySH on the total cell density (□) and live (▨) and dead (■) cells for the three experiments from which the cell cycle data were taken is shown in panel B. Also shown in panel B is the increase in density of the untreated control population after 24 hr.

CySH concentrations, of up to 10^{-3} M, were toxic (data not shown).

In contrast to the effects of CySH on cell number, this drug did not inhibit thymidine incorporation to the same extent, indicating that DNA synthesis was still operating in these cells despite their proliferative arrest (Fig. 1). This observation could be explicable if the action of CySH was to slow the passage of CCRF-CEM cells through S phase. Thus, the effects of CySH on the cell cycle distribution of CCRF-CEM cells were investigated over 96 hr. CySH caused an increase in the proportion of S phase cells, at the expense of G_1 cells, within 12 hr of their application to CCRF-CEM (Fig. 2A). The increase in S phase cells indicates that this agent acts to slow the passage of cells

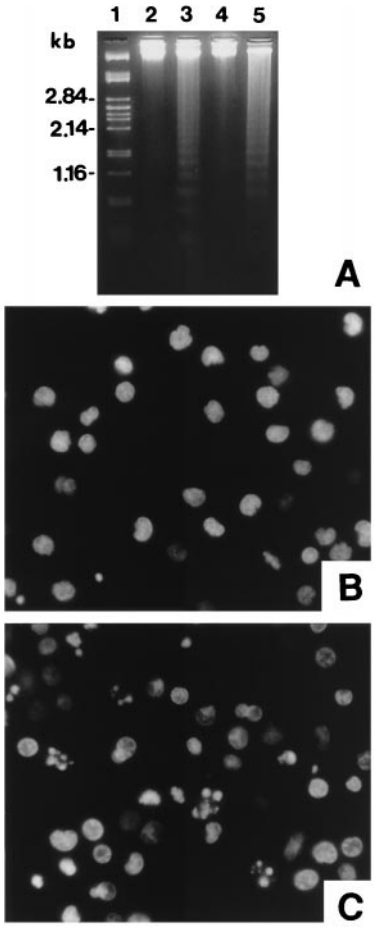

FIG. 3. Induction of apoptosis in CCRF-CEM cells by CySH. (A) Gel electrophoresis of DNA from CCRF-CEM cells: lane 1, λ/*Pst*I marker; lane 2, CCRF-CEM control at 4 hr; lane 3, 80 μM CySH at 4 hr; lane 4, 10 μM dexamethasone at 4 hr; and lane 5, 10 μM dexamethasone at 48 hr. Lanes 3 and 5 reveal DNA fragmentation characteristic of apoptosis. (B and C) Photomicrographs of CCRF-CEM cells stained with Hoechst 33342: control (B) and 80 μM CySH-treated CCRF-CEM cells at 4 hr (C).

through this phase of the cycle. This slowing of S phase does not represent a block in the cell cycle because cells enter both G_1 and G_2, with a concomitant loss of cells from S phase, by 24 hr. The proportion of S phase cells continues to decline in favour of increases in G_1 phase cells with increasing incubation time. These changes cannot be accounted for by the entry of S phase cells into G_2/M and G_1 alone, because the cell density does not increase appreciably from 24 to 48 hr (Fig. 2B). However, the number of non-viable cells increases from 24 to 72 hr, indicating that some of the CySH-induced loss of S phase cells is due to cell death.

The regular pattern of cell loss suggested that a subpopulation of cells were undergoing apoptosis following the administration of CySH, and this was evident within 4 hr, as indicated by internucleosomal DNA cleavage (Fig. 3A). Furthermore, the chromatin of CySH-treated cells stained with Hoechst 33342 appeared condensed and fragmented (Fig. 3C). This staining pattern of chromatin was comparable to that of glucocorticoid-induced apoptotic CCRF-CEM cells [35]. In contrast, the nuclei of untreated CCRF-CEM cells showed clear nuclear staining with Hoechst 33342 (Fig. 3B; [35]). Thus, CySH acts to inhibit the proliferation of CCRF-CEM cells by reducing their passage through the S phase and to induce apoptosis in a subpopulation of cells.

Role of Superoxide and Peroxide in the Antiproliferative Actions of CySH

The ability of CySH to generate peroxide in RPMI medium was demonstrated by polarography. CySH caused a significant increase in the rate of oxygen consumption over that seen with RPMI medium alone (Table 1). This increase in O_2 consumption was blocked, in part (61%), by EDTA, indicating the involvement of divalent cations. Oxygen consumption in RPMI was also increased significantly by the addition of FBS. The addition of CySH to serum-containing medium further increased O_2 consumption above that seen with FBS and RPMI alone, in a concentration-dependent manner. Dithiocarbamate attenuated the O_2 consumption by CySH and FBS (93.5%, Table 1), demonstrating a catalytic role for copper in the generation of peroxide by CySH. The major source for copper in serum is ceruloplasmin, and this protein was able to stimulate O_2 consumption by CySH, in the absence of serum (Table 1). The addition of 20–100 μg/mL catalase restored the O_2 consumed in the presence of CySH and FBS or ceruloplasmin, confirming that peroxide had been generated (data not shown).

The role of H_2O_2 and $O_2^{\cdot-}$ in the antiproliferative actions of CySH was investigated with catalase and SOD. Neither SOD nor catalase, alone or in combination, significantly altered the proliferation of CCRF-CEM cells (Table 2). However, catalase abolished the ability of CySH to inhibit proliferation, indicating that the production of H_2O_2 by this aminothiol significantly contributes to its cytostatic actions. It is unlikely that CySH produced an equimolar amount of H_2O_2 because 80 μM H_2O_2 was toxic to the cells (Table 2). Peroxide results from SOD acting on $O_2^{\cdot-}$, and this enzyme significantly potentiated the ability of CySH to both kill and inhibit the proliferation of CCRF-CEM cells (Table 2). Catalase reversed the cellular effects of the combination of SOD and CySH, indicating that H_2O_2 was the likely product of these two agents and that CySH generates $O_2^{\cdot-}$.

Inhibition of the Proliferation of CCRF-CEM Cells by Congeners of CySH

The ability of submillimolar concentrations of CySH to generate peroxide is shared by a variety of low molecular weight thiol-bearing compounds [21], including *N*-acetylcysteine [22, 26], WR 1065 [23, 26], and penicillamine [24–26]. Therefore, we hypothesized that congeners of

TABLE 1. Oxygen consumption by CySH-containing solutions

Treatment	O_2 (nM/min)
RPMI medium	44.0 ± 2.2 (3)
80 μM CySH + RPMI	152 ± 26.7* (3)
EDTA + RPMI	41.8 ± 5.8 (3)
80 μM CySH + EDTA + RPMI	83.7 ± 5.8† (3)
FBS + RPMI	173 ± 22.9‡ (16)
80 μM CySH + FBS + RPMI	314 ± 18.2§ (6)
200 μM CySH + FBS + RPMI	465 ± 45.3‖ (5)
400 μM CySH + FBS + RPMI	730 ± 43.0¶ (4)
Dithiocarbamate + FBS + RPMI	254 ± 13.6 (4)
400 μM CySH + dithiocarbamate + FBS + RPMI	291 ± 35.7** (4)
Ceruloplasmin + RPMI	0 ± 0 (4)
400 μM CySH + ceruloplasmin + RPMI	894 ± 43.2 (4)
400 μM CySH + ceruloplasmin + dithiocarbamate + RPMI	0 ± 0 (4)

In these experiments, RPMI medium was supplemented with either 10% FBS, 1 mM EDTA, 5 mM dithiocarbamate, 274 units/L of ceruloplasmin or combinations thereof. Values are means ± SEM; the number of experiments is given in parentheses.

* $P < 0.005$, significantly different from RPMI.

† $P < 0.05$, significantly different from 80 μM CySH + RPMI.

‡ $P < 0.05$, significantly different from RPMI.

§ $P < 0.05$, significantly different from FBS + RPMI.

‖ $P < 0.005$, significantly different from FBS + RPMI.

¶ $P < 0.001$, significantly different from FBS + RPMI.

** $P < 0.0005$, significantly different from 400 μM CySH + FBS + RPMI.

CySH that did not bear free thiol groups would not inhibit proliferation, whereas those that did, would. Neither ethanolamine, which has a hydroxyl in place of the thiol group of CySH, nor WR 638, which has the thiol phosphorylated, was able to influence the density of CCRF-CEM cells at concentrations up to 800 μM (data not shown). WR 638 differs from other aminothiophosphoesters in that it is not a substrate for cellular phosphatases [1]. However, all compounds bearing free thiol groups (CySH, *N*-acetylcysteine, *N*-acetylcysteamine, L-penicillamine, D-penicillamine, WR 1065, 2-, 3- and 4-aminothiophenol) or those that can be exposed following metabolic activation (cystamine, D-penicillamine methyl ester, WR 2721, WR 2822, and WR 2823) were able to inhibit the proliferation of CCRF-CEM cells (Table 3). Furthermore, molecular modeling of the fifteen antiproliferative thiol congeners revealed that a terminal thiol, rather than any other moiety, was most likely to be responsible for causing the inhibition of proliferation. For example, the charge on the sulfur of the terminal thiol correlates with the inhibition of proliferation of the congeners according to the expression $^1/_{IC_{50}} = 1.86 \times$ (charge on the sulfur) ($r = 0.78$, Fig. 4, Table 3). Thus, as the charge of the thiol becomes more electronegative, the ability of the thiol-bearing compound to inhibit proliferation becomes proportionately less. The charge on the sulfur also influences the amount of proton dissociation from the thiol group and the ability to inhibit cell density was found to correlate linearly ($r = 0.99$) with the known pK_a of four of the thiol-bearing compounds (Table 3). Taken together, these studies indicate that the thiol moiety is essential for the antiproliferative effects of the tested compounds [36].

TABLE 2. Effect of catalase and SOD on the inhibition of CCRF-CEM proliferation by CySH

Conditions	Proliferation (% control)	Eosin-negative cells (% viability)
Control	100 ± 0.0	96.5 ± 0.5
CySH	21.0 ± 2.3*	91.7 ± 0.5*
SOD	90.5 ± 5.0	95.9 ± 0.5
CySH + SOD	8.1 ± 4.1*†	84.6 ± 1.8*‡
Catalase	101 ± 7.7	96.6 ± 0.3
CySH + catalase	94.9 ± 4.9§	94.8 ± 0.5‖
SOD + catalase	89.8 ± 2.7§	95.9 ± 0.6
CySH + SOD + catalase	98.8 ± 3.2	96.0 ± 1.0‡
H_2O_2	0.00	0.00

CCRF-CEM cells at 2.5×10^5/mL were treated with either 80 μM CySH, 80 μM H_2O_2, 20 μg/mL SOD, or 20 μg/mL catalase, alone or in combination, for 24 hr. Shown are means ± SEM for the cell density and viability measurements from six experiments.

* $P < 0.0001$, significantly different from control.

† $P < 0.01$, significantly different from CySH.

‡ $P < 0.005$, significantly different from CySH.

§ $P < 0.0001$, significantly different from CySH.

‖ $P < 0.001$, significantly different from CySH.

The conformational studies also showed that, in addition to the thiol group, the other major determinant of the antiproliferative capacity of these congeners was the overall conformation of the compounds. With the exception of 2,3-dimercapto-1-propane sulfonic acid, all of the compounds tested were either primary or secondary amines. The most active compounds were those that have the amino acid moiety and the thiol in close proximity, producing a "closed conformation." The optimal distance between these

TABLE 3. Inhibition of the proliferation of CCRF-CEM and B4D cells by CySH congeners

Common name	Formula	IC_{50} (μM) CCRF-CEM	IC_{50} (μM) B4D	Calculated change at S (eV)	$pK_{a(SH)}$
2-Aminothiophenol	$H_2N(C_6H_4)SH$	15.2 ± 1.55 (3) *14.3 ± 1.12* (6)*	13.3 ± 0.41 (3)	0.1281	
3-Aminothiophenol	$H_2N(C_6H_4)SH$	19.4 ± 2.86 (4) *14.9 ± 1.44† (6)*	19.5 ± 1.73 (7)	0.1626	6.6
WR 1065	$H_2N(CH_2)_3NH(CH_2)_2SH$	44.0 ± 4.31‡ (7) *19.6 ± 4.24† (7)*	51.7 ± 4.85 (5)	0.0478	
N-Acetylcysteamine	$H_3CCONH(CH_2)_2SH$	59.7 ± 7.55§ (9) *19.7 ± 4.44† (4)*	78.8 ± 6.13 (4)	0.0548	
4-Aminothiophenol	$H_2N(C_6H_4)SH$	37.4 ± 3.31§ (6) *21.7 ± 2.92‖ (6)*	38.5 ± 4.89 (3)	0.161	
Penicillamine methyl ester	$(H_3C)_2C(SH)CH(NH_2)COOCH_3$	37.2 ± 4.03¶ (11) *24.0 ± 4.42 (12)*	106 ± 14.4 (5)	0.0401	
2,3-Dimercapto-1-propane sulfonic acid	$HO_3SCH_2CH(SH)CH_2SH$	15.6 ± 2.36 (10) *24.7 ± 6.17 (7)*	7.38 ± 0.21 (6)	0.0527	
CySH	$H_2N(CH_2)_2SH$	53.6 ± 4.00** (23) *33.7 ± 2.63 (19)*	52.3 ± 4.37 (6)	0.0477	8.5
N-Acetylcysteine	$HOOCCH(NHOCCH_3)CH_2SH$	80.6 ± 7.28‡ (8) *45.2 ± 4.15‖ (6)*	73.1 ± 5.15 (11)	0.0668	9.5
L-Penicillamine	$(H_3C)_2C(SH)CH(NH_2)COOH$	67.4 ± 7.59 (11) *47.6 ± 6.58‖ (6)*	93.2 ± 12.5 (5)	0.075	10.4
D-Penicillamine	$(H_3C)_2C(SH)CH(NH_2)COOH$	55.7 ± 5.06 (6)		0.075	10.4
Cystamine	$[NH_2(CH_2)_2S\text{-}]_2$	119 ± 5.75** (4) *49.0 ± 4.66† (6)*	111 ± 16.2 (5)	0	
WR 2822	$NH_2(CH_2)_4NH(CH_2)_2SH_2PO_3$	152 ± 7.65¶ (5) *89.2 ± 8.60‖ (7)*		0.01495	
WR 2721	$NH_2(CH_2)_3NH(CH_2)_2SH_2PO_3$	*153 ± 33.8‖ (9)*		0.0119	
WR 2823	$NH_2(CH_2)_5NH(CH_2)_2SH_2PO_3$	*316 ± 67.3‖ (8)*		0.015	
WR 638	$H_2N(CH_2)_2SH_2PO_3$	NI††			
Ethanolamine	$NH_2(CH)_2OH$	NI			
D- and L-Lipoic acid	$HOOC(CH_2)_4\text{-}CH\text{-}CH_2CH_2\text{-}S\text{-}S\text{-}$	NI			

CCRF-CEM or B4D cells seeded at 2.5×10^5/mL were treated with graded concentrations of thiol-bearing compounds for 24 hr, and the IC_{50} values [means ± SEM of (N) determinations] for both thymidine incorporation and cell density measurements (italicized) were determined. The charge at the sulfur was calculated as described in the text, and $pK_{a(SH)}$ values for 3-aminothiophenol, *N*-acetylcysteine, and penicillamine are taken from Jencks and Regenstein [36] and for CySH from Vergroesen *et al.* [37].

* $P < 0.001$, significant difference vs CySH.
† $P < 0.01$, significant difference vs CySH.
‡ $P < 0.001$, cell density vs thymidine measurements.
§ $P < 0.01$, cell density vs thymidine measurements.
‖ $P < 0.05$, significant difference vs CySH.
¶ $P < 0.05$, cell density vs thymidine measurements.
** $P < 0.0001$, cell density vs thymidine measurements.
†† Not inhibitory at concentrations between 0 and 2 mM.

two moieties is between 2 and 5 carbon atoms, or 3.17 Å and 5.9 Å. This is particularly exemplified by the difference in activity of the aminothiophenol compounds. The most active of these compounds, 2-aminothiophenol, has the most "closed conformation" while the least active, 4-aminothiophenol, is the most open in conformation. It would seem that this distance criterion is most effective when combined with the presence of a relatively rigid benzene ring in the structure of the compound. Of the alkyl aminothiol compounds (CySH, *N*-acetylcysteine, *N*-acetylcysteamine, L-penicillamine, D-penicillamine, WR 1065, WR 2721, WR 2822, WR 2823), the ideal degree of separation between the thiol and terminal amino groups for antiproliferative ability was exemplified by WR 1065 (Table 3).

A number of dithiol compounds (D-lipoate, *L*-lipoate and 2,3-dimercapto-1-propane sulfonic acid) were also tested. The lipoate isomers were toxic to CCRF-CEM cells, rather than antiproliferative at millimolar concentrations (data not shown). In contrast, 2,3-dimercapto-1-propane sulfonic acid was an efficient inhibitor of leukemia cell proliferation with an IC_{50} of 25 μM (Table 3), which lies between values obtained for CySH (IC_{50} = 33.7 μM) and 4-aminothiophenol (IC_{50} = 21.7 μM, Table 3). One interesting feature of the antiproliferative effects of 2,3-dimercapto-1-propane sulfonic acid on CCRF-CEM is that the inhibition of thymidine incorporation (IC_{50} = 15.6 μM) was comparable to its inhibition of increases in cell number (IC_{50} = 24.7 μM) (Table 3). The alkyl aminothiols and 4-aminothiophenol all inhibited the proliferation-dependent increase in cell number to a greater extent than they inhibited thymidine incorporation (Table 3, Fig. 1).

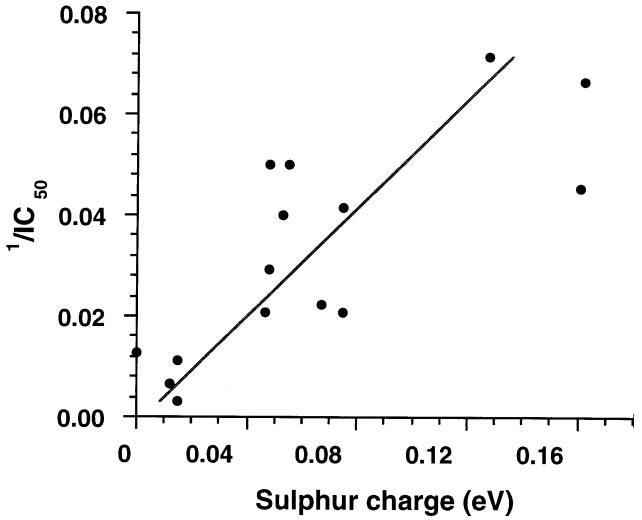

FIG. 4. Inhibition of proliferation of CCRF-CEM cells by thiol-bearing compounds as a function of the calculated charge on the sulfur. The data for this figure were taken from Table 3.

Comparison of Drug-Naive and -Resistant Leukemia Cell Lines

The ability of CySH to inhibit proliferation appears to correlate with its ability to generate peroxide (Tables 1 and 2), a property of many thiol-bearing compounds that also inhibit proliferation (Table 3). Thus, we hypothesized that these compounds would also inhibit the proliferation of other leukemia cell lines, including those made resistant to anticancer drugs. In support of this hypothesis, WR 1065, L-penicillamine, cystamine, and the aminothiophenols all inhibited the proliferation of B4D cells, a methotrexate-resistant clone of CCRF-CEM cells [27], with approximately the same efficacy as their effects on CCRF-CEM cells, as measured by thymidine incorporation (Table 3). Interestingly, 2,3-dimercapto-1-propane sulfonic acid arrested the proliferation of B4D cells (IC_{50} = 7.4 ± 0.2) with significantly greater efficacy than that of CCRF-CEM cells (IC_{50} = 16 ± 2.4, $P < 0.01$, Table 3). In addition, 2,3-dimercapto-1-propane sulfonic acid was more effective at inhibiting thymidine incorporation into the DNA of B4D cells relative to CCRF-CEM cells (Table 3). B4D cells were confirmed to be ~100 times more resistant to methotrexate than the parent cell line (data not shown). In addition to its effects on B4D cells, CySH arrested the proliferation of VB 1000, an extremely vinblastine-resistant CCRF-CEM subline (~10^3-fold resistance to vinblastine [28]), with an IC_{50} of 550 ± 17 μM (N = 4), which is approximately 10-fold higher ($P < 0.0001$) than the IC_{50} values obtained with B4D or CCRF-CEM cells. Finally, 2,3-dimercapto-1-propane sulfonic acid also inhibited the proliferation of a B cell-derived leukemia cell line, Namalwa, with an IC_{50} of 4.2 ± 0.9 μM (N = 6).

DISCUSSION

In this study, thiol-bearing compounds were shown to inhibit the proliferation of both drug-naive and -resistant leukemia cell lines. Thiol-bearing compounds can potentially arrest proliferation by scavenging reduced oxygen species necessary for growth and by catalyzing the production of peroxide. The involvement of reduced oxygen species in proliferation has been inferred from experiments where scavengers of reactive oxygen species arrest both proliferation and the activity of transcription factors necessary for mitogenesis [38]. In support of this hypothesis, CySH has been shown to inhibit both the recruitment of mitogenically stimulated human peripheral blood lymphocytes into G_1 from G_0 and the DNA binding activity of NF-κB and AP-1 [13, 14]. However, the inhibition of leukemia cell lines by thiol-bearing compounds differs from that of normal lymphocytes. CySH slows the passage of CCRF-CEM cells through S phase (Fig. 2), whereas it arrests mitogenically stimulated lymphocytes during the G_0–G_1 transition [13]. WR 1065, WR 151326, and CySH have also been shown to affect the transit of other transformed cell lines through S phase [15, 39], which suggests that thiol-bearing compounds act to retard DNA synthesis. Indeed, CySH and its congeners do attenuate DNA replication [9, 15, 40] and the activity of two enzymes involved in this process, thymidine kinase [40] and DNA polymerase [41]. One of the consequences of arresting CCRF-CEM cells was to induce apoptosis in a subpopulation of cells, most likely the CySH-affected S phase cells. It is not known if the induction of apoptosis was simply a consequence of attenuating DNA synthesis in these cells or a direct action of CySH and its products, but it suggests that some elements of this process are sensitive to actions of thiol-bearing compounds.

We hypothesized that the difference between normal and neoplastic cells resided in their abilities to cope with a peroxidative stress, based on the observations that neoplastic cells have a lower complement of peroxide-catabolizing enzymes [42], that S phase cells are preferentially sensitive to oxidative stress, and that submillimolar concentrations of thiol generate peroxide. Cytotoxic and cytostatic concentrations of CySH (Table 1) [22, 43], WR 1065 [23, 26, 44], penicillamine [24–26], and *N*-acetylcysteine [22, 26] all generate H_2O_2. Moreover, catalase ameliorates both the cytotoxic [22, 26, 43] and the cytostatic action of thiol-bearing compounds (Table 2) [25, 26, 45]. The production of H_2O_2 by CySH is not likely to be equimolar as predicted from stoichiometry of reactions 1–3, because 80 μM H_2O_2 was toxic to CCRF-CEM cells and probably relates to the reaction of thiols with superoxide and peroxide, albeit at a low rate, at micromolar thiol concentrations [26, 46]. Dithiol compounds react slowly with peroxide and may account for the toxicity of the lipoates [26] and the ability of 2,3-dimercapto-1-propane sulfonic acid to inhibit both thymidine incorporation and the proliferation-dependent increase in cell number with equal efficacy (Table 3).

Thiol-mediated peroxide production is thought to result from the reduction of serum-associated transition metals by thiols (reaction 1) because H_2O_2 production can be inhibited by transition metal chelators, in particular the copper chelator dithiocarbamate (Table 1) [22, 23, 25, 45]. Moreover, the substitution of copper or ceruloplasmin for serum is sufficient to support both the production of peroxide (Table 1) [22, 23, 25, 45] and cellular effects of thiol-bearing compounds [25, 26, 45, 47]. Thiol-dependent reduction of transition metals requires the concomitant oxidation of the thiol (reaction 1), and this has been demonstrated for CySH [26], dithiothreitol [26, 47], penicillamine [25, 26], WR 1065 [23, 26], *N*-acetylcysteine, 2,3-dimercaptopropane sulfonic acid, and lipoate [26]. The reduced metal resulting from reaction 1 is thought to then donate an electron to oxygen, generating $O_2^{\cdot-}$ (reaction 2), which, in turn, dismutases to H_2O_2 (reaction 3). This reaction is supported by the observations that the addition of thiols to solutions containing transition metals results in the consumption of O_2 (Table 1) [21–23, 25] and that WR 1065 [48] and penicillamine [25] reduce either cytochrome *c* or nitro blue tetrazolium in an SOD-inhibitable manner. The generation of $O_2^{\cdot-}$ by CySH in serum is supported by the observation that the potentiation of the cytostatic actions of CySH by SOD was blocked by catalase, inferring the production of H_2O_2 from $O_2^{\cdot-}$ (reaction 3).

In addition to the above reactions, WR 1065 can also generate H_2O_2 via its degradation to CySH and acrolein by copper-dependent amine oxidases according to the following reaction [44]:

$$H_2N(CH_2)_3NH(CH_2)_2SH + O_2 + H_2O \rightarrow CH_2 = CH\text{-}CHO + H_2N(CH_2)_2SH + H_2O_2 + NH_3 \quad (4)$$

CySH as the major product of this reaction would further generate H_2O_2 according to reactions 1–3. Thus, these two series of H_2O_2-generating reactions might account for the increased efficacy of WR 1065 as compared to CySH with respect to inhibition of the proliferation of leukemia cell lines (Table 3). It should be noted that, in general, the ability of thiols to generate H_2O_2 is limited to submillimolar concentrations of these agents. At higher concentrations, thiols react rapidly with both peroxide and superoxide, thereby limiting H_2O_2 production [46]. In addition, the previously mentioned ability of thiol-bearing compounds to detoxify the harmful effects of radiation, xenobiotics, and excess transition metals [2–6] relates more to their actions as hydroxyl radical scavengers, chelators, and disulfide reductants than to generation of H_2O_2.

The ability to produce peroxide is a function of the thiol group, and all of the compounds tested that were shown to be antiproliferative bear either free thiol groups or those that can be exposed via metabolism (Table 3, Fig. 4). Moreover, the acidity or sulfur charge of the thiol is an important determinant of the antiproliferative activity of the thiol-bearing compound (Table 3). Vergroesen *et al.* [37] also noted that the toxicity of thiol-bearing compounds is related to the $pK_{a(SH)}$. The metal-catalyzed oxidation of thiols (reaction 1) is favored at pH values close to and greater than the $pK_{a(SH)}$, indicating that proton lability is an important determinant in the efficacy of these compounds [21]. Thus, thiol-bearing compounds with $pK_{a(SH)}$ values close to or less than physiological pH are more likely to reduce available transition metals [21] and inhibit proliferation (Table 3) or kill cells [37]. The acidity of the thiol group increases in the presence of a nearby electron-withdrawing group such as the amino moiety and, in the case of alkyl thiols, decreases with increasing chain length and chain branching [49].

Previous studies [13] have shown that the amino moiety also influences the cytostatic activity of thiol-bearing compounds. Here we have shown that the ideal conformation of the thiol and amino moieties was one in which these two groups were in close proximity producing a "closed conformation," as exemplified by the aminothiophenols. Increasing the distance between the terminal amino and thiol and the flexibility of the molecule decreased the efficacy of the aminothiol as shown with the series of aminothiophosphoesters (Table 3, Fig. 4). The results obtained with D- and L-penicillamine demonstrate that optical isomerization is not an important property with respect to the ability to inhibit proliferation, at least in the case of this thiol. However, the addition of a methyl ester to D-penicillamine improved the antiproliferative ability of this compound as measured by inhibition of [^{3}H]thymidine incorporation (Table 3). Since the addition of a methyl ester group decreases the polarity of the parent compound, this suggests that the uptake of the compounds may be an important determinant of their antiproliferative actions. The compounds that required metabolic conversion to the corresponding free thiol, WR 2721 to WR 1065 and cystamine to CySH, were less effective in inhibiting the proliferation of CCRF-CEM cells than their corresponding free thiol (Table 3), suggesting that either (i) the metabolic conversion is not completely efficient, (ii) there are differences in the uptake of the analogs, or (iii) the kinetics of peroxide production and metabolism may play an important role in determining the efficacy of these compounds. The difference in the efficacy between cystamine and CySH is particularly pertinent because each mol of the disulfide can theoretically be reduced to 2 mol of the thiol.

Pharmacokinetic studies have shown that CySH can attain plasma concentrations of between 30 and 60 μM, for 2 hr after a single oral dose of 26 mg/kg [50]. These concentrations correspond approximately to the inhibitory concentrations of CySH reported here (Fig. 1) and so might account for the *in vivo* effects of this agent [10, 16–20]. It is unlikely that it will be possible to administer CySH at high enough concentrations to kill neoplastic cells because of the toxicity associated with high-dose CySH treatment [51]. Indeed, CySH treatment has not been shown to eliminate neoplastic cells in any study [17–20] except one [16]. While CySH might not be an efficacious antineoplas-

tic agent, other thiol-bearing compounds with the optimum physico-chemical characteristic determined in this study may be. These studies are important given the selective nature of some of these congeners and their ability to act on both drug-naive and -resistant cells.

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