Cyclic Urea and Thiourea Derivatives as Inducers of Murine Erythroleukemia Differentiation

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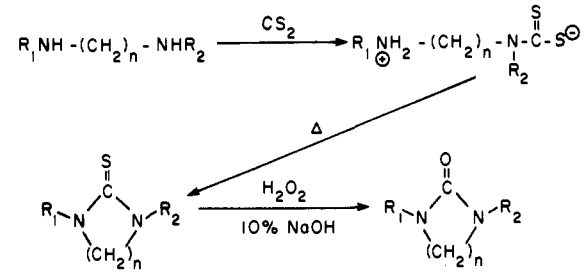
A series of derivatives of tetramethylurea, a known inducer of the differentiation of Friend erythroleukemia cells, has been synthesized and tested for its capacity to induce erythroid maturation, as measured by the synthesis of hemoglobin. Cyclic urea and thiourea derivatives consisting of five-, six-, and seven-membered ring systems containing *N*-alkyl substituents were prepared. Most of these agents were relatively effective inducers of differentiation, with *N*-alkyl substitution appearing to be essential for maximum response. The most potent agents developed were *N,N'*-dimethyl cyclic ureas. Exposure to concentrations of 2 to 4 mM of these derivatives resulted in more than 90% of the cell population achieving a differentiated state. Under these conditions, the parent compound, tetramethylurea, was slightly less efficacious, causing differentiation of only 68% of the population at its maximum effective level of 4 mM.

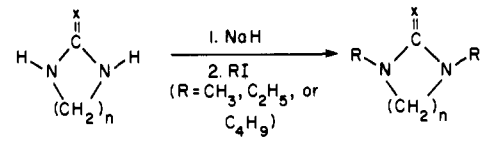
Friend murine leukemia cells in culture can be induced to differentiate along the erythroid pathway by dimethyl sulfoxide (Me_2SO)[1] and a variety of structurally diverse chemical classes (see ref 2–8 for representative inducers). The maturation process is characterized by a series of morphological and biochemical events that mimic normal erythropoiesis,[1,8–11] including a marked decrease in cell volume,[1,12] changes in membrane properties,[13–18] the accumulation of erythroid markers such as globin mRNA, enzymes of heme biosynthesis, and hemoglobin,[1,19–22] and a loss of proliferative capacity.[23–26]

Scheme I



12, n = 2; $R_1 = R_2 = CH_3$
13, n = 2; $R_1 = R_2 = C_2H_5$
20, n = 3; $R_1 = H$, $R_2 = CH_3$
21, n = 3; $R_1 = R_2 = CH_3$
22, n = 3; $R_1 = R_2 = C_2H_5$
26, n = 4; $R_1 = R_2 = H$

15, n = 3; $R_1 = H$, $R_2 = CH_3$
23, n = 4; $R_1 = R_2 = H$



8, x = O; n = 2; $R = CH_3$
9, x = O; n = 2; $R = C_2H_5$
16, x = O; n = 3; $R = CH_3$
17, x = O; n = 3; $R = C_2H_5$
18, x = O; n = 3; $R = n\text{-}C_4H_9$
24, x = O; n = 4; $R = CH_3$
25, x = O; n = 4; $R = C_2H_5$
27, x = S; n = 4; $R = CH_3$

As part of a program to develop new and potent inducers of differentiation and to examine the relationship between structure and the capacity to initiate the maturation of Friend erythroleukemia cells, we have synthesized a variety of agents with structural similarities to tetramethylurea, an effective inducer of differentiation of murine erythro-

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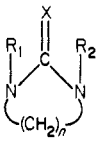
Table I. Synthetic Methodology and Physicochemical Properties of Cyclic Ureas and Thioureas



no.	n	X	R_1	R_2	method	bp (mm) or mp, °C	yield, %	formula
8	2	O	CH_3	CH_3	D	46-49 (0.5)	80	$C_5H_{10}N_2O$
9	2	O	C_2H_5	C_2H_5	D	66-67 (0.1)	68	$C_7H_{14}N_2O$
12	2	S	CH_3	CH_3	A	111-112	72	$C_5H_{10}N_2S$
13	2	S	C_2H_5	C_2H_5	A	62-63	63	$C_7H_{14}N_2S$
15	3	O	H	CH_3	C	91-92	61	$C_5H_{10}N_2O$
16	3	O	CH_3	CH_3	D	60-61 (0.05)	67	$C_6H_{12}N_2O$
17	3	O	C_2H_5	C_2H_5	D	58-61 (0.1)	62	$C_8H_{16}N_2O$
18	3	O	n-C_4H_9	n-C_4H_9	D	96-100 (0.1)	60	$C_{12}H_{24}N_2O$
20	3	S	H	CH_3	A	121-122	70	$C_5H_{10}N_2S$
21	3	S	CH_3	CH_3	A	79	56	$C_6H_{12}N_2S$
22	3	S	C_2H_5	C_2H_5	A	56-57	30	$C_8H_{16}N_2S$
23	4	O	H	H	C	175-177	60	$C_5H_{10}N_2O$
24	4	O	CH_3	CH_3	D	45-47 (0.1)	67	$C_7H_{14}N_2O$
25	4	O	C_2H_5	C_2H_5	D	81-86 (0.5)	62	$C_9H_{18}N_2O$
26	4	S	H	H	A	178	65	$C_5H_{10}N_2S$
27	4	S	CH_3	CH_3	E	63-65	21	$C_7H_{14}N_2S$

leukemia cells, and have compared the biological potency of these compounds in this system.[27,28] The agents synthesized have included cyclic ureas and thioureas consisting of five-, six-, and seven-membered ring systems N-substituted with CH_3 and C_2H_5 groups. These agents were relatively good inducers of differentiation, as measured by the accumulation of hemoglobin, with *N*-alkyl substitution, in general, appearing to enhance the efficacy of the compounds as initiators of erythroid maturation. The most potent compounds of this class were the *N,N'*-dimethyl cyclic ureas; these agents caused 90% of the cell population to synthesize hemoglobin at concentrations of inducer 50 to 100 times lower than Me_2SO and compared favorably with tetramethylurea, which converted only 68% of the cellular population to the differentiated state at its maximally effective concentration.

Chemistry. The procedures employed for the synthesis of cyclic urea and thiourea derivatives are outlined in Scheme I. Various polymethylenediamines were refluxed with carbon disulfide in aqueous ethanol to form thiocarbamic acid intermediates, which were heated continuously to give cyclic thioureas. A lengthening of the alkyl chain and the number of *N*-alkyl substitutions on the diamines led to a decrease in the tendency to form cyclic derivatives. Thus, the thiocarbamic acid intermediates of dimethyltetramethylenediamine (6) and dimethylhexamethylenediamine (7) failed to cyclize to form the desired thioureas. Certain cyclic ureas, such as *N*-methyltrimethyleneurea (15) and tetramethyleneurea (23), were obtained in 60% yield by the oxidation of their corresponding thioureas with 30% H_2O_2 in NaOH solution. Disubstituted cyclic ureas and the cyclic thiourea 27 were prepared by alkylation of the sodium salts of their unsubstituted derivatives. The N-alkylation of cyclic ureas generally occurred in good yields, ranging from 60 to 80%, whereas yields of comparable cyclic thioureas were low (about 20%). The physicochemical properties and the synthetic methodologies employed for the compounds that were prepared are summarized in Table I.

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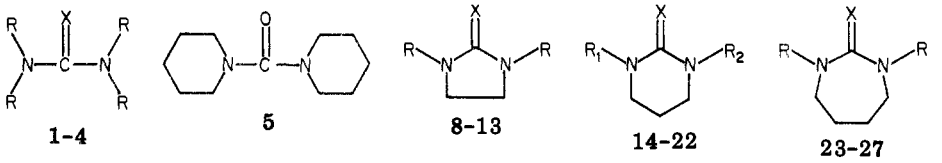
Biological Results and Discussion

Cyclic urea and thiourea derivatives were compared to Me_2SO and to tetramethylurea as inducers of the differentiation of murine erythroleukemia cells; concurrently, their effects on cellular replication were measured. These measurements were accomplished by exposing exponentially growing murine erythroleukemia cells to a wide range of concentrations of each agent to determine the optimal level required for maximum differentiation. The degree of erythroid maturation was measured by assessing the proportion of benzidine-positive cells (i.e., hemoglobin-containing cells) 6 days after continuous exposure to the agent under test. Cell numbers were determined on days 3 and 6 of incubation.

The results shown in Table II indicate that tetraalkylureas and -thioureas were generally effective initiators of maturation, producing 68–79% benzidine-positive cells at optimal concentrations of 2–4 mM. The alkyl substituents tested consisted of *N*-methyl or *N*-ethyl groups. Tetraethylthiourea (4) was exceedingly toxic to leukemia cells throughout the range of concentrations tested (i.e., 0.1–10 mM); this agent was not an effective inducer of erythroid differentiation at the concentrations tested, possibly because the level required for effective induction exceeded the amount needed to initiate maturation. Attachment of bulky ring systems to the nitrogen atoms of urea to form bispentamethyleneurea (5) markedly decreased the capacity to induce differentiation. Ethyleneurea (8) and ethylenethiourea (11) were relatively weak inducers of differentiation, while the disubstituted ethyleneureas (9 and 10) and -thioureas (12 and 13) were more efficacious initiators of cell maturation. In the trimethyleneurea series, a direct correlation was obtained between the number of *N*-alkyl substituents on the ring nitrogen and the percentage of cells in the population induced to synthesize hemoglobin. Thus, the percentage of benzidine-positive cells rose from 15% in the absence of N-methylation of trimethyleneurea (14) to 50 and 92% when the *N*-methyl (15) and *N,N'*-dimethyl (16) substituted derivatives, respectively, were employed. A similar relationship was observed with trimethylenethiourea derivatives. An increase in the size of the alkyl substituent on the ring nitrogen atoms led to a decrease in the capacity of the compounds to initiate cellular differentiation. Thus, the dibutyl derivative 18 was a noninducer of maturation,

Table II. Effects of Ureas and Thioureas on the Replication and Erythroid Differentiation of Friend Leukemia Cells



no.	X	R	concn, mM	% cell growth on day 3[a]	% cell growth on day 6[a]	benzidine-positive cells, %
1	O	CH_3	4	104	115	68
2	O	C_2H_5	2	69	77	68
3	S	CH_3	2	123	98	79
4	S	C_2H_5[b]	0.1-10	<4	<1	<1
5			4	28	33	10
8	O	H	4	88	108	4
9	O	CH_3	4	70	73	92
10	O	C_2H_5	16	36	140	81
11	S	H	4	128	62	21
12	S	CH_3	2	102	95	66
13	S	C_2H_5	2	60	120	84
14	O	$R_1 = R_2 = H$	4	124	121	15
15	O	$R_1 = H; R_2 = CH_3$	2	46	136	50
16	O	$R_1 = R_2 = CH_3$	2	90	87	92
17	O	$R_1 = R_2 = C_2H_5$	4	160	127	70
18	O	$R_1 = R_2 = n\text{-}C_4H_9$[b]	0.1-10	<5	<1	<1
19	S	$R_1 = R_2 = H$	2	144	44	14
20	S	$R_1 = H; R_2 = CH_3$	2	107	166	22
21	S	$R_1 = R_2 = CH_3$	4	75	135	83
22	S	$R_1 = R_2 = C_2H_5$	1	45	97	61
23	O	H	2	93	104	66
24	O	CH_3	4	25	61	92
25	O	C_2H_5	4	40	131	31
26	S	H	2	54	82	42
27	S	CH_3	4	64	73	79
Me_2SO[c]			105	70	120	75
Me_2SO[c]			210	72	110	94

[a] Percent of untreated control cell growth. [b] These agents were lethal to cell growth in the range of concentrations tested (0.1-10 mM). [c] Me_2SO was included as a control in all experiments.

being lethal to cells in the range of concentrations tested. In this series, the dimethylurea derivative **16** was the most effective inducer, causing 92% of Friend cells to differentiate at a level of 2 mM. A relatively high degree of cellular maturation was induced by the unsubstituted cyclic urea **23** and cyclic thiourea **26**; however, in a manner analogous to the other ring systems tested, the *N,N'*-dimethyl substituted derivative **24** was the most potent, producing 92% benzidine-positive cells at a concentration of 4 mM. The findings demonstrate that there is a wide degree of latitude in the structural requirements for an effective inducer of differentiation in the tetramethylurea series, implying that the receptor(s) for these agents is relatively nonspecific.

Experimental Section

Melting points were taken on a Thomas-Hoover capillary apparatus and are uncorrected. The boiling points were recorded at various reduced pressures. Elemental analyses were performed by the Baron Consulting Co., Orange, CT. NMR spectra were obtained using a Varian Model T-60A spectrometer; tetramethylsilane was used as an internal standard in $CDCl_3$ and as an external reference in dimethyl-d_6 sulfoxide. Prepared compounds were homogeneous when analyzed by micro-thin-layer chromatography on silica gel, and NMR spectra and elemental analyses were consistent with the expected chemical structures.

Method A. Preparation of Cyclic Thioureas. CS_2 (7 mL) was added dropwise with stirring to a solution of polymethylenediamine or an *N,N'*-dialkyl derivative thereof (0.1 M) in 50% aqueous ethanol (60 mL). Reactions were initiated by gradual heating, which led to the formation of thiocarbamic acid intermediates as white precipitates. After refluxing for 1 h, reaction mixtures were treated with concentrated HCl (2–3 mL) and refluxed continuously for an additional 9 to 10 h. The products were collected by filtration after cooling. Recrystallization from aqueous ethanol gave the desired pure thiourea.[29]

Method B. Intermediate Dithiocarbamate Salts 6 and 7. Compounds **6** and **7** were obtained by treatment of either *N,N'*-dimethyltetramethylenediamine or *N,N'*-dimethylhexamethylenediamine with CS_2 following the procedure outlined in method A. NMR spectra and elemental analyses supported the expected chemical structures for the recrystallized white precipitates that were obtained. Compound **6**: yield 60%; mp 209–211 °C. Anal. ($C_7H_{16}N_2S_2$) C, H, N. Compound **7**: yield 75%; mp 235–237 °C. Anal. ($C_{17}H_{16}N_2S_2$) C, H, N.

Method C. *N*-Methyltrimethyleneurea (15) and Tetramethyleneurea (23). The cyclic thioureas **20** and **26** (0.1 M) were treated with 30% H_2O_2 (70 mL) in 10% aqueous NaOH (20 mL) with stirring at 70 °C.[30] The thioureas dissolved in the alkaline solution and the reaction mixtures were kept at 70 °C for 30 min. After the residue was removed by filtration, the filtrates were evaporated to dryness in vacuo. Recrystallization from $CHCl_3$ gave the expected products in 60–65% yield.

Method D. *N,N'*-Dialkylalkyleneureas. Freshly distilled dioxane (250 mL) was added slowly with stirring to a mixture of an alkyleneurea (0.1 M) and 61% NaH dispersion (13 g) under N_2. The reaction mixtures were kept between 55 and 60 °C for 4 h and then were cooled to room temperature. After an alkyl

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iodide (0.6 M) was added, the reaction mixtures were refluxed for 3.5 h and filtered to remove precipitated NaI, and the solvent was evaporated. Vacuum distillation of residual oils gave the desired products.

Method E. *N,N'*-Dimethyltetramethylenethiourea (27). Compound **27** was prepared by a modification of method D. After CH_3I (0.2 M) was added to the sodium salt of the cyclic thiourea **26**, the reaction mixture was maintained at 55 to 60 °C for 2 h. Vacuum distillation provided an oily product which recrystallized from ether–heptane to give pure **27**.[31]

Method F. Cell Culture and Induction Experiments. Murine erythroleukemia cells (clone 745A), established originally by Friend et al.,[1] were kindly donated by Dr. A. S. Tsiftsoglou of the Massachusetts Institute of Technology Center for Cancer Research. Cells were maintained in suspension culture at 37 °C in a 10% CO_2 humidified atmosphere by weekly passage of 10^5 cells/mL in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum (GIBCO), streptomycin (100 μg/mL), and penicillin (100 units/mL). The capacity of each agent to induce erythroid differentiation was measured in exponentially growing cells. Agents were added at the time of cell seeding (10^5 cells/mL), and each agent was tested over a range of concentrations in at least two separate experiments, employing graded twofold increases in concentration; the average value for the optimum effective concentration of each agent tested is given in Table II. Me_2SO-treated cultures served as positive controls in all experiments. On days 3 and 6, cell numbers were measured using a Coulter Model ZBI electronic particle counter. On day 6, the proportion of differentiated cells was determined cytologically by measuring the number of hemoglobin-containing cells which stained blue with an acid solution of benzidine peroxide as described by Orkin et al.[32]

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Induction of Differentiation of Leukemia Cells in Vitro by N-Substituted Amides, Lactams, and 2-Pyridones

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N-Substituted amides, lactams, and 2-pyridones were examined for their ability to induce leukemic cell differentiation in vitro. These substituted amides were effective inducers of the differentiation of murine erythroleukemia (Friend) cells, as judged by the cellular accumulation of hemoglobin. N-Substitution increases the effectiveness of all classes of agents as inducers of maturation as judged by an increase in potency. *N*-Cyclohexylacetamide appeared to be a particularly effective compound, causing 92% of the population to synthesize hemoglobin at a concentration which was not inhibitory to cell replication. In the lactam series, an increase in ring size was paralleled by an increase in the potency of unsubstituted compounds, but ring size had less effect on N-substituted lactams. Increasing the chain length of the *N*-alkyl substituent had little additional effect on a series of lactam and 2-pyridone derivatives; however, some decrease in maximal induction occurred with butyl- and hexyl-substituted 2-pyridones. *N*-Isopropyl-2-pyridone, selected for further study, was as good an inducer of the HL60 human promyelocytic leukemia as it was of the Friend murine erythroleukemia.

Friend murine erythroleukemia cells induced to differentiate along the erythroid pathway,[1] and HL60 human leukemia cells induced to undergo maturation from a primitive promyelocyte or myeloblast to an end stage granulocytic form,[2] represent two in vitro experimental model systems for the development of agents capable of inducing terminal cellular differentiation of neoplastic cells. Many of the compounds that cause differentiation of the Friend erythroleukemia are also effective inducers of HL60 maturation,[2-4] implying that similarities exist in the mechanism by which these neoplastic cells can be initiated to enter a differentiation program.

Since the first report of chemically induced differentiation of murine leukemic cells by dimethyl sulfoxide[1] (Me_2SO), a variety of classes of compounds that are effective initiators of maturation have been described. These include highly polar compounds,[5] cryoprotective agents,[6] short-chain fatty acids[7] and aliphatic carbonyls,[8] purines,[9] and polymethylene diamides.[10,11] Since many of the effective inducing agents contain one or more amide functional groups, this study was designed to examine the effectiveness of a variety of amide-containing derivatives to determine the structural features required for induction of leukemia cell differentiation.

Experimental Section

Chemical agents were either prepared by previously reported procedures or obtained from commercial sources; structures were confirmed by NMR spectra and elemental analyses. Acetylation of amines containing either pyridyl, phenyl, or cyclohexyl functions by appropriate acid anhydrides provided various amide derivatives;[12] pure amides were obtained by recrystallization of the crude products. Picolinamides 11 and 14 were synthesized according to the procedure of Morkved and Cronyn[13] by refluxing a mixture

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