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]

Acknowledgment. This research was supported in part by U.S. Public Health Service Grants CA-02817 and CA-16359 from the National Cancer Institute.

(31) E. J. Lien and W. D. Kumler, *J. Med. Chem.*, 11, 214 (1968).

(32) S. H. Orkin, F. I. Harosi, and P. Leder, *Proc. Natl. Acad. Sci. U.S.A.*, 72, 98 (1975).

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.

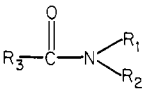
Experimental Section

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

(1) C. Friend, W. Scher, J. G. Holland, and T. Sato, *Proc. Natl. Acad. Sci. U.S.A.*, 68, 378 (1971).
(2) S. J. Collins, F. W. Ruscetti, R. E. Gallagher, and R. C. Gallo, *Proc. Natl. Acad. Sci. U.S.A.*, 75, 2458 (1978).
(3) J. Lotem and L. Sachs, *Int. J. Cancer*, 25, 561 (1980).
(4) S. J. Collins, A. Brodner, R. Ting, and R. C. Gallo, *Int. J. Cancer*, 25, 213 (1980).
(5) M. Tanaka, J. Levy, M. Terada, R. Breslow, R. A. Rifkind, and P. A. Marks, *Proc. Natl. Acad. Sci. U.S.A.*, 72, 1003 (1975).
(6) H. D. Preisler, G. Christoff, and E. Taylor, *Blood*, 47, 363 (1976).
(7) A. Leder and P. Leder, *Cell*, 5, 319 (1975).
(8) G. I. Malinin and P. S. Ebert, *FEBS Lett.*, 109, 264 (1980).
(9) J. F. Gusella and D. Housman, *Cell*, 8, 263 (1976).
(10) R. C. Reuben, R. L. Wife, R. Breslow, R. A. Rifkind, and P. A. Marks, *Proc. Natl. Acad. Sci. U.S.A.*, 73, 862 (1976).
(11) T. Hozumi, J. Nomura, and M. Ishizawa, *Int. J. Cancer*, 23, 119 (1979).
(12) C. Li, L. S. Rittmann, A. S. Tsiftsoglou, K. K. Bhargava, and A. C. Sartorelli, *J. Med. Chem.*, 21, 874 (1978).

0022-2623/81/1824-1092$01.25/0

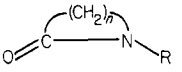
Table I. Murine Leukemia Cell Growth and Differentiation in the Presence of Substituted Alkyl and Aryl Amides



no.	R_1	R_2	R_3	optimum concn,[a] mM	cell growth,[b] % of control: day 3	cell growth,[b] % of control: day 6	benzidine-positive cells,[c] %
1	H	CH_3	CH_3	40	121	55	90
2	CH_3	CH_3	CH_3	40	32	21	84
3	H	2-pyridyl	CH_3	2	144	58	85
4	H	3-pyridyl	CH_3	5	183	167	86
5	H	4-pyridyl	CH_3	5	37	60	34
6	H	phenyl	CH_3	4	75	65	71
7	H	cyclohexyl	CH_3	4	95	150	92
8	pentamethylene		CH_3	8	50	138	85
9	tetramethylene		CH_3	4	54	99	60
10	2-pyridyl	2-pyridyl	CH_3	2	74	85	36
11	H	2-pyridyl	2-pyridyl	1	35	50	33
12	H	2-pyridyl	CF_3	1	98	89	69
13	H	2-pyridyl	CH_2Cl	2	98	119	83
14	H	CH_3	2-pyridyl	2	50	80	60
15	H	CH_3	3-pyridyl	4	94	78	93
16	H	CH_3	phenyl	1	54	99	62

[a] Concentration producing the maximum percentage of benzidine-positive cells after 6 days of continuous exposure. [b] Murine erythroleukemia cells were initially exposed to each agent at a cell concentration of 1×10^5 cells/mL. Cell numbers were measured on day 3 and day 6 using a Coulter Model ZBI particle counter and were expressed as a percentage of control cell growth. [c] The percentage of benzidine-positive cells was ascertained on day 6 by counting blue-stained cells using a light microscope. Under the conditions employed, solvent-treated and Me_2SO-treated (210 mM) cultures produced 3 and 94% benzidine-positive cells, respectively.

Table II. Murine Leukemia Cell Growth and Differentiation in the Presence of *N*-Alkyl Lactams[a]



no.	*n*	R	optimum concn, mM	cell growth, % of control: day 3	cell growth, % of control: day 6	benzidine-positive cells, %
17	3	H	100	26	100	89
18	3	CH_3	10	97	83	87
19	3	C_2H_5	10	53	81	90
20	3	$C(=O)CH_3$	20	156	116	73
21	4	H	40	72	67	64
22	4	CH_3	5	97	69	89
23	4	C_2H_5	4	186	84	87
24	4	$C(=O)CH_3$	20	170	74	86
25	5	H	20	95	81	82
26	5	CH_3	2	109	68	90
27	5	C_2H_5	4	106	63	96
28	5	$C(=O)CH_3$	4	102	85	53

[a] Cells were treated and evaluated as described in Table I.

of picolinic acid and thionyl chloride for 1 h. The resulting picolinoyl chloride was used immediately by reaction with either 40% aqueous methylamine or 2-aminopyridine. *N*-Ethyl lactams **19**, **23**, and **27** were obtained by treatment of the unsubstituted lactams with sodium hydride under reflux. The sodium salts that formed were alkylated with ethyl iodide to give the corresponding *N*-ethyl lactams.[14] Similarly, the reaction of 2-pyridone and potassium hydroxide under reflux provided the potassium salt of 2-pyridone. N-Alkylation of the resulting salts by an appropriate alkyl bromide gave the *N*-alkyl-2-pyridones **33**–**35**.

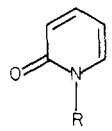
Friend erythroleukemia cells (clone 745A) and HL60 cells were employed as previously described.[12,15] Water-soluble agents were used as aqueous solutions, and water-insoluble compounds were dissolved in either ethanol or acetone. At the final concentration of solvent used, no effect on cell growth or differentiation occurred.[12] Erythroid differentiation, used as the primary screen for the capacity of synthesized compounds to induce maturation, was assessed by measuring the percentage of benzidine-positive (i.e., hemoglobin-containing) cells.[12] Differentiation of HL60 cells was monitored by morphological criteria and by the percentage of cells demonstrating phagocytosis-associated oxidative metabolism.[15,16] Each compound was assayed in duplicate over at least a 10-fold range of concentrations. Variability between duplicates was generally less than 10%.

Biological Results and Discussion

Substituted amides, lactams, and 2-pyridones were monitored for their capacities to induce maturation by ascertaining the concentration of each agent required to cause maximum differentiation of murine erythroleukemia cells, as measured by the percentage of hemoglobin-con-

(13) E. H. Morkved and M. W. Cronyn, *Acta Chem. Scand., Ser. B*, **32**, 231 (1978).
(14) C. S. Marvel and W. W. Moyer, Jr., *J. Am. Chem. Soc.*, **22**, 1065 (1957).
(15) R. J. Papac, A. E. Brown, E. L. Schwartz, and A. C. Sartorelli, *Cancer Lett.*, **10**, 33 (1980).
(16) S. J. Collins, F. W. Ruscetti, R. E. Gallagher, and R. C. Gallo, *J. Exp. Med.*, **149**, 969 (1979).

Table III. Murine Leukemia Cell Growth and Differentiation in the Presence of *N*-Alkyl-2-pyridones[a]



no.	R	optimum concn, mM	cell growth, % of control: day 3	cell growth, % of control: day 6	benzidine-positive cells, %
29	H	16	114	77	95
30	CH_3	4	74	62	89
31	C_2H_5	4	78	79	86
32	$CH(CH_3)_2$	2	96	80	90
33	C_3H_7	2	112	129	82
34	C_4H_9	1	103	125	76
35	C_6H_{13}	1	101	75	58

[a] Cells were treated and evaluated as described in Table I.

taining cells present in the population (Tables I–III). *N*-Methylacetamide (1) and *N*,*N*-dimethylacetamide (2), previously reported as inducers of Friend cell differentiation,[5,10] were confirmed as effective initiators of maturation in this system. Replacement of an *N*-methyl group by a bulky function, such as pyridyl, phenyl, or cyclohexyl, increased the potency of the acetamides by 8- to 20-fold. Substitution with the cyclohexyl moiety produced an acetamide virtually as effective as an inducer as the potent amide hexamethylenebisacetamide, reported earlier by Reuben et al.[10] Furthermore, *N*-cyclohexylacetamide produced maximum induction of differentiation at a concentration that did not inhibit cellular replication. The introduction of a cyclic ring structure at the nitrogen atom (8 and 9) only slightly reduced the capability of these compounds to induce differentiation. The presence of two bulky functions as part of the amide structure (10 and 11) lessened inducing ability, whereas compounds substituted with electron-withdrawing functions (12 and 13) retained their ability to function as initiators of maturation. No predictable effect was observed when a bulky group was placed on the carbonyl site instead of on the nitrogen atom. Thus, agents 14–16 exerted moderate to excellent induction (i.e., 60 to 93% benzidine-positive cells) at concentrations of 1 to 4 mM.

The relationship between the structure of lactam derivatives and their capacity to induce maturation of Friend erythroleukemia cells is summarized in Table II. In a manner analogous to the *N*-alkylacetamides, *N*-alkyl substitution of lactams, in general, enhanced potency. Furthermore, increasing the size of the lactam ring progressively enhanced the activity of non-N-alkylated lactams to serve as inducers of maturation; ring size, however, had less effect on the efficacy of N-substituted derivatives.

N-Alkyl-2-pyridones represent a previously unreported class of inducers of differentiation; again, N-alkylation increased the potency of these compounds by at least a factor of four, although some reduction in activity occurred with the largest alkyl substitutions tested. These *N*-alkyl-2-pyridones were quite effective inducers of erythroid differentiation of Friend leukemia cells at relatively low concentrations (1 to 4 mM); these levels caused little or no inhibition of cell proliferation. A representative derivative of this class was tested for its capacity to initiate maturation of HL60 cells (Figure 1). This compound, *N*-isopropyl-2-pyridone (32), produced morphologic and functional differentiation of the human promyelocytes to the mature granulocytes that are characteristic of this line.[4]

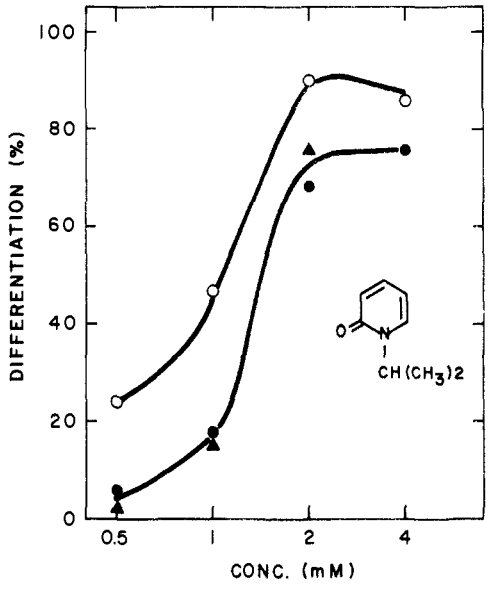


Figure 1. Leukemia cell differentiation by *N*-isopropyl-2-pyridone (32). Friend erythroleukemia cells or HL60 human promyelocytic leukemia cells were exposed to *N*-isopropyl-2-pyridone for 6 or 10 days, respectively. HL60 cells were resuspended in fresh medium containing 32 after the first 5 days. Friend murine cell differentiation (○) was assessed by determining the percentage of benzidine-positive cells. HL60 cell differentiation was measured by morphological criteria (▲) or by the percentage of cells containing formazan granules (●), observed by incubation for 20 min with 0.1% nitroblue tetrazolium and 1 μg of 12-*O*-tetradecanoylphorbol-13-acetate, as a measure of phagocytosis-associated oxidative metabolism.

Reuben et al.[10] have reported that polymethylene diamides, but not the corresponding diamines, are good inducers of murine erythroleukemia differentiation. The present study demonstrates that incorporation of a single *N*-alkyl or *N*-aryl amide functionality into a variety of chemical structures results in agents capable of inducing leukemic cell differentiation. Based on the wide variety of active agents, no rigid structural requirements appeared to be essential for erythroid maturation of murine leukemic cells by amides. The findings, however, suggest that other compounds containing N-substituted amides should be tested as inducers of leukemia cell differentiation.

Acknowledgment. Supported in part by U.S. Public Health Service Grants CA-02817 and CA-16359 from the National Cancer Institute. E.L.S. is a fellow in cancer research supported by Grant DRG-408F of the Damon Runyon–Walter Winchell Cancer Fund.