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Pyridine Derivatives as Potent Inducers of Erythroid Differentiation in Friend Leukemia Cells

Chau-der Li, Lana S. Rittmann, Asterios S. Tsiftsoglou, Kuldeep K. Bhargava, and Alan C. Sartorelli*

Department of Pharmacology and Developmental Therapeutics Program, Comprehensive Cancer Center, Yale University School of Medicine, New Haven, Connecticut 06510. *Received March 15, 1978*

Friend erythroleukemia cells in culture can be induced to differentiate along the erythroid pathway by dimethyl sulfoxide and a variety of organic polar compounds. Since this system appears to be a reasonable model to uncover agents with the potential to decrease the malignant phenotype of certain cancer cells through maturation, we have instituted a search for new and effective inducers of differentiation. To this end, we wish to report on the capacity of a series of pyridine derivatives to induce erythroid differentiation, which is monitored by the attainment of specialized function (i.e., the ability to synthesize hemoglobin detected as benzidine-stained cells). Three types of derivatives, acetamidopyridines, acetamidopyridine *N*-oxides, and acetamido-1-methyl-2-pyridones, were prepared and characterized. All but one of the compounds in these classes induced an accumulation of hemoglobin by Friend cells, although pyridine alone was not an effective inducer of differentiation. 2-Acetamidopyridine, 1-methyl-2-pyridone, and 1-methyl-5-acetamido-2-pyridone caused induction of maturation equivalent to dimethyl sulfoxide at concentrations approximately 100-fold lower than the polar solvent. It is interesting to note that all of the compounds tested which caused differentiation in this cell system contained the sequence $-C(=O)-N(R)-$ (R = H or CH_3) either attached to the heterocyclic ring or as part of the ring structure itself.

Friend and her co-workers[1] have reported that murine virus transformed leukemia cells grown in culture can be induced to differentiate along the erythroid pathway by treatment with dimethyl sulfoxide (Me_2SO). The process of differentiation is characterized by the synthesis and accumulation of globin mRNA[2] and hemoglobin,[3] the appearance of erythroid antigens on the cell surface,[4] the induction of spectrin,[5] a large variety of morphological alterations similar to those associated with the differentiation of normal proerythroblasts to orthochromatic normoblasts,[6] and loss of cell proliferative capacity.[7-9] *N*,*N*-Dimethylformamide and a wide variety of other organic polar compounds,[10,11] as well as some polymethylene bisacetamides, such as hexamethylene bisacetamide,[12] have been shown to be potent inducers of differentiation in this system. These compounds have in common a hydrophilic or polar group covalently joined to a hydrophobic portion of the molecule. Other structurally unrelated molecules, including butyric acid,[13] hypoxanthine and some purine analogues,[14] and ouabain,[15] can also stimulate erythroid differentiation, suggesting that there may be more than one mechanism involved in this process.

Furthermore, there is evidence[1] that Me_2SO-treated cells, which have attained specialized function, possess lower malignant potential as compared to untreated cells. Thus, Friend erythroleukemia cells in culture provide a valuable system to study the design of agents which are capable of inducing cell differentiation and decreasing the malignant potential of a tumor. A continued search for new potent inducers and a study of the relationship between structure and the capacity to induce differentiation would appear to be useful to gain an understanding of the mechanisms of erythroid differentiation in the erythroleukemia cells. It is a primary objective of this laboratory to develop new inducing agents which (a) function at low concentrations and (b) induce a high percentage of cells to differentiate. Since certain amide-containing agents and pyridine *N*-oxide have been reported[10-12] to be effective inducers of the differentiation of Friend leukemia cells, we have synthesized and studied the biological activities of amides of three classes of pyridine derivatives, including pyridines, pyridine *N*-oxides, and 2-pyridones. The biological findings demonstrate that many derivatives of pyridine, an inactive agent in this system, are capable of inducing differentiation of the Friend erythroleukemia. 2-Acetamidopyridine, 1-methyl-2-pyridone, and 1-methyl-5-acetamido-2-pyridone were among the most potent inducers studied, being effective at more than $^1/_{100}$th the concentration of Me_2SO required for equivalent induction.

Chemistry. Various acetamidopyridine derivatives were prepared by treatment of corresponding aminopyridines with pyridine and acetic anhydride; their *N*-oxides were obtained by reaction with *m*-chloroperbenzoic acid in chloroform.[16] Using the Chichibabin reaction,[17] a mixture of 4-phenylpyridine and sodium amide in dimethylaniline was heated at 110 °C to give 2-amino-4-phenylpyridine (**3**) as the major product. Following the procedure of Caldwell and Kornfeld,[18] β-acetamidopyridine derivatives were obtained from corresponding β-nitropyridines. Acetylation of 2-hydroxy-6-aminopyridine and partial hydrolysis of the resulting diacetate product afforded 2-hydroxy-6-acetamidopyridine[19] which exists predominantly in the oxo form, 6-acetamido-2(1*H*)-pyridone (**11**).[20] N-Methylation of 2-hydroxy-5-nitropyridine with dimethyl sulfate gave 1-methyl-5-nitro-2-pyridone (**13**).[20,21] 1-Methyl-6-acet-

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Scheme I

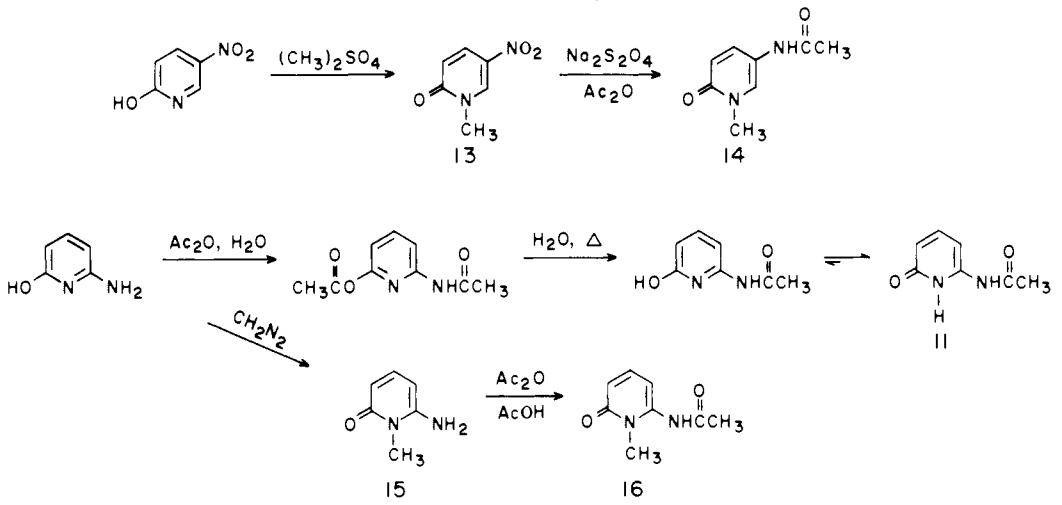
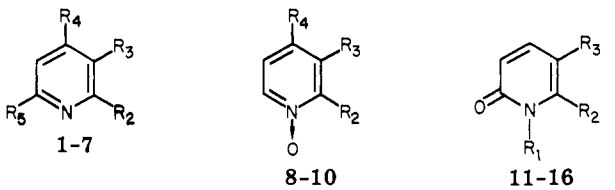


Table I. Methods and Physical Properties of Pyridine Derivatives



agent	R_1	R_2	R_3	R_4	R_5	method[a]	mp or bp (mm), °C	yield, %	formula
1		$NHC(=O)CH_3$	H	H	H	A	71	92	$C_7H_8N_2O$
2		H	$NHC(=O)CH_3$	H	H	A	133	85	$C_7H_8N_2O$
3		NH_2	H	C_6H_5	H	C	162-163	50	$C_{11}H_8N_2$
4		$NHC(=O)CH_3$	H	C_6H_5	H	A	143-145	87	$C_{13}H_{12}N_2O$
5		$CH_2NHC(=O)CH_3$	H	H	H	A	143-147 (2.5)	92	$C_8H_{10}N_2O_2$
6		$CH_2CH_2NHC(=O)CH_3$	H	H	H	A	163 (4)	90	$C_9H_{12}N_2O_2$
7		$NHC(=O)CH_3$	H	H	$NHC(=O)CH_3$	A	201	84	$C_9H_{11}N_2O_2$
8		$NHC(=O)CH_3$	H	H		B	141	78	$C_7H_9N_2O_2$
9		H	$NHC(=O)CH_3$	H		B	208-210	70	$C_7H_9N_2O_2$
10		$NHC(=O)CH_3$	H	C_6H_5		B	178-179	76	$C_{13}H_{13}N_2O_2$
11	H	$NHC(=O)CH_3$	H			F	212-215	80	$C_7H_8N_2O_2$
12	H	H	$NHC(=O)CH_3$			G	232-233	70	$C_7H_8N_2O_2$
13	CH_3	H	NO_2			D	171-172	80	$C_6H_6NO_3$
14	CH_3	H	$NHC(=O)CH_3$			G	140-142	70	$C_8H_{10}N_2O_2$
15	CH_3	NH_2	H			E	165-167	40	$C_6H_8N_2O_2$
16	CH_3	$NHC(=O)CH_3$	H			H	148	47	$C_8H_{10}N_2O_2$

[a] See the Experimental Section.

amido-2-pyridone (**16**) was prepared by methylation of 2-hydroxy-6-aminopyridine with diazomethane[22] and subsequent acetylation with acetic anhydride and acetic acid. These reactions are outlined in Scheme I. The compounds prepared, including the methodology employed, melting points, and reaction yields, are summarized in Table I.

Biological Results and Discussion. The pyridine derivatives were tested for their effects on cell growth and differentiation of the Friend erythroleukemia. Cells were exposed to varying concentrations of each agent to determine the optimal level required for maximum differentiation. Erythroid differentiation was measured by assessing the proportion of benzidine-positive cells (i.e., hemoglobin-containing cells) on day 6 following the addition of the potential inducer. The total amount of hemoglobin per 10^7 cells was also determined at this time. Cell numbers were determined on days 3 and 6 of incubation.

The results, indicated in Table II, show that all of the pyridine derivatives tested, except 4-phenylpyridine *N*-oxide, induced erythroid differentiation in this system. 2-Acetamidopyridine (**1**), 1-methyl-2-pyridone (**18**), and 1-methyl-5-acetamido-2-pyridone (**14**) were the most effective inducers; approximately 70% benzidine-positive cells were obtained after incubation with these agents at concentrations as low as 2 mM. 1-Methyl-6-acetamido-2-pyridone (**16**), 2-acetamidomethylpyridine (**5**), and 2-(2-acetamidoethyl)pyridine (**6**) were also effective inducers, causing 60–73% cell differentiation at higher concentrations (10–16 mM). The studies of the relationship between structure and activity revealed that 2-acetamidopyridine was a more effective inducer than the corresponding isomer substituted at the 3 position. Acetamidopyridines were more potent inducers of differentiation than their corresponding *N*-oxides. However, 2- and 3-acetamidopyridine *N*-oxides (**8** and **9**, respectively) were effective inducers at concentrations tenfold less than that of pyridine *N*-oxide, a relatively potent inducer of differentiation in this system.[11] Increasing the distance between the acetamido group and the pyridine ring by insertion of a methylene or ethylene chain resulted in less potent inducing agents (**5** and **6**, respectively). The introduction of two acetamido functions onto the pyridine

Table II. Optimal Concentration of Agents Required for the Maximum Proportion of Benzidine-Positive Cells

agent	optimum concn, mM	% cell growth[a,b] on day 3	benzidine-positive cells, %	μg of hemoglobin per 10^7 cells
1	2.0	65	72	30.6
5	15	36	68	30.0
6	10	33	60	28.0
2	8.0	58	43	14.4
7	4.0	80	28	8.6
4	0.04	21	8	ND[c]
8	10	74	43	22.6
9	10	74	35	15.6
10	1.0	17	17	12.2
17[d]	0.4	26	<1	<0.5
18[d]	2.0	23	74	ND[c]
16	16	55	73	22.2
14	2.0	91	67	33.8
19[d]	8.6	11	26	ND[c]
12	1.0	26	16	ND[c]
11	2.0	22	4	ND[c]
Me_2SO	210	60	80	31.2
control		100	<1	<0.05

[a] The percent cell growth of inducer-treated cultures is based on the cell numbers of untreated control cultures. [b] The cell numbers on day 6 of treated cells ranged from 95 to 100% of untreated control values. [c] Not determined. [d] 4-Phenylpyridine *N*-oxide (17), 1-methyl-2-pyridone (18), and 2(1*H*)-pyridone (19) were purchased from Aldrich Chemical Co., Inc., and were purified by crystallization or distillation before use.

ring (7) also did not result in a further enhancement of inducing ability.

Increasing the hydrophobicity of 2-acetamidopyridine by the addition of a phenyl group at the 4-pyridyl position (compound 4) did not enhance the ability of the parent compound to induce differentiation. In contrast, 1-methyl-2-pyridone (18) was very active at a concentration fivefold lower than that reported for the analogous compound, 1-methyl-2-piperidone.[11] Furthermore, the relatively high degree of induction obtained by 1-methyl-5-acetamido-2-pyridone (14) and 1-methyl-6-acetamido-2-pyridone (16) (Table II) at concentrations of 2 and 16 mM, respectively, indicated that the addition of an acetamido group to the active ring system, 1-methyl-2-pyridone, did not alter the potency of the compound as an inducer of differentiation. Thus, the results presented demonstrate the existence of a new group of pyridine derivatives which are potent inducers of erythroid differentiation in Friend leukemia cells. Although pyridine itself is not an inducer in this system, it appears that derivatives which contain the chemical structure -C-(=O)-N(R)- (R = H or CH_3) either attached directly on the ring or as a part of the ring structure itself are active inducers of differentiation.

The results shown in Table II indicate that, except for compound 14, transient inhibition of cell growth is associated with the capacity of these agents to induce differentiation. These data are consistent with the finding[23] that Me_2SO-induced cell differentiation is associated with a transient inhibition of DNA synthesis and prolongation of the G_1 phase of the cell cycle. Drug-treated cells recover from the growth inhibition by day 6; therefore, no difference in cell number is observed between control and treated cell populations at this time.

The biochemical mechanism by which Me_2SO, the pyridine derivatives, and other chemical agents induce erythroid differentiation of Friend leukemia cells is unknown. Although evidence exists to suggest that Me_2SO may act at the chromosomal level by affecting DNA transcription,[24-26] the physical properties of the various classes of compounds suggest possible interactions with the plasma membrane. This hypothesis is supported by recent studies indicating a relationship between membrane-inducer interactions and the differentiation process.[27-29] Furthermore, the expression of erythroid function induced by ouabain[15] also implicates a membrane-mediated mechanism in Friend leukemia cell maturation.

Experimental Section

Melting points were taken on a Thomas-Hoover capillary apparatus and are uncorrected. Elemental analyses were performed by the Baron Consulting Co., Orange, Conn. IR spectra were measured using a Perkin-Elmer Model 257 spectrophotometer, and NMR spectra were obtained with a Varian T-60A NMR spectrometer. Prepared compounds were homogeneous when analyzed by micro thin-layer chromatography on silica gel, and NMR spectra and elemental analyses were consistent with the reported chemical structures.

A. Acetylation of Aminopyridines. Aminopyridines (50 mmol) were dissolved in a mixture of pyridine (15 mL) and acetic anhydride (20 mL), and the solution was kept at room temperature for 24 h. Water (20 mL) was added and the solution was evaporated in vacuo to dryness. The desired pure products were obtained by recrystallization from the appropriate solvents.

B. Formation of Pyridine *N*-Oxides. Pyridine derivatives (10 mmol) were dissolved in chloroform (20 mL) and cooled in an ice bath. *m*-Chloroperbenzoic acid (13 mmol) was added to the solution gradually and the mixture was stirred at room temperature overnight. Anhydrous K_2CO_3 was added and stirred for 1 h, the resulting precipitate was removed by filtration, and the filtrate was evaporated to dryness. Recrystallization from appropriate solvents gave pure *N*-oxides.

C. 2-Amino-4-phenylpyridine (3). Following Leffler's procedure,[17] 4-phenylpyridine (12 g, 80 mmol) and sodium amide (3.9 g, 100 mmol) in dry dimethylaniline (15 mL) were heated at 110 °C for 10 h. NaOH (5%, 50 mL) was added carefully to the cold reaction mixture and water (200 mL) was then added. The solid was collected by filtration and was recrystallized from benzene to give 3 (6.8 g, 50%), mp 162–163 °C.

D. 1-Methyl-5-nitro-2-pyridone (13). 2-Hydroxy-5-nitropyridine (2.8 g, 20 mmol) was added in portions to a mixture of dimethyl sulfate (14 mL, 110 mmol) and 3 N NaOH (30 mL). The mixture was stirred at room temperature for 2 h and acidified with 1 N HCl. The yellow precipitate which formed was collected by filtration; recrystallization from ethanol gave the pure product 13 (2.5 g, 80%), mp 171–172 °C.

E. 1-Methyl-6-amino-2-pyridone (15). Employing a modification of the procedure of Peresleni et al.,[22] 2-hydroxy-6-aminopyridine (6.6 g, 60 mmol) in methanol was added to a solution of diazomethane (6 g) in ether (300 mL) and kept at 5–10 °C for 24 h. After evaporation, addition of ether to the resultant syrup gave a solid which was recrystallized from water to give 15 (3 g, 40%), mp 165–167 °C (lit.[22] 163–165 °C).

F. 6-Acetamido-2(1*H*)-pyridone (11). Using the procedure of Buu-Hoi et al.,[19] 2-hydroxy-6-aminopyridine (5.5 g, 50 mmol) in water (20 mL) was treated with acetic anhydride (20 mL) at 50 °C for 2 h. The resulting diacetate was isolated by evaporation and refluxed in water to yield 11 after recrystallization from ethanol (6.1 g, 80%): mp 213–215 °C (lit.[19] 212–213 °C).

G. 5-Acetamido-2(1*H*)-pyridone (12) and 1-Methyl-5-acetamido-2-pyridone (14). 2-Hydroxy-5-nitropyridine and 1-methyl-5-nitro-2-pyridone were used as the starting materials, respectively. The nitropyridines (10 mmol), sodium hydrosulfite (34 mmol), and acetic anhydride (25 mmol) in water were kept overnight at room temperature. The crude products were recrystallized from water to give the desired products in 70% yield.

H. 1-Methyl-6-acetamido-2-pyridone (16). 1-Methyl-6-amino-2-pyridone (1.5 g, 12 mmol) and acetic anhydride (20 mL) in acetic acid (15 mL) were stirred at 50 °C for 2 days. The reaction was evaporated to dryness in vacuo and the residue was decolorized with charcoal. Recrystallization from water gave 16 (0.94 g, 47%), mp 148 °C.

I. Friend Erythroleukemia Cell Culture. The cells employed in this work were Friend murine virus induced erythroleukemia cells of DBA/2J origin, established originally by Friend et al.,[30] and kindly supplied by Dr. N. Gabelman of Mount Sinai School of Medicine, N.Y. Cells were grown in suspension culture at 37 °C in 10% CO_2 humidified atmosphere by weekly passage of 10^5 cells/mL in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin (GIBCO, Grand Island, N.Y.).

J. Induction Experiments and Determination of Hemoglobin. Cells in log phase were cultured at 37 °C at a level of 10^5 cells/mL in T-25 cm^2 Corning polyethylene flasks containing 10 mL of medium. Compounds were added at the time of seeding using a Hamilton syringe. Each compound was tested over a range of concentrations in two separate experiments employing twofold increases in concentration. Cultures incubated with Me_2SO (210 mM) served as a positive control. Water-insoluble agents were dissolved in either 50% ethanol or acetone; the final concentration of these solvents was kept severalfold below the level at which cell proliferation and differentiation were affected. After 3 days of incubation, the cell concentration was determined using a Coulter counter, Model ZBI. On day 6, the cell cultures were scored for the proportion of benzidine-positive cells using a benzidine peroxide stain as described by Orkin et al.[31] In those cultures in which benzidine-positive cells were formed, cells were collected by centrifugation at 350g for 10 min and washed twice with phosphate-buffered 0.9% NaCl, pH 7.4, and cell extracts were prepared by sonication and assayed for total hemoglobin content by measuring the absorbancy at 515 nm of the cell lysate which had been incubated with benzidine as described.[32]

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Stereoelectronic Factors in the Binding of Substrate Analogues and Inhibitors to Purine Nucleoside Phosphorylase Isolated from Human Erythrocytes

Frank Jordan* and Annie Wu

Carl A. Olson Laboratories of Chemistry, Rutgers, The State University, Newark, New Jersey 07102.
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Several aspects of the stereoelectronic requirements of substrates of human erythrocytic purine nucleoside phosphorylase (E.C. 2.4.2.1) were elucidated providing the following information: (a) the N^1 position cannot have a nonhydrogen substituent; (b) the 5′-OH group must be present for catalytic activity to be exhibited but is not an essential functional group for inhibitory action to be observed; (c) on the C^8 position groups larger than $-NH_2$ or $-Br$ cannot be accommodated; (d) the *syn*-glycosyl conformation (i.e., 8-bromoguanosine) is acceptable but may not be an absolute requirement for phosphorolysis; (e) among nucleic base inhibitors methylation at N^3, N^7, or N^9 vastly decreases the inhibitory properties as does a nitrogen in lieu of C–H in the 8 position. The results clearly indicate that this enzyme differs in its stereoelectronic requirements from the *Escherichia coli* enzyme.

Levene and Medigreceann first reported on enzymatic degradation of purine nucleosides.[1] Klein later recognized[2] that phosphate and arsenate activate the enzymatic splitting of the nucleoside glycosyl bond. Kalckar finally demonstrated that the process was accompanied by the uptake of inorganic phosphate and the formation of a

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