Synthesis and Antitumor Activity of Dihydro-5-azacytidine, a Hydrolytically Stable Analogue of 5-Azacytidine[1]

John A. Beisler,* Mohamed M. Abbasi,[2] James A. Kelley, and John S. Driscoll

Drug Design and Chemistry Section, Laboratory of Medicinal Chemistry and Biology, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014.
Received November 18, 1976

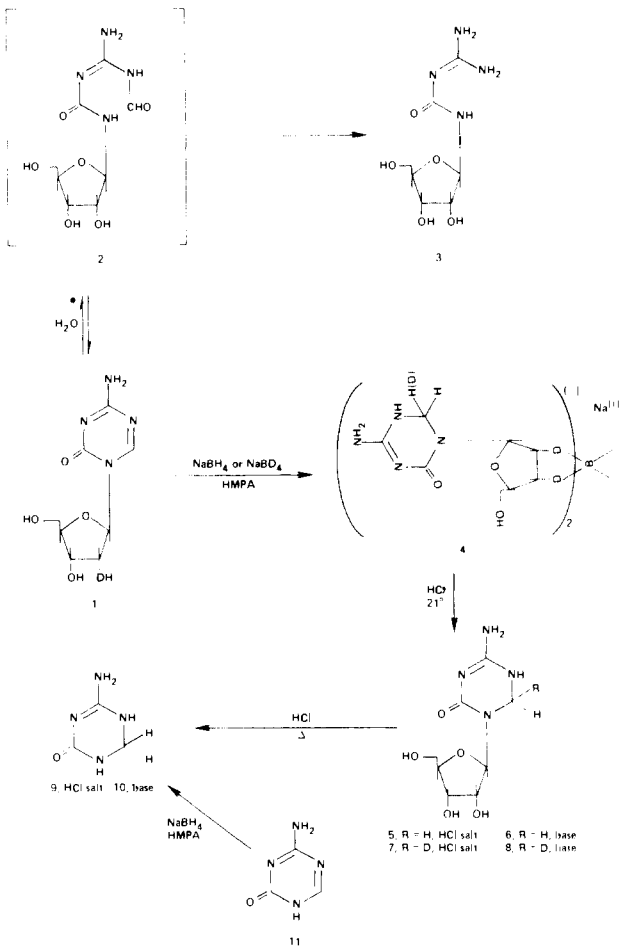
Full clinical utility of the antileukemic drug, 5-azacytidine (1), is hampered by its facile hydrolysis in aqueous formulations. The present study sought to improve the stability of the parent drug while retaining the antitumor attributes through the synthesis of a reduced analogue of 1. Borohydride reduction of 1 gave 5,6-dihydro-5-azacytidine hydrochloride (5) after acid hydrolysis of a boron-containing intermediate. The structure proof and characterization of 5 was achieved primarily with UV, NMR, and GC–MS with the aid of a deuterated derivative (7) prepared by using borodeuteride in the initial reduction step. Vigorous treatment of 5 with acid gave the aglycon 9 which was independently synthesized from 5-azacytosine (11). The dihydro analogue 5 was completely stable at room temperature in aqueous solutions over a broad pH range for up to 3 weeks. In comparative antitumor assays 5 showed good activity in L1210 systems when administered intraperitoneally or orally. Although higher dose levels were necessary, 5 had approximately 80% of the antitumor efficacy shown by 1. Neither 5 nor 1 showed a dependency on administration schedule. Cross resistance between 5 and 1 was demonstrated using an L1210 subline resistant to 1. 5 was found to be superior to 1 in therapeutic index and in its ability to cross the blood-brain barrier in sufficient quantity to be therapeutic against intracranially implanted L1210 cells. Subjective evidence is given which suggests 5 is a prodrug of 1.

5-Azacytidine (1) is a nitrogen bioisostere of cytidine conceived as a potential inhibitor of nucleic acid biosynthesis and synthesized by Piskala and Sorm[3] in 1964; later it was also isolated from the culture filtrates of *Streptoverticilium ladakanus*.[4] The nucleoside was reported to have antibacterial properties[4,5] and to be a potent inhibitor of rapidly proliferating murine neoplasms.[4] 5-Azacytidine is incorporated into both RNA and DNA[6] and disrupts protein synthesis, probably through its incorporation into messenger RNA.[7] The noteworthy activity of 1 in experimental biological systems and antitumor assays encouraged clinical trials of 1 in the treatment of leukemia.[8-10] Although of apparent limited value in the treatment of solid tumors,[11,12] 5-azacytidine has been particularly effective against acute myelogenous leukemia.[9,10]

The primary dose-limiting toxicity revealed in clinical studies was severe nausea and vomiting (regardless of dose or administration route) which were mitigated somewhat by giving the drug in divided doses.[9,11] Nevertheless, the high frequency among patients of nausea and vomiting dimmed the clinical future of this otherwise promising drug.[11] Using a technique of continuous iv infusion of the drug over a 5-day period, however, virtually eliminated the nausea and vomiting toxicity[13,14] while preserving the full therapeutic effect.[13] Although continuous infusion of 1 provides an effective resolution of a toxicity problem, the instability of 1 with respect to hydrolysis adds a complicating factor not important in single dose administration.

An early report from the Czech group[15] described the hydrolysis of 1 in aqueous solutions (see Scheme I) which leads to a guanylurea ribose (3) via an unstable intermediate (2) at a rate which is dependent on pH and temperature.[16] The best clinical formulation of 1 for maximum stability[17] utilizes lactated Ringer's solution for reconstitution of a clinical vial, but even so, 15% of the drug is lost after 4.7 h, when stored at 25 °C, to products of unknown therapeutic value and unknown toxicity.[18] As a consequence, strict control over dosages and drug purity using the continuous infusion technique is difficult to achieve even if the drug is freshly prepared at 4-h intervals as has been recommended.[13]

Scheme I



It was our intent to modify the structure of 1 so as to (i) overcome the water instability problem while (ii) preserving the same antitumor action of the parent. These goals would be ideally achieved using the prodrug concept[19] of drug modification such that the clinical experience gained with 1 would be largely applicable to a solution stable analogue.

We would like to report the synthesis of 5,6-dihydro-5-azacytidine, which is stable for weeks at room tem-

perature in aqueous solution over a broad pH range, and which has substantial activity in mouse leukemia test systems.[20]

Chemistry. The electron density at the 6-carbon atom of the triazine ring system of 1 is significantly lower than in cytosine as shown by quantum chemical calculations.[15] This finding is consistent with the observation that 1 is vulnerable to nucleophilic attack at position 6 which, when the nucleophile is water, results in the fission of the triazine ring with the formation of intermediate 2. A reasoned approach toward making a hydrolytically more stable analogue of 1 would either impede nucleophilic attack on carbon 6 or increase the electron density at that position. For our initial effort we chose the latter alternative through the simple expediency of saturating the 5,6 double bond of 1. While oxidation of the reduced nucleoside to 1 in vivo is a stringent requirement, it could conceivably be accomplished by enzymatic hydroxylation at the C-6 position of the triazine followed by loss of the elements of water to give the aromatic system.

The synthesis of 5,6-dihydro-5-azacytidine hydrochloride (5, Scheme I) was accomplished via a sodium borohydride reduction of 1 in hexamethylphosphoramide (HMPA) solution. The reduction provided an intermediate, boron-containing complex which could be purified by recrystallization from water giving a material that appeared homogeneous by TLC. However, elemental analysis gave variable results but did consistently suggest the presence of one boron atom for every two nucleoside units. By contrast, reduction of 5-azacytosine (11) with borohydride in the same way did *not* result in a boron-containing product but rather gave the reduced base directly. The UV spectrum of the boron complex was similar to that of 5 when measured in two different buffers indicating the lack of involvement of boron with the triazinone chromophore. Structure 4 is proposed for the complex wherein the *cis*-glycol functions of two ribosyl groups participate in a borate anion. Similar borate complexes of ribonucleosides have been used as protecting groups in synthetic schemes.[21]

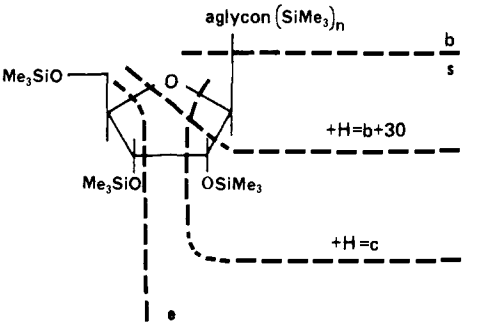
Acid hydrolysis of the complex (4) liberated the reduced nucleoside as the hydrochloride salt (5) which could be converted to the free base (6) either with an anion-exchange column or by treatment with ammonium hydroxide solution. The NMR spectrum of 5 showed the anomeric proton as a doublet at δ 5.54 and a singlet at higher field (δ 4.69) due to the two methylene protons at C-6; the aromatic proton shown by 1 at δ 8.7 was absent. Substitution of sodium borodeuteride for borohydride in the reduction of 1 gave 7 after mild acid hydrolysis. The NMR spectrum of 7 was identical with that of 5 except the singlet due to the C-6 methylene group integrated for only one proton.

Vigorous treatment of 5 with 6 N hydrochloric acid caused cleavage of the glycosidic bond allowing isolation of the aglycon as the hydrochloride salt (9). The salt was converted to the free base (10) using an anion-exchange column. Reduction of 5-azacytosine (11) with sodium borohydride in HMPA solution gave 10 which showed a singlet (δ 4.54) in the NMR spectrum as the only resonance signal due to nonexchangeable protons. The hydrochloride salt (9) was prepared by treatment of 10 with ethanolic hydrogen chloride. Mixture melting points of 9 and 10 with the corresponding reduced triazines obtained from the hydrolysis of the nucleoside (5) gave no depression.

After rendering sufficiently volatile by trimethylsilylation with bis(trimethylsilyl)trifluoroacetamide (BSTFA),[22] the triazine aglycons (10 and 11), the triazine nucleosides (1 and 6), and the deuterated nucleoside (8) were analyzed via gas chromatography–mass spectrometry (GC–MS). 5-Azacytosine (11) as the bis(trimethylsilyl) derivative gave an isothermal retention index[23] (IRI) of 1480 in the GC (3% SE-30 liquid phase) and the mass spectrum showed an intense parent ion at m/e 256. In the case of dihydro-5-azacytosine (10) the molecular ion (m/e 330) is quite diagnostic since reduction of the 5,6 double bond of 11 produces an additional site for trimethylsilylation. Loss of hydrogen from the molecular ion is also much enhanced since it results in formation of an aromatic immonium ion. The IRI of 10 as the tris(trimethylsilyl) derivative was 1670.

Silylation of 5-azacytidine (1) with BSTFA smoothly gave a tetrakis(trimethylsilyl) derivative that gave a single peak in the GC (IRI 2620) and gave a peak for the molecular ion at m/e 532. The reduced nucleoside (6) has an additional site subject to trimethylsilylation which leads to a pentakis(trimethylsilyl) derivative (IRI 2465) exhibiting a peak at m/e 606 due to the molecular ion. Selected ions from the mass spectra of the derivatized nucleosides, which are compared in Table I, possess several sets of diagnostic ions:[24,25] (i) ions resulting from loss of neutral species from the intact molecular ion such as the M – CH_3 ion, (ii) ions resulting from the derivatized base (b) and its rearrangements, and (iii) ions resulting from the trimethylsilylated ribosyl moiety(s). All the ions produced by derivatized 6 which are associated with the triazine nucleus attest to its reduced nature. The deuterated nucleoside (8) from $NaBD_4$ reduction when trimethylsilylated with BSTEA gives a molecular ion one atomic mass unit higher (m/e 607) than the corresponding nondeuterated material, and the shift of all base series ions (b, b + H, b + 2H, b + 30, c, e) one unit higher indicates incorporation has occurred in the triazine moiety of the nucleoside.

Table I. Mass Spectrometry Fragmentation Patterns for Silylated 5-Azacytidine (1) and Dihydro-5-azacytidine (6)



Ion	m/e (rel intensity) 5-Azacytidine·4Me3Si	Dihydro-5-azacytidine·5Me3Si
M^+	532 (0.14)	606 (1.2)
M – H		605 (1.6)
M – 15	517 (0.77)	591 (1.6)
e	314 (0.82)	388 (2.4)
c	299 (0.38)	373 (1.7)
s – H	348 (3.4)	348 (3.0)
b + 30		287 (2.3)
b + 2H	185 (3.1)	259 (8.8)[a]
b		257 (9.7)
Base peak	73 (100)	73 (100)

[a] This mass also represents the loss of Me_3SiOH from the intact sugar fragment as well as the +1 isotope peak for the isobaric b + H and s – H – Me_3SiOH ions. Analysis of the mass spectrum of trimethylsilylated 8 where the b + 2H peak occurs at m/e 260 indicates that the b + 2H ion is only responsible for a minor fraction of this peak.

Table II. Comparative Survival Times of Mice Treated with 5-Azacytidine (1) or Dihydro-5-azacytidine (5) in Antitumor Test Systems[a]

System no.	Tumor	Schedule[b]	Compd	Expt no.[c]	Dose[d] range	% ILS at optimal dose	Min active dose	Optimal active dose	Max active dose	T − C[e]
1	L1210	QD1	5	8891	300-1600	34	300	600	1600	−3.1
				8892	300-1600	39	300	1600	1600	−1.5
			1	8891	12.5-100	73	12.5	50	100	−3.5
				8892	12.5-100	75	12.5	50	100	−3.8
2	L1210	Q4D	5	8887	150-600	104	150	600	600	−4.3
				8895	12.5-800	96	12.5	800	800	−1.5
			1	8887	5-40	138	5	20	40	−1.2
				8895	5-40	94	5	10	20	−1.5
3	L1210	Q4D(Q6H)	5	8891	9.4-150	68	9.4	150	150	−1.5
				8892	9.4-150	72	18.7	125	150	−2.5
			1	8891	1.25-10	126	1.25	5	5	−0.5
				8892	1.25-10	135	1.25	5	5	−1.5
4	L1210	QD1-9	5	8892	6.25-400	116	6.25	100	300	−3.5
				R002	0.78-600	128	3.13	100	200	−3.9
			1	8892	0.75-6	128	0.75	3	6	0.9
				R002	0.78-12.5	172	0.78	3.13	3.13	−3.0
5	L1210/AZA	QD1-9	5	01	0.78-600	9		25		0.1
				02	0.39-200	0				
			1	01	0.78-12.5	0				
				02	0.78-12.5	0				
6	PO-L1210	QD1-9	5	8903	12.5-1000	50	200	400	400	−4.4
				8904	12.5-1000	82	400	1000	1000	−2.2
			1	8903	1.56-25	24		25		−4.1
				8904	1.56-25	125	1.56	12.5	25	−3.1
7	IC-L1210	QD1-9	5	47	6.25-400	46	25	100	100	−1.9
				48	6.25-400	44	25	100	200	−0.8
			1	47	0.75-6	27	0.75	0.75	0.75	0.7
				48	0.75-6	28	0.75	0.75	0.75	−0.5
8	P388	QD1-9	5	5768	1.56-400	85	12.5	100	200	−2.8
				3968	0.75-600	100	12.5	200	200	−4.3
			1	5768	0.75-6	127	0.75	3	3	−0.4
				3968	0.75-12.5	162	0.75	3.13	3.13	−4.3
9	B16	QD1-9	5	326	1.56-400	19		200		−3.3
				327	1.56-400	25		100		−2.0
			1	326	0.75-6	20		3		−1.5
				327	0.75-6	42	1.5	3		−0.5

[a] Test compounds in 0.9% saline solution were administered intraperitoneally (ip) according to the specified schedule except in the PO-L1210 system (system 6) where the compounds were administered orally to nonfasting animals. The mice were tumored ip except in the IC-L1210 system (system 7) where tumor cells were implanted intracerebrally (ic). [b] In the QD1 schedule drug was administered only on the first day after tumor implantation (one injection). Drug was given on days 1, 5, and 9 with the Q4D schedule (three injections) and on days 1-9 with the QD1-9 schedule (nine injections). The Q4D(Q6H) schedule required drug administration every 6 h on days 1, 5, and 9 (12 injections). [c] Where more than two experiments were carried out for a compound in a specific system, the two experiments which produced the highest ILS values were selected for tabulation. [d] All doses are expressed in milligrams per kilogram of body weight per injection. [e] The difference of the average body weight change in grams of the optimal dose level test group (T) and the control group (C) measured on day 5.

Antitumor Evaluations. Dihydro-5-azacytidine hydrochloride (**5**) was evaluated for antitumor activity in comparative studies (Table II) with 5-azacytidine (**1**) according to protocols[26] devised by the Division of Cancer Treatment, National Cancer Institute, using the following mouse tumor test systems: lymphoid leukemia L1210, lymphocytic leukemia P388, and melanotic melanoma B16. In each system dose–response assays were conducted where each successive dose was half of the preceding higher dose. The increase in life-span of the test animals beyond the survival time of the untreated control animals expressed as a percentage (% ILS) was used in all the test systems to evaluate antitumor activity. Activity is defined here as a % ILS value $\geq$25% for L1210 and P388 and $\geq$40% for B16.

The L1210 assay, which was most sensitive to **5**, was used to elucidate several questions. Systems 1–4 (Table II) sought to reveal a possible schedule dependency on the activity of **5** when administered intraperitoneally (ip). The effectiveness of oral administration of **5** was explored in system 6 (PO-L1210). To probe the ability of **5** to cross the blood–brain barrier, system 7 (IC-L1210) was employed wherein tumor cells were implanted in the cranial cavity and drug was administered ip. An L1210 subline resistant to 5-azacytidine (L1210/AZA) was used to test (system 5) a possible cross resistance of the dihydro analogue with the parent.

The P388 tumor was used to assess the activity of **5** against a second leukemia model and the B16 test system was utilized to uncover any potential activity of **5** in a solid tumor model (systems 8 and 9, respectively).

Results and Discussion

Of the two objectives set forth for this study (vide supra), the first has been clearly met. We have reported[20] that buffered solutions (pH 2 and 6) of dihydro-5-azacytidine (**5**) showed no decomposition at 25 °C over a 3-week observation period as determined by a quantitative NMR assay. Moreover, **5** can be isolated unchanged (as the free base) after storing for 7 days at room temperature in 1 N ammonium hydroxide solution. Thus, the reduced nucleoside (**5**) is completely stable in aqueous solutions allowing, if necessary, administration by the continuous infusion technique without concern for drug hydrolysis in

Table III. L1210 Leukemia Antitumor Evaluation of Incidental Compounds[a]

Compd	Schedule	Dose range, mg/kg	% ILS[b] (dose)	T - C[c] (dose)
3	Q4D	5-365	0 (240)	-2.2 (240)
4	Q4D	25-400	80 (400)	-1.5 (400)
6	QD1-9	1.56-400	86 (50)	-3.2 (400)
9	QD4	6.25-400	0 (400)	1.0 (400)

[a] Test compounds in 0.9% saline solution were administered intraperitoneally (ip) according to the specified schedule following ip tumor implantation. [b] The tabulated dose (mg/kg) was the highest nontoxic dose given producing the indicated % ILS in a dose-response assay or the dose producing the maximum % ILS in the case of active compounds. [c] The difference of the average body weight change in grams of the test group (T) and the control group (C) measured on day 5 at the highest nontoxic dose tested.

aqueous formulation. By comparison, the parent drug (**1**) has a half-life of 48 h at pH 7 (25 °C).[18] Moreover, it is completely decomposed in a few hours in 1 N ammonium hydroxide solution giving mainly a guanylurea ribose (**3**), which is considerably less inhibitory to the growth of *Escherichia coli* than **1**,[15] and as the picrate salt, has no in vivo antitumor activity against L1210 leukemia (Table III).

Several salient points of interest can be derived from systems 1-4 of the comparative L1210 testing outlined in Table II. First, although both drugs exhibit good activity, the efficacy of **5**, in the main, is somewhat less than that of **1**. Second, the dose level of **5** necessary to produce maximum activity is about 35 times the optimum dose of **1**. This is most clearly in evidence when the results of the QD1-9 schedule (system 4) are considered. The apparent potency differential could also be explained by a 1-3% contamination of the reduced nucleoside (**5**) by the parent drug (**1**) which served as a starting material. However, using a high-pressure liquid chromatography analytical method the concentration of **1** (if present at all) in samples of **5** was shown to be considerably less than 0.05%[20] and would not contribute to the observed antitumor response of **5**. When **5** was stored overnight in 1 N ammonium hydroxide solution at room temperature the free base (**6**) thus obtained exhibited antitumor activity (Table III) within the anticipated % ILS range. Since **1** is readily decomposed by ammonium hydroxide, one could assume its selective destruction if it were present in **5** so treated. Therefore, it can be concluded that there is no detectable **1** in the reduced nucleoside, and **5** is solely responsible for the antitumor activity observed. Third, the activity of both **1** and **5** is not very sensitive to changes in administration schedules. This is a somewhat surprising observation since it has been reported[27,28] that **1** appears to be cell cycle specific to cultured L1210 cells. Initial testing results led us to believe **5** was rapidly excreted due to its good water solubility (40 mg/mL, pH 6) so that if the drug were administered every 6 h on days 1, 5, and 9, an improvement in % ILS might be realized. In addition, if there were some cell cycle specificity, the Q4D(Q6H) schedule might better be able to exploit it than the Q4D regimen. On the contrary, the more intensive schedule (system 3) appeared to be less effective.

The data from the two L1210 QD1-9 experiments (system 4) presented in abbreviated form in Table II were used along with data generated from three additional L1210 QD1-9 experiments determined during the course of this study to develop dose-response relationships for **1** and **5** in order to calculate their respective therapeutic indices (TI). Using the method of Skipper and Schmidt[29] (TI = optimal dose for maximum % ILS/lowest dose giving 40% ILS) one obtains TI = 10 for **5** (100 mg/kg ÷ 10 mg/kg) and TI = 7.5 for **1** (3 mg/kg ÷ 0.4 mg/kg). Alternatively, the method of Goldin[30] (TI = highest dose giving 40% ILS/lowest dose giving 40% ILS) yields TI = 40 for **5** (400 mg/kg ÷ 10 mg/kg) and TI = 14 for **1** (5.7 mg/kg ÷ 0.4 mg/kg). Therefore, it appears that **5** has a more favorable therapeutic index than **1** as found by two methods of calculation.

The remaining portion of Table II gives the results of some special studies done with the L1210 model (systems 5-7) as well as the testing results of **5** using two other tumor models (systems 8 and 9).

In an attempt to approach the question of a possible prodrug nature of **5**, a subline of L1210 leukemia which is resistant to **1** was used to establish a possible cross resistance between **1** and **5** (system 5, L1210/AZA). In comparative experiments the L1210/AZA tumor was completely resistant to both **1** and **5**, a result which is consistent with **5** being a prodrug of **1**, although the results could also be explained in terms of similar mechanisms of drug action.

Unlike **1**, the dihydro analogue (**5**) is stable to hydrolysis at pH 2 and would be capable of withstanding acidic stomach conditions making oral administration a possible route. Therefore, **5** was tested in L1210 where drug was given orally to the test animals (system 6, PO-L1210). Reproducible activity was observed which suggests the feasibility of this administration route in clinical usage, perhaps as part of remission maintenance therapy. Although **1** was found to be marginally active in this system, its known instability in the low pH[15,16] range would probably preclude any serious consideration for clinical use using the oral administration route.

Implantation of L1210 cells intracerebrally (system 7, IC-L1210) provided a good model to determine the ability of **5** to cross the blood-brain barrier. In this test **5** was clearly superior to **1** in terms of the % ILS parameter. **1** is known to penetrate only slightly into the central nervous system (CNS)[31] and therefore is unable to reach the tumor cells in effective concentration. The reproducible activity of **5** in the brain tumor model might serve as an indicator of a special advantage it might have relative to **1** in the clinical treatment of leukemia. Infiltration of the CNS by leukemic cells occurs in a high percentage of leukemic children, and incidence increases with increasing survival time of the child. Accordingly, intrathecal administration of cytostatic agents and/or radiation usually constitutes part of treatment whether or not CNS involvement is diagnosed.[32] It is possible **5** could find a place in the treatment of leukemia where it not only treats the primary disease site but also provides prophylaxis for the CNS.

Antitumor testing of **5** was extended to P388 leukemia (system 8) and the solid tumor model, B16 melanoma (system 9). As was anticipated, **5** had good activity against the P388 system. On the other hand, both **1** and **5** were essentially inactive against the murine solid tumor which is commensurate with the clinical trials[11,12] of **1** in treatment of solid tumors.

In addition to the in vivo comparative bioassays of **1** and **5** described above, comparative cytotoxicity studies on cultured L1210 cells have also been conducted.[33] Solutions of **5** were found to be cytotoxic but required a tenfold higher concentration than that found necessary for **1** to cause cessation of growth. Protection against the effects of **1** or **5** is afforded the L1210 cells by either cytidine or

uridine added to the culture medium at the same time as the drug. While the in vitro cytotoxicity of **5** is difficult to rationalize in terms of a prodrug role for **5** where metabolic activation is required, **5** may be intrinsically cytotoxic. On the other hand, the following subjective evidence indicates that the dihydro analogue may be a prodrug form of 5-azacytidine: (a) both are active in the same murine leukemia test systems but neither is active against the B16 solid tumor; (b) neither has any apparent schedule dependency; and (c) in experiments with the L1210/5-azacytidine resistant tumor, a cross resistance was observed. Definitive information to resolve the prodrug hypothesis will best come from in vitro and in vivo metabolite studies.

Table III gives the antitumor data for some synthetic intermediates and other compounds peripheral to this study. There was occasion to discuss compounds **3** and **6** above. Compound **4**, which is the boron-containing intermediate from the borohydride reduction of **1**, has activity in L1210 leukemia which approximates the level of activity shown by **5** in the same system (Table II, system 2). The most probable explanation for this result involves in vivo hydrolysis of the borate ester to give **5** which acts by itself or through a metabolic product to exert the observed antileukemic effect. In view of the potential use of boron-containing compounds in cancer therapy of the CNS,[34] **4** was tested in the murine ependymoblastoma brain tumor model.[35,36] The results, however, were negative perhaps due to the inability of a charged molecule to penetrate the blood–brain barrier in sufficient quantity.

Conclusion

It is felt that dihydro-5-azacytidine (**5**) has sufficient merit as a potential antitumor drug to warrant further preclinical study. Formulation should cause a minimum of difficulty due to the good water solubility of **5** and its stability over a broad pH range. Although the dose levels necessary for optimal effects are high relative to **1**, and might prove to be disadvantageous, the antitumor activity of **5** is, nevertheless, quite substantial in the L1210 model which has been used as an indicator system for clinical effectiveness.[37] The stability of the reduced nucleoside to acid in addition to its demonstrated activity in L1210 leukemia via oral administration might constitute an advantage in clinical usage over the parent drug. Similarly, the activity of **5** in the IC-L1210 model perhaps signals a potential CNS prophylaxis role in the treatment of acute leukemia. Finally, the more favorable therapeutic index of **5** suggests it might be used with a greater margin of safety than **1**.

Experimental Section

The GC–MS system consisted of a Varian Aerograph 2740 gas chromatograph coupled to a Du Pont 21-492 mass spectrometer via a glass transfer line and jet separator maintained at 255 °C. The mass spectrometer was operated with the ion source at 240 °C, a 300-μA ionizing current, and a 75-eV ionizing voltage. The injector port of the chromatograph was equilibrated at 250 °C and the flame ionization detector (FID) was maintained at 275 °C. A 5 ft × $^1/_8$ in. stainless steel column packed with 3% SE-30 on 120 mesh Variport Q was operated isothermally at 220 °C. The GC column effluent was directed to both the mass spectrometer ion source and FID through a fixed-ratio split so that a gas chromatogram, a total-ion current chromatogram, and a mass spectrum could be recorded simultaneously. Proton NMR spectra were recorded with a Varian T-60 or a Varian HA-100D spectrometer in Me_2SO-d_6 or D_2O solution using Me_4Si or TSP, respectively, as internal standards. When required, labile protons were completely exchanged by dissolving the samples in D_2O and lyophilizing the solution. Repeating the D_2O dissolution and lyophilization a total of three times gave the NMR sample. UV spectra ($\epsilon \times 10^{-3}$) were recorded on a Cary Model 15 spectrometer using pH 2 (potassium chloride–hydrochloric acid) or pH 8 (monobasic potassium phosphate–sodium hydroxide) buffers. Specific rotations were measured in a 1-dm cell with a Perkin-Elmer Model 141 polarimeter. Elemental analyses were carried out by the Section on Microanalytical Services and Instrumentation, NIAMDD, NIH, and by Galbraith Laboratories, Inc., Knoxville, Tenn. Where analyses are reported only by the symbols of the elements, results were within ±0.4% of the theoretical values. Compound purity was routinely checked by TLC using 5 × 20 cm plates coated with Baker 1B2-F silica gel. Four solvent systems were employed: butanol–ethanol–water (40:11:19), butanol–acetic acid–water (5:2:3), 2-propanol–ammonia–water (7:1:2), isobutyric acid–ammonia–water (66:33:1.5). Spot visualization was achieved with UV light or by charring after spraying the plate with concentrated sulfuric acid. Melting points were determined with a Thomas-Hoover capillary apparatus and are uncorrected.

1-β-D-Ribofuranosyl-3-guanylurea (3). The procedure of Pithova et al.[15] was followed for the hydrolysis of **1** with 1 N aqueous ammonia solution to give **3** as a syrup. When a water solution of the syrup was lyophilized a fluffy white material was obtained which was very hygroscopic: mp 103–105 °C dec (sinters at 82–86 °C).

A methanol solution of the syrup gave the picrate in 70% yield when treated with ethanolic picric acid: mp 172–174 °C (lit.[15] 172–174 °C). The melting point was raised to 177–178 °C by recrystallization from methanol. Anal. ($C_7H_{14}N_4O_5 \cdot C_6H_3N_3O_7$, 463.3) C, H, N.

4-Amino-5,6-dihydro-1-β-D-ribofuranosyl-1,3,5-triazin-2(1*H*)-one Hydrochloride (5). A solution of 4.88 g (0.02 mol) of **1** in 20 mL of HMPA was treated with 1.52 g (0.04 mol) of sodium borohydride and stirred at 50 °C for 1 h and then at room temperature for 3 h. The reaction was hydrolyzed with 50 mL each of methanol and water and allowed to stand at 0 °C overnight. Concentration of the solution under vacuum (bath 30 °C) gave a syrup which was washed with ether and the residue taken up in 70 mL of methanol. The boron complex (**4**) as a white powder (3.81 g) was precipitated by careful addition of ether (70 mL). Evaporation of the supernatant and subjecting the residue to another ether–methanol precipitation provided an additional 0.33 g of **4**: total yield 79%; mp >330 °C (darkens at 260 °C). Recrystallization could be effected from concentrated water solutions. In large-scale preparations the combined crops from ether–methanol precipitation are best recrystallized from water after adjusting the pH to 7 with a few drops of dilute hydrochloric acid: UV λ_{max} (pH 2) end absorption; UV λ_{max} (pH 8) 233 nm (7.37).

A solution of **4** (4.60 g, 0.017 mol) in 40 mL of 6 N hydrochloric acid was stirred at 21 °C for 4 h. Ethanol (70 mL) was added and stirring continued at −10 °C for 1 h to give a white precipitate which was removed by filtration and washed successively with ethanol and ether. On standing the reaction solution gave a second crop bringing the total yield of **5** to 4.05 g (85%): mp 180–181 °C dec. Recrystallization from methanol–ethanol gave colorless needles: mp 180–181 °C dec; $[\alpha]^{26}_D$ −29° (*c* 1.0, H_2O); UV λ_{max} (pH 2) end absorption; UV λ_{max} (pH 8) 233 nm (4.98); NMR (Me_2SO-d_6) δ 5.54 (d, *J* = 6 Hz, 1, C_1·H), 4.69 (s, 2, C_6H). Anal. ($C_8H_{14}N_4O_5$·HCl, 282.7) C, H, N, Cl.

4-Amino-5,6-dihydro-1-β-D-ribofuranosyl-1,3,5-triazin-2(1*H*)-one (6). A solution of 1.50 g (5.3 mmol) of the hydrochloride **5** in 150 mL of 1 N ammonium hydroxide was stirred at room temperature for 5 h, stored overnight at 25 °C, and then concentrated under vacuum (25 °C bath) to ca. 20 mL. Addition of 100 mL of absolute ethanol to the concentrate gave 1.15 g (89%) of crystals which when recrystallized from absolute ethanol provided 0.82 g of **6**, mp 216–218 °C dec. The analytical sample (methanol) melted at 218–220 °C dec: $[\alpha]^{29}_D$ −23° (*c* 1.0, H_2O); UV λ_{max} (pH 2) end absorption; UV λ_{max} (pH 8) 233 nm (6.69); NMR (Me_2SO-d_6) δ 5.59 (d, *J* = 6 Hz, C_1·H), 4.57 (s, 2, C_6H); NMR (D_2O) δ 5.75 (d, *J* = 6 Hz, 1, C_1·H), 4.64 (s, 2, C_6H). Anal. ($C_8H_{14}N_4O_5$, 246.2) C, H, N.

The free base (**6**) could also be formed by passing an aqueous solution of **5** slowly through a Dowex 1-X2 (OH^- form) column. Lyophilization of the aqueous eluates and recrystallization from methanol gave **6** in good yield: mp 218–219 °C.

Reduction of 1 with Sodium Borodeuteride. 4-Amino-5,6-dihydro-1-β-D-ribofuranosyl-1,3,5-triazin-2(1*H*)-one-6-*d* Hydrochloride (7). A solution of 1 (976 mg, 4.0 mmol) in 5 mL of HMPA was reduced with 168 mg (4.0 mmol) of sodium borodeuteride using the procedure described above for the preparation of 5. The boron complex, thus obtained, was recrystallized from water to give 887 mg of white crystals, mp >300 °C. The complex was hydrolyzed with 6 N hydrochloric acid (6 mL) for 4 h at room temperature as before affording 570 mg of 7 after recrystallization from ethanol: mp 180–181 °C dec.

The free base (8) was liberated from an aqueous solution of 7 (220 mg) run through a Dowex 1-X2 (OH^- form) column. After lyophilization of the eluates and recrystallization from ethanol, 145 mg of 8 was obtained: mp 218–220 °C. The integrated NMR spectrum was best observed free from the interference of the signal due to traces of water. To that end 25 mg was silylated in an NMR tube with 0.6 mL of a BSTFA–acetonitrile-d_3 solution (1:2) by sonication at room temperature until solution was complete. The observed integrated areas were 1:1:5 for the anomeric proton, the C-6 methylene proton, and the ribosyl protons, respectively.

Hydrolysis of 5. 5,6-Dihydro-5-azacytosine Hydrochloride (9). A solution of 5 (141 mg, 0.5 mmol) in 5 mL of 6 N hydrochloric acid was heated on a steam bath for 30 min. The darkened reaction solution was treated with charcoal and filtered. Concentration of the filtrate under reduced pressure to approximately 1 mL caused crystals (50 mg, 66%) to separate which were washed with absolute ethanol, mp 255–260 °C dec. Recrystallization from ethanol (10 mL) raised the melting point to 259–261 °C dec. Mixture melting point with 9 prepared by reduction of 11 (see below) gave no depression and spectral properties were identical.

A methanol solution of 9 from the nucleoside hydrolysis when passed through a Dowex 1-X2 column (OH^- form) gave the free base (10): mp 186–188 °C dec. Mixture melting point with 10 prepared from 11 was 188–189 °C dec.

5,6-Dihydro-5-azacytosine (10) from the Reduction of 11. To a solution of 5-azacytosine (11, 1.12 g, 10 mmol) in 7 mL of HMPA was added, with stirring at room temperature, 1.0 g (26 mmol) of sodium borohydride in small portions over 15 min. The reaction mixture was stirred at 21 °C for 1 h and then heated at 50 °C for 2 h. After storing the solution overnight at room temperature, 10 mL of methanol was added (stirred 2 h), followed by 10 mL of water. The following day solvents were removed in vacuo (30 °C bath) to afford a syrup which was triturated successively with portions of ether and portions of absolute ethanol. A solid separated (0.9 g) which was recrystallized three times from absolute ethanol giving 0.4 g (35%) of 10 as white crystals: mp 189–190 °C dec; UV λ_{max} (pH 2) end absorption; UV λ_{max} (pH 8) 233 nm (4.14); NMR (D_2O) δ 4.54 (s, C_6H); GC–MS (trimethylsilyl derivative) *m/e* (rel intensity) 330 (12), 329 (25), 257 (28), 256 (13), 241 (12), 171 (100), 142 (9), 100 (57), 99 (28). Anal. ($C_3H_6N_4O$, 114.1) C, H, N.

The starting material (11) gave UV λ_{max} (pH 2) 246 nm (5.13); UV λ_{max} (pH 8) 245 nm (3.54, shoulder); GC–MS (trimethylsilyl derivative) *m/e* (rel intensity) 256 (98), 255 (2), 214 (5), 198 (3), 171 (100), 142 (34), 100 (33), 99 (50).

The hydrochloride salt of 10 was prepared by treating a solution of 10 (30 mg, 0.26 mmol) in absolute ethanol (5 mL) with 2 mL of absolute ethanol saturated with gaseous hydrogen chloride. The reaction solution was warmed at 50 °C for 10 min and then allowed to cool to room temperature causing the salt (9) to crystallize from solution (30 mg, 77%): mp 259–261 °C dec. Recrystallization from absolute ethanol gave the analytical sample: mp 260–261 °C dec; NMR (D_2O) δ 4.65 (s, C_6H). Anal. ($C_3H_6N_4O$·HCl, 150.6) C, H, N, Cl.

Silylation Procedure. Nucleosides and their aglycons (1, 6, 8, 10, and 11) were derivatized for gas chromatography (GC) and combined gas chromatography–mass spectrometry (GC–MS) by trimethylsilylation with bis(trimethylsilyl)trifluoroacetamide (BSTFA). A 1:2 solution of BSTFA–acetonitrile was allowed to react with 1–3 mg of the appropriate free base in a 14 × 48 mm screw cap vial with a Teflon-lined rubber septum by sonication at room temperature until solution occurred (usually 10–15 min). Aliquots of these solutions were used directly for analysis. In the case of 6, 8, and 10, heating the silylation mixture initiated an oxidative decomposition of the trimethylsilylated derivative to an extent which was proportional to temperature and duration of heating.

Acknowledgment. The authors express their appreciation to Mr. L. Dudeck and Mr. G. Congleton, Hazleton Laboratories, Vienna, Va., and Dr. I. Wodinsky, Arthur D. Little, Inc., Cambridge, Mass., for biological evaluations. The generous supply of 5-azacytidine made available by Dr. H. B. Wood, National Cancer Institute, Bethesda, Md., is gratefully acknowledged.

References and Notes

(1) Presented in part before the Division of Medicinal Chemistry at the 172nd National Meeting of the American Chemical Society, San Francisco, Calif., August 1976, MEDI 72.
(2) NIH Visiting Postdoctoral Fellow from the National Research Center, Dokki, Cairo, Egypt.
(3) A. Piskala and F. Sorm, *Collect. Czech. Chem. Commun.*, **29**, 2060 (1964).
(4) L. J. Hanka, J. S. Evans, D. J. Mason, and A. Dietz, *Antimicrob. Agents Chemother.*, 619 (1966).
(5) F. Sorm, A. Piskala, A. Cihak, and J. Vesely, *Experientia*, **20**, 202 (1964).
(6) V. Paces, J. Doskocil, and F. Sorm, *Biochim. Biophys. Acta*, **161**, 352 (1968).
(7) J. Doskocil, V. Paces, and F. Sorm, *Biochim. Biophys. Acta*, **145**, 771 (1967).
(8) O. Hrodek and J. Vesely, *Neoplasma*, **18**, 493 (1971).
(9) M. Karon, S. Sieger, S. Leimbrock, J. E. Finklestein, M. Nesbit, and J. J. Swaney, *Blood*, **42**, 359 (1973).
(10) K. B. McCredie, G. P. Bodey, M. A. Burgess, J. U. Gutterman, V. Rodriguez, M. P. Sullivan, and E. T. Freireich, *Cancer Chemother. Rep.*, **57**, 319 (1973).
(11) C. G. Moertel, A. J. Schutt, R. J. Reitemeier, and R. G. Hahn, *Cancer Chemother. Rep.*, **56**, 649 (1972).
(12) R. E. Bellet, M. J. Mastrangelo, P. F. Engstrom, J. G. Strawitz, A. J. Weiss, and J. W. Yarbo, *Cancer Chemother. Rep.*, **58**, 217 (1974).
(13) P. L. Lomen, L. H. Baker, G. L. Neil, and M. K. Samson, *Cancer Chemother. Rep.*, **59**, 1123 (1975).
(14) Z. H. Israili, W. R. Vogler, E. S. Mingioli, J. L. Pirkle, R. W. Smithwick, and J. H. Goldstein, *Cancer Res.*, **36**, 1453 (1976).
(15) P. Pithova, A. Piskala, J. Pitha, and F. Sorm, *Collect. Czech. Chem. Commun.*, **30**, 2801 (1965).
(16) The decomposition pathway indicated by 1 → 2 → 3 is a valid first approximation of the events occurring at 25 °C and pH 7. However, additional hydrolysis products have been isolated or postulated [see R. E. Notari and J. L. DeYoung, *J. Pharm. Sci.*, **64**, 1148 (1975), and ref 15] and other pathways come into play under different pH and temperature conditions.
(17) Division of Cancer Treatment, National Cancer Institute, NIH.
(18) J. A. Beisler, unpublished results. The analysis was carried out using high-pressure liquid chromatography which will be described in full in a future communication.
(19) T. Higuchi and V. Stella, "Pro-drugs as Novel Drug Delivery Systems", ACS Symposium Series 14, American Chemical Society, Washington, D.C., 1975.
(20) A preliminary report of this work has been published: J. A. Beisler, M. M. Abbasi, and J. S. Driscoll, *Cancer Treat. Rep.*, **60**, 1671 (1976).
(21) M. Eto, M. Iio, Y. Kobayashi, H. Omura, and M. Eto, *Agric. Biol. Chem.*, **38**, 2081 (1974).
(22) S. Y. Chang, D. B. Lakings, R. W. Zumwalt, C. W. Gehrke, and T. P. Waalkes, *J. Lab. Clin. Med.*, **83**, 816 (1974).
(23) E. Kovats, *Helv. Chim. Acta*, **41**, 1915 (1958).
(24) J. A. McCloskey, A. M. Lawson, K. Tsuboyama, P. M. Krueger, and R. N. Stillwell, *J. Am. Chem. Soc.*, **90**, 4182 (1968).
(25) J. A. McCloskey in "Basic Principles in Nucleic Acid Chemistry", Vol. 1, P. O. P. Ts'o, Ed., Academic Press, New York, N.Y., 1974, p 209.
(26) R. I. Geran, N. H. Greenberg, M. M. Macdonald, A. M. Schumacher, and B. J. Abbott, *Cancer Chemother. Rep.*,

Part 3, 3, 1 (1972).
(27) H. H. Lloyd, E. A. Dulmadge, and L. J. Wilkoff, *Cancer Chemother. Rep.*, **56**, 585 (1972).
(28) L. H. Li, E. J. Olin, T. J. Fraser, and B. K. Bhuyan, *Cancer Res.*, **30**, 2770 (1970).
(29) H. E. Skipper and L. H. Schmidt, *Cancer Chemother. Rep.*, **17**, 1 (1962).
(30) A. Goldin, *Methods Cancer Res.*, **4**, 228 (1968).
(31) K. Raska, Jr., M. Jurovcik, Z. Sormova, and F. Sorm, *Collect. Czech. Chem. Commun.*, **30**, 3001 (1965).
(32) S. Thunold and P. J. Moe, *Acta Pathol. Microbiol. Scand., Sect. A, Suppl.*, **236**, 84 (1973).
(33) P. Voytek, J. A. Beisler, M. M. Abbasi, and M. K. Wolpert-DeFilippes, *Cancer Res.*, in press.
(34) G. L. Brownell, A. H. Soloway, and W. H. Sweet in "Modern Trends in Radiotherapy", T. J. Deeley and C. A. P. Wood, Ed., Butterworths, London, 1967.
(35) R. I. Geran, G. F. Congleton, L. E. Dudeck, B. J. Abbott, and J. L. Gargus, *Cancer Chemother. Rep., Part 2*, **4**, 53 (1974).
(36) P. C. Merker, I. Wodinsky, and R. I. Geran, *Cancer Chemother. Rep.*, **59**, 729 (1975).
(37) A. Goldin, A. A. Serpick, and N. Mantel, *Cancer Chemother. Rep.*, **50**, 173 (1966).

Synthesis and Antileukemic Activity of 5-Substituted 2,3-Dihydro-6,7-bis(hydroxymethyl)-1*H*-pyrrolizine Diesters

Wayne K. Anderson* and Paul F. Corey

Department of Medicinal Chemistry, School of Pharmacy, State University of New York at Buffalo, Buffalo, New York 14214.
Received November 2, 1976

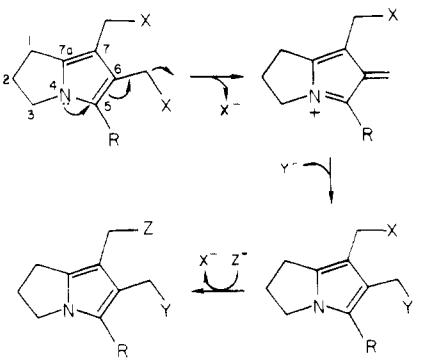
Treatment of *N*-acylproline derivatives, **2**, with acetic anhydride–dimethyl acetylenedicarboxylate (DMAD) gave 5-substituted derivatives of dimethyl 2,3-dihydro-1*H*-pyrrolizine-6,7-dicarboxylate (**5**). The reaction proceeds via a 1,3-dipolar addition of DMAD with the mesoionic oxazalone intermediate **3**, generated in situ, with concomitant elimination of carbon dioxide. Reduction of **5** gave the diols **6** which upon subsequent acylation gave **1**. The bis(*N*-methylcarbamate) **1d** and the diacetate **1i** show a modest level of in vivo antileukemic activity in the L1210 assay. A majority of the diesters, **1**, showed significant antileukemic activity in the in vivo P-388 assay. The bis(carbamate) **1d** afforded "cures" at dose levels as low as 12.5 mg/kg; **1q** showed potent activity at doses as low as 0.78 mg/kg. Several other compounds showed potent activity against P-388 over a greater than fourfold dose range with no acute toxicity. Half-lives for several diacetate derivatives of **1** were determined for aqueous Me_2SO solutions. The preparation of **7** and **8** shows that **1** may react by *O*-alkyl ester cleavage.

A large number of structurally diverse naturally occurring tumor inhibitory compounds have been isolated and identified over the past several years and a major proportion of these compounds contains at least one, often two or three, reactive electrophilic centers in the molecule in addition to several nonelectrophilic moieties.[1] The polyfunctionality is significant because it is the complex interrelationship of these functional groups that contributes to the antitumor activity, cell specificity, and toxicity which these compounds exhibit overall.[2]

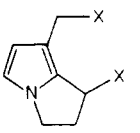
Structure–activity relationship studies with many of these natural products are often limited by the small quantities of material available and by the relatively limited number of modifications which actually can be performed on these complex molecules. Insight into the relationships between structure and activity can, in many instances, only be gained through studies with simpler molecules. The design of these simpler molecules uses the natural product as the base template.[3-5]

During the course of our continuing effort to prepare simple polyfunctional compounds for antitumor evaluation, we synthesized a series of substituted 2,3-dihydro-6,7-bis(hydroxymethyl)-1*H*-pyrrolizine diesters (**1**). Compounds of this type were chosen for study on the basis of certain similarities with the tumor inhibitory mitomycins[6] and pyrrolizidine alkaloids.[7] The pyrrole metabolites of various pyrrolizidine alkaloids can act as alkylating agents but are too reactive and too toxic for drug use. Mitomycin, on the other hand, possesses similar reactive electrophilic centers but is sufficiently stable to reach the cell nucleus where it can react with DNA. Both the mitomycins and

Scheme I[12]



the pyrrolizidine alkaloid pyrrole metabolites possess the general partial structure



Since the pyrrole metabolites of the pyrrolizidine alkaloids appear to be too reactive to give useful cancer chemotherapeutic activity, it should be possible to modulate this reactivity downward toward that of the mitomycins. The potential electrophilic reactivity of the allylic esters in **1** (via *O*-alkyl cleavage[8]) will be enhanced by participation of the ring nitrogen (Scheme I) similar to the mitomycins and pyrrolizidine alkaloid pyrrole metabolites. Fur-