Antiviral Activity of C-5 Substituted Tubercidin Analogues

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The pyrrolo[2,3-*d*]pyrimidine nucleoside antibiotics tubercidin, toyocamycin, and sangivamycin and the synthetic analogues 5-chloro-, 5,6-dichloro-, 5-bromo-, 6-bromo-, 5,6-dibromo-, 5-iodo-, 5-(1-hydroxyethyl)-, 5-(1-methoxyethyl)-, (*E*)-5-(2-bromoethenyl)-, (*E*)-5-(2-cyanoethenyl)-, 5-(2-buten-1-yl)-, 5-(3-hydroxypropyl)-, and 5-butyltubercidin were evaluated for their antiviral properties against six RNA viruses and three DNA viruses in HeLa cell, primary rabbit kidney cell, and Vero cell cultures. Most of the derivatives had substantial activity against the RNA viruses, with the least activity shown by 6-bromo-, 5,6-dichloro-, and 5,6-dibromotubercidin. The C-5 substituted derivatives were quite toxic for the host cells. 5-(1-Hydroxyethyl)-, 5-(1-methoxyethyl)-, and 5-(2-buten-1-yl)tubercidin were more selective against reovirus type 1, parainfluenza virus type 3 and Coxsackie virus B4 than tubercidin and the 5-halotubercidins. When tested for in vivo activity against Coxsackie B4 virus infection in newborn NMRI mice, 5-(1-hydroxyethyl)- and 5-(1-methoxyethyl)tubercidin caused a significant decrease in the mortality rate at a dose level of 100 μg per mouse. The inhibitory effects on L-1210 cell growth were also determined, and toyocamycin (ID_{50} = 0.006 μg/mL) was found to be the most active compound. This study demonstrates the significance of structural modification at C-5 and the potential of C-5 substituted analogues of tubercidin as biologically active agents.

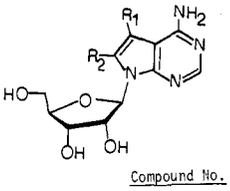
The pyrrolo[2,3-*d*]pyrimidine ribonucleoside antibiotics tubercidin, toyocamycin, and sangivamycin (Chart I) exhibit an interesting range of biological activity. Tubercidin is a highly cytotoxic adenosine analogue that interferes with numerous cellular processes, including mitochondrial respiration, de novo purine synthesis, rRNA processing, methylation of tRNA, and protein and nucleic acid syntheses.[1,2] The C-5 substituted derivatives of tubercidin, toyocamycin, and sangivamycin are more selective in their biological activity. Sangivamycin shows substantial antileukemic[3] and antitumor activity in vitro[4,5] and in vivo.[6,7] Toyocamycin also shows significant antitumor properties[8] but has more recently been of interest because of its ability to inhibit viral RNA replication. Among the viruses known to be inhibited by toyocamycin are vesicular stomatitis virus, adenovirus, murine retrovirus, and other oncornaviruses.[9–13]

Unlike the parent nucleoside tubercidin, which appears to be cytotoxic because of inhibition of a vital step in the glycolytic pathway,[14] the C-5 substituted analogues appear to be antiviral agents because of their incorporation into RNA.

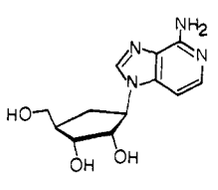
The exact mechanism by which toyocamycin inhibits viral replication may differ significantly for different viruses. Swart and Hodge[10] report that toyocamycin blocks adenovirus growth by preventing the polyadenylation of adenovirus heteronuclear RNA. In the case of vesicular stomatitis virus, the mRNAs are apparently processed normally in toyocamycin-treated cells, and the resulting mRNAs are functional despite the incorporated toyocamycin. However, intracellular genome-length 42S RNA synthesis is inhibited and, as a consequence, so is viral replication.[9] Genome-length RNA is produced in murine Eveline cells infected with Friend virus and treated with toyocamycin. However, the released virus progeny is neither infectious nor endowed with endogenous reverse transcriptase activity.[11] In this case, toyocamycin incorporation alters the ability of the RNA to either be transcribed or translated.

From studies on murine retrovirus, Hamelin and coworkers suggested that RNA containing toyocamycin in place of adenosine was less stable and more sensitive to S_1 nuclease action.[12] The same authors have looked at the effect of toyocamycin on oncornaviral production in acutely

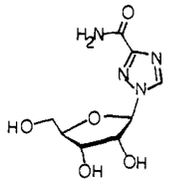
Chart I



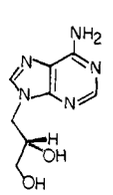
Compound	Compound No.	R_1	R_2
Tubercidin	1	H	H
5-Chlorotubercidin	2	Cl	H
5-Bromotubercidin	3	Br	H
5-Iodotubercidin	4	I	H
6-Bromotubercidin	5	H	Br
5,6-Dichlorotubercidin	6	Cl	Cl
5,6-Dibromotubercidin	7	Br	Br
Toyocamycin	8	CN	H
Sangivamycin	9	$CONH_2$	H
5-(1-Hydroxyethyl)tubercidin	10	$CHOHCH_3$	H
5-(1-Methoxyethyl)tubercidin	11	$CH(OCH_3)CH_3$	H
(E)-5-(2-Bromoethenyl)tubercidin	12	(E)-CH=CHBr	H
(E)-5-(2-Cyanoethenyl)tubercidin	13	(E)-CH=CHCN	H
5-(2-Buten-1-yl)tubercidin	14	$CH_2CH=CHCH_3$	H
5-(3-Hydroxypropyl)tubercidin	15	$(CH_2)_3OH$	H
5-Butyltubercidin	16	$(CH_2)_3CH_3$	H



3-Deazaaristeromycin 17



Ribavirin 18



S-DHPA 19

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infected cells[13] and found results similar to those reported by Moyer and Holmes[9] for vesicular stomatitis virus. The viral mRNA and the proteins for which they code are synthesized, but there is no virus release.

The effects of toyocamycin are not limited to viral RNAs. The analogue is also incorported into mammalian cell RNA and inhibits the processing of the 45S RNA precusor into 28S and 18S ribosomal RNA.[15] At least one other C-5 substituted pyrrolo[2,3-*d*]pyrimidine ribonucleoside shows similar biological activity. The synthetic analogue 5-bromotubercidin blocks the synthesis of Rous sarcoma virus RNA and after a period of exposure inhibits viral protein synthesis and mature virion formation.[16] The cellular synthesis of heterogeneous nuclear RNA and rRNA are also inhibited.

It should be noted as well that sangivamycin is an effective inhibitor of nuclear RNA synthesis in L-1210 cells.[17] In Ehrlich ascites cells, premature transcription of ribosomal precursor RNA appears to be a result of sangivamycin incorporation.[18] Ritch and Glazer found a correlation between cell death and incorporation of sangivamycin into poly(A) RNA of sarcoma 180 cells.[19]

As potential drugs, one factor that favors the utility of pyrrolo[2,3-*d*]pyrimidine nucleosides is their stability to deamination by adenosine deaminase and to glycosidic bond cleavage by purine nucleoside phosphorylases, the two major pathways by which bioactive purine nucleoside analogues are inactivated.[20]

Very few comparative studies on the ability of structurally different C-5 substituted pyrrolo[2,3-*d*]pyrimidine nucleosides to inhibit RNA synthesis have been done. Comparison of sangivamycin, toyocamycin, thiosangivamycin, tubercidin-5-carboxamidine, and tubercidin-5-carboxamidoxime in Ehrlich ascites cells in vitro showed that thiosangivamycin was the most effective inhibitor of nuclear RNA synthesis, although all its congeners showed substantial activity.[21]

Ritch and Helmsworth have determined the relationship between the cytotoxicity of these analogues and their effect on RNA synthesis in sarcoma 180 cells.[22] They found an inverse correlation between cytotoxicity and potency in RNA synthesis inhibition. Since fraudulent incorporation into RNA appears responsible for the lethal effects of C-5 substituted pyrrolo[2,3-*d*]pyrimidine nucleosides, this relationship seems consistent. The less the compound inhibits RNA synthesis, the more rapidly it is taken up into cellular RNA with resultant cytotoxic activity.

There is a significant parallelism between the effect that C-5 substituted pyrrolo[2,3-*d*]pyrimidine ribonucleosides have on RNA function after incorporation and the effect that some C-5 substituted pyrimidine 2′-deoxyribonucleosides have on DNA function. The potent antiherpes agent (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BV-dUrd) is incorporated into the HSV-1 genome via the 5′-triphosphate, although less efficiently than the natural substrate thymidine, and inhibits viral replication as a result of this incorporation.[23-25] The key to the selectivity of BV-dUrd lies in two viral-encoded enzymes, thymidine kinase and DNA polymerase. Both viral enzymes are tolerant of the C-5 substituent, and, in particular, the viral DNA polymerase binds BV-dUrd more tightly than do the cellular DNA polymerases α, β, and γ.[26] An uninfected cell is unable to convert BV-dUrd to its 5′-triphosphate, since the cytosol thymidine kinase is significantly more selective and intolerant of side chains much larger than methyl in the C-5 position. This structure-dependent selectivity and the resultant vast range of antiviral activity exhibited by different C-5 substituted pyrimidine 2′-deoxyribonucleosides suggest that a similar group of substituted compounds should be examined for the parallel case of the pyrrolo[2,3-*d*]pyrimidine nucleosides.

A limiting factor is the lack of availability of C-5 modified derivatives of tubercidin. In addition to the naturally occurring nucleoside antibiotics and the synthetic modifications described by Townsend and co-workers[27] and tested by Saffer and Glazer,[21] only the 5-halotubercidins,[28,29] 5-(hydroxymethyl)tubercidin,[30] and a group of hypermodified derivatives[31] have been evaluated for biological activity. Recent advances in synthetic chemistry, particularly in the area of direct substitution of tubercidin at C-5,[28,32,33] have provided sufficient structural variations to warrant a broad evaluation for antiviral activity. If selective activity against RNA viruses is possible within this class of analogues, an indication of this potential should be observed. The present report describes the result of our antiviral tests with the tubercidin derivatives

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Table I. Cytotoxicity and Antiviral Activity of Nucleoside Analogues in HeLa Cell Cultures

no.	compound	min cytotoxic concn,[a] μg/mL	min inhibitory concn,[b] μg/mL: vesicular stomatitis virus	Coxsackie virus B4	polio virus type 1
1	tubercidin	0.4	0.007	0.2	0.007
2	5-chlorotubercidin	4	>1	>0.4	>1
3	5-bromotubercidin	1	>0.4	>0.4	>0.4
4	5-iodotubercidin	1	>0.4	>0.4	>0.4
5	6-bromotubercidin	400	400	>200	150
6	5,6-dichlorotubercidin	40	>10	>10	>10
7	5,6-dibromotubercidin	40	>10	>10	>10
8	toyocamycin	≥0.4	0.07	>0.01	>0.01
9	sangivamycin	0.4	0.04	>0.04	0.07
10	5-(1-hydroxyethyl)tubercidin	40	0.2	0.7	0.2
11	5-(1-methoxyethyl)tubercidin	10	>10	0.7	0.7
12	(*E*)-5-(2-bromoethenyl)tubercidin	4	>1	>1	>1
13	(*E*)-5-(2-cyanoethenyl)tubercidin	4	0.4	1	4
14	5-(2-buten-1-yl)tubercidin	1	0.07	0.2	0.2
15	5-(3-hydroxypropyl)tubercidin	40	2	7	7
16	5-butyltubercidin	>40	>40	>40	>40
17	3-deazaaristeromycin	>400	0.7	>400	>400
18	ribavirin	>400	4	20	7
19	(*S*)-DHPA	>400	10	>400	>400

[a] Required to cause a microscopically detectable alteration of normal cell morphology. [b] Required to reduce virus-induced cytopathogenicity by 50%.

in cell cultures infected with Coxsackie virus B4, herpes simplex virus type 1 or 2, parainfluenza virus type 3, poliovirus type 1, reovirus type 1, Sindbis virus, vaccinia virus, and vesicular stomatitis virus.

Experimental Section

Compounds. The majority of the tubercidin analogues were synthesized. Tubercidin was purchased from the Upjohn Co., Fine Chemicals Division (Kalamazoo, MI). 5-Chlorotubercidin, 5-bromotubercidin, 6-bromotubercidin, and the 5,6-dihalogenated analogues (compounds **5** and **6**) were obtained by the reaction of tubercidin with *N*-chlorosuccinimide and *N*-bromosuccinimide.[28] 5-Iodotubercidin was synthesized from 5-mercuritubercidin.[32] The nucleoside antibiotics toyocamycin and sangivamycin were gifts of the National Cancer Institute (Bethesda, MD). Nucleosides **7**–**18** were synthesized via the organopalladium intermediate derived from tubercidin.[32]

Viruses. The origin of the viruses was as follows: herpes simplex virus type 1 (strain KOS) and type 2 (strain G), see ref 35; vaccinia virus, vesicular stomatitis virus, measles virus, Sindbis virus, Coxsackie type B4 virus, and polio virus type 1, see ref 34; reovirus type 1 (ATCC VR-230) and parainfluenza virus type 3 (ATCC VR-93) were obtained from the American Type Culture Collection (Rockville, MD). The virus stocks were grown in either primary rabbit kidney cells (herpes simplex virus types 1 and 2 and vesicular stomatitis virus), Vero cells (measles virus, reovirus, parainfluenza virus, and Coxsackie B4 virus), HeLa cells (poliovirus), chick embryo cells (Sindbis virus), or chorio-allantoic membrane cells (vaccinia virus).

Antiviral Assays. The antiviral assays were based upon an inhibition of virus-induced cytopathogenicity in either HeLa cell, Vero (African green monkey kidney) cell, or primary rabbit kidney cell cultures, following previously established procedures.[35]

Cytotoxicity. In tests that were run in parallel with the antiviral assays, the compounds were examined for their effects on the morphology of normal, uninfected cell cultures, which had been in contact with the compounds for the same time period as the virus-infected cells. A microscopically detectable disruption of normal cell morphology was taken as the end point of cytotoxicity.

Cytotoxicity was also assessed by the inhibition of cell proliferation; these tests were carried out with murine leukemia L-1210 cells in their exponential growth phase. This procedure has been described previously.[36]

Antiviral Activity in Vivo. A selected number of compounds (**1**, **10**, **11**, **14**, and **15**) was examined for their in vivo activities against Coxsackie B4 virus and vesicular stomatitis virus infections in newborn NMRI mice. When about 48 h old, the mice were infected subcutaneously with 10 times the LD_{50} of either Coxsackie B4 virus or vesicular stomatitis virus. They were treated intraperitoneally with a single dose of the nucleoside analogue 1 h after virus infection. Both virus and compound were administered in a volume of 0.1 mL per mouse. Mortality was recorded daily for a period of 20 days.

Results

In Vitro Activity toward Coxsackie Virus B4. As shown in Table I, tubercidin and all of the C-5 substituted analogues derived from it showed activity against Coxsackie virus B4 in HeLa cell cultures, with concentrations required to reduce virus-induced cytopathogenicity by 50% ranging from 0.2 μg/mL for tubercidin and 5-(2-buten-1-yl)tubercidin to 10 μg/mL for 5,6-dichloro- and 5,6-dibromotubercidin. The least activity was found for the two derivatives that had a halogen atom substituted in the C-6 position as well as at C-5. 6-Bromotubercidin, the only example in this study of an analogue with a group at C-6 but no substitution at C-5, was virtually inactive, with a minimum inhibitory concentration greater than 200 μg/mL. Nucleoside **5** was also relatively nontoxic toward HeLa cells compared to the C-5 substituted derivatives. As compared to ribavirin, the C-5 substituted tubercidin analogues were far more potent against Coxsackie virus B4, but they were also significantly more cytotoxic. The minimum inhibitory concentration for ribavirin in HeLa cell cultures was 70 μg/mL, while the minimal cytotoxic concentration was greater than 400 μg/mL. Although most of the tubercidin analogues showed an antiviral index (ratio of the minimum toxic concentration, as determined by a microscopically detectable alteration of normal cell morphology, over the minimum antiviral concentration) of less than 10, the antiviral selectivity for nucleosides **10** and **11** was 57 and 14, respectively.

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Table II. Cytotoxicity and Antiviral Activity of Nucleoside Analogues in Vero Cell Cultures

no.	min cytotoxic concn,[a] μg/mL	min inhibitory concn,[b] μg/mL: reovirus type 1	parainfluenza virus type 3	Sindbis virus	Coxsackie virus B4
1	0.4	0.07	0.07	0.2	0.2
2	4	0.7	>1	>1	>1
3	0.4	>0.4	>0.4	>0.4	>0.4
4	1	>0.4	>0.4	>0.4	>0.4
5	400	70	70	>200	70
6	40	>10	>10	>10	>10
7	40	10	>10	>10	10
8	0.04	>0.01	>0.01	>0.04	>0.01
9	0.4	>0.1	0.02	0.2	0.02
10	40	0.4	0.7	7	2
11	100	>1	2	>40	2
12	4	1	1	>1	>1
13	100	20	7	>40	>10
14	100	2	>1	>40	2
15	40	2	2	>10	7
16	>100	>40	20	>40	>40
17	>100	2	2	20	2
18	>400	20	20	150	>400
19	>400	70	20	>400	400

[a] Required to cause a microscopically detectable alteration of normal cell morphology. [b] Required to reduce virus-induced cytopathogenicity by 50%.

Table III. Cytotoxicity and Antiviral Activity of Nucleoside Analogues in Primary Rabbit Kidney Cell Cultures

no.	minimal cytotoxic concn,[a] μg/mL	min inhibitory concn,[b] μg/mL: HSV-1 (KOS)	HSV-2 (G)	vaccinia virus	vesicular stomatitis virus
1	0.4	0.07	0.2	0.02	0.007
2	4	≥1	>1	>1	>1
3	1	>1	>1	0.2	>1
4	1	0.02	>1	0.07	>0.4
5	>400	>400	>400	300	300
6	40	>40	>40	>40	>40
7	100	>100	>40	20	>40
8	≥0.04	>0.04	0.07	>0.01	>1
9	≥10	0.07	0.07	0.02	0.02
10	10	2	7	0.7	0.4
11	100	20	>40	0.7	>40
12	≥4	>1	>1	>1	>1
13	100	2	20	0.7	20
14	200	20	150	0.7	>40
15	≥40	7	>10	2	7
16	40	>40	>40	>40	>40
17	≥200	>400	300	0.1	0.07
18	≥400	>400	>400	10	70
19	≥400	>400	>400	70	10

[a] Required to cause a microscopically detectable alteration of normal cell morphology. [b] Required to reduce virus-induced cytopathogenicity by 50%.

The nucleoside antibiotics toyocamycin and sangivamycin, which, along with 5-bromotubercidin, have previously been studied as antiviral agents, were exceptionally cytotoxic (≤0.04 μg/mL) to HeLa cells and showed no antiviral selectivity. 5-Bromotubercidin showed a similar lack of selectivity (antiviral index ~2) against Coxsackie virus B4 in HeLa Cells.

Two analogues with selectivity indexes of less than 5 in HeLa cells were somewhat more selective in Vero cell cultures (Table II). (*E*)-5-(2-Cyanoethenyl)tubercidin showed a minimal inhibitory concentration of 10 μg/mL against Coxsackie virus B4 and an antiviral index of 10, while (*E*)-5-(2-buten-1-yl)tubercidin was active at 2 μg/mL and had an antiviral index of 50. The two analogues **10** and 11 which showed selectivity in HeLa cell cultures showed similar selectivity in Vero cells (antiviral indexes of 20 and 50, respectively). But the most potent antiviral agent in Vero B cell cultures was sangivamycin, which reduced virus-induced cytopathogenicity by 50% at 0.02 μg/mL and had an antiviral index of 20.

In Vitro Activity toward Herpes Simplex Virus Types 1 and 2. A similar range of activity was observed against the DNA viruses HSV-1 and HSV-2 (Table III). In this respect, the C-5 substituted tubercidin derivatives differed from the antiviral agent ribavirin, which did not prove active against either HSV-1 or HSV-2. Again, the least activity was shown by the three analogues that possess a substituent at C-6. The minimal inhibitory concentration for nucleosides 5–7 against HSV-1 (KOS) and HSV-2 (G) in primary rabbit kidney cell cultures was greater than 40 μg/mL.

The three nucleoside antibiotics tubercidin, sangivamycin, and toyocamycin were particularly potent against HSV-1 and HSV-2 (minimum inhibitory concentrations in the range of 0.07 μg/mL), while the 5-halo analogues were less cytotoxic and less inhibitory toward HSV-1 and HSV-2 by a factor of 10. For the majority of the tubercidin analogues the antiviral indexes were less

than 10. The most potent analogue against HSV-1, 5-iodotubercidin, reduced virus-induced cytopathogenicity by 50% at 0.02 μg/mL and had an antiviral index of 50.

Nucleoside 13 showed activity toward HSV-1 at a concentration (2 μg/mL) similar to that for which activity against Coxsackie virus B4 in HeLa cells was observed (>1 μg/mL) but was significantly less toxic toward primary rabbit kidney cell cultures than toward HeLa cell cultures.

In Vitro Activity toward Parainfluenza Virus Type 3. Twelve of the sixteen compounds tested were active toward parainfluenza virus type 3 in Vero cells at a concentration of less than 10 μg/mL (Table II). However, of these compounds only five showed selectivity indexes greater than 20.

5-(1-Hydroxyethyl)- and 5-(1-methoxyethyl)tubercidin were again among the group of the active and rather selective compounds with antiviral indexes of 57 and 50, respectively. Both were significantly more potent than (*S*)-9-(2,3-dihydroxypropyl)adenine [(*S*)-DHPA] and ribavirin toward parainfluenza virus type 3, but they were also somewhat more toxic toward Vero cell cultures. Their activity was more closely matched by 3-deazaaristeromycin, which was active at a concentration of 2 μg/mL. Tubercidin, toyocamycin, and sangivamycin were again among the three most active compounds tested (minimal inhibitory concentration ≤ 0.07 μg/mL), but they were also the most toxic toward Vero cell cultures (toxicity at 0.4 μg/mL).

5-(2-Buten-1-yl)tubercidin was the most selective compound, since its minimal inhibitory concentration was 1 μg/mL and cytotoxicity was not apparent until a concentration of 100 μg/mL was reached. Nucleoside 14 was about as active against Coxsackie virus B4, but because of its toxicity for HeLa cells, it did not show nearly as great a selectivity in HeLa cell culture.

In Vitro Activity against Polio Virus Type 1. The nucleosides 1, 8, and 9 again showed the most potent activity against polio virus type 1 (Table I). They were all active at a concentration below 0.1 μg/mL. Yet, 5-(1-hydroxyethyl)- and 5-(1-methoxyethyl)tubercidin, which were active at 0.2 and 0.7 μg/mL, respectively, had greater antiviral indexes (200 and 14, respectively) in HeLa cell culture. 5-(2-Buten-1-yl)tubercidin was also quite active (inhibitory at 0.2 μg/mL) but did not show much selectivity in HeLa cell cultures because of its cytotoxicity. Ribavirin was active against polio virus type 1 but at not nearly as low a concentration as the active tubercidin analogues.

In Vitro Activity against Reovirus Type 1. Eleven tubercidin analogues were active against reovirus type 1 in Vero cell culture at a concentration of 2 μg/mL or less (Table II), but tubercidin, toyocamycin, sangivamycin, and the 5-halotubercidins showed very little selectivity because of their high toxicity toward Vero cells. Increasing the size of the C-5 side chain did not necessarily decrease antiviral activity, but cytotoxicity decreased significantly. 5-(1-Hydroxyethyl)- and 5-(1-methoxyethyl)tubercidin again stood out in this respect. Both showed antiviral indexes of 100 against reovirus type 1 in Vero cell culture and activity was observed at a concentration of 0.4 and 1 μg/mL, respectively. Nucleosides 14 and 15 were not quite as active (inhibitory at 2 μg/mL) but showed similar toxicity toward Vero cells. Neither (*S*)-DHPA nor ribavirin was of comparable activity, but 3-deazaaristeromycin was active at 0.7 μg/mL.

In Vitro Activity against Vaccinia Virus. Many of the analogues showed significantly higher activity against vaccinia virus than against HSV-1 and HSV-2 in primary rabbit kidney cells (Table III). 5-(1-Methoxyethyl)- and 5-(2-buten-1-yl)tubercidin, which were both active against HSV-1 at 20 μg/mL, inhibited vaccinia virus at 0.7 μg/mL. As a result, the antiviral index for both for these compounds in primary rabbit kidney cells was greater than 100. 5-Iodotubercidin, although not as active against vaccinia virus (inhibitory at 0.07 μg/mL) as against HSV-1, appeared to be relatively more inhibitory for these DNA viruses than for the RNA viruses in comparison to the other C-5 substituted tubercidin derivatives. From the standpoint of activity and lack of cytotoxicity, 3-deazaaristeromycin (17), which was active at 0.1 μg/mL but nontoxic to primary rabbit kidney cells at 400 μg/mL, appeared more interesting as an antiviral agent than the tubercidin analogues.

In Vitro Activity against Sindbis Virus. With one exception, only those derivatives with fairly small C-5 substituents were as active against Sindbis virus in Vero cell culture as against other RNA viruses (Table II). Tubercidin, sangivamycin, toyocamycin, and the 5-halotubercidins were all active at a concentration of 1 μg/mL or less. 5-(2-Bromoethenyl)tubercidin, which was cytotoxic at a concentration of 4 μg/mL toward Vero cells, HeLa cells, and primary rabbit kidney cells, inhibited the majority of viruses, including Sindbis virus, at a concentration greater than 1 μg/mL. Of all the viruses examined, Sindbis virus was the least inhibited by (*S*)-DHPA, ribavirin, and 3-deazaaristeromycin.

In Vitro Activity against Vesicular Stomatitis Virus. The activity of the analogues against vesicular stomatitis virus was determined in both HeLa cell and primary rabbit kidney cell cultures (Table I and III). Although two of the compounds, 13 and 14, were much more active against vesicular stomatitis virus in HeLa cell culture, they were also more cytotoxic, with antiviral indexes in the range of 10–15. Only two compounds, 5-(1-hydroxyethyl)tubercidin and tubercidin, showed better antiviral indexes in either cell culture. The antiviral index for 5-(1-hydroxyethyl)tubercidin was 200 in HeLa cell culture and 25 in primary rabbit kidney cell culture. Tubercidin, which was by far the most active compound (inhibitory at 0.007 μg/mL), had a therapeutic index of 57 in both types of cell culture. As observed with most of the other RNA viruses examined, the C-5 substituted derivatives with relatively small substituents were active against vesicular stomatitis virus at concentrations slightly lower than the cytotoxic concentrations.

In Vivo Activity against Coxsackie B4 Virus. Tubercidin (1) and the four analogues 10, 11, 14, and 15 were tested against Coxsackie B-4 virus infections in newborn mice. The dose level was limited by the toxicity of the derivatives. Tubercidin had an LD_{50} of 54 μg per mouse, while the LD_{50} for each of the other derivatives was of the order of 300 μg per mouse. Of 120 control mice inoculated subcutaneously with Coxsackie B4 virus, 87% died (Figure 1). Nucleosides 14 and 15 were ineffective in reducing the mortality rate when given at a dose up to 100 μg per mouse. For 14 and 15, 18 of 20 (90%) and 16 of 20 mice (80%) died, respectively, within the observation period of 20 days (data not shown). The highest dose level at which tubercidin was tested was 20 μg per mouse, and at this dosage level it caused a 95% mortality rate (19 out of 20 mice; data not shown).

When injected at a dose of 100 μg per mouse, 5-(1-hydroxyethyl)tubercindin (10) reduced the mortality rate of newborn mice infected with Coxsackie B4 virus from 87 to 33% (Figure 1A), and 5-(1-methoxyethyl)tubercidin (11) reduced the mortality rate from 87 to 27% (Figure

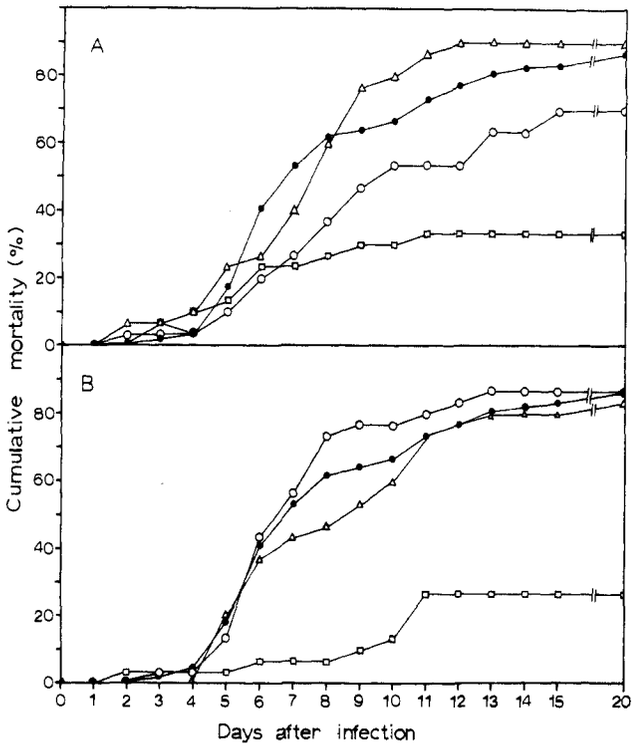

Figure 1. Effects of nucleosides **10** [5-(1-hydroxyethyl)tubercidin] and **11** [5-(1-methoxyethyl)tubercidin] on the mortality of newborn NMRI mice inoculated subcutaneously with Coxsackie B4 virus. (A) 5-(1-Hydroxyethyl)tubercidin at 5 (○), 20 (△), or 100 μg/mouse (□); control = ●. (B) 5-(1-Methoxyethyl)tubercidin at 5 (○), 20 (△), or 100 μg/mouse (□); control = ●. The compounds were injected intraperitoneally as a single dose in a volume of 0.1 mL per mouse 1 h after virus infection. There were 120 mice for the control group and 30 mice for each of the experimental groups.

1B). The differences in the mortality rate of the control group and the 5-(1-hydroxyethyl)- or 5-(1-methoxyethyl)tubercidin group were highly significant: $p < 0.001$, as assessed by the X^2 test (with Yates' correction). When administered at 5 or 20 μg per mouse, neither nucleoside **10** nor **11** proved effective in reducing the mortality rate of Coxsackie B4 virus infected mice (Figure 1).

No in vivo activity was noted for any of the analogues, when administered at a dose up to 100 μg per mouse, to newborn mice infected subcutaneously with vesicular stomatitis virus.

Antitumor Cell Activity. In view of the activity of tubercidin, sangivamycin, and toyocamycin as antitumor agents, it was considered important to compare the effects of other analogues described here on the proliferation of L-1210 cells.

The most active compound was toyocamycin, which with an ID_{50} of 0.006 μg/mL was two times more active than 5-chlorotubercidin and three times more active than sangivamycin (Table IV). It is of interest to note that 5-chlorotubercidin appeared 100 times less toxic toward Vero cells and primary rabbit kidney cells and 10 times less toxic toward HeLa cells than toyocamycin. However, 5-chlorotubercidin (NSC 124149) was found to be toxic toward mice at a dose level of 5 mg/kg but inactive against L-1210 in mice at 2.5 mg/kg. Tubercidin was not as active against L-1210 cell proliferation (ID_{50} = 0.040 μg/mL) but, like sangivamycin and toyocamycin, showed substantial cytotoxicity against HeLa cell, primary rabbit kidney cell, and Vero cell cultures. For the other analogues tested, the activity against L-1210 cell proliferation in vitro was not nearly as great. 5-(1-Hydroxyethyl)- and 5-(1-methoxyethyl)tubercidin, which ranked among the more selective antiviral agents, were not nearly as active against L-1210 (ID_{50} = 0.111 and 0.404 μg/mL, respectively) as toyocamycin, sangivamycin, and 5-chlorotubercidin.

Table IV. Effects of Tubercidin Analogues on the Proliferation of L-1210 Cells

compound	no.	ID_{50},[a] μg/mL
tubercidin	1	0.040 ± 0.022
5-chlorotubercidin	2	0.014 ± 0.007
5-bromotubercidin	3	0.587 ± 0.161
5-iodotubercidin	4	1.25 ± 0.06
6-bromotubercidin	5	36.5 ± 7.53
5,6-dichlorotubercidin	6	10.1 ± 1.21
5,6-dibromotubercidin	7	2.31 ± 0.54
toyocamycin	8	0.006 ± 0.001
sangivamycin	9	0.019 ± 0.003
5-(1-hydroxyethyl)tubercidin	10	0.111 ± 0.018
5-(1-methoxyethyl)tubercidin	11	0.404 ± 0.092
(*E*)-5-(2-bromoethenyl)tubercidin	12	7.84 ± 2.05
(*E*)-5-(2-cyanoethenyl)tubercidin	13	1.36 ± 0.17
5-(2-buten-1-yl)tubercidin	14	0.63 ± 0.14
5-(3-hydroxypropyl)tubercidin	15	1.28 ± 0.14
5-butyltubercidin	16	81.6 ± 38.4
3-deazaaristeromycin	17	not determined
ribavirin	18	3.18 ± 0.7
(*S*)-DHPA	19	170 ± 48

[a] Dose inhibiting cell proliferation by 50% (plus or minus standard deviation).

Discussion

The primary question that we sought to answer through this study was whether there exists a C-5 substituted analogue of tubercidin that inhibits the replication of RNA viruses and yet is less cytotoxic than the parent nucleoside tubercidin or the nucleoside antibiotics toyocamycin and sangivamycin. The activity of these antibiotics resulting from their incorporation into RNA has been recognized, but no systematic antiviral evaluation has been reported. From previous studies, toyocamycin, sangivamycin, and 5-bromotubercidin appear to be more selective than tubercidin, which is both antiviral and cytotoxic. Because these analogues are still cytotoxic, it is of interest to see what additional structural modification could be made at C-5 to decrease cytotoxicity and possibly retain antiviral activity.

To further establish the significance of C-5 substitution, we compared the activity of a C-6 halogenated analogue, 6-bromotubercidin, and two 5,6-disubstituted analogues, 5,6-dichlorotubercidin and 5,6-dibromotubercidin.

A direct comparison between 5-bromo- and 6-bromotubercidin indicated the latter to be 4×10^2 to 10^3 times less cytotoxic than 5-bromotubercidin toward Vero, HeLa, and primary rabbit kidney cell cultures. Comparative lack of cytotoxicity also appeared from the studies on inhibition of L-1210 cell growth. Furthermore, 6-bromotubercidin had insignificant antiviral activity against any of the DNA or RNA viruses tested. Although 5,6-dichloro- and 5,6-dibromotubercidin were more cytotoxic than 6-bromotubercidin, neither was nearly as toxic nor as active as 5-chloro- and 5-bromotubercidin.

An obvious rationale for this behavior is that the C-6 substituent may prevent these molecules from obtaining the anti conformation about the glycosidic bond. For comparison, 8-bromoadenosine, which is structurally equivalent to 6-bromotubercidin, has been demonstrated to exist solely in the syn conformation, since the large bromo substitutent is prevented by interaction with the C-2′ hydrogen to assume an anti conformation.[37] The 5′-triphosphates are, accordingly, not substrates for the

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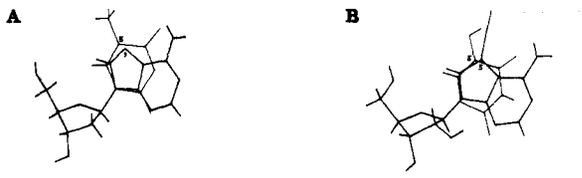

Figure 2. (A) Structure of 2′-deoxyadenosine superimposed on 2′-deoxythymidine and (B) toyocamycin superimposed on 5-hydroxyuridine.

RNA polymerase, since nucleoside 5′-triphosphates must be in the anti conformation to be recognized, bind to the active site, and be incorporated into the growing RNA chain.[38,39]

On the other hand, it has already been demonstrated that DNA polymerase can tolerate fairly large substitutents in other positions of the heterocyclic base. DNA polymerase I from *Escherichia coli* incorporates dUTP analogues possessing nonpolar C-5 substitutents as large as *n*-pentyl.[40] That even larger polar substituents can be tolerated was demonstrated by the incorporation of dUTP derivatives linked to biotin through an aminopropenyl group at C-5.[41]

When the carbohydrate moieties of nucleosides are superimposed, the C-5 position of pyrimidine nucleosides is observed to be spatially equivalent to the N-7 position of purine nucleosides, which in turn is equivalent to C-5 of pyrrolo[2,3-*d*]pyrimidine nucleosides (tubercidin). Superimposed projections of thymidine and 2′-deoxyadenosine in the preferred anti conformation are shown in Figure 2A.[42] A substituent on N-7 would project only slightly further away from the sugar in comparison to the C-5 methyl of 2′-deoxythymidine. This spatial relationship extends to ribonucleosides and pyrrolo[2,3-*d*]pyrimidines as shown in Figure 2B where a structure for toyocamycin is superimposed on 5-hydroxyuridine. The identity of the substituent at C-5 of the uracil ring does not appreciably affect its orientation in space relative to the rest of the molecule. 5-Bromo-, 5-carbamoyl- and 5-(carboxymethyl)uridine[43,44] have virtually identical structures. On the basis of this information it is a reasonable working hypothesis that at least some of the C-5 substituted analogues of tubercidin described here have antiviral activity because they are incorporated into RNA and effect processing or further transcription as already described for toyocamycin. To be active via incorporation into RNA, clearly these analogues must first be transformed to their 5′-triphosphates, probably via adenosine kinase, adenylate kinase, and nucleoside diphosphokinase. Tubercidin, toyocamycin, and sangivamycin are known to be converted to their 5′-triphosphates in vitro and in vivo.[2,45,46]

Since expression of activity likely depends on conversion of these analogues to the triphosphate, the differences in activity exhibited by the various C-5 modifications could be as much a result of a bottleneck at a kinase as it could be the result of differences in the amounts incorporated into RNA or effects on RNA structure and function after incorporation. Further studies in this direction are warranted.

The nature of the C-5 substituent, as expected, has a definite effect on antiviral activity. The least active and cytotoxic of the C-5 substituted analogues examined was 5-butyltubercidin (**16**), which suggests that a polarizable group in the side chain is important. A substituent of similar size, 2-butenyl, increased cytotoxicity toward HeLa cell cultures by a factor of 40 and activity toward vesicular stomatitis virus, Coxsackie virus B4, and polio virus type 1 by a factor of greater than 200. In Vero cell cultures, compounds **14** and **16** were of equivalent cytotoxicity; however, against reovirus type 1, parainfluenza virus type 3, and Coxsackie virus B4, nucleoside **14** was 20 times more active. Other polar groups effect the activity differently. When the C-5 substituent was 3-hydroxypropyl, toxicity toward HeLa and primary rabbit kidney cell cultures was equivalent to the *n*-butyl analogue, but the antiviral activity was slightly higher (5–20 times) against each of the viruses.

Two C-5 substituents that were slightly smaller than the butyl group but more rigid, 2-bromoethenyl and 2-cyanoethenyl, showed relatively poor selectivity. (*E*)-5-(2-Bromovinyl)tubercidin (**12**) was toxic to all three cell cultures at 4 μg/mL and in no case inhibitory toward virus-induced cytopathogenicity at less than 1 μg/mL. 5-(2-Cyanoethenyl)tubercidin (**13**) was less toxic (100 μg/mL) toward Vero and primary rabbit kidney cells than compound **12** and was active toward parainfluenza virus type 3 at 7 μg/mL and Coxsackie B4 at ≥10 μg/mL. Nucleosides **4**, **13**, and **14** were active and more selective toward inhibition of the two DNA viruses vaccinia and HSV-1 than the other analogues. It may be noteworthy that the most active of these toward HSV-1 (0.02 μg/mL), 5-iodotubercidin (**4**), is an exceptionally potent inhibitor of adenosine kinase.[47] In contrast, the three well-established broad-spectrum antiviral agents (*S*)-DHPA, ribavirin, and 3-deazaaristeromycin were active against most of the RNA viruses examined in this study but not against HSV-1 or HSV-2.

Two analogues with oxygen at C-1 of the side chains, 5-(1-hydroxyethyl)- and 5-(1-methoxyethyl)tubercidin, showed antiviral activity and cytotoxicity very similar to 5-(2-buten-1-yl)tubercidin, but the methoxyethyl derivative was much less active against vesicular stomatitis virus.

One may conclude from these results that C-5 substituted tubercidin analogues can be build that are significantly less cytotoxic than the nucleoside antibiotics tubercidin, toyocamycin, and sangivamycin and still retain activity against both RNA and DNA viruses. The primary objective of this paper has been to point out the potential significance of C-5 substituted tubercidin analogues. Substantial claims regarding antiviral activity must await further in vivo testing and quantitative toxicity studies. In addition, other structural analogues clearly need to be examined. Synthetic techniques are available to create C-5 substituted tubercidin analogues with longer and more

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complex side chains as well as to create further structural variation at the level of two- and three-carbon side-chain substituents.

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Pyridonecarboxylic Acids as Antibacterial Agents. 2.[1] Synthesis and Structure–Activity Relationships of 1,6,7-Trisubstituted 1,4-Dihydro-4-oxo-1,8-naphthyridine-3-carboxylic Acids, Including Enoxacin, a New Antibacterial Agent[2]

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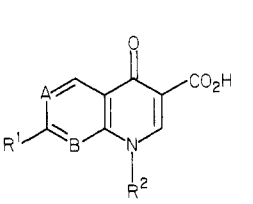
The title compounds having nitro, amino, cyano, chloro, or fluoro as the C-6 substituent were prepared. Introduction of the chloro and cyano groups at C-6 was accomplished by the Sandmeyer reaction of 6-amino-1,8-naphthyridine derivatives **9** via their 6-diazonium salts **10**. The reaction was extended to the synthesis of the 6-fluoro analogues **20**, involving the Balz–Schiemann reaction of the diazonium tetrafluoroborate **19**. Furthermore, a series of the 1-ethyl (**24** and **27–30**), 1-vinyl (**35–37**), 1-(2-fluoroethyl) (**38** and **39**), and 1-(difluoromethyl) (**40**) analogues of 7-substituted 6-fluoro-1,4-dihydro-4-oxo-1,8-naphthyridine-3-carboxylic acids was prepared. 1-Pyrrolidinyl and, particularly, N-substituted or unsubstituted 1-piperazinyl groups were introduced as the C-7 variants. As a result of this study, 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-1,8-naphthyridine-3-carboxylic acid (**24b**; named enoxacin, originally AT-2266) was found to show the most broad and potent in vitro antibacterial activity, an excellent in vivo efficacy on systemic infections, and a weak acute toxicity. Structure–activity relationships of compounds with variations of substituents at C-1, C-6, and C-7 are also discussed.

In earlier papers, we had reported the synthesis and antibacterial activity of pyrido[2,3-*d*]pyrimidines (**1**),[3,4] 1,6- and 1,8-naphthyridines (**2** and **3**),[1] and quinolines (**4**),[5] all of which possess a 1,4-dihydro-4-oxopyridine-3-carboxylic acid moiety as a common structure. This class of compounds has increasingly attracted attention as a source of new antibacterial agents.[6] It has been reported that nalidixic acid (**3**, $R^1 = CH_3$; $R^2 = C_2H_5$)[7] and oxolinic acid [**4**, R^1 = 6,7-(-OCH_2O-), $R^2 = C_2H_5$][8] are a specific inhibitor of DNA synthesis in susceptible bacterial cells, and this inhibition is due to the prevention of DNA gyrase activity.[9] Recent findings indicate that piromidic acid (**1a**, $R^2 = C_2H_5$)[3] and pipemidic acid (**1b**, $R^2 = C_2H_5$)[4] also act by the same mechanism.[10]

Chart I[a]



1, A = B = N
2, A = N; B = CH
3, A = CH; B = N
4, A = B = CH
5, A = CR^3; B = N
6, A = CF; B = CH; $R^2 = C_2H_5$

[a] a, R^1 =  ; b, R^1 =  ; c, R^1 =  .

A continuing interest in this field led us to an investigation of 1,6,7-trisubstituted 1,4-dihydro-4-oxo-1,8-naphthyridine-3-carboxylic acids (**5**), with the C-6 substituent being either nitro, amino, chloro, or fluoro; little information about the effect of the C-6 substituent upon antibacterial activity has been available thus far.

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