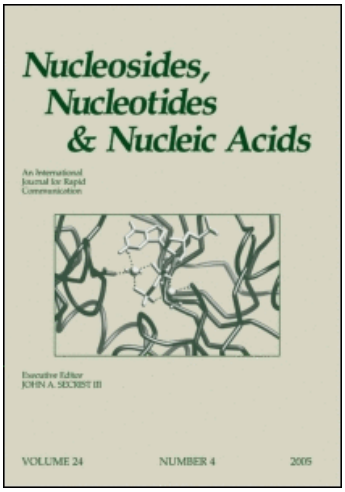

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ACYCLIC SUGAR ANALOGS OF TRICIRIBINE: LACK OF ANTIVIRAL AND ANTIPROLIFERATIVE ACTIVITY CORRELATE WITH LOW INTRACELLULAR PHOSPHORYLATION

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ABSTRACT: Triciribine and triciribine monophosphate have antiviral and antiproliferative activity at low or submicromolar concentrations. In an effort to improve and better understand this activity, we have synthesized a series of acyclic analogs and evaluated them for activity against select viruses and cancer cell lines. We conclude that the rigid ribosyl ring system of triciribine must be intact in order to be phosphorylated and to obtain significant antiviral and antiproliferative activity.

INTRODUCTION

Triciribine (TCN) is a tricyclic nucleoside that was first synthesized by Schram and Townsend in 1971.[1] Initial testing of triciribine and the water soluble 5′-monophosphate[2] (TCN-P) against murine leukemic L1210 cells revealed their potential as antineoplastic agents. This discovery led to extensive *in vitro*[3-17] and *in vivo*[18-22] studies of TCN and TCN-P as novel antiproliferative agents. Phase I clinical trials were completed with TCN-P[23-29] and it was advanced to phase II studies as a potential antiproliferative agent.[27, 29-32]

Early studies revealed that TCN is converted intracellularly to TCN-P by adenosine kinase.[4,6-7] Phosphorylation is essential for antiproliferative activity as demonstrated by the absence of growth inhibition when adenosine kinase deficient cells were treated with TCN.[3,11,12] The activity of TCN-P also requires adenosine kinase because extracellular TCN-P is a charged species and does not cross the cell membrane and therefore must be dephosphorylated to TCN by extracellular phosphatases or cellular ecto-5′-nucleotidase and rephosphorylated to TCN-P by intracellular adenosine kinase.[7,12] Unlike other nucleoside analogs, TCN is metabolized only to the monophosphate and not to di- or triphosphate forms.[9] Furthermore, no incorporation of TCN into nucleic acids has been observed.[17] TCN-P inhibits both DNA and protein synthesis[16,17], but the exact mechanism of antiproliferative activity is unknown.

More recently, we have found TCN and TCN-P are selective and potent inhibitors of HIV-1 and HIV-2 in acutely and persistently infected cells.[33] These studies also found no cross resistance to TCN or TCN-P in AZT- or TIBO-resistant HIV strains.[33] Similar to its antiproliferative activity in L1210 cells, TCN must be phosphorylated to TCN-P to be active against HIV.[34] Together with the data on the lack of cross resistance, the results suggest that TCN and TCN-P have an entirely different mode of action than AZT or TIBO. Furthermore, cytotoxicity such as that observed in murine L1210 cells appears to be highly cell line specific[3] and was not observed in human cell lines used to propagate HIV and human cytomegalovirus (HCMV).[33] Studies on the antiviral mechanism of action of TCN are currently underway.

Since 1971, only 7-aza analogs of TCN have been synthesized and evaluated.[35-39] This prompted us to initiate specific structure activity relationship studies to determine the structural requirements and mode of action of TCN and TCN-P. This study is the first in a series that explores the structural requirements for the sugar residing at the N-8 position of TCN for biological activity and was designed to explore the rigidity requirements of the ribosyl moiety. Knowing the importance of the 5′-hydroxyl group, we designed analogs to maintain the relative availability and position of the 5′-hydroxyl group. Scission of the bond between the 2′ and 3′ carbons of TCN was the most obvious of these modifications, followed by analogs lacking the 2′-carbon, and the 2′,3′-carbons (Figure 1). Analysis of these analogs has provided important insight into the rigidity requirements

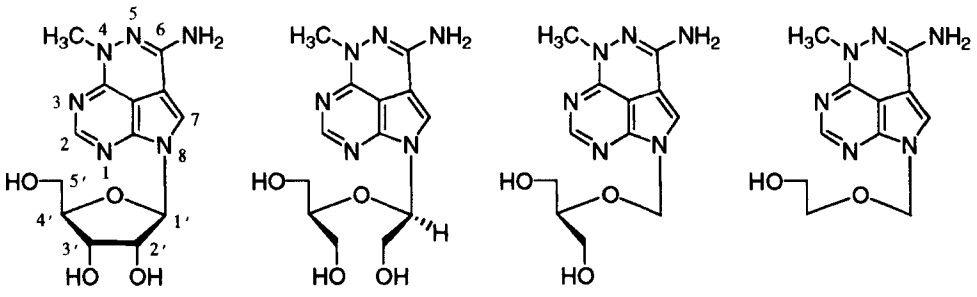


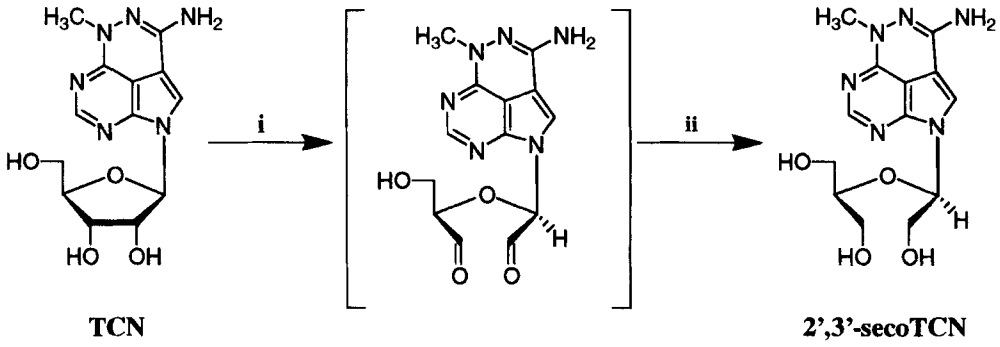
Figure 1. Acyclic Analogs of Triciribine (TCN).

of TCN with regard to phosphorylation and *in vitro* activity against selected viruses and cancer cell lines.

RESULTS AND DISCUSSION

2-Amino-8-[2-hydroxy-1-(1,3-dihydroxy-2-propoxy)ethyl]-4-methylpyrrolo-[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (2',3'-secoTCN) (Scheme 1), was synthesized by dissolving TCN[1] in methanol and sequentially treating it with aqueous sodium periodate and solid sodium borohydride. Each reaction was complete within 15 min, with the formation of a white precipitate as a byproduct of each reaction. Filtration of the precipitate from each reaction and column chromatography of the filtrate afforded pure 2',3'-secoTCN.

2-Amino-8-[(2-hydroxyethoxy)methyl]-4-methylpyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]-pyridazine (HEM-TCN) (Scheme 2), was synthesized from 4-amino-6-bromo-5-cyano-7-(2-hydroxyethoxy)methylpyrrolo[2,3-*d*]pyrimidine[40] (**1**). Acetylation of compound **1** with acetic anhydride gave 7-(2-acetoxyethoxy)methyl-4-amino-6-bromo-5-cyanopyrrolo-[2,3-*d*]pyrimidine (**2**). Compound **2** was diazotized with sodium nitrite in aqueous acetic acid to furnish 7-(2-acetoxyethoxy)methyl-6-bromo-5-cyanopyrrolo[2,3-*d*]pyrimidin-4-one (**3**). 7-(2-Acetoxyethoxy)methyl-6-bromo-5-cyano-4-(N-1-methylhydrazino)pyrrolo-[2,3-*d*]pyrimidine (**5**) was then prepared by first chlorinating compound **3** with phosphorous oxychloride to give 7-(2-acetoxyethoxy)methyl-6-bromo-4-chloro-5-cyano-



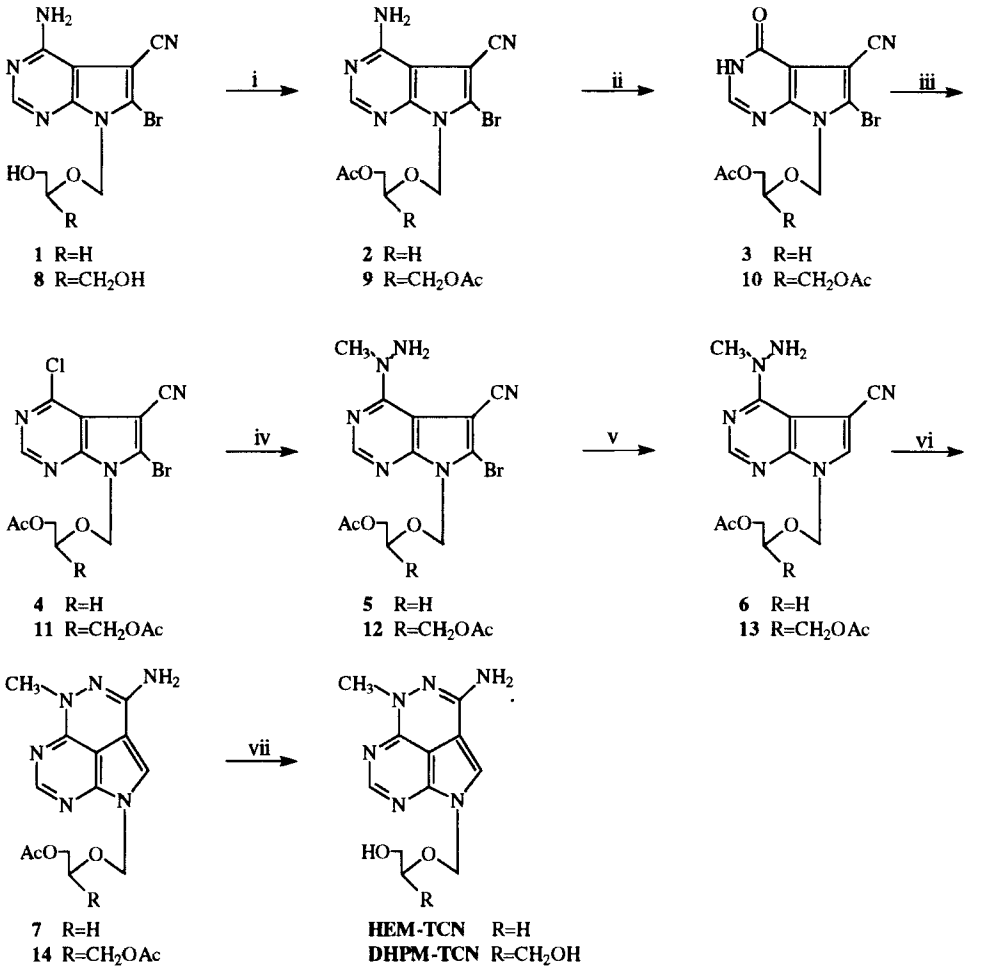
[a]Reagents: i. aq. $NaIO_4$, MeOH, RT, 15 min; ii. $NaBH_4$(s), MeOH, RT, 15 min.

Scheme 1[a]. Synthesis of 2',3'-secoTCN.

pyrrolo[2,3-*d*]pyrimidine (**4**) and then displacing the 4-chloro group with methylhydrazine in ethanol. Removal of the 6-bromo group by catalytic hydrogenation with 10% palladium on charcoal under 50 psi of hydrogen gas provided 7-(2-acetoxyethoxy)methyl-5-cyano-4-(N-1-methylhydrazino)pyrrolo[2,3-*d*]pyrimidine (**6**). Acid catalyzed ring annulation of compound **6** to afford 8-(2-acetoxyethoxy)methyl-6-amino-4-methylpyrrolo[4,3,2-*de*]-pyrimido[4,5-*c*]pyridazine (**7**) was accomplished in ethanol at reflux temperature. The desired product, HEM-TCN, was obtained after deprotection of compound **7** with methanolic ammonia at room temperature.

2-Amino-8-[(1,3-dihydroxypropoxy)methyl]-4-methylpyrrolo[4,3,2-*de*]pyrimido-[4,5-*c*]pyridazine (DHPM-TCN) (Scheme 2) was prepared from 4-amino-6-bromo-5-cyano-7-[(1,3-dihydroxy-2-propoxy)methyl]pyrrolo[2,3-*d*]pyrimidine[40] (**8**) by the same procedure as described above for the preparation of HEM-TCN.

These TCN analogs (2',3'-secoTCN, DHPM-TCN and HEM-TCN) were evaluated in an assay that uses growth rate and growth inhibition of murine L1210 cells as indicators of cytotoxicity. For each compound, it was found that after four days of incubation with L1210 cells, no inhibition of cell proliferation (cytotoxicity) was observed at concentrations as high as 100 μM (Table 1). Likewise, these acyclic analogs were not cytotoxic to KB cells, human foreskin fibroblasts (HFF) and CEM-SS cells (Table 1).



[a]Reagents: i. Ac_2O, pyridine; ii. $AcOH/H_2O$, $NaNO_2$; iii. $POCl_3$; iv. CH_3NHNH_2, EtOH; v. H_2, 10% Pd/C, EtOAc/EtOH; vi. HCl, EtOH, reflux; vii. NH_3, MeOH.

Scheme 2[a]. Synthesis of HEM-TCN and DHPM-TCN.

Thus, in contrast to TCN and TCN-P, which are potent inhibitors of L1210 cell growth, the acyclic analogs inhibited neither murine nor human cell growth.

The three target compounds also were evaluated for activity against three viruses. Activity against HIV-1 was measured in an assay that employs reverse transcriptase (RT) in culture supernatants as a marker for HIV-1. Activity against HSV-1 was determined in

Table 1. Antiproliferative and Antiviral Activity of Acyclic Analogs of TCN.

	50% inhibitory concentration (μM)						
	antineoplastic activity in murine L1210 cells[a]	cytotoxicity in human cells[b]			antiviral activity[c]		
Compound	cytotoxicity	KB growth	HFF visual	CEM-SS visual	HIV-1 RT	HSV-1 ELISA	HCMV plaque
TCN	0.035	>100	100	>100	0.04	23	2.5
TCN-P	0.025	10	19	>1.0	0.04	20	0.8
2′,3′-secoTCN	>100	>100	>100	>100	>100	>100	>100
DHPM-TCN	>100	>100	>100	>100	>100	>100	>100
HEM-TCN	>100	>100	>100	>100	>100	>100	>100

[a]Antiproliferative activity was determined in murine L1210 cells as described in the text. Growth rate was calculated as the percent of control in the presence of 100 μM compound concentration. Cytotoxicity is the concentration required to reduce L1210 cell growth rate to 50% of control rate. [b]Inhibition of KB cell growth was measured as described in the text in quadruplicate assays. Visual cytotoxicity was scored on uninfected HFF and CEM-SS cells used in HCMV plaque and HIV RT assays. [c]Antiviral activity was determined using an ELISA assay in quadruplicate for HSV-1, a plaque assay in duplicate for HCMV and amount of reverse transcriptase (RT) activity in culture supernatants in triplicate for HIV-1 as described in the text.

an ELISA and against HCMV in a plaque assay. For each compound, it was found that production of virus was equal to that of control, even at concentrations as high as 100 μM (Table 1) thereby demonstrating that 2′,3′-secoTCN, DHPM-TCN and HEM-TCN had no activity against these viruses.

Given the results of the biological assays, it was unclear whether the lack of activity was inherent to the molecule (e.g. the rigidity of the ribosyl ring system is necessary for TCN or TCN-P to exert their mode of action) or was due to the inability of these molecules to be phosphorylated to an active species. Therefore, the three TCN analogs were incubated at a concentration of 100μM with CEM-SS cells for 24 h. Intracellular nucleotides were extracted, separated, and quantitated by the same reverse

Table 2. Intracellular Phosphorylation of TCN and Acyclic Analogs in Uninfected CEM-SS Cells.

compound	extracellular concentration (μM)	incubation time (h)	intracellular monophosphate concentration (μM)[a]
TCN[b]	100	5	323
	100	12	307[c]
2',3'-secoTCN	100	24	<3[d]
DHPM-TCN	100	24	9
HEM-TCN	100	24	<3[d]

[a]Nucleotide extractions and quantitation by HPLC was performed as described in the text. [b]Control data for TCN phosphorylation previously published in ref. 34. [c]Average from two to five experiments. [d]Not detected; limit of detection was 3-5μM.

phase HPLC techniques used to separate TCN-P from TCN. No metabolites of 2',3'-secoTCN and HEM-TCN were detected. A peak in the HPLC chromatograph consistent with a small amount of DHPM-TCN monophosphate was observed, but the amount was very low compared to the amounts of TCN-P detected when TCN was incubated with CEM-SS cells for shorter periods of time (Table 2). Thus, either the acyclic nucleosides were not transported across the cell membrane or, more likely, they were not recognized as substrates for adenosine kinase. As a result, it is unknown whether these compounds, if phosphorylated, would exert the same mode of action as TCN-P inside the cell. Nonetheless, it is clear that the rigid ribosyl ring system of TCN and TCN-P must be intact in order to maintain antiproliferative and antiviral activity.

EXPERIMENTAL

Melting points were taken on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Thin-layer chromatography (TLC) was run on silica 60F-254 plates (Analtech, Inc.) using 5:1:1 chloroform:methanol:ethyl acetate, unless otherwise specified, and the plates were observed using UV light absorption at 254 nm. Nuclear magnetic resonance spectra were determined at 200, 300, 360 MHz with a BRUKER WP 200/300/360 SY or at 500 MHz on a Bruker Avance. The chemical shift values are

expressed in δ values (parts per million) relative to the chemical shift of the residual DMSO-d_5 (δ 2.50 ppm) contained in the solvent (DMSO-d_6). UV spectra were obtained with a Kontron UVIKON 860 ultraviolet spectrometer. Elemental analyses were performed by the Chemistry Department, University of Michigan, Ann Arbor, Michigan and are within ±0.4% of the theoretical values. E. Merck silica gel (230-400 mesh) was used for gravity or flash column chromatography. All evaporations were carried out on a rotary evaporator under reduced pressure (water aspirator) at temperatures less than 55° C.

6-Amino-8-[2-hydroxy-1-(1,3-dihydroxy-2-propoxy)ethyl]-4-methylpyrrolo-[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (2′,3′-secoTCN). TCN[1] (0.80 g, 2.5 mmol) was dissolved in methanol (150 mL). Aqueous sodium periodate (0.64 g, 3 mmol in 10 mL of water) was added and the reaction mixture was allowed to stir at room temperature. After 15 min, a white precipitate had formed and TLC showed the presence of a new spot at R_f = 0.45 (2:8 methanol:chloroform). The white precipitate was collected by filtration and discarded. Sodium borohydride (0.095 g, 2.5 mmol) was added to the filtrate and the reaction mixture was allowed to stir at room temperature. After 15 min, a white precipitate had formed and TLC showed the presence of a new spot at R_f = 0.38 (2:8 methanol:chloroform). The white precipitate was collected by filtration and discarded. The filtrate was evaporated and the residue was eluted from a silica gel column (4.5 cm (d) x 7 cm (h)) using a 1:9 methanol:chloroform solvent system. Fractions containing a spot at R_f = 0.38 (2:8 methanol:chloroform) were combined to give 2′,3′-secoTCN (0.64 g, 79% yield). 2′,3′-secoTCN (200 mg) was dissolved in hot acetone and recrystallized from a mixture of acetone and hexane. The crystals were collected by filtration and dried at 80 °C under reduced pressure (90 mg, 45% yield). mp 124-126°C; UV [λ_{max} (ε)] (pH 1) 287 (10217), 278 (10217), (pH 7) 292 (11053), (pH 11) 291 (12075); ^{1}H NMR (DMSO-d_6) δ 8.0 (1H, s, H-2), 7.0 (1H, s, H-7), 6.2 (2H, bs, NH_2), 5.9 (1H, t, H-1′), 5.1 (1H, t, OH), 4.7 (1H, t, OH), 4.5 (1H, t, OH), 3.9 (1H, m, H-2′), 3.7 (1H, m, H-2′), 3.6 (1H, m, H-3′ or H-5′), 3.4 (1H, m, H-3′ or H-5′), 3.4 (3H, s. CH_3), 3.3 (2H, m, H-4′), 3.2 (1H, m, H-3′ or H-5′), 3.1 (1H, m, H-3′ or H-5′); ^{13}C NMR (DMSO-d_6) δ 157.0 (C2), 152.1 (C8a), 147.9 (C3a), 146.7 (C6a), 109.6 (C7), 109.2 (C8b), 104.4 (C6), 84.9 (C1′), 80.2 (C4′), 63.5 (C2′), 61.4 (C3′ and C5′), 36.0

(CH_3). Anal. Calcd. For $C_{13}H_{18}N_6O_4 \cdot 0.25\ H_2O$: C, 47.78; H, 5.67; N, 25.73. Found: C, 47.75; H, 6.02; N, 25.60.

7-[(2-Acetoxyethoxy)methyl]-4-amino-6-bromo-5-cyanopyrrolo-[2,3-*d*]pyrimidine (**2**). A mixture of 4-amino-6-bromo-5-cyano-7-[(2-hydroxyethoxy)-methyl]pyrrolo[2,3-*d*]pyrimidine[40] (**1**) (3.5 g, 11.2 mmol) and acetic anhydride (3.43 g, 33.6 mmol) in dry pyridine (150 mL) was allowed to stir for 24 h at room temperature, under argon. The pyridine was removed and the residue was coevaporated with toluene (2 x 100 mL) to yield 3.5 g (88%) of crude **2** as a brown solid. This product was used in the next reaction without further purification. A sample was recrystallized from an ethyl acetate:hexane mixture to yield pure **2** as a yellow powder: $R_f = 0.72$; mp 158-160°C; ^{1}H NMR (DMSO-d_6) δ 8.2 (1H, s, H-2), 7.0 (2H, br s, NH_2), 5.6 (2H, s, NCH_2), 4.0 (2 H, m, $AcOCH_2$), 3.7 (2H, m, CH_2O), 1.9 (3H, s, $COCH_3$). Anal. Calcd. For $C_{12}H_{12}N_5O_3Br$: C, 40.68; H, 3.39; N, 19.77. Found: C, 41.08; H, 3.53; N, 19.68.

7-[(2-Acetoxyethoxy)methyl]-6-bromo-5-cyanopyrrolo[2,3-*d*]-pyrimidin-4-one (**3**). 7-[(2-Acetoxyethoxy)methyl]-4-amino-6-bromo-5-cyanopyrrolo-[2,3-*d*]pyrimidine (**2**) (2.65 g, 7.5 mmol) was suspended in a mixture of glacial acetic acid (15 mL), water (145 mL), and sodium nitrite (0.6 g, 0.8 mmol). The reaction mixture was stirred at 70 °C and three additional portions of sodium nitrite (0.6 g, 0.8 mmol) were added over a period of 45 min. The reaction mixture was allowed to stir for 18 h at 90 °C before the solvent was evaporated at 50 °C. The orange residue was resuspended in a saturated sodium bicarbonate solution (150 mL) and stirred until all gas formation had ceased. The mixture was extracted with ethyl acetate (2 x 150 mL) and the extracts were dried over magnesium sulfate. After filtration, the solvent was removed to yield 2.4 g (91%) of crude **3**. This product was used in the next reaction without further purification. A sample was recrystallized from an ethyl acetate:hexane mixture to yield pure **3** as a yellow powder: $R_f = 0.44$; mp 160-162°C; ^{1}H NMR (DMSO-d_6) δ 8.1 (1H, s, H-2), 5.6 (2H, s, NCH_2), 4.1 (2H, m, $AcOCH_2$), 3.7 (2H, m, CH_2O), 1.9 (3H, s, $COCH_3$). Anal. Calcd. For $C_{12}H_{11}N_4O_4Br$: C, 40.55; H, 3.10; N, 15.77. Found: C, 40.88; H, 3.03; N, 15.65.

7-[(2-Acetoxyethoxy)methyl]-6-bromo-4-chloro-5-cyanopyrrolo[2,3-*d*]-pyrimidine (**4**). 7-[(2-Acetoxyethoxy)methyl]-6-bromo-5-cyanopyrrolo[2,3-*d*]pyrimidin-

4-one (**3**) (1.4 g, 4 mmol) was suspended in phosphorous oxychloride (80 mL) and heated at reflux temperature for 30 min under an atmosphere of argon. The reaction system was then fitted with a distillation apparatus and the reaction mixture was concentrated to 5 mL at 35 °C under reduced pressure (water aspirator). Pouring the reaction mixture into ice water (250 mL) destroyed the remaining phosphorous oxychloride. The yellow precipitate was collected by filtration and the solid was dried in a vacuum oven at 60 °C to yield 1.4 g (95%) of crude **4**. This product was used in the next reaction without further purification. A sample was recrystallized from an ethyl acetate:hexane mixture to yield pure **4** as a yellow powder: $R_f = 0.75$; mp 150-152°C; ^{1}H NMR (DMSO-d_6) δ 8.9 (1H, s, H-2), 5.8 (2H, s, NCH_2), 4.1 (2H, m, $AcOCH_2$), 3.7 (2H, m, CH_2O), 1.9 (3H, s, $COCH_3$). Anal. Calcd. For $C_{12}H_{10}N_4O_3BrCl$: C, 38.54; H, 2.68; N, 14.99. Found: C, 38.24; H, 2.76; N, 14.88.

7-[(2-Acetoxyethoxy)methyl]-6-bromo-5-cyano-4-(N-1-methylhydrazino)-pyrrolo[2,3-*d*]pyrimidine (**5**). 7-[(2-Acetoxyethoxy)methyl]-6-bromo-4-chloro-5-cyano-pyrrolo[2,3-*d*]pyrimidine (**4**) (0.40 g, 1.1 mmol) was dissolved in a mixture of chloroform (30 mL) and ethanol (50 mL). Methylhydrazine (0.06 g, 1.32 mmol) was added and the reaction mixture stirred for 12 h, under an atmosphere of argon. The reaction mixture was concentrated to 20 mL and the white solid was collected by filtration, washed with ethanol (50 mL), and then dried in a vacuum oven (60 °C) to yield 0.38 g (90%) of crude **5**. This product was used in the next reaction without further purification. A sample was recrystallized from an ethyl acetate:hexane mixture to yield pure **5** as a white powder: $R_f =$ 0.63; mp 161-163°C; ^{1}H NMR (DMSO-d_6) δ 8.2 (1H, s, H-2), 5.6 (2H, s, NCH_2), 5.2 (2H, br s, NH_2), 4.0 (2H, m, $AcOCH_2$), 3.3 (3H, s, NCH_3), 1.9 (3H, s, $COCH_3$). Anal. Calcd. For $C_{13}H_{15}N_6O_3Br$: C, 40.75; H, 3.95; N, 21.93. Found: C, 40.75; H, 3.86; N, 21.55.

7-[(2-Acetoxyethoxy)methyl]-5-cyano-4-(N-1-methylhydrazino)pyrrolo-[2,3-*d*]pyrimidine (**6**). 7-[(2-Acetoxyethoxy)methyl]-6-bromo-5-cyano-4-(N-1-methyl-hydrazino)pyrrolo[2,3-*d*]pyrimidine (**5**) (0.61 g, 1.6 mmol) and 10% palladium on carbon (0.12 g) were added to a mixture of ethyl acetate (50 mL), ethanol (25 mL) and 1N NH_4OH (1.8 mL). The reaction mixture was shaken at room temperature on a Parr hydrogenator for 18 h under 50 psi of hydrogen gas. The suspension was filtered through

a pad of Celite and the pad of Celite was washed with ethyl acetate (50 mL) at room temperature. The solvent was removed and the residue was eluted from a silica gel column (4.5 cm (d) x 7 cm (h)) using a 1:9 methanol:chloroform solvent system. Fractions containing a spot at $R_f = 0.56$ were combined and evaporated to yield 0.40 g (83%) of crude **6**. This product was used in the next reaction without further purification. A sample was recrystallized from an ethyl acetate:hexane mixture to yield pure **6** as a white powder: $R_f = 0.56$; mp 120-122°C; ^{1}H NMR (DMSO-d_6) δ 8.3 (1H, s, H-2), 8.2 (1H, s, H-6), 5.6 (2H, s, NCH_2), 5.2 (2H, br s, NH_2), 4.0 (2H, m, $AcOCH_2$), 3.7 (2H, m, CH_2O), 3.3 (3H, s, NCH_3), 1.9 (3H, s, $COCH_3$). Anal. Calcd. For $C_{13}H_{16}N_6O_3$: C, 51.43; H, 5.28; N, 27.70. Found: C, 51.44; H, 5.08; N, 27.67.

8-[2-Acetoxyethoxy)methyl]-6-amino-4-methylpyrrolo[4,3,2-*de*]pyrimido-[4,5-*c*]pyridazine (**7**). 7-[(2-Acetoxyethoxy)methyl]-5-cyano-4-(N-1-methylhydrazino)-pyrrolo[2,3-*d*]pyrimidine (**6**) (0.4 g, 1.32 mmol) was suspended in ethanol (40 mL) with 1 drop of conc. HCl and heated at reflux temperature for 18 h. The solution was cooled to room temperature and the ethanol was removed to yield 0.33 g (83%) of crude **7**. This product was used in the next reaction without further purification. A sample was recrystallized from an ethyl acetate:hexane mixture to yield pure **7** as a tan powder: $R_f =$ 0.50; mp 156-158°C; ^{1}H NMR (DMSO-d_6) δ 8.1 (1H, s, H-2), 7.0 (1H, s, CH-6), 6.3 (2H, br s, NH_2), 5.5 (2H, s, NCH_2), 4.0 (2H, m, $AcOCH_2$), 3.6 (2H, m, CH_2O), 3.4 (3H, s, NCH_3), 1.9 (3H, s, $COCH_3$). Anal. Calcd. For $C_{13}H_{16}N_6O_3$: C, 51.43; H, 5.28; N, 27.70. Found: C, 51.77; H, 5.34; N, 27.59.

6-Amino-8-[(2-hydroxyethoxy)methyl]-4-methylpyrrolo[4,3,2-*de*]pyrimido-[4,5-*c*]pyridazine (HEM-TCN). Methanolic ammonia (200 mL), saturated at 0 °C, was added to a pressure bottle containing 8-[2-acetoxyethoxy)methyl]-6-amino-4-methylpyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (**7**) (0.20 g, 0.66 mmol). The sealed reaction vessel was warmed to room temperature and stirred for 18 h. The solvent was then removed to yield 0.16 g (94%) of crude HEM-TCN, which was eluted from a silica gel column (2.5 cm (d) x 7 cm (h)) using a 1:9 methanol:chloroform solvent system. Fractions containing a spot at $R_f = 0.30$ were combined and evaporated. The residue was recrystallized from an ethyl acetate:hexane mixture to yield 0.11g (65 % yield) of pure HEM-TCN as a tan powder: $R_f = 0.30$; mp 185-187°C; IR (KBr) no peak at 2250 cm^{-1};

UV [λ_{max} (ε)] (pH 1) 287 (9967), 278 (9967), (pH 7) 292 (13034), (pH 11) 291 (12118); ^{1}H NMR (DMSO-d_6) δ 8.1 (1H, s, H-2), 7.0 (1H, s, H-7), 6.3 (2H, s, NH_2), 5.5 (2H, s, NCH_2), 4.7 (1H, t, OH), 3.5 (4H, s, OCH_2CH_2O), 3.4 (3H, s, NCH_3); ^{13}C NMR (DMSO-d_6) δ 157.0 (C2), 152.1 (C8a), 147.8 (C3a), 146.6 (C6a), 112.1 (C7), 109.1 (C8b), 104.6 (C6), 74.7 (C1′), 71.3 (C4′), 60.7 (C5′), 36.1 (CH_3). Anal. Calcd. For $C_{11}H_{14}N_6O_2$: C, 50.38; H, 5.34; N, 32.06. Found: C, 50.43; H, 5.38; N, 32.18.

4-Amino-6-bromo-5-cyano-7-[(1,3-diacetoxy-2-propoxy)methyl]pyrrolo-[2,3-*d*]pyrimidine (9). A mixture of 4-amino-6-bromo-5-cyano-7-[(1,3-dihydroxy-2-propoxy)methyl]pyrrolo[2,3-*d*]pyrimidine[40] (**8**) (5.1 g, 15 mmol) and acetic anhydride (9.2 g, 90 mmol) in dry pyridine (200 mL) was allowed to stir for 24 hr at room temperature, under argon. The pyridine was removed and the residue was coevaporated with toluene (2 x 100 mL). The solid was resuspended in toluene (150 mL), collected by filtration and dried at 50°C under reduced pressure to yield 6.1 g (95%) of compound **9** as a tan solid. This product was used in the next reaction without further purification. A sample was recrystallized from an ethyl acetate:hexane mixture to yield pure **9** as a white powder: R_f = 0.82; mp 170-172°C; ^{1}H NMR (DMSO-d_6) δ 8.3 (1H, s, H-2), 7.1 (2H, br s, NH_2), 5.7 (2H, s, NCH_2), 4.1-3.9 (5H, m, OCH_2CHCH_2O), 1.9 (6H, s, $COCH_3$). Anal. Calcd. For $C_{15}H_{15}N_4O_6Br$: C, 42.25; H, 3.75; N, 16.43. Found: C, 42.33; H, 3.92; N, 16.27.

6-Bromo-5-cyano-7-[(1,3-diacetoxy-2-propoxy)methyl]pyrrolo[2,3-*d*]-pyrimidin-4-one (10). 4-Amino-6-bromo-5-cyano-7-[(1,3-diacetoxy-2-propoxy)methyl]-pyrrolo[2,3-*d*]pyrimidine (**9**) (3.0 g, 7.0 mmol) was suspended in a mixture of glacial acetic acid (20 mL), water (100 mL), and sodium nitrite (3.45 g, 50 mmol). This suspension was allowed to stir at 80 °C for 30 min. Additional sodium nitrite (3.45 g, 50 mmol) was added and the reaction mixture was allowed to stir for 18 h at 80 °C. The solvent was evaporated at 55 °C. The orange residue was resuspended in a saturated sodium bicarbonate solution (150 mL) and stirred until gas formation had ceased. The mixture was extracted with ethyl acetate (3 x 150 mL) and the extracts were dried over magnesium sulfate. After filtration, the solvent was removed to yield 2.2 g (73%) of crude compound **10**. This product was used in the next reaction without further purification. A sample was recrystallized from an ethyl acetate:hexane mixture to yield

pure compound **10** as a yellow powder: $R_f = 0.45$; mp 163-165°C; ^{1}H NMR (DMSO-d_6) δ 8.2 (1H, s, H-2), 5.7 (2H, s, NCH_2), 4.1-3.8 (5H, m, OCH_2CHCH_2O), 1.9 (6H, s, $COCH_3$). Anal. Calcd. For $C_{12}H_{11}N_4O_4Br$: C, 42.15; H, 3.51; N, 13.11. Found: C, 42.13; H, 3.72; N, 13.25.

6-Bromo-4-chloro-5-cyano-7-[(1,3-diacetoxy-2-propoxy)methyl]pyrrolo-[2,3-*d*]pyrimidine (11). 6-Bromo-5-cyano-7-[(1,3-diacetoxy-2-propoxy)methyl]pyrrolo-[2,3-*d*]pyrimidin-4-one (**10**) (1.5 g, 3.5 mmol) was suspended in phosphorous oxychloride (70 mL) and heated at reflux temperature for 30 min under an atmosphere of argon. The reaction system was then fitted with a distillation apparatus and the reaction mixture was concentrated to 5 mL at 35 °C under reduced pressure (water aspirator). Pouring the concentrated reaction mixture into ice water (250 mL) destroyed the remaining phosphorous oxychloride. The yellow precipitate was collected by filtration and dried in a vacuum oven at 60 °C to yield 1.4 g (90%) of crude **11**. This product was used in the next reaction without further purification. A sample was recrystallized from an ethyl acetate:hexane mixture to yield pure **11** as a yellow powder: R_f = 0.72 (9:1 chloroform:methanol); mp 113-115°C; ^{1}H NMR (DMSO-d_6) δ 8.9 (1H, s, H-2), 5.9 (2H, s, NCH_2), 4.1-3.9 (5H, m, OCH_2CHCH_2O), 1.9 (6H, s, $COCH_3$). Anal. Calcd. For $C_{15}H_{14}N_4O_5BrCl$: C, 40.31; H, 3.13; N, 12.54. Found: C, 40.51; H, 3.24; N, 12.21.

6-Bromo-5-cyano-7-[(1,3-diacetoxy-2-propoxy)methyl]-4-(N-1-methyl-hydrazino)pyrrolo[2,3-*d*]pyrimidine (12). 6-Bromo-4-chloro-5-cyano-7-[(1,3-diacetoxy-2-propoxy)methyl]pyrrolo[2,3-*d*]pyrimidine (**11**) (1.40 g, 3.1 mmol) was dissolved in a mixture of chloroform (30 mL) and ethanol (50 mL). Methylhydrazine (0.18 g, 3.72 mmol) was added and the reaction mixture stirred for 4 h, under argon. The solvent was removed leaving 1.4 g (98% yield) of crude **12** as a yellow solid. This product was used in the next reaction without further purification. A sample was recrystallized from an ethyl acetate:hexane mixture to yield pure **12** as a yellow powder: $R_f = 0.56$ (9:1 chloroform:methanol); mp 130-132°C; ^{1}H NMR (DMSO-d_6) δ 8.2 (1H, s, H-2), 5.7 (2H, s, NCH_2), 5.2 (2H, br s, NH_2), 4.0 (5H, m, OCH_2CHCH_2O), 3.3 (3H, s, NCH_3), 1.9 (6H, s, $COCH_3$). Anal. Calcd. For $C_{16}H_{19}N_6O_5Br$: C, 42.10; H, 4.17; N, 18.42. Found: C, 42.44; H, 4.22; N, 18.09.

5-Cyano-7-[(1,3-diacetoxy-2-propoxy)methyl]-4-(N-1-methylhydrazino)-pyrrolo[2,3-*d*]pyrimidine (13). 6-Bromo-5-cyano-7-[(1,3-diacetoxy-2-propoxy)methyl]-

4-(N-1-methylhydrazino)pyrrolo[2,3-*d*]pyrimidine (**12**) (0.35 g, 0.76 mmol) and 10% palladium on carbon (0.12 g) were added to a mixture of ethyl acetate (50 mL), ethanol (25 mL) and 1N NH_4OH (1.8 mL). The reaction mixture was shaken at room temperature on a Parr hydrogenator for 3 h under 50 psi of hydrogen gas. The suspension was filtered through a pad of Celite and the pad of Celite was washed with ethyl acetate (50 mL). The solvent was removed to give 0.20 g (70% yield) of crude **13**. This product was used in the next reaction without further purification. A sample was recrystallized from an ethyl acetate:hexane mixture to yield pure **13** as a white powder: R_f = 0.53 (9:1 chloroform:methanol); mp 114-116°C; ^{1}H NMR (DMSO-d_6) δ 8.3 (1H, s, H-2), 8.2 (1H, s, H-6), 5.6 (2H, s, NCH_2), 5.2 (2H, br s, NH_2), 4.1-3.9 (5H, m, OCH_2CHCH_2O), 3.4 (3H, s, NCH_3), 1.9 (6H, s, $COCH_3$). Anal. Calcd. For $C_{16}H_{20}N_6O_5$: C, 51.06; H, 5.32; N, 22.34. Found: C, 51.02; H, 5.33; N, 22.12.

6-Amino-4-methyl-8-[(1,3-diacetoxy-2-propoxy)methyl]pyrrolo[4,3,2-*de*]-pyrimido[4,5-*c*]pyridazine (**14**). 5-Cyano-7-[(1,3-diacetoxy-2-propoxy)methyl]-4-(N-1-methylhydrazino)pyrrolo[2,3-*d*]pyrimidine (**13**) (1.0 g, 2.85 mmol) was suspended in ethanol (40 mL) with 1 drop of conc. HCl and heated at reflux temperature for 18 h. The solution was cooled to room temperature and the ethanol was removed. The residue was eluted from a silica gel column (4.5 cm (d) x 7 cm (h)) using a 9:1 chloroform:methanol solvent system. Fractions containing a spot at R_f = 0.43 (9:1 chloroform:methanol) were combined and evaporated to yield 0.60 g (60% yield) of compound **14**. This product was used in the next reaction without further purification. A sample was recrystallized from an ethyl acetate:hexane mixture to yield pure **18** as a yellow solid: R_f = 0.43 (9:1 chloroform:methanol); mp 160-162°C; ^{1}H NMR (DMSO-d_6) δ 8.1 (1H, s, H-2), 7.0 (1H, s, H-6), 6.3 (2H, br s, NH_2), 5.6 (2H, s, NCH_2), 4.1-3.9 (5H, m, OCH_2CHCH_2O), 3.4 (3H, s, NCH_3), 1.9 (6H, s, $COCH_3$). Anal. Calcd. For $C_{16}H_{20}N_6O_5$: C, 51.06; H, 5.32; N, 22.34. Found: C, 51.12; H, 5.32; N, 22.02.

6-Amino-8-[(1,3-dihydroxy-2-propoxy)methyl]-4-methylpyrrolo[4,3,2-*de*]-pyrimido[4,5-*c*]pyridazine (DHPM-TCN). Methanolic ammonia (200 mL), saturated at 0°C, was added to a pressure bottle containing 6-amino-4-methyl-8-[(1,3-diacetoxy-2-propoxy)methyl]pyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (**14**) (0.19 g, 0.53 mmol). The sealed reaction vessel was warmed to room temperature and the mixture was stirred

for 24 h. The solvent was removed and the solid was recrystallized from ethanol to yield 0.11 g (73%) of pure DHPM-TCN as a tan powder: $R_f = 0.11$ (9:1 chloroform:methanol); mp 220-222°C; UV [λ_{max} (ε)] (pH 1) 287 (11277), 278 (11277), (pH 7) 292 (13543), (pH 11) 291 (13115); ^{1}H NMR (DMSO-d_6) δ 8.1 (1H, s, CH-2), 7.0 (1H, s, H-7), 6.3 (2H, s, NH_2), 5.6 (2H, s, NCH_2), 4.6 (2H, t, OH), 3.5 (1H, m, H-4′), 3.4 (5H, m, NCH_3 and H-3′ or H-5′), 3.30 (2H, m, H-3′ or H-5′); ^{13}C NMR (DMSO-d_6) δ 157.3 (C2), 152.1 (C8a), 147.5 (C3a), 146.6 (C6a), 112.1 (C7), 109.1 (C8b), 104.5 (C6), 74.1 (C1′), 74.1 (C4′), 61.6 (C3′ and C5′), 36.1 (CH_3). Anal. Calcd. For $C_{12}H_{16}N_6O_3$: C, 49.32; H, 5.48; N, 28.76. Found: C, 49.76; H, 5.81; N, 28.57.

***In Vitro* Antineoplastic Studies.** The *in vitro* cytotoxicity against murine L1210 leukemic cells was determined in a cell growth assay described previously.[41] L1210 cells were grown in Fischer's medium supplemented with 10% heat inactivated (56 °C, 30 min) horse serum and subcultured by serial dilution. Growth rates were calculated from determinations of the number of cells at 0, 24, 48, 72, and 96 h in the presence of selected concentrations of the test compound. The 50% inhibitory concentration (IC_{50}) was defined as the concentration required to decrease the growth rate to 50% of the untreated control cells. Growth rate was calculated from the slope of a semilogarithmic plot of cell number against time for the treated culture as a percent of the control.

***In Vitro* Antiviral Studies. Cell culture procedures.** The routine growth and passage of KB, HFF and BSC-1 cells was performed in monolayer cultures using minimal essential medium (MEM) with either Hanks salts [MEM(H)] or Earle salts [MEM(E)] supplemented with 10% calf serum as detailed previously.[42] The sodium bicarbonate concentration was varied to meet the buffering capacity required. Cells were passaged at 1:2 to 1:10 dilutions according to conventional procedures by using 0.05% trypsin plus 0.02% EDTA in a HEPES buffered salt solution. Similar suspension culture conditions were employed for CEM cells.

HIV-1 Assay. The HIV strain III_B producer cell line $H9III_B$ was obtained through the courtesy of Dr. R. C. Gallo. HIV strain III_B was propagated in CEM-SS cells as described previously by Kucera *et al.*[33,43] To evaluate the activities of compounds in cells acutely infected with HIV, reverse transcriptase (RT) was employed as a marker for HIV-1. CEM-SS cells were infected at a m.o.i. of approximately 0.001 p.f.u. per cell with

strain III_B of HIV-1. Test compounds were added to triplicate wells at seven concentrations ranging from 100 μM to 0.14 μM. After six days incubation, supernatant samples were taken and the amount of RT activity was measured by the incorporation of [^{3}H]dTTP into acid insoluble material using the assay described by White *et al.*[44]

HCMV plaque assay. The Towne strain, plaque-purified isolate P_O, of HCMV was kindly provided by Dr. Mark Stinski, University of Iowa. HFF cells in 24-well cluster dishes were infected with approximately 100 plaque forming units (p.f.u.) of HCMV per cm^2 cell sheet and compounds assayed in duplicate using four to eight concentrations using the procedures detailed earlier.[42]

HSV-1 ELISA. Selected drug concentrations in triplicate and HSV-1 (KOS strain kindly provided by Dr. Sandra K. Weller, University of Connecticut) at a concentration of 100 p.f.u./well were added to individual wells in 96-well cluster dishes. Following a 3-day incubation at 37°C, an enzyme-linked immunosorbent assay (ELISA)[45] was employed to detect HSV-1. Drug effects were calculated as a percentage of the reduction in absorbance in the presence of each drug concentration compared to absorbance obtained with virus in the absence of drug.

Cytotoxicity assays. Several different assays were used to explore the cytotoxicity of selected compounds using methods we have detailed previously. (*i*) Cytotoxicity produced in stationary HFF cells and in CEM-SS cells was determined by microscopic inspection of cells not affected by the virus used in the respective assays.[42] (*ii*) The effect of compounds during two population doublings of KB cells was determined by crystal violet staining and spectrophotometric quantitation of dye eluted from stained cells as described earlier.[46]

Data analysis. Dose-response relationships were constructed by linearly regressing the percent inhibition of parameters derived in the preceding sections against log drug concentrations. Fifty-percent inhibitory (IC_{50}) concentrations were calculated from the regression lines. Samples containing positive controls [acyclovir, ganciclovir and zidovudine (AZT), respectively] for HSV-1, HCMV, and HIV were used in all assays.

Extraction Procedures For Chromatographic Analysis. CEM-SS cells were grown in suspension culture with concentrations of compounds given in Table 2 for 5 to 24 h. Cells were harvested by centrifugation (250 x g, 5 min) at 25° C, the resulting cell

pellet was resuspended in 10 mL of cold Puck's saline with glucose (Gibco), and the cell suspension was again centrifuged (250 x g, 5 min). The extraction procedures used to obtain the aqueous phase of supernatant were previously described.[47] The aqueous phase was frozen at –76 °C for high performance liquid chromatography (HPLC) analysis.

Nucleotide Analysis. TCN-P was separated and quantitated by either anion exchange HPLC as detailed by Wotring et al.[12] or by ion pair reverse-phase HPLC as described by Walseth *et al.*[48] A Spectra-Physics system was employed consisting of a model SP8800 ternary pump, a SP8500 dynamic mixer, a SP8780 autosampler and a SP8490 variable wavelength detector. Peaks were integrated on a model SP4270 integrator. A Compaq model 386 computer with WINner 386 software (Spectra-Physics) was used for system and data management.

In an initial experiment, analysis was performed using anion exchange HPLC but superior resolution of monophosphates was achieved by reverse phase HPLC; consequently it was adopted in all subsequent experiments. Ion pair reverse-phase chromatography was performed on a 3.9 x 300 mm μBondapak C18 column (Waters). The solvent was 5 mM tetrabutylammonium hydroxide (TBA), 5% methanol adjusted to pH 2.5 with formic acid. Separation of nucleotide monophosphates was carried out at a flow rate of 1 mL/min. with dual wavelength detection set at 254 and 290 nm. TCN-P metabolite identification was based on comparison of unknown peaks to retention time of TCN-P standard and on the ratio of 290/254 nm due to the absorbance maximum of TCN and TCN-P at 290 nm.[12]

Intracellular concentrations were calculated based upon the integrated area under HPLC peaks of TCN-P, conversion of area to nmoles of TCN-P based upon the area under TCN-P peaks from standards of known concentration, and conversion of concentration from nmoles/10^6 cells to micromolarity based upon a cell volume of 1.9 pL/cell. Volumes of CEM-SS cells were determined by microscopic examination of cells in a hemacytometer, measurement of the diameter of 10-20 representative cells, and assumption of spherical geometry.

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