Synthesis and Antiviral Activity of Novel 5-(1-Cyanamido-2-haloethyl) and 5-(1-Hydroxy(or methoxy)-2-azidoethyl) Analogues of Uracil Nucleosides

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A new class of 5-(1-cyanamido-2-haloethyl)-2′-deoxyuridines (**4**−**6**) and arabinouridines (**7**, **8**) were synthesized by the regiospecific addition of halogenocyanamides (X-NHCN) to the 5-vinyl substituent of the respective 5-vinyl-2′-deoxyuridine (**2**) and 2′-arabinouridine (**3**). Reaction of **2** with sodium azide, ceric ammonium nitrate, and acetonitrile−methanol or water afforded the 5-(1-hydroxy-2-azidoethyl)-(**10**) and 5-(1-methoxy-2-azidoethyl)-2′-deoxyuridines (**11**). In vitro antiviral activities against HSV-1-TK$^+$ (KOS and E-377), HSV-1-TK$^-$, HSV-2, VZV, HCMV, and DHBV were determined. Of the newly synthesized compounds, 5-(1-cyanamido-2-iodoethyl)-2′-deoxyuridine (**6**) exhibited the most potent anti-HSV-1 activity, which was equipotent to acyclovir and superior to 5-ethyl-2′-deoxyuridine (EDU). In addition, it was significantly inhibitory for thymidine kinase deficient strain of HSV-1 (EC_{50} = 2.3−15.3 μM). The 5-(1-cyanamido-2-haloethyl)-2′-deoxyuridines (**4**−**6**) all were approximately equipotent against HSV-2 and were ∼1.5- and 15-fold less inhibitory for HSV-2 than EDU and acyclovir, respectively. Compounds **4**−**6** were all inactive against HCMV but exhibited appreciable antiviral activity against VZV. Their anti-VZV activity was similar or higher to that of EDU and approximately 5−12-fold lower than that of acyclovir. The 5-(1-cyanamido-2-haloethyl)-(**7**,**8**) analogues of arabinouridine were moderately inhibitory for VZV and HSV-1 (strain KOS), whereas compounds **10** and **11** were inactive against herpes viruses. Compounds **5** and **6** also demonstrated modest anti-hepatitis B virus activity against DHBV (EC_{50} = 19.9−23.6 μM). Interestingly, the related 5-(1-azido-2-bromoethyl)-2′-deoxyuridine (**1n**) analogue proved to be markedly inhibitory to DHBV replication (EC_{50} = 2.6−6.6 μM). All compounds investigated exhibited low host cell toxicity to several stationary and proliferating host cell lines as well as mitogen-stimulated proliferating human T lymphocytes.

Introduction

New development in the synthesis of 2′-deoxyuridine and arabinouridine that possess novel 2-carbon substituents at the C-5 position and exhibit potent antiviral activity represents an important area of drug design. Among many 5-substituted pyrimidine nucleosides that have been investigated, (*E*)-5-(2-halovinyl)- (**1a**, IVDU; **1b**, BVDU; **1c**, CVDU)[1], and 5-(2-chloroethyl)- (**1d**, CEDU) 2′-deoxyuridines[2] are the most potent and selective in their action against herpes simplex virus type-1 (HSV-1). CEDU is effective against systemic HSV-1 infection and HSV-1 encephalitis in mice at 5−15-fold lower dose than BVDU.[3] Herpes simplex type-2 virus (HSV-2) replication is affected only at considerably higher concentrations of BVDU or CEDU. The less potent 5-ethyl-2′-deoxyuridine (**1e**, EDU) is approximately equiactive against HSV-1 and HSV-2.[1] In contrast, 5-(hydroxyethyl)-2′-deoxyuridine (**1f**) is an inactive antiviral agent.[2]

Shiau et al.[4] reported that the 5-(hydroxymethyl)- (**1g**) and 5-(azidomethyl)- (**1h**) 2′-deoxyuridines have good affinity for HSV-1 encoded thymidine kinase (TK) and are strong inhibitors of HSV-1 replication. However, they were cytotoxic to host cells. In earlier studies, the synthesis of 5-[1-hydroxy (or methoxy)-2-haloethyl]-2′-deoxyuridines (**1i**−**l**)[5,6] was reported, which exhibited significant in vitro activity against HSV-1 (EC_{50} = 0.24−31.2 μM range). In further studies, the 5-(1-azido-2-haloethyl) derivatives of 2′-deoxyuridine (**1m**−**o**)[7] were found to exhibit broad-spectrum antiviral activity against herpes viruses [HSV-1 (EC_{50} = 0.06−6.4 μM range), HSV-2 (EC_{50} = 2.9−13.2 μM range), VZV (EC_{50} = 4.5−12.3 μM range), and EBV (EC_{50} = 4.0−11.1 μM range)]. These compounds were more active than the 5-(1-hydroxy (or methoxy)-2-haloethyl) analogues (**1i**−**l**) against HSV-1 and HSV-2. These studies indicate that an azido substituent at C-1 in the 5-(1-azido-2-haloethyl) side chain is an important determinant for potent antiviral activity against several herpes viruses. To synthesize more effective broad-spectrum antiviral agents, a cyanamido (NHCN) moiety located at the C-1 position of the 5-substituent attracted our attention. The NHCN moiety is structurally and electronically related to N_3 group and appears to be an efficient bioisostere of both N_3 and OH groups.[8,9] The cyanamido group, like the azido group, retains nitrogen atoms at the N-α and N-γ positions. The resonance contribution for the cyanamido group may resemble the π-electron delocalization for azido resonance species. Bioisosteres modulate biological activity by virtue of subtle differences in physicochemical properties and define some of the essential requirements of the pharmacophores. It was therefore of interest to investigate the hitherto unknown

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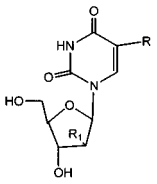
5-(1-cyanamido-2-haloethyl)-2′-deoxyuridines (**4**−**6**) to determine whether the substitution of the N_3 at the C-1 by a NHCN group could result in a new class of compounds more potent and selective than 5-(1-azido-2-haloethyl)-2′-deoxyuridines (**1m**−**o**).

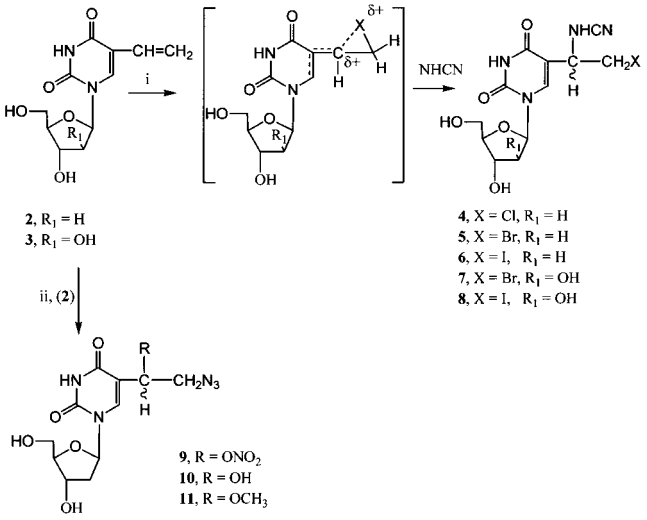
To continue our efforts to study the structure−activity relationships (SARs) of modified nucleosides, besides novel 5-(C-1) substituted derivatives (**4**−**6**), we were interested in investigating the effect of bioisosteric azido moiety at the C-2 carbon of 5-substituent on biological activity. Therefore, we have now synthesized novel 5-(1-hydroxy-2-azidoethyl)- (**10**) and 5-(1-methoxy-2-azidoethyl)- (**11**) 2′-deoxyuridines. The rationale for the synthesis of **10** and **11** came from the observation that the halogen atom at the C-2 position of the 5-substituent contributes significantly to the anti-herpes activity.[1−3] We also observed previously that 5-(1-azido-2-haloethyl) analogues (**1m**−**o**) exhibited potent antiviral activity in contrast to the 5-(1-azidoethyl) analogue of 2′-deoxyuridine (**1q**), which was devoid of antiviral activity.[7] The electronic (inductive effect, *F* value), steric (molecular refractive value), and lipophilic effect (π-value) of an azido group are close to that of halogens, viz: N_3, 0.30, 10.2, 0.46; Cl, 0.41, 6.03, 0.71; Br, 0.44, 8.88, 0.86; I, 0.40, 13.94, 1.12, respectively.[10] Thus the azido substituent possesses desirable physicochemical properties to be considered as a bioisosteric replacement of halogen substituents (Cl, Br, I) at the C-2 position of the 5-substituent. Furthermore, the azido group may be capable of electrostatic binding to an enzyme, such as TK, that is not possible with a halogen such as I, Br, or Cl. An interesting property of azido substituents containing pyrimidine nucleosides is that they can provide highly reactive functional groups upon exposure to UV light or radiation, which can interact with the target enzyme susceptible to photoaffinity labeling, resulting in enzyme inactivation.[11]



1a, R = CH=CHI,	R_1 = H		**1i**, R = CH(OH)CH_2X,	R_1 = H
1b, R = CH=CHBr,	R_1 = H		**1j**, R = CH(OMe)CH_2Cl,	R_1 = H
1c, R = CH=CHCl,	R_1 = H		**1k**, R = CH(OMe)CH_2Br,	R_1 = H
1d, R = CH_2CH_2Cl,	R_1 = H		**1l**, R = CH(OMe)CH_2I,	R_1 = H
1e, R = CH_2CH_3,	R_1 = H		**1m**, R = CH(N_3)CH_2Cl,	R_1 = H
1f, R = CH_2CH_2OH,	R_1 = H		**1n**, R = CH(N_3)CH_2Br,	R_1 = H
1g, R = CH_2OH,	R_1 = H		**1o**, R = CH(N_3)CH_2I,	R_1 = H
1h, R = CH_2N_3,	R_1 = H		**1p**, R = CH(N_3)CH_2X,	R_1 = OH
			1q, R = CH(N_3)CH_3,	R_1 = H

In this report we describe the synthesis and antiviral activity for the new series of 5-(1-cyanamido-2-haloethyl)-2′-deoxyuridines (**4**−**6**), arabinouridines (**7**, **8**) and 5-[1-hydroxy (or methoxy)-2-azidoethyl]- (**10**, **11**) analogues of 2′-deoxyuridine. It was postulated that compounds (**4**−**8**, **10**, **11**), in contrast to EDU (**1e**),[12] would be resistant to metabolic hydroxylation at the C-1 position of the 5-substituent due to obstructive substitution by the cyanamido, hydroxy, or methoxy substituents. Of the newly synthesized compounds, 5-(1-cyanamido-2-haloethyl)- (**5**, **6**) derivatives were found to exhibit appreciable anti-HBV activity (EC_{50} = 19.9−23.6 μM). These results suggested that 5-(1-azido-2-haloethyl) analogues (**1m**−**o**) could be potential anti-HBV agents and encouraged us to resynthesize **1m**−**o** to evaluate them for their anti-HBV activity. Interestingly, among these compounds, 5-(1-azido-2-bromoethyl)-2′-deoxyuridine (**1n**) was found to be markedly inhibitory for DHBV replication (EC_{50} = 2.6−6.6 μM). These results further prompted us to resynthesize the related 5-(1-methoxy-2-haloethyl)-2′-deoxyuridines (**1j**−**l**) in order to determine their anti-HBV activity and to identify structure−activity correlationship. However, they did not prove to be inhibitory to DHBV replication at 25 μM.

Scheme 1[a]



[a] Reagents: (i) *N*-chlorosuccinimide, NH_2CN, CH_3CN, 0−25 °C (**4**); *N*-bromosuccinimide (**5**, **7**), *N*-iodosuccinimide (**6**, **8**), NH_2CN, DME, 0 °C; (ii) ceric ammonium nitrate, NaN_3, CH_3CN-H_2O, −5 °C [**10** (method A)]; ceric ammonium nitrate, NaN_3, dry CH_3CN, −15 °C [**10** (method B), **11**].

Chemistry

The target 5-(1-cyanamido-2-haloethyl) derivatives of 2′-deoxyuridine (**4**−**6**, 15−42%), and arabinouridine (**7**, **8**, 18−19%) were synthesized by the reaction of respective 5-vinyl analogues (**2**, **3**) with *N*-halosuccinimide and cyanamide as illustrated in Scheme 1. The ^{13}C NMR (*J* modulation) spectrum of **4** provided conclusive evidence for the regiospecific addition of XNHCN (X = Cl, Br, I) across the 5-vinyl substituent of **2** and **3**. For example, the chlorine atom in **4** is attached to a methylene carbon that exhibited resonance at δ 45.4, whereas the cyanamido substituent is attached to a chiral methine carbon that exhibited dual resonances at δ 55.9 and 56.2. The regiospecific addition is consistent with reports that unsymmetrical olefins, capable of halonium ion formation, were found to favor an unsymmetrical bridged intermediate of the type illustrated in Scheme 1, even in solvents having a high dipole moment.[13] Similar regioselectivity has been also observed for the addition of hypobromous acid (BrOH),[14,15] methyl hypobromite ($BrOCH_3$),[16] and bromoazide (BrN_3)[17] to unsymmetrically substituted alkenes. Compounds **4**−**8** are mixtures of two diastereomers (1:1 ratio), which differ in configuration (*R* and *S*) at the 1-carbon atom of the 5-(1-

cyanamido-2-haloethyl) moiety. Attempts to separate the diastereomers of **4**−**8** by flash column chromatography or multiple-development TLC techniques were unsuccessful.

In the reaction of **2** with *N*-chlorosuccinimide and cyanamide in acetonitrile, a mixture of (*E*)-5-(2-chlorovinyl)-2′-deoxyuridine (**1c**) and 5-(1-methoxy-2-chloroethyl)-2′-deoxyuridine (**1j**) was also produced in addition to the target 5-(1-cyanamido-2-chloroethyl) product (**4**). Further, reactions of **2** and **3** employing *N*-halosuccinimide and cyanamide provided complex reaction mixtures of starting materials and unidentified compounds, which were difficult to separate, leading to low yields of the desired compounds.

Reaction of 5-vinyl-2′-deoxyuridine (**2**) with 2 equiv of ceric ammonium nitrate and 1 equiv of sodium azide in CH_3CN-H_2O yielded 5-(1-hydroxy-2-azidoethyl)-2′-deoxyuridine (**10**, 31.5% (Scheme 1)). In a related reaction of **2** in dry acetonitrile, followed by quenching with methanol, 5-(1-methoxy-2-azidoethyl)-2′-deoxyuridine (**11**, 25%) was obtained. Although a similar reaction of **2** in dry acetonitrile proceeded instantaneously, showing one major product by analytical TLC, a second less polar compound was present after the reaction mixture was quenched with aqueous acetonitrile. Separation of this reaction mixture by silica gel column chromatography afforded 5-(1-hydroxy-2-azidoethyl)-2′-deoxyuridine (**10**, 32%). The less polar compound which arose after the aqueous acetonitrile quench was identical (^{1}H NMR) to the compound **10** as described before. Compounds **10** and **11** were mixtures of two diastereomers. The ^{1}H NMR spectrum of **10** and **11** exhibited overlapping multiplets for the CH_2N$_3$ resonances at δ 3.30 (**10**) and 3.56 (**11**), a multiplet assigned to the C*H*OH resonance at δ 4.60 (**10**), a multiplet assigned to the C*H*OMe resonance at δ 4.48 (**11**), a singlet assigned to the CHO*H* resonance at δ 5.72 (exchanges with deuterium oxide) (**10**), two closely spaced singlets for the OCH_3 protons at δ 3.34 and 3.36 (**11**), and two closely spaced singlets for H-6 at δ 7.80 and 7.82 (**10**) and 7.90 and 7.92 (**11**). The ^{13}C NMR spectrum of **10** and **11** provided further evidence for the assigned structures based on the resonances at δ 55.01 (*C*H$_2$N$_3$ of **10**), 53.84 (*C*H$_2$N$_3$ of **11**), 66.14 (*C*HOH of **10**), 76.54 (*C*HOCH$_3$ of **11**), and 57.46 and 57.52 (OCH_3 of **11**). The signals assigned for protons at the C-1 substituent and the carbon (C-1) bearing the hydroxy or methoxy group are consistent with the shifts reported for 5-[1-hydroxy (or methoxy)-2-haloethyl]-2′-deoxyuridines.[5,6] The IR spectrum of **10** and **11** showed very strong bands at 2118 and 2104 cm^{-1}, respectively, for the azide group, whereas the bands for the nitrate group were not present. In both of these instances (**10** and **11**), there was no evidence for the presence of the 5-(1-nitrato-2-azidoethyl)-2′-deoxyuridine (**9**). This is in contrast to a previous report where reaction of olefins with ceric ammonium nitrate and sodium azide in dry or aqueous acetonitrile produced α-azido-*β*-nitratoalkanes as addition products.[18,19]

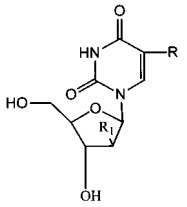
A possible mechanism for the formation of **10** and **11** involves the decomposition of 5-(1-nitrato-2-azidoethyl)-2′-deoxyuridine (**9**), which is formed at lower temperature, to a carbonium ion intermediate at 25 °C which underwent reaction with methanol or water to yield the respective products that were isolated. The intermediacy of related carbonium ions has been previously proposed.[20]

Results and Discussion

The antiviral activities for the new class of 5-(1-cyanamido-2-haloethyl)- (**4**−**8**) and 5-(1-hydroxy(or methoxy)-2-azidoethyl)- (**10**, **11**) uracil nucleosides were determined in culture against herpes simplex virus type 1 (HSV-1, strains KOS and E-377), herpes simplex virus type 2 (HSV-2, strain MS), varicella zoster virus (VZV, strain Ellen), and human cytomegalovirus (HCMV, strain AD-169) infected human foreskin fibroblast (HFF) or Vero cells. In addition, the compounds were also evaluated against a thymidine kinase-deficient (TK$^-$) mutant HSV-1 strain (KOSSB) in Vero cells. The results are summarized in Table 1. The 5-(1-cyanamido-2-haloethyl)-2′-deoxyuridines (**4**−**6**) exhibited significant inhibitory activity against HSV-1 (strains KOS and E-377), HSV-2, and VZV replication. The most potent compound of this series 5-(1-cyanamido-2-iodoethyl)-2′-deoxyuridine (**6**) inhibited HSV-1 strains (EC_{50} = 1.6−9.4 μM range) at concentrations which compared favorably with that of reference drug acyclovir (EC_{50} = 2.2−4.4 μM range) and exhibited superior activity to that of 5-ethyl-2′-deoxyuridine (**1e**) (EC_{50} = 19.5−42.9 μM range). The 5-(1-cyanamido-2-bromoethyl)-2′-deoxyuridine (**5**) also proved to be a potent inhibitor of HSV-1 replication, it is 3−10-fold (depending on the strain and cell line) less active than acyclovir, and approximately equiactive to EDU against HSV-1, whereas the corresponding chloro (**4**) analogue was much less inhibitory, i.e., 17−48-fold less inhibitory than acyclovir and 2−5 times less active than EDU. The 5-(1-cyanamido-2-bromoethyl)- (**5**) and 5-(1-cyanamido-2-iodoethyl)- (**6**) derivatives were found to exhibit 16- and 6-fold superior anti-HSV-1 activity, respectively, relative to their C-1 azido counterparts (**1n**,**o**),[7] when compared with acyclovir as a reference. In contrast, the anti-HSV-1 activity of 5-(1-cyanamido-2-chloroethyl)- (**4**) was significantly reduced relative to the 5-(1-azido-2-chloroethyl)-2′-deoxyuridine (**1m**).[7] The compounds **5** and **6** also exhibited superior anti-HSV-1 activity relative to their corresponding C-1 methoxy (**1k**,**l**) and C-1 hydroxy derivatives (**1i**, X = Br, I), compounds **5**,**6** being 1−10-fold less active than acyclovir whereas **1k**,**l** were 10−100 fold less active and **1i** (X = Br, I) was 1000-times less active than acyclovir.[5,6] In the 5-(1-cyanamido-2-haloethyl)- (**4**−**6**) series of compounds, the relative anti-HSV-1 activity order parallels that of the 5-(1-methoxy-2-haloethyl)- (**1j**−**l**) series, (I > Br > Cl),[5,6] and differs from that of 5-(1-azido-2-haloethyl)-2′-deoxyuridines (**1m**−**o**) (Cl > I > Br).[7] These results suggest that the C-1 substituent, in conjunction with the halogen atom at the C-2 of the 5-side chain, collectively determine the anti-HSV-1 activity.

Acyclovir is a widely used drug for the treatment of opportunistic herpes virus infections in immunocompromised patients. However, resistance to acyclovir is increasing, due to altered or deficient thymidine kinase enzymes in the resistant viruses.[21] Herpes virus thymidine kinase (HSV-TK) leads to initial selective phosphorylation of acyclovir resulting in monophosphate derivative of the drug. However, the herpes virus

Table 1. In Vitro Anti-Herpes Activity of 5-Substituted 2′-Deoxyuridines and Arabinouridines



			EC_{50} (μM)[a]					
no.	R	R_1	HSV-1[b] KOSSB (TK^-)	HSV-1[b] KOS (TK^+)	HSV-1 E-377 (TK^+)	HSV-2[c] MS	VZV[c] Ellen	HCMV[c] AD-169
4	$CH(NHCN)CH_2Cl$	H	>150	37.5	210[d]	105[d]	31.5[d]	274
5	$CH(NHCN)CH_2Br$	H	>133	6.6	42.5	87.7[d]	20.7	>266
6	$CH(NHCN)CH_2I$	H	2.3−15.3	1.6	9.4[d]	77.8[c]	14.6	>236
7	$CH(NHCN)CH_2Br$	OH	>125	62.5	>256	>256	32.0	>256
8	$CH(NHCN)CH_2I$	OH	>114	22.8	>228	>228	11.4	>228
10	$CH(OH)CH_2N_3$	H	>161	>80.0	>322	>322	>322	>322
11	$CH(OMe)CH_2N_3$	H	>150	>75.0	>300	>300	>300	>300
1e	CH_2CH_3(EDU)[e]	H	>780	19.5	42.9	58.5	32.7	>390
BVDU[f]	CH═CHBr	H	>150	1.5	2.7	ND	0.003	>150
ACV[g]			177.6	2.2	4.4	6.2	2.6	ND
GCV[h]			ND	ND[i]	ND	ND	ND	0.23

[a] Mean of two to three assays. [b] The concentration (μM) required to inhibit plaque formation in monolayers of Vero cells by 50%. [c] The drug concentration (μM) required to reduce the viral cytopathic effect (CPE) in infected human foreskin fibroblast (HFF) cell monolayers to 50% of untreated infected controls. [d] Plaque reduction assay. [e] 5-Ethyl-2′-deoxyuridine. [f] (*E*)-5-(2-Bromovinyl)-2′-deoxyuridine. [g] Acyclovir. [h] Ganciclovir. [i] ND = not determined.

infected cells still contain host encoded TK, and this is the reason that nucleoside analogues, which work as anti-herpes agents independent of HSV-TK, can be identified and potentially used for acyclovir resistant HSV infections. Therefore, we examined the activity of newly designed compounds **4**−**8**, **10**, and **11** in HSV-TK negative strain of HSV-1 (KOSSB). Interestingly, the compound 5-(1-cyanamido-2-iodoethyl)-2′-deoxyuridine (**6**) exhibited marked activity against HSV-TK^- HSV-1 infected cells (EC_{50} = 2.3−15.3 μM), as compared to acyclovir (EC_{50} = 177.6 μM). The exact mechanism of action of the compound **6** is not yet known. However, it can be postulated that compound **6** may be phosphorylated by cellular kinases followed by selective inhibition of viral DNA polymerase.

The anti-HSV-2 activity of compounds **4**−**6** is about 1.5- and ∼15-fold less than that of EDU and acyclovir, respectively, but they exhibited greater anti-HSV-2 activity than the corresponding 5-(1-azido-2-haloethyl) series of analogues (**1m**−**o**), which were 32−146-fold less potent than acyclovir.[7] The halogen atom was not a determinant of anti-HSV-2 activity in compounds **4**−**6** since they were all equiactive against HSV-2, whereas in 5-(1-azido-2-haloethyl)- (**1m**−**o**) series of compounds,[7] the relative anti-HSV-2 activity order was Br ≥ Cl > I.

The 5-(1-cyanamido-2-haloethyl)-2′-deoxyuridines (**4**−**6**), like 5-(1-azido-2-haloethyl)-2′-deoxyuridines (**1m**−**o**), were inactive against HCMV and exhibited appreciable activity against VZV (EC_{50} = 14.6−31.5 μM range). The anti-VZV activity of compounds **4**−**6** was similar or higher than that of EDU (EC_{50} = 32.7 μM), approximately 5−12-fold lower than that of acyclovir (EC_{50} = 2.6 μM), and approaching that of 5-(1-azido-2-haloethyl) derivatives (**1m**−**o**) (EC_{50} = 4.5−12.3 μM range).[7] The nature of C-1 or C-2 substituents in the compounds **4**−**6** and **1m**−**o** did not alter their relative anti-VZV activity, since compounds **4**−**6** and **1m**−**o**[7] were almost equiactive.

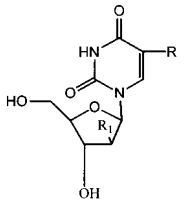
Thus the above observations indicate that a C-1 cyanamido functional group in the ethyl side chain at the 5-position of the pyrimidine ring not only influences the antiviral potency but also modulates the antiviral spectrum.

The 5-(1-cyanamido-2-haloethyl)- (**7**, **8**) analogues of arabinouridine were inactive for HSV-1 (strain E-377), HSV-2, and HCMV. However, they were moderately inhibitory for VZV (EC_{50} = 11.4−32.0 μM range) and HSV-1 (strain KOS) (EC_{50} = 22.8−62.5 μM range). Thus the anti-HSV-1 activity of these compounds is dependent on the virus strain used in the assays.

Intriguingly, 5-(1-hydroxy-2-azidoethyl)- (**10**) and 5-(1-methoxy-2-azidoethyl)- (**11**) 2′-deoxyuridines, in contrast to 5-(1-hydroxy (or methoxy)-2-haloethyl)-2′-deoxyuridines (**1i**−**l**),[5,6] did not show increased antiviral activity but were found to be inactive. This indicates that an azido group is not preferred to a halogen atom at the C-2 position of the 5-substituent. The observed lack of activity of **10**, **11** against TK^+ herpes viruses and TK^- herpes virus may be attributed to their insufficient recognition by cellular/herpes thymidine kinases and/or poor affinity of their 5′-triphosphate derivatives for viral DNA polymerases.

The anti-HBV activity for new compounds **4**−**8**, as well as **1j**−**o**,[5−7] along with the reference antiviral compound (−)-β-L-2′,3′-dideoxy-3′-thiacytidine (3-TC), was also assessed in confluent cultures of primary duck hepatocytes obtained from chronically infected Pekin ducks. These cells chronically produce DHBV DNA, therefore antiviral activity was determined by analysis of viral DNA by dot blot hybridization (Table 2). In the new class of 5-(1-cyanamido-2-haloethyl)-2′-deoxyuridines, compounds **5** and **6** exhibited considerable inhibition of DHBV replication (EC_{50} = 19.9−23.6 μM) in vitro, in contrast to their 2′-arabinouridine analogues (**7** and **8**), which were not active at 25 μM. These results are consistent with the previous observations that changing

Table 2. In Vitro Anti-Hepatitis B Virus Activity of 5-Substituted 2′-Deoxyuridines and Arabinouridines



no.	R	R_1	EC_{50} (μM) DHBV[a]
1j[b]	$CH(OMe)CH_2Cl$	H	>30.0
1k	$CH(OMe)CH_2Br$	H	>30.0
1l	$CH(OMe)CH_2I$	H	>25.0
1m	$CH(N_3)CH_2Cl$	H	15.0
1n	$CH(N_3)CH_2Br$	H	2.6−6.6
1o	$CH(N_3)CH_2I$	H	11.8
4	$CH(NHCN)CH_2Cl$	H	>30.0
5	$CH(NHCN)CH_2Br$	H	19.9
6	$CH(NHCN)CH_2I$	H	23.6
7	$CH(NHCN)CH_2Br$	OH	>25.0
8	$CH(NHCN)CH_2I$	OH	>25.0
10	$CH(OH)CH_2N_3$	H	ND[d]
11	$CH(OMe)CH_2N_3$	H	ND
1e	CH_2CH_3(EDU)[c]	H	29.2
3-TC[e]			0.04

[a] The drug concentration (μM) required to reduce the viral DNA in HBV infected primary duck hepatocytes to 50% of untreated infected controls. [b] Compounds **1j**−**o** are reported in refs 5, 6, and 8. [c] 5-Ethyl-2′-deoxyuridine. [d] ND = not determined. [e] (−)-β-L-2′,3′-Dideoxy-3′-thiacytidine.

the 2′-deoxyribose in 5′-tri-phosphate derivative of 5-propenyl-2′-deoxyuridine to an arabinose moiety resulted in reduced inhibitory activity against DHBV DNA polymerase.[22] The corresponding 5-(1-methoxy-2-haloethyl)-2′-deoxyuridine derivatives (**1j**−**l**) exhibited less than 50% inhibitory activity against DHBV replication at 25−30 μM. Surprisingly, the 5-(1-azido-2-haloethyl)- (**1m**−**o**) series of compounds demonstrated a significant anti-HBV activity with 50% effective concentration (EC_{50}) of 2.6−15.0 μM, the most potent being 5-(1-azido-2-bromoethyl) derivative (**1n**) with an EC_{50} of 2.6−6.6 μM. The marked anti-HBV activity of 5-(1-azido-2-bromoethyl)-2′-deoxyuridine (**1n**) is in contrast to other 5-substituted-2′-deoxyuridines. For example, BVDU (**1b**) is a potent anti-herpes agent but did not show anti-HBV activity (EC_{50} = >150 μM) as measured in the DHBV infected primary duck hepatocyte cultures.[23] Thus, it was noted that the 5-(1-azido-2-haloethyl) moiety at the 5-position of the pyrimidine nucleosides contributes significantly to the potent anti-HBV activity. Although the exact mechanism of action of these compounds is not known, it is possible that they may be phosphorylated by host cell kinases to their triphosphate derivatives, which then inhibit DHBV DNA polymerase like other nucleoside analogues. The compounds **4**−**8** and **1j**−**o** did not have any visual effect on the morphology and viability of uninfected duck hepatocytes up to a concentration of 150 μM.

The cytotoxic activities of compounds **4**−**8** were also determined by the National Cancer Institute (NCI) using an in vitro assay[24] in which compounds were evaluated against approximately 60 human tumor cell lines (e.g., melanoma, leukemia, nonsmall cell lung, small cell lung, colon, central nervous system, ovarian, prostate, and renal cancers). None of the compounds showed any activity or selectivity in these assays up to the highest concentration tested (100 μM) (data not shown), indicating that novel compounds **4**−**8** do not have cytotoxicity against various human cell lines nor any anti-cancer activity.

The compounds **4**−**8**, **10**, and **11** were tested in vitro for their toxicity against several other cell lines. None of these compounds displayed significant in vitro toxicity against stationary phase cells [Vero cells (CC_{50} >228 to >600 μM), HFF cells (CC_{50} >228 to >322 μM)] and proliferating [HFF (IC_{50} = >118 to >322 μM)] cell lines up to the highest concentration tested. In rapidly proliferating fresh human T lymphocyte cell culture, cellular DNA synthesis, as monitored by the incorporation of [methyl-^{3}H]-thymidine into DNA, was also not affected by compounds **4**−**8**, **10**, and **11** up to concentrations of >114 to >150 μM.

The observation that compounds **4**−**6** exhibited significant anti-HSV-1, HSV-2, and VZV activity and no toxicity to several host cells suggests that they may be selectively phosphorylated in virus infected cells by HSV-TK or VZV-TK, respectively, and inhibit viral DNA polymerases. Alternatively, compounds **4**−**6** may be phosphorylated by cellular kinases in both virus infected and uninfected cells, but selectively inhibit viral DNA polymerase, since they show activity against TK-deficient strain of HSV-1 (**6**) as well as DHBV (**5**, **6**).

Summary

The hitherto unknown 5-(1-cyanamido-2-haloethyl) analogues of 2′-deoxyuridine (**4**−**6**) represent novel inhibitors of HSV-1, HSV-2, VZV, and DHBV in cell culture. 5-(1-Cyanamido-2-iodoethyl)-2′-deoxyuridine (**6**) was found to be higher or equally active for HSV-1, 1.3- and 12.5-fold less active for HSV-2, 2-fold more to 5-fold less active for VZV, compared to the reference drugs EDU and acyclovir, respectively. It is noteworthy that compound **6** was also active against thymidine kinase negative strain of HSV-1 and therefore could serve as a useful lead compound for the development of improved anti-herpes drugs. It was observed that replacement of the C-1 azido moiety in **1m**−**o** with a cyanamido group (**4**−**6**) at the 5-position of the pyrimidine ring led to compounds with an altered antiviral spectrum, specifically increased anti-HSV-1 and HSV-2 activities. In contrast, it is encouraging that 5-(1-azido-2-haloethyl)-2′-deoxyuridine analogues (**1m**−**o**) emerged as a new series of potent anti-HBV agents that exhibited superior anti-HBV activity compared to those of 5-(1-cyanamido-2-haloethyl) analogues (**4**−**6**). Our observations reported here support the design and investigation of novel bioisosteric molecules. Further structure−activity relationship studies are ongoing on these new series of compounds.

Experimental Section

Melting points were determined with a Buchi capillary apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were determined for solutions in Me_2SO-d_6, MeOH-d_4, or D_2O on a Bruker AM 300 spectrometer using Me_4Si as an internal standard (^{1}H NMR). The assignment of all exchangeable protons (OH, NH) was confirmed by the addition of the D_2O. ^{13}C NMR spectra were acquired using the *J* modulated spin−echo technique where methyl and methine carbon resonances appear as positive peaks, and methylene and quaternary carbons appear as negative peaks. Infrared spectra were recorded using a Nicolet Magna 750 FT spectrometer. Micro-

analyses were within ±0.4% of theoretical values for all elements listed, unless otherwise indicated. Preparative thin-layer chromatography (PTLC) was performed using Whatman PLK 5F plates, 1.0 mm in thickness, and silica gel column chromatography was carried out using Merck 7734 silica gel (100−200 μM particle size). 5-Vinyl-2′-deoxyuridine (**2**) and 5-vinylarabinouridine (**3**)[6,25] were prepared by using the published procedures.

5-(1-Cyanamido-2-chloroethyl)-2′-deoxyuridine (4), (*E*)-5-(2-Chlorovinyl)-2′-deoxyuridine (1c), and 5-(1-Methoxy-2-chloroethyl)-2′-deoxyuridine (1j). *N*-Chlorosuccinimide (79.8 mg, 0.6 mmol) was added slowly to a pre-cooled (0−5 °C) solution of 5-vinyl-2′-deoxyuridine (101 mg, 0.4 mmol) and cyanamide (67.2 mg, 1.6 mmol) in acetonitrile (25 mL). The resulting reaction mixture was stirred for 4 h at 0−5 °C and then stirred for 20 h at room temperature. Removal of the solvent in vacuo gave a residue that was purified by silica gel column chromatography using chloroform−methanol (92:8, v/v) as eluent. The first fraction eluted (20 mg) was found to be a mixture of (*E*)-5-(2-chlorovinyl)-2′-deoxyuridine (**1c**) and 5-(1-methoxy-2-chloroethyl)-2′-deoxyuridine (**1j**) in a ratio of 1:2, based on the ^{1}H NMR spectral data. The compounds **1c** and **1j** were identical (^{1}H NMR) to authentic samples.[5,26]

Further elution using chloroform−methanol (90:10, v/v) solvent yielded 5-(1-cyanamido-2-chloroethyl)-2′-deoxyuridine (**4**) as a viscous syrup (25 mg, 19%); ^{1}H NMR (Me_2SO-d_6) (mixture of two diastereomers in a ratio of 1:1) δ 2.14 (m, 2H, H-2′), 3.60 (m, 2H, H-5′), 3.80 (m, 1H, H-4′), 3.90 (m, 2H, CH_2-Cl), 4.28 (m, 2H, H-3′, C*H*NHCN), 5.0−5.40 (br, 2H, 3′-OH, 5′-OH), 6.14 and 6.18 (two overlapping t, J = 6.0 Hz, 1H total H-1′), 7.50 (m, 1H, N*H*CN; exchanges with deuterium oxide), 7.98 and 8.0 (2s, 1H total, H-6), 11.60 (s, 1H, NH, exchanges with deuterium oxide); ^{13}C NMR (MeOH-d_4) 41.73 (C-2′), 45.48 (*C*H$_2$Cl), 55.99 and 56.29 (*C*HNHCN), 62.71 (C-5′), 72.08 and 72.30 (C-3′), 86.90 (C-1′), 89.16 (C-4′), 111.20 (C-5), 116.73 (CN), 140.85 and 140.92 (C-6), 151.69 (C-2, *C*═O), 164.19 (C-4, *C*═O). Anal. ($C_{12}H_{15}N_4O_5Cl$) C, H, N.

5-(1-Cyanamido-2-bromoethyl)-2′-deoxyuridine (5). *N*-Bromosuccinimide (35.6 mg, 0.2 mmol) was added slowly to a pre-cooled (0 °C) solution of 5-vinyl-2′-deoxyuridine (50.8 mg, 0.2 mmol) and cyanamide (33.6 mg, 0.8 mmol) in dimethoxyethane (20 mL). The initial yellow color produced upon addition of each NBS aliquot quickly disappeared when all the NBS had reacted. The reaction mixture was stirred (2 h) at 0 °C, and the solvent was removed in vacuo. The resulting residue was purified by silica gel column chromatography using chloroform−methanol (90:10, v/v) as eluent to afford 5-(1-cyanamido-2-bromoethyl)-2′-deoxyuridine (**5**) as a syrup (32 mg, 42.5%): ^{1}H NMR (Me_2SO-d_6) (mixture of two diastereomers in a ratio of 1:1) δ 2.14 (m, 2H, H-2′), 3.58 (m, 2H, H-5′), 3.70 (m, 2H, CH_2Br), 3.82 (m, 1H, H-4′), 4.25 (m, 1H, H-3′), 4.30 (m, 1H, C*H*NHCN), 5.06 and 5.28 (2 br s, 1H each, 3′-OH, 5′-OH), 6.16 and 6.18 (two overlapping t, J = 6.0 Hz, 1H total H-1′), 7.50 (m, 1H, N*H*CN; exchanges with deuterium oxide), 7.98 and 8.0 (2s, 1H total, H-6), 11.60 (s, 1H, NH, exchanges with deuterium oxide). Anal. ($C_{12}H_{15}N_4O_5Br$) C, H, N.

5-(1-Cyanamido-2-iodoethyl)-2′-deoxyuridine (6). *N*-Iodosuccinimide (116 mg, 0.51 mmol) was added slowly to a pre-cooled (0 °C) solution of 5-vinyl-2′-deoxyuridine (120 mg, 0.47 mmol) and cyanamide (80 mg, 1.9 mmol) in dimethoxyethane (20 mL). The reaction mixture was stirred for 1 h at 0 °C. The reaction was completed, and the product was purified as described for **2** to yield 5-(1-cyanamido-2-iodoethyl)-2′-deoxyuridine (**6**) as a syrup (45 mg, 22.5%): ^{1}H NMR (D_2O) (mixture of two diastereomers in a ratio of 1:1) δ 2.38 (m, 2H, H-2′), 3.60−3.82 (complex m, 4H, H-5′, CH_2I), 4.08 (m, 1H, H-4′), 4.50 (m, 2H, H-3′, C*H*NHCN), 6.30 and 6.32 (two overlapping t, J = 6.0 Hz, 1H total H-1′), 8.06 and 8.08 (2s, 1H total, H-6). Anal. ($C_{12}H_{15}N_4O_5I$) C, H, N.

5-(1-Cyanamido-2-bromoethyl)-arabinouridine (7). *N*-Bromosuccinimide (89 mg, 0.5 mmol) was added slowly with stirring to a pre-cooled (0 °C) solution of 5-vinyl-arabinouridine (135 mg, 0.5 mmol) and cyanamide (84 mg, 2.0 mmol) in dimethoxyethane (50 mL) during a period of 5 min. The reaction was allowed to proceed for 2 h at 0 °C with stirring, and the solvent was removed in vacuo. The product was purified by PTLC using chloroform−methanol (90:10, v/v) as the development solvent to yield 5-(1-cyanamido-2-bromoethyl)-arabinouridine (**7**) as a syrup (36 mg, 18%): ^{1}H NMR (MeOH-d_4) (mixture of two diastereomers in a ratio of 1:1) δ 3.55 (m, 2H, CH_2Br), 3.80 (m, 2H, H-5′), 3.92 (m, 1H, H-4′), 4.08 (m, 1H, H-3′), 4.15 (m, 1H, H-2′), 4.70 (m, 1H, C*H*NHCN), 6.14 (d, $J_{1',2'}$ = 3.0 Hz, 1H, H-1′), 7.90 and 7.92 (2s, 1H total, H-6). Anal. ($C_{12}H_{15}N_4O_6Br$) C, H, N.

5-(1-Cyanamido-2-iodoethyl)-arabinouridine (8). *N*-Iodosuccinimide (119 mg, 0.53 mmol) was added slowly with stirring to a pre-cooled (0 °C) solution of 5-vinyl-arabinouridine (143 mg, 0.53 mmol) and cyanamide (88.2 mg, 2.1 mmol) in dimethoxyethane (100 mL), and the reaction was allowed to proceed for 2 h at 0 °C. Removal of the solvent in vacuo gave a residue, which was purified by PTLC using chloroform−methanol (90:10, v/v) as the development solvent. Extraction of the ultraviolet visible spot with chloroform:methanol (88:12, v/v) yielded 5-(1-cyanamido-2-iodoethyl)-arabinouridine (**8**) as a syrup (45 mg, 19.2%): ^{1}H NMR (D_2O) (mixture of two diastereomers in a ratio of 1:1) δ 3.50 (m, 2H, CH_2I), 3.80 (m, 2H, H-5′), 3.95 (m, 1H, H-4′), 4.06 (m, 1H, H-3′), 4.20 (m, 1H, H-2′), 4.42 (m, 1H, C*H*NHCN), 6.20 and 6.22 (two overlapping d, $J_{1',2'}$ = 3.0 Hz, 1H total, H-1′), 7.88 and 7.90 (two s, 1H total, H-6). Anal. ($C_{12}H_{15}N_4O_6I$) C, H, N.

5-(1-Hydroxy-2-azidoethyl)-2′-deoxyuridine (10). Method A. Ceric ammonium nitrate (550 mg, 1.0 mmol) was added slowly to a pre-cooled suspension (−5 °C) prepared by mixing a solution of 5-vinyl-2′-deoxyuridine (130 mg, 0.51 mmol) and sodium azide (39 mg, 0.6 mmol) in acetonitrile (80 mL) and water (0.5 mL). The reaction mixture was stirred for 2 h at −5 °C, and the solvent was removed in vacuo. Purification of the product by elution from a silica gel column using chloroform−methanol (90:10, v/v) as the eluent afforded 5-(1-hydroxy-2-azidoethyl)-2′-deoxyuridine (**10**) as a syrup (50 mg, 31.5%): ^{1}H NMR (Me_2SO-d_6) (mixture of two diastereomers in a ratio of 1:1) δ 2.08 (m, 2H, H-2′), 3.30 (m, 2H, CH_2N_3), 3.54 (m, 2H, H-5′), 3.78 (m, 1H, H-4′), 4.22 (m, 1H, H-3′), 4.60 (m, 1H, C*H*OH), 5.0 and 5.28 (two s, 1H each, 3′-OH, 5′-OH, exchanges with deuterium oxide), 5.72 (s, 1H, CHO*H*, exchanges with deuterium oxide), 6.18 and 6.20 (two overlapping t, J = 6.0 Hz, 1H total H-1′), 7.80 and 7.82 (two s, 1H total, H-6), 11.45 (br, 1H, NH, exchanges with deuterium oxide); ^{13}C NMR (Me_2SO-d_6) δ 39.78 and 40.06 (C-2′), 55.01 (*C*H$_2$N$_3$), 61.62 and 61.90 (C-5′), 66.14 (*C*HOH), 70.74 and 70.80 (C-3′), 84.59 and 84.64 (C-1′), 87.51 and 87.56 (C-4′), 114.32 (C-5), 137.67 (C-6), 150.40 (C-2, *C*═O), 164.5 (C-4, *C*═O); IR 2118 (N_3) cm^{-1}. Anal. ($C_{11}H_{15}N_5O_6$) C, H, N.

5-(1-Hydroxy-2-azidoethyl)-2′-deoxyuridine (10). Method B. Ceric ammonium nitrate (876 mg, 1.6 mmol) was added slowly to a cooled (ca. −15 °C) flask containing a suspension of 5-vinyl-2′-deoxyuridine (203 mg, 0.8 mmol) and sodium azide (57 mg, 0.88 mmol) in dry acetonitrile (200 mL) with stirring. The reaction was allowed to proceed stirred for 1 h at −15 °C. The reaction mixture was evaporated in vacuo, and the residue obtained was redissolved in aqueous acetonitrile (50 mL). Purification of the product by elution from a silica gel column using chloroform−methanol (90:10, v/v) as eluent yielded 5-(1-hydroxy-2-azidoethyl)-2′-deoxyuridine (**10**) as a syrup (80 mg, 32%). The ^{1}H NMR, ^{13}C NMR, and FT-IR spectra for **10** were identical to the spectral data described in the method A.

5-(1-Methoxy-2-azidoethyl)-2′-deoxyuridine (11). Ceric ammonium nitrate (219 mg, 0.4 mmol) was added slowly to a cooled (ca. −15 °C) flask containing a suspension of 5-vinyl-2′-deoxyuridine (50.4 mg, 0.2 mmol) and sodium azide (13 mg, 0.2 mmol) in dry acetonitrile (50 mL) with stirring. The reaction mixture was stirred for 30 min at −15 °C. The reaction mixture was evaporated in vacuo, and the residue obtained was dissolved in methanol (1 mL) and purified on a PTLC using chloroform−methanol (90:10, v/v) as the development solvent. Extraction of the ultraviolet visible spot with chloroform:methanol (85:15, v/v) yielded 5-(1-methoxy-2-azidoethyl)-

2′-deoxyuridine (**11**) as a viscous syrup (16 mg, 25%): ^{1}H NMR (D_2O) (mixture of two diastereomers in a ratio of 1:1) δ 2.42 (m, 2H, H-2′), 3.34 and 3.36 (two s, 3H total, OCH_3), 3.56 (m, 2H, CH_2N_3), 3.80 (m, 2H, H-5′), 4.08 (m, 1H, H-4′), 4.48 (m, 2H, H-3′, CHOCH_3), 6.30 and 6.32 (two overlapping t, J = 6.0 Hz, 1H total H-1′), 7.90 and 7.92 (two s, 1H total, H-6); ^{13}C NMR (D_2O) δ 39.89 and 40.01 (C-2′), 53.84 (CH_2N_3), 57.46 and 57.52 (OCH_3), 61.46 and 61.79 (C-5′), 71.13 and 71.20 (C-3′), 76.54 ($CHOCH_3$), 86.48 and 86.55 (C-1′), 87.62 and 87.67 (C-4′), 111.63 (C-5), 140.52 (C-6), 152.12 (C-2, C=O), 165.23 (C-4, C=O); IR 2104 (N_3) cm^{-1}. Anal. ($C_{12}H_{17}N_5O_6$) C, H, N.

In Vitro Antiviral Assays [HSV-1 (E-377), HSV-2, HCMV, VZV]. Plaque reduction and cytopathic effect (CPE) inhibition assays for HSV-1, HSV-2, HCMV, and VZV were performed under the NIH Antiviral Research Branch Testing Program using standard procedures described previously.[7]

In Vitro Antiviral Assays [HSV-1 (KOS, TK$^+$), (KOSSB, TK$^-$)]. African green monkey kidney (Vero) cells were grown in DMEM supplemented with 5% FBS. Cells were seeded into 24-well plates 1 day prior to the assay. Wild-type HSV-1, KOS, and a thymidine kinase deficient mutant, KOSSB,[27] were used to infect at ~100 PFU's per well for 1 h at 37 °C. After infecting, the inoculum was replaced with serial dilutions of media containing diluted drug. All drug dilutions were done in duplicate, initially using the following concentrations: 25, 10, 5, and 1 μg/mL (for TK$^+$ virus) and 50, 25, 10, and 1 μg/mL (for TK$^-$ virus). Once an activity range was determined, compounds were again tested at 1:2 serial dilutions to obtain a more precise EC_{50}. Controls included infected wells that were not treated with drugs, as well as infected wells treated with acyclovir at 5 and 1 μg/mL (for TK$^+$ virus) and 50, 25, 10, and 1 μg/mL (for TK$^-$ virus). Plates were incubated for 48 h at 37 °C. To visualize plaques, wells were fixed by incubation with methanol for 10 min at room temperature. The methanol was aspirated and replaced with 1× Giemsa stain (Sigma) for 1 h at room temperature. Plaques were counted and compared to the number of plaques in the no-drug controls in order to calculate EC_{50}.

In Vitro Antiviral Assay (Duck Hepatitis B Virus, DHBV). Pekin duck eggs were obtained from a duck colony maintained at the University of Alberta farm and were stored in a 37 °C egg incubator until hatching occurred. Newly hatched ducklings were infected with a 50 μL intravenous injection of duck serum containing DHBV. Persistently infected ducks were identified by detection of DHBV DNA in sera by Dot hybridization.[28]

Primary cultures of duck hepatocytes were prepared from 9 to 14 day old DHBV-infected ducklings using a modified method.[29] Cells were cultured in 60 mm cell culture dishes in 4 mL of L-15 medium containing 5% fetal bovine serum, penicillin G Sodium (10 IU/mL), streptomycin sulfate (10 μg/mL), and nystatin (25 U/mL). The test compounds were always added in triplicate to the hepatocyte cultures on day 2 and were maintained in culture with media changed every second day until day 12. Cells were harvested at day 14. Initially, the test compounds were screened at a 10 μg/mL final concentration. Inhibition in DHBV replication at 10 μg/mL was calculated as the average of triplicate wells. Standard deviations were within 10% of the average values. After initial testing, test compounds were serially diluted to determine more precise anti-DHBV EC_{50} values. The hepatocytes were lysed with 1.0 mL of lysis buffer containing 10 mM Tris-HCl and 1% SDS. The lysate was digested with 0.5 mg/mL proteinase K and extracted with an equal volume of phenol saturated with Tris-HCl EDTA and 0.1% 8-hydroxyquinoline, followed by extraction with chloroform. Concentrated NaCl (5M) was added to the aqueous phase to yield a final concentration of 0.5 M NaCl, and the DNA was precipitated with two volumes of 95% ethanol. The DNA pellet was washed with 70% ethanol and dried. The dried DNA was dissolved in 100 μL of a solution containing Tris-HCl EDTA.

DNA samples were applied to a nylon filter (Hybond-N, Amersham) using a Bio-Dot (Bio-Rad Laboratories) microfiltration apparatus. DNA on the filter was denatured with NaOH/NaCl at room temperature for 30 min and neutralized in Tris-HCl/NaCl. The filters were exposed to ultraviolet irradiation for 3 min. DNA hybridization was initiated by adding a recently prepared DHBV (^{32}P) DNA probe at 10^6 CPM/mL and incubating overnight. Filters were washed twice in 1xSSC (20xSSC in 3 M NaCl plus 0.3 M sodium citrate, pH 7.0)−0.1% SDS at 65 °C for 2 h and 1xSSC at room temperature for 30 min. After an autoradiographic image was obtained, the filters were exposed in a phosphoimaging screen for 1−2 h and samples were quantitated by a Fujix BAS1000 and the percentage density of phosphoimaging units were calculated.[28] 3-TC was used as the reference compound. Tests were repeated 2−3 times, and the data for each test compound were compared with a positive and negative control performed at the same time under identical conditions. For the compounds where the EC_{50} obtained from three experiments was within 10% standard deviation, average values are shown, otherwise a range of EC_{50} values are shown. Percent inhibition was calculated by using the following formula: (untreated positive control − treated test sample) × 100/untreated positive control.

Cell Cytotoxicity: Neutral Red Uptake Assay. Cytotoxicity of test compounds on human foreskin fibroblast (HFF) cells was determined using neutral red uptake assay as reported previously.[7]

MTT Assay. Cell viability was measured using the cell proliferation kit 1 (MTT; Boehringer Mannheim) as per manufacturer's instructions. Briefly, a 96-well plate was seeded with Vero cells at a density of 2.5×10^4 cells per well. Cells were allowed to attach for 6−8 h, the media was replaced with media containing drugs at concentrations of 200, 100, 50, 25, 12.5, 6.3, and 1.5 μg/mL. DMSO was also included as control. Plates were incubated for 3 days at 37 °C. The color reaction involved adding 10 μL MTT reagent per well, incubating 4 h at 37 °C, and then adding 100 μL of solubilization reagent. Plates were read on an ELISA plate reader (Abs 560−650 nm) following an overnight incubation at 37 °C.

Cell Proliferation Assay: HFF Cells. The effect of test compounds on proliferation of HFF cells was determined according to previously reported procedure.[7]

Human T Cells. Enriched T cell populations were purified from buffy coats obtained from normal Canadian Blood Service donors, by using nylon wool columns according to published procedures.[30] Test compounds dissolved in DMSO at 10 mg/mL were prepared as stock solutions. In 96-well flat bottom plates, test compounds were plated in triplicate wells starting from 50 μg/mL final concentrations. Serial dilutions of compounds were prepared at 1:2, 1:5, or 1:10 dilution for up to eight dilutions. All of the dilutions were made with serum free AIM V media for lymphocytes (Life Technologies, ON). Control wells with the same DMSO concentration as those with test compounds were also prepared in triplicate wells. A total of 2×10^5 T cells in AIM V media were added to each of these prepared wells. This was followed by the addition of phytohemagglutinin (PHA, Sigma Chemical Company) to make up a final concentration of 1 μg/mL of PHA. Positive control wells with PHA and T cells, and negative control wells with T cells in media alone, were also prepared on each plate. The cultures were incubated for 3−4 days in 5% CO_2 and 95% humidity at 37 °C. After 3−4 days, [^{3}H] thymidine (1 μCi/well) was added to each well. Eighteen hours after pulsing with radioactive thymidine, the cells were harvested onto nylon filters, and [^{3}H] thymidine incorporation into the DNA of proliferating cells was measured by liquid scintillation counting. CPM of wells containing test compounds were compared with CPM of the wells containing stimulated T cells and corresponding DMSO concentration.

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