Synthesis and Antiviral (HIV-1, HBV) Activities of 5-Halo-6-methoxy(or azido)-5,6-dihydro-3′-fluoro-3′-deoxythymidine Diastereomers. Potential Prodrugs to 3′-Fluoro-3′-deoxythymidine

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A new class of 5-halo-6-methoxy(or azido)-5,6-dihydro-3′-fluoro-3′-deoxythymidines (**4**–**13**) were investigated as potential anti-AIDS drugs. These 5,6-dihydro derivatives, which are also potential prodrugs to 3′-fluoro-3′-deoxythymidine (FLT), were designed to have properties which would enhance their duration of action, lipophilicity, and cephalic delivery to the central nervous system. The 5-halo-6-methoxy(or azido)-5,6-dihydro-3′-fluoro-3′-deoxythymidines, which differ in configuration at the C-5 and C-6 positions, were synthesized by the regiospecific addition of XR (X = Br, Cl, I; R = OMe, N_3) to the 5,6-olefinic bond of FLT. These 5-halo-6-methoxy-5,6-dihydro derivatives are more lipophilic (P = 1.5–5.15 range) than the parent compound FLT (P = 0.5). Regeneration of the 5,6-olefinic bond to give FLT, upon incubation of the 5-halo-6-methoxy-5,6-dihydro compounds with glutathione, was dependent on the nature of the 5-halo substituent (I > Br > Cl). The ability of these 5-halo-6-methoxy(or azido)-5,6-dihydro compounds (**4**–**13**) to protect CEM cells against HIV-induced cytopathogenicity was evaluated. The C-5 halo substituent was a determinant of anti-HIV-1 activity where the approximately equipotent 5-iodo and 5-bromo were generally more potent than the 5-chloro derivatives of FLT. Compounds having the (5*S*,6*S*)-configuration were more potent than the corresponding (5*R*,6*R*)-diastereomer. The most potent anti-HIV-1 agents, which included the (5*R*,6*R*)-5-Br,6-OMe (**4**), (5*S*,6*S*)-5-Br,6-OMe (**5**), and (5*S*,6*S*)-5-I,6-OMe (**10**) derivatives of FLT, exhibited comparable activities to the reference drugs AZT and FLT. Although (5*R*,6*R*)-5-bromo-6-methoxy-5,6-dihydro-3′-fluoro-3′-deoxythymidine (**4**) inhibited hepatitis B virus replication at a 5–6-fold higher concentration (EC_{50}) than the reference drug 2′,3′-dideoxycytidine (DDC), it was 3–5-fold less cytotoxic (CC_{50}) than DDC.

There are currently four antiretroviral drugs approved for use in HIV disease, *viz* 3′-azido-3′-deoxythymidine (AZT, zidovudine, Retrovir), 2′,3′-dideoxyinosine (ddI, didanosine, Videx), 2′,3′-dideoxycytidine (DDC, zalcitabine, HVID), and 2′,3′-didehydro-3′-deoxythymidine (d4T, stavudine, Zerit). The failure of existing drugs to stop progression of acquired immunodeficiency syndrome (AIDS), which leads to AIDS dementia and other neurological manifestations of HIV infection, is due in part to their inability to maintain adequate drug levels at the site of replication over extended periods due to their relatively short half-lives.[1–4] Other factors which contribute to failure include the development of drug resistance and unacceptable clinical toxicities. The design of anti-HIV drugs that provide a therapeutic cephalic concentration has presented a significant challenge to medicinal chemists[5–7] since the brain is a sanctuary for HIV. Thymidine analogs possessing a 3′-azido, 3′-fluoro, or 2′,3′-unsaturated moiety are particularly potent and selective anti-HIV agents.[7–13] Although 3′-fluoro-3′-deoxythymidine (**3**, FLT) bears a close structural relationship to 3′-azido-3′-deoxythymidine (**1**, AZT), the 3′-substituent may be a determinant of clinical toxicity. For example, AZT induces a dose related bone marrow toxicity that is manifested as severe anemia and leukopenia.[14] It was subsequently reported that 3′-amino-3′-deoxythymidine (AMT) is a highly toxic catabolite to AZT.[15] FLT 5′-triphosphate is also a potent inhibitor of endogenous hepatitis B virus (HBV) DNA polymerase and the production of HBV in HepG2 cells transfected with HBV DNA *in vitro*.[16] Although, there are distinct differences between HBV and HIV infections, HBV infection has been suggested as a potential cofactor for HIV infection.[17]

AZT is one of the more lipophilic (P = 1.29) compounds investigated clinically, and it readily enters the cerebrospinal fluid (CSF).[18,19] However, the entry of AZT into brain tissue from the CSF is likely not sufficient to provide a therapeutic concentration that suppresses viral replication in the brain. Less than 1% of AZT injected via the carotid artery in rats penetrated into brain.[20] These results suggest that the low lipophilicity of FLT (P = 0.5) may retard its entry into the brain by passive diffusion, resulting in an inadequate therapeutic brain concentration. The further development of FLT was recently discontinued, suggesting that significant antiviral activity was not observed at plasma concentrations that were well tolerated in a controlled phase II study.[1] Therefore, it is of interest to prepare more lipophilic derivatives of FLT for anti-HIV evaluation.

The toxicity of AZT can be reduced by using lower doses,[21] but this protocol is not adequate for children and for cerebral infections[22] since low doses do not provide effective intracellular drug concentrations. In such cases, prodrugs which are more lipophilic than the parent drug such as FLT and that have a long blood half-life possess the ability to cross the blood–brain

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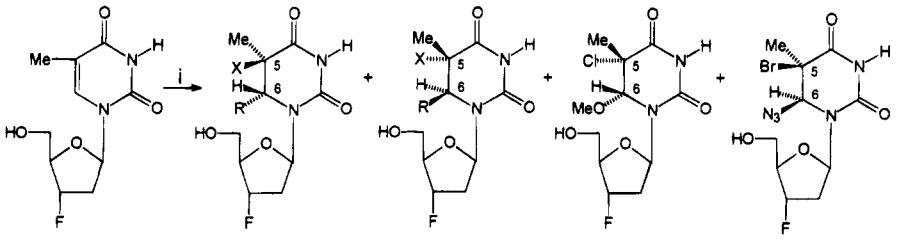
Scheme 1[a]



3, FLT (+)-*trans*-(5R,6R) (-)-*trans*-(5S,6S) (+)-*cis*-(5S,6R) (-)-*cis*-(5R,6S)

4 (X=Br, R=OMe) **5** (X=Br, R=OMe)

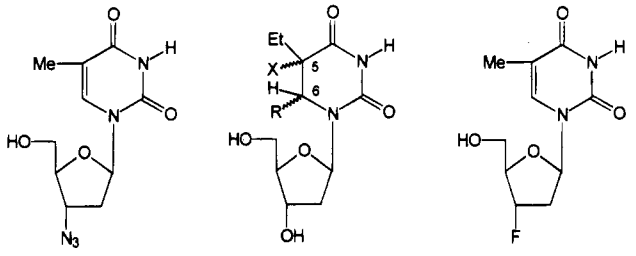
6 (X=Cl, R=OMe) **7** (X=Cl, R=OMe) **8**

9 (X=I, R=OMe) **10** (X=I, R=OMe)

11 (X=Br, R=N_3) **12** (X=Br, R=N_3) **13**

[a] Reagents: (i) Br_2, MeOH, 25 °C (**4**, **5**); *N*-chlorosuccinimide, MeOH, HOAc, 25 °C (**6**–**8**); *N*-iodosuccinimide, MeOH, HOAc, 25 °C (**9**, **10**); *N*-bromosuccinimide, sodium azide, 1,2-dimethoxyethane, 25 °C (**11**–**13**).

barrier (BBB), provide a sustained release of the parent drug, and reduced toxicity to nontarget tissues are needed.



1, AZT **2a**, R = OEt, X = Br **2b**, R = OMe, X = Cl 3, FLT

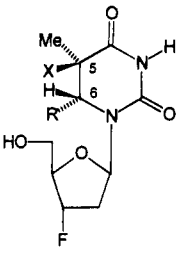
Many investigations have focused on the development of agents which can more readily penetrate into the CNS due to their enhanced ability to cross the BBB. Esterification of the 5'-hydroxyl group is a common approach that has been used to improve the brain uptake and *in vivo* efficacy of anti-HIV nucleoside[23–26] derivatives. However, these modifications have not resulted so far in compounds with a clear-cut therapeutic efficacy.

We recently described some *in vitro* properties for a new class of 5-halo-6-alkoxy-5,6-dihydro derivatives (**2**) that were designed as lipophilic brain-targeted prodrugs to the anti-herpes drug 5-ethyl-2'-deoxyuridine (EDU).[27,28] [4-^{14}C]-(5*R*,6*R*)-5-Bromo-5-ethyl-6-ethoxy-5,6-dihydro-2'-deoxyuridine (**2a**)[28] provided a significantly higher brain concentration of EDU than did its parent compound EDU in mice after iv dosing. 5-Chloro-5-ethyl-6-methoxy-5,6-dihydro-2'-deoxyuridine (**2b**) provided a longer blood residence time compared to EDU.[27] These 5,6-dihydro compounds may act as reservoirs for the slow release of the parent nucleoside *in vivo* which may arise by a spontaneous regeneration of the double bond to give EDU or by consecutive dehalogenation elimination reactions upon reaction with a tissue nucleophile such as glutathione. The advantageous combination of desirable physicochemical properties and favorable pharmacokinetic characteristics of the 5,6-dihydro compounds **2a**–**b** prompted us to investigate 5-halo-6-methoxy(or azido)-5,6-dihydro analogs of FLT as potential anti-HIV drugs. We now report the synthesis, anti-HIV activity, and some biochemical properties of 5-halo-6-methoxy-(or azido)-5,6-dihydro-3'-fluoro-3'-deoxythymidines (**4**–**13**) as potential anti-HIV agents and/or prodrugs to FLT that may penetrate across the BBB more effectively by passive diffusion to provide a therapeutic brain concentration of FLT.

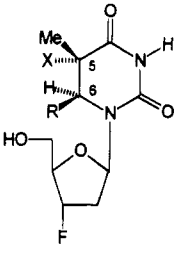
Chemistry

Reaction of FLT with molecular bromine in methanol at 25 °C afforded the (+)-*trans*-(5*R*,6*R*)-**4** and (−)-*trans*-(5*S*,6*S*)-**5** diastereomers of 5-bromo-6-methoxy-5,6-dihydro-3'-fluoro-3'-deoxythymidine in 56 and 28% yields, respectively (see Scheme 1). A similar reaction of FLT with *N*-chlorosuccinimide in methanol in the presence of glacial acetic acid gave the (+)-*trans*-(5*R*,6*R*)-**6**, (−)-*trans*-(5*S*,6*S*)-**7** (11%), and (+)-*cis*-(5*S*,6*R*)-**8** (22%) diastereomers of 5-chloro-6-methoxy-5,6-dihydro-3'-fluoro-3'-deoxythymidine. The reaction of FLT with *N*-iodosuccinimide in methanol in the presence of glacial acetic acid yielded the (+)-*trans*-(5*R*,6*R*)-**9** (37%) and (−)-*trans*-(5*S*,6*S*)-**10** (37%) diastereomers of 5-iodo-6-methoxy-5,6-dihydro-3'-fluoro-3'-deoxythymidine. Reaction of FLT with *N*-bromosuccinimide and sodium azide in 1,2-dimethoxyethane at 25 °C afforded a mixture of the (+)-*trans*-(5*R*,6*R*)-**11**, (−)-*trans*-(5*S*,6*S*)-**12**, and (−)-*cis*-(5*R*,6*S*)-**13** diastereomers of 5-bromo-6-azido-5,6-dihydro-3'-fluoro-3'-deoxythymidine in 55% yield. These 5-halo-6-methoxy(or azido)-5,6-dihydro derivatives **4**–**13** of FLT most likely arise via the initial formation of a 5,6-halonium ion intermediate which is susceptible to regiospecific nucleophilic attack by methanol, or azide anion, at the sterically less hindered C-6 position. The configuration of compounds **4**–**13** at the C-5 and C-6 positions was assigned by comparing the optical rotation and ^{1}H NMR spectral data with that of similar compounds, for which the absolute configuration is known, such as 5-bromo-6-methoxy(or hydroxy)-5,6-dihydrothymidine[29,30] diastereomers. The most distinct differences in ^{1}H NMR chemical shift positions for these diastereomers occur for the H-1', H-2', and H-2'' protons in the sugar moiety and the H-6 proton of the base. The 5,6-dihydro compounds **4**–**13** are stable products that were separated by preparative thin layer chromatography (PTLC) or silica gel column chromatography, with the exception of the 5-bromo-6-azido-5,6-dihydro diastereomers **11**–**13** that could not be separated by either of these chromatographic techniques, which were isolated as a mixture.

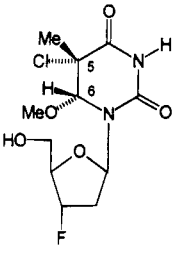
Table 1. *In Vitro* Anti-HIV Activity,[a] Partition Coefficients (*P*), and *in Vitro* Incubation Studies with Glutathione of 5-Halo-6-methoxy(or azido)-5,6-dihydro-3′-fluoro-3′-deoxythymidines



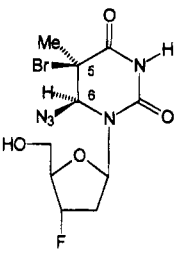
4, 6, 9, 11
(+)-*trans*-(5R,6R)



5, 10, 12
(-)-*trans*-(5S,6S)



8
(+)-*cis*-(5S,6R)



13
(-)-*cis*-(5R,6S)

no.	X	R	configuration	IC_{50} (M)[b]	EC_{50} (M)[c]	SI[d]	*P*[e]	% FLT[f]
4	Br	OMe	5*R*,6*R*	1.72×10^{-6}	5.25×10^{-9}	328	4.71	10
5	Br	OMe	5*S*,6*S*	9.72×10^{-6}	3.25×10^{-9}	2991	3.44	10
6	Cl	OMe	5*R*,6*R*	$>8.0 \times 10^{-4}$	5.55×10^{-6}	>144	5.15	0
8	Cl	OMe	5*S*,6*R*	$>8.0 \times 10^{-4}$	3.79×10^{-5}	>21	1.50	0
9	I	OMe	5*R*,6*R*	5.73×10^{-5}	5.00×10^{-5}	1	2.81	50
10	I	OMe	5*S*,6*S*	1.22×10^{-5}	3.75×10^{-9}	3253	4.0	90
11–13[g]	Br	N_3	5*R*,6*R*; 5*S*,6*S*; 5*R*,6*S*	1.0×10^{-4}	1.45×10^{-8}	7143	ND[h]	95
AZT				5×10^{-4}	3×10^{-9}	166666	1.29	
AZT[i]					4×10^{-9}			
FLT[i]					1×10^{-9}		0.50	

[a] Testing was performed by the National Cancer Institute's Developmental Therapeutics Program, AIDS antiviral screening program. All of the data listed were compared to the corresponding test results for AZT which served as the treated control, performed at the same time. [b] The IC_{50} value is the test drug concentration which results in a 50% survival of uninfected untreated control CEM cells (e.g. cytotoxicity of the test drug). [c] The EC_{50} value is the test drug concentration which produces a 50% survival of HIV-1 infected cells relative to uninfected untreated controls (e.g., *in vitro* anti-HIV-1 activity). [d] SI = selectivity index (IC_{50}/EC_{50}). [e] *P* = concentration in octanol/concentration in water, $n = 2$. [f] The percent of 3′-fluoro-3′-deoxythymidine (FLT) formed upon incubation of the test 5,6-dihydro compound with glutathione using a test compound:glutathione molar ratio of 1:2 at 37 °C for 30 min. [g] Tested as a mixture of diastereomers. [h] ND = not determined. [i] The EC_{50} value, defined as the dose required to effect a 50% reduction in the cytopathic effect of HIV for MT4 cells was taken from ref 44.

Results and Discussion

The objective of this study involved the design of 5-halo-6-methoxy(or azido)-5,6-dihydro-3′-fluoro-3′-deoxythymidines (**4**–**13**) as brain-targeted drugs, and/or prodrugs to FLT, for evaluation as anti-HIV agents. The partition coefficients, which are an indicator of the ability of a compound to cross the BBB[31] for these 5-halo-6-methoxy-5,6-dihydro analogs of FLT were all larger ($P = 1.5–5.15$) than that of FLT ($P = 0.5$) or AZT ($P = 1.29$). In the absence of an active nucleoside transport system, the enhanced lipophilicity of these 5-halo-6-methoxy-5,6-dihydro analogs, relative to FLT, should increase their ability to cross the BBB by a diffusion mechanism. This postulate is based on the observation that increasing the lipophilicity of compounds with a molecular weight of less than 400 has been reported to improve brain permeability.[32] Furthermore, the parabolic relationship between log *P* values and brain extractability for a group of ^{11}C-labeled compounds suggests an optimal log *P* range of 0.9–2.5 for radiopharmaceuticals designed to cross the BBB by virtue of their lipid solubility.[33] The differences between the *P* values for the (+)-*trans*-(5*R*,6*R*)- and (−)-*trans*-(5*S*,6*S*)-diastereomers possessing a C-5 bromo (**4**, **5**) or iodo (**9**, **10**) substituent was smaller than that between the (+)-*trans*-(5*R*,6*R*)-**6** ($P = 5.15$) and (+)-*cis*-(5*S*,6*R*)-**8** ($P = 1.5$) 5-chloro diastereomers.

The utility of 5-halo-6-methoxy(or azido)-5,6-dihydro derivatives of FLT (**4**–**13**) as potential prodrugs to FLT would be dependent upon their pharmacokinetic properties, tissue biodistribution, and rate of bioconversion to FLT. The *in vitro* incubation of the 5,6-dihydro compounds **4**–**13** with the model thiol glutathione (GSH) was therefore investigated (substrate:GSH ratio = 1:2 for a 30 min incubation at 37 °C) to determine the ability of GSH to regenerate the 5,6-olefinic bond present in FLT. In mammalian tissues, the GSH concentration is in the 0.5–1 mM range, whereas the cysteine concentration is in the 0.03–0.1 mM range.[34,38] It is possible that dehalogenation and elimination to generate FLT *in vivo* could occur by a chemical reaction with GSH or cysteine and/or an enzymatic reaction with a thiol-containing enzyme. Regeneration of the 5,6-olefinic bond to afford FLT, upon incubation with GSH, was dependent upon the nature of the C-5 halo substituent in the 5-halo-6-methoxy-5,6-dihydro series **4**–**10** where the relative 5,6-olefinic double bond regeneration order was I > Br > Cl (see Table 1). The nature of the C-6 substituent was also a determinant of 5,6-double bond regeneration since a mixture of the (5*R*,6*R*)-**11**, (5*S*,6*S*)-**12**, and (5*R*,6*S*)-**13** 5-bromo-6-azido-5,6-dihydro-3′-fluoro-3′-deoxythymidine diastereomers afforded FLT (95%), whereas the (5*R*,6*R*)-**4** and (5*S*,6*S*)-**5** 5-bromo-6-methoxy analogs each gave FLT (10%). In contrast, the (5*R*,6*R*)-**6** and (5*S*,6*R*)-**8** diastereomers of 5-chloro-6-methoxy-5,6-dihydro derivatives did not undergo conversion to FLT upon incubation with GSH. The reaction of the 5,6-dihydro analogs of FLT with GSH (R-SH) could occur by two mechanisms (see Figure 1). Nucleophilic attack by R-SH on the C-5 halo substituent (X) would give the carbanion or enolate anion **ii** (E2 Hal mechanism, pathway A). Alternatively, a S_N2 displacement of X by R-SH to give **iii** (S_N2 mechanism, pathway B) and a subsequent reaction with GSH would also yield carbanion **ii**. Elimination of methoxide, or azide, anion from the carbanion intermediate **ii** would regenerate the

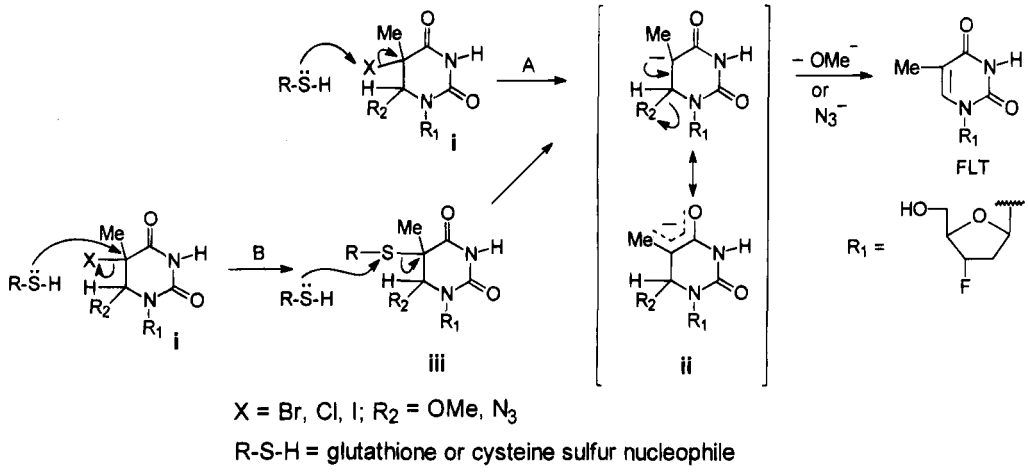


Figure 1. Proposed mechanisms for the conversion of 5-halo-6-methoxy(or azido)-5,6-dihydro-3'-fluoro-3'-deoxythymidines to FLT. Pathway A (E2 Hal mechanism), elmination under the influence of a nucleophile; pathway B ($\bar{S}_N2$ mechanism), nucleophilic substitution of X by R-SH, followed by elimination of R-S under the influence of a second nucleophile.

Table 2. *In Vitro* Anti-Hepatitis B Virus Activities of 5-Bromo-6-methoxy(or azido)-5,6-dihydro-3'-fluoro-3'-deoxythymidines

		EC_{50} (μM)[b]		EC_{90} (μM)[c]		SI[d]	
no.	CC_{50} (μM)[a]	extracellular DNA	intracellular DNA	extracellular DNA	intracellular DNA	extracellular DNA	intracellular DNA
4	765 ± 98	6 ± 0.7	13 ± 2	36 ± 5	42 ± 6	21	18
5	1044 ± 44	21 ± 2	26 ± 3	67 ± 6	118 ± 16	16	8.8
11–13[e]	176 ± 16	5.6 ± 0.9	13 ± 2	42 ± 4	57 ± 5	4.2	3.1
DDC[f]	219 ± 28	1.3 ± 0.2	2.1 ± 0.3	8.1 ± 1.7	12 ± 2.4	27	18

[a] CC_{50} = 50% cytotoxic concentration. [b] EC_{50} = 50% effective concentration. [c] EC_{90} = 90% effective concentration. [d] Selectivity index (CC_{50}/EC_{50}). [e] Tested as a mixture of diastereomers. [f] DDC = 2',3'-dideoxycytidine.

5,6-olefinic bond to afford FLT. Similar mechanisms have been proposed for the dehalogenation of 5-bromo-6-methoxy-5,6-dihydrothymine by cysteine.[35] Nucleophilic attack on halogen at C-5 and sulfur attack at sulfur bonded to C-5 in 5,6-dihydrouracils have been reported previously.[36-38]

A number of 5-halo-6-alkoxy(or azido)-5,6-dihydro-3'-fluoro-3'-deoxythymidines (**4–6, 8–13**) were evaluated by the U.S. National Cancer Institute Antiviral Evaluations Branch in an *in vitro* anti-HIV screen using HIV-1 infected T4 lymphocytes (CEM cell line) (see results in Table 1). The C-5 substituent (I, Br, Cl) was a determinant of anti-HIV-1 activity where the approximately equipotent 5-iodo (**10**) and 5-bromo (**4, 5**) analogs were more active than the corresponding 5-chloro (**6, 8**) compounds. An exception to this activity profile was the weakly active (5*R*,6*R*)-5-iodo-6-methoxy-5,6-dihydro-3'-fluoro-3'-deoxythymidine (**9**) for which the anti-HIV-1 EC_{50} and the cell cytoxicity IC_{50} values were similar. In the 5-bromo series of compounds, the difference in anti-HIV-1 activity between the 6-methoxy diastereomers (**4** or **5**) and the mixture of 6-azido diastereomers (**11–13**) was small, although the 6-azido compounds **11–13** undergo greater bioconversion (95%), relative to the 6-methoxy diastereomers **4–5** (10%), to FLT upon incubation with GSH. The configuration at the C-5 and C-6 positions of diastereomers was a determinant of anti-HIV-1 activity where the potency order was (5*S*,6*S*)-**10** > (5*R*,6*R*)-**9** (X = iodo); (5*S*,6*S*)-**5** ≥ (5*R*,6*R*)-**4** (X = bromo), and (5*R*,6*R*)-**6** > (5*S*,6*R*)-**8** (X = chloro). The observation that the 5-chloro-6-methoxy-5,6-dihydro diastereomers (**6, 8**) did not undergo conversion to FLT upon *in vitro* incubation with GSH suggests that their *in vitro* anti-HIV-1 activity is likely due to the 5,6-dihydro compounds, rather than the parent compound FLT. This postulate is consistent with results from a previous study which showed that the (5*R*,6*R*)- and (5*S*,6*R*)-diastereomers of 5-chloro-5-ethyl-6-methoxy-5,6-dihydro-2'-deoxyuridine exhibited antiviral activity despite having very long blood residence times (that indicate low or no conversion) of 7 and 6 h, respectively, following intravenous administration to rats.[27] Balzarini *et al.*[39] have reported that all three 5-halogeno (Cl, Br, I) derivatives of 3'-fluoro-2',3'-dideoxyuridine (FddClUrd, FddBrUrd, FddIUrd) inhibited HIV-1 replication in MT4 cells with an effective dose (ED_{50}) in the 0.16–0.41 μM range. FddClUrd was markedly more selective with respect to its anti-HIV-1 activity than FddBrUrd or FddUrd (selectivity indexes of 1408, 59, and 14, respectively) due to the low cytotoxicity of FddClUrd relative to that of FddBrUrd or FddIUrd (CD_{50} values of 535, 24, and 2.2 μM, respectively). The 5-chloro-5,6-dihydro diastereomers **6** and **8** investigated in this study were likewise less cytotoxic to CEM cells ($IC_{50} > 8.0 \times 10^{-4}$ M) relative to the 5-iodo-5,6-dihydro **9** and **10** ($IC_{50} = 5.7 \times 10^{-5}$ and 1.2×10^{-5} M) and 5-bromo-5,6-dihydro diastereomers **4** and **5** ($IC_{50} = 1.7 \times 10^{-6}$ and 9.7×10^{-6} M). The most potent anti-HIV-1 agents (5*R*,6*R*)-5-Br,6-OMe-**4**, (5*S*,6*S*)-5-Br,6-OMe-**5**, and (5*S*,6*S*)-5-I,6-OMe-**10** exhibited comparable activities to the reference drugs AZT and FLT.

The ability of the (5*R*,6*R*)-**4** and (5*S*,6*S*)-**5** 5-bromo-6-methoxy-5,6-dihydro diastereomers, and a mixture of the (5*R*,6*R*)-**11**, (5*S*,6*S*)-**12**, and (5*R*,6*S*)-**13** 5-bromo-6-azido-5,6-dihydro diastereomers, of FLT to inhibit hepatitis B virus (HBV) replication in chronically HBV-producing human liver cells (2.2.15 cells) was determined (see results in Table 2). The (5*R*,6*R*)-5-bromo-6-methoxy-5,6-dihydro compound **4** and a mixture of the three 5-bromo-6-azido-5,6-dihydro diastereomers **11–13** in-

hibited replication (EC_{50}, EC_{90} values) at a 5–6-fold higher concentration than the reference drug 2′,3′-dideoxycytidine (DDC). In contrast, the (5*R*,6*R*)-**4** and (5*S*,6*S*)-**5** 5-bromo-6-methoxy-5,6-dihydro derivatives of FLT, were 3–5-fold less cytotoxic (CC_{50}) than DDC.

Summary

A new class of 5-halo-6-methoxy(or azido)-5,6-dihydro-3′-fluoro-3′-deoxythymidines (**4**–**13**) have been designed. Their enhanced lipophilicity (P = 1.5–5.15 range), relative to the parent compound FLT (P = 0.5), may enhance their ability to cross the BBB to provide a higher concentration of active drug in the brain. Compounds possessing the 5-bromo or 5-iodo substituent may serve as prodrugs to FLT due to regeneration of the 5,6-double bond. It may be possible to control the rate of FLT release *in vivo*, which would influence the blood half-life and toxicity, by selecting the appropriate combination of C-5 and C-6 substituents. The 5-halo-6-methoxy(or azido)-5,6-dihydro compounds described, some of which are equipotent to AZT and FLT, could serve as useful lead compounds for the development of a drug, or prodrug to FLT, that possesses superior pharmacokinetic and biodistribution (enhanced brain and decreased bone uptake) properties.

Experimental Section

Nuclear magnetic resonance spectra (^{1}H NMR, ^{13}C NMR) were determined on a Bruker AM-300 spectrometer. The assignment of all exchangeable protons (OH, NH) was confirmed by the addition of 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 carbon resonances appear as negative peaks. Thin layer chromatography (TLC) was performed using Whatman MK6F silica gel microslides (250 μm thickness). Preparative thin layer chromatography (PTLC) was carried out using Whatman PLK5F plates (1 mm thickness). Silica gel column chromatography was performed using Merck 7734 (60–200 mesh) silica gel. Microanalyses were within ±0.4% of theoretical values for all elements listed, unless otherwise indicated. 3′-Fluoro-3′-deoxythymidine (**3**, FLT) was prepared using a literature procedure.[40] **Warning**: Halogenated solvents such as dichloromethane must not be used in certain reactions, such as those described for the preparation of products **11**–**13**, since its reaction with sodium azide may produce potentially explosive polyazidomethane.

(+)-*trans*-(5*R*,6*R*)-5-Bromo-6-methoxy-5,6-dihydro-3′-fluoro-3′-deoxythymidine (4) and (−)-*trans*-(5*S*,6*S*)-5-Bromo-6-methoxy-5,6-dihydro-3′-fluoro-3′-deoxythymidine (5). A freshly prepared solution of methyl hypobromite (bromine in methanol) was added to a solution of FLT (50 mg, 0.2 mmol) in methanol (15 mL) at 25 °C with stirring until the light yellow color of the reaction mixture persisted. The reaction was allowed to proceed at 25 °C for 10 min prior to neutralization to pH 6 using a solution of methanolic sodium hydroxide. The solvent was removed *in vacuo*, and the residue obtained was separated by PTLC using chloroform–methanol (92:8, v/v) as development solvent. The desired product band was extracted to yield **4** (40 mg, 56%) as a viscous oil: R_f 0.58 ($CHCl_3$–MeOH, 9:1, v/v); $[\alpha]^{25}_D$ = +67.2° (*c* 0.23, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.96 (s, 3H, C*H*$_3$), 2.44 (ddd, $J_{2',F}$ = 22.2, J_{gem} = 15.6, $J_{1',2'}$ = 7.2 Hz, 1H, H-2′), 2.64 (dddd, $J_{2'',F}$ = 41.4, J_{gem} = 15.6, $J_{1',2''}$ = 9.6, $J_{2'',3'}$ = 5.4 Hz, 1H, H-2″), 2.97 (br s, 1H, O*H*), 3.45 (s, 3H, OC*H*$_3$), 3.80 and 3.88 (two dd, J_{gem} = 13.8, $J_{4',5'}$ = 3.2 Hz, 1H each, H-5′), 4.26 (dt, $J_{4',F}$ = 28.2, $J_{4',5'}$ = 3.2 Hz, 1H, H-4′), 4.81 (s, 1H, H-6), 5.24 (dd, $J_{3',F}$ = 55.2, $J_{2'',3'}$ = 5.4 Hz, 1H, H-3′), 5.92 (dd, $J_{1',2''}$ = 9.6, $J_{1',2'}$ = 7.2 Hz, 1H, H-1′), 8.34 (s, 1H, N*H*); ^{13}C NMR ($CDCl_3$) δ 22.8 (*C*H$_3$), 37.3 (C-2′, $J_{2',F}$ = 20.3 Hz), 52.9 (C-5), 57.5 (O*C*H$_3$), 62.5 (C-5′, $J_{5',F}$ = 11.2 Hz), 84.7 (C-4′), $J_{4',F}$ = 24.7 Hz), 88.1 (C-1′), 90.2 (C-6), 94.3 (C-3′, $J_{3',F}$ = 176.4 Hz), 150.7 (C-2 *C*=O), 167.0 (C-4 *C*=O). Anal. ($C_{11}H_{16}BrFN_2O_5$) C, H, N.

Extraction of the product band having R_f 0.70 ($CHCl_3$:MeOH, 9:1, v/v) afforded **5** as a viscous oil (20 mg, 28%); $[\alpha]^{25}_D$ = −72.5° (*c* 0.16, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.97 (s, 3H, C*H*$_3$), 2.36 (ddd, $J_{2',F}$ = 22.2, J_{gem} = 15.0, $J_{1',2'}$ = 6.6 Hz, 1H, H-2′), 2.96 (dddd, $J_{2'',F}$ = 40.8, J_{gem} = 15.0, $J_{1',2''}$ = 9.6, $J_{2'',3'}$ = 6.6 Hz, 1H, H-2″), 3.6 (s, 3H, OC*H*$_3$), 3.73 and 3.88 (dd, J_{gem} = 13.8, $J_{4',5'}$ = 1.6 Hz, 1H each, H-5′), 4.28 (d, $J_{4',F}$ = 28.8 Hz, 1H, H-4′), 4.54 (s, 1H, H-6), 5.32 (dd, $J_{1',2''}$ = 9.6, $J_{1',2'}$ = 6.6 Hz, 1H, H-1′), 5.34 (dd, $J_{3',F}$ = 54.6, $J_{2'',3'}$ = 6.6 Hz, 1H, H-3′), 8.2 (s, 1H, N*H*); ^{13}C NMR ($CDCl_3$) δ 22.7 (*C*H$_3$), 35.1 (C-2′, $J_{2',F}$ = 20.2 Hz), 53.3 (C-5), 57.3 (O*C*H$_3$), 62.7 (C-5′, $J_{5',F}$ = 11.3 Hz), 85.9 (C-4′, $J_{4',F}$ = 23.0 Hz), 93.6 (C-1′), 95.3 (C-3′, $J_{3',F}$ = 174.2 Hz), 95.8 (C-6), 150.5 (C-2 *C*=O), 166.7 (C-4 *C*=O). Anal. ($C_{11}H_{16}BrFN_2O_5$) C, H, N.

(+)-*trans*-(5*R*,6*R*)-5-Chloro-6-methoxy-5,6-dihydro-3′-fluoro-3′-deoxythymidine (6), (−)-*trans*-(5*S*,6*S*)-5-Chloro-6-methoxy-5,6-dihydro-3′-fluoro-3′-deoxythymidine (7), and (+)-*cis*-(5*S*,6*R*)-5-Chloro-6-methoxy-5,6-dihydro-3′-fluoro-3′-deoxythymidine (8). *N*-Chlorosuccinimide (100 mg, 0.7 mmol) was added to a solution of FLT (75 mg, 0.3 mmol) in methanol (15 mL) and glacial acetic acid (0.3 mL) with stirring. After stirring the reaction mixture at 25 °C for 20 h, an additional aliquot of *N*-chlorosuccinimide (200 mg, 1.5 mmol) and glacial acetic acid (0.6 mL) was added. The reaction was allowed to proceed at 25 °C for 40 h with stirring prior to neutralization using a solution of methanolic sodium hydroxide. Removal of the solvent *in vacuo* gave a residue which was dissolved in ethyl acetate (25 mL), the ethyl acetate layer was washed with cold water (2 × 5 mL), and dried (Na_2SO_4), and the solvent was removed *in vacuo*. The residue obtained was separated by PTLC using chloroform–methanol (92:8, v/v) as development solvent. The desired product band was extracted to yield **6** as a viscous oil (40 mg, 43%): R_f 0.61 ($CHCl_3$:MeOH, 9:1, v/v); $[\alpha]^{25}_D$ = +56.6° (*c* 0.17 MeOH); ^{1}H NMR ($CDCl_3$) δ 1.78 (s, 3H, C*H*$_3$), 2.40 (ddd, $J_{2',F}$ = 22.8, J_{gem} = 15.0, $J_{1',2'}$ = 6.6 Hz, 1H, H-2′), 2.58 (dddd, $J_{2'',F}$ = 38.1, J_{gem} = 15.0, $J_{1',2''}$ = 9.2, $J_{2'',3'}$ = 5.2 Hz, 1H, H-2″), 2.9 (br s, 1H, O*H*), 3.43 (s, 3H, OC*H*$_3$), 3.75–3.92 (m, 2H, H-5′), 4.26 (d, $J_{4',F}$ = 27.8 Hz, 1H, H-4′), 4.74 (s, 1H, H-6), 5.25 (dd, $J_{3',F}$ = 53.4, $J_{2'',3'}$ = 5.2 Hz, 1H, H-3′), 5.92 (dd, $J_{1',2''}$ = 9.2, $J_{1',2'}$ = 6.6 Hz, 1H, H-1′), 8.32 (s, 1H, N*H*); ^{13}C NMR ($CDCl_3$) δ 21.6 (*C*H$_3$), 37.3 (C-2′, $J_{2',F}$ = 20.3 Hz), 57.4 (O*C*H$_3$), 60.7 (C-5), 62.4 (C-5′, $J_{5',F}$ = 11.3 Hz), 84.7 (C-4′, $J_{4',F}$ = 24.9 Hz), 87.9 (C-1′), 89.8 (C-6), 93.3 (C-3′, $J_{3',F}$ = 176.3 Hz), 150.7 (C-2 *C*=O), 166.5 (C-4 *C*=O). Anal. ($C_{11}H_{16}ClFN_2O_5$) C, H, N.

Extraction of the product band having R_f 0.60 ($CHCl_3$:MeOH, 9:1, v/v) afforded **7** (10 mg, 11%) as a syrup: $[\alpha]^{25}_D$ = −40.5° (*c* 0.13, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.80 (s, 3H, C*H*$_3$), 2.30 (ddd, $J_{2',F}$ = 22.2, J_{gem} = 15.0, $J_{1',2'}$ = 5.4 Hz, 1H, H-2′), 2.92 (dddd, $J_{2'',F}$ = 42.0, J_{gem} = 15.0, $J_{1',2''}$ = 9.6, $J_{2'',3'}$ = 5.4 Hz, 1H, H-2″), 3.6 (s, 3H, OC*H*$_3$), 3.74 and 3.88 (dd, J_{gem} = 13.6, $J_{4',5'}$ = 1.6 Hz, 1H each, H-5′), 4.26 (d, $J_{4',F}$ = 28.8 Hz, 1H, H-4′), 4.44 (s, 1H, H-6), 5.32 (dd, $J_{1',2''}$ = 9.6, $J_{1',2'}$ = 5.4 Hz, 1H, H-1′), 5.34 (dd, $J_{3',F}$ = 55.2, $J_{2'',3'}$ = 5.4 Hz, 1H, H-3′), 7.92 (s, 1H, N*H*). Anal. ($C_{11}H_{16}ClFN_2O_5{\cdot}^1/_2H_2O$) C, H, N.

Extraction of the product band having R_f 0.55 ($CHCl_3$:MeOH 9:1, v/v) afforded **8** (20 mg, 22%) as a viscous oil: $[\alpha]^{25}_D$ = +32.2° (*c* 0.16, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.81 (s, 3H, C*H*$_3$), 2.40 (ddd, $J_{2',F}$ = 20.4, J_{gem} = 14.7, $J_{1',2'}$ = 5.7 Hz, 1H, H-2′), 2.72 (dddd, $J_{2'',F}$ = 39.6, J_{gem} = 14.7, $J_{1',2''}$ = 9.0, $J_{2'',3'}$ = 5.4 Hz, 1H, H-2″), 3.1 (br s, 1H, O*H*), 3.52 (s, 3H, OC*H*$_3$), 3.78 and 3.85 (dd, J_{gem} = 13.2, $J_{4',5'}$ = 1.6 Hz, 1H each, H-5′), 4.26 (d, $J_{4',F}$ = 26.4 Hz, 1H, H-4′), 4.62 (s, 1H, H-6), 5.26 (dd, $J_{3',F}$ = 54.6, $J_{2'',3'}$ = 5.4 Hz, 1H, H-3′), 5.70 (dd, $J_{1',2''}$ = 9.0, $J_{1',2'}$ = 5.7 Hz, 1H, H-1′), 8.04 (s, 1H, N*H*); ^{13}C NMR ($CDCl_3$) δ 26.0 (*C*H$_3$), 37.2 (C-2′, $J_{2',F}$ = 20.3 Hz), 58.2 (O*C*H$_3$), 62.6 (C-5′), $J_{5',F}$ = 11.1 Hz), 66.8 (C-5), 85.1 (C-4′, $J_{4',F}$ = 24.7 Hz), 89.5 (C-1′), 92.2 (C-6), 94.6 (C-3′, $J_{3',F}$ = 175.9 Hz, 150.7 (C-2 *C*=O), 167.1 (C-4 *C*=O). Anal. ($C_{11}H_{16}ClFN_2O_5$) H, N; C: calc, 42.51; found, 42.09.

(+)-*trans*-(5*R*,6*R*)-5-Iodo-6-methoxy-5,6-dihydro-3′-fluoro-3′-deoxythymidine (9) and (−)-*trans*-(5*S*,6*S*)-5-Iodo-6-methoxy-5,6-dihydro-3′-fluoro-3′-deoxythymidine (10). *N*-Iodosuccinimide (75 mg, 0.33 mmol) was added slowly with

stirring to a solution of FLT (75 mg, 0.3 mmol) in methanol (15 mL) and glacial acetic acid (0.2 mL) during a period of 5 min. After the reaction mixture was stirred at 25 °C for 10 h, an additional aliquot of *N*-iodosuccinimide (25 mg, 0.11 mmol) and glacial acetic acid (0.2 mL) was added. The reaction was allowed to proceed for 20 h at 25 °C prior to neutralization with methanolic sodium hydroxide. Removal of the solvent *in vacuo* gave a residue which was separated by PTLC using chloroform–methanol (92:8, v/v) as development solvent. The desired product band was extracted to yield **9** (45 mg, 37%) as an oil: R_f 0.58 ($CHCl_3$:MeOH, 9:1, v/v); $[\alpha]^{25}_D = +74.1°$ (*c* 0.14, MeOH); ^1H NMR ($CDCl_3$) δ 2.18 (s, 3H, CH_3), 2.48 (ddd, $J_{2',F}$ = 21.8, J_{gem} = 14.8, $J_{1',2'}$ = 5.8 Hz, 1H, H-2'), 2.72 (dddd, $J_{2'',F}$ = 39.6, J_{gem} = 14.8, $J_{1',2''}$ = 9.2, $J_{2'',3'}$ = 5.2 Hz, 1H, H-2''), 2.97 (br s, 1H, OH), 3.45 (s, 3H, OCH_3), 3.78 (dd, J_{gem} = 13.4, $J_{4',5'}$ = 3.2 Hz, 1H, H-5'), 3.88 (dd, J_{gem} = 13.4, $J_{4',5''}$ = 3.2 Hz, 1H, H-5''), 4.28 (dt, $J_{4',F}$ = 27.8, $J_{4',5'}$ = $J_{4',5''}$ = 3.2 Hz, 1H, H-4'), 4.85 (s, 1H, H-6), 5.26 (dd, $J_{3',F}$ = 54.4, $J_{2'',3'}$ = 5.2 Hz, 1H, H-3'), 5.86 (dd, $J_{1',2''}$ = 9.2, $J_{1',2'}$ = 5.8 Hz, 1H, H-1'), 8.1 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 25.7 (CH_3), 34.0 (C-5), 37.3 (C-2', $J_{2',F}$ = 20.4 Hz), 57.3 (OCH_3), 62.5 (C-5', $J_{5',F}$ = 11.0 Hz), 84.8 (C-4', $J_{4',F}$ = 24.0 Hz), 88.4 (C-1'), 92.2 (C-6), 94.3 (C-3', $J_{3',F}$ = 176.5 Hz), 150.8 (C-2 *C*=O), 169.0 (C-4 *C*=O). Anal. ($C_{11}H_{16}IFN_2O_5$) C, H, N.

Extraction of the product band having R_f 0.66 ($CHCl_3$:MeOH, 9:1, v/v) afforded **10** (45 mg, 37%) as an oil: $[\alpha]^{25}_D$ = −83.0° (*c* 0.35, MeOH); ^1H NMR ($CDCl_3$) δ 2.12 (s, 3H, CH_3), 2.32 (ddd, $J_{2',F}$ = 22.0, J_{gem} = 14.2, $J_{1',2'}$ = 5.6 Hz, 1H, H-2'), 2.97 (dddd, $J_{2'',F}$ = 40.4, J_{gem} = 14.2, $J_{1',2''}$ = 9.6, $J_{2'',3'}$ = 4.8 Hz, 1H, H-2''), 3.60 (s, 3H, OCH_3), 3.76 and 3.88 (dd, J_{gem} = 12.4, $J_{4',5'}$ = 1.6 Hz, 1H each, H-5'), 4.28 (d, $J_{4',F}$ = 28.8 Hz, 1H, H-4'), 4.6 (s, 1H, H-6), 5.32 (dd, $J_{1',2''}$ = 9.6, $J_{1',2'}$ = 5.6 Hz, 1H, H-1'), 5.34 (dd, $J_{3',F}$ = 54.0, $J_{2'',3'}$ = 4.8 Hz, 1H, H-3'), 8.35 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 25.5 (CH_3), 34.6 (C-5), 35.2 (C-2', $J_{2',F}$ = 20.2), 57.2 (OCH_3), 62.7 (C-5', $J_{5',F}$ = 11.3 Hz), 85.8 (C-4', $J_{4',F}$ = 24.4 Hz), 93.5 (C-1'), 95.3 (C-3', $J_{3',F}$ = 174.2 Hz), 97.4 (C-6), 150.8 (C-2 *C*=O), 168.9 (C-4 *C*=O). Anal. ($C_{11}H_{16}IFN_2O_5 \cdot {}^1/_2H_2O$) C, H, N.

(+)-*trans*-(5*R*,6*R*)-5-Bromo-6-azido-5,6-dihydro-3'-fluoro-3'-deoxythymidine (11), (−)-*trans*-(5*S*,6*S*)-5-Bromo-6-azido-5,6-dihydro-3'-fluoro-3'-deoxythymidine (12), and (−)-*cis*-(5*R*,6*S*)-5-Bromo-6-azido-5,6-dihydro-3'-fluoro-3'-deoxythymidine (13). *N*-Bromosuccinimide (0.06 g, 0.33 mmol) was added in aliquots to a precooled −5 °C suspension prepared by mixing a solution of FLT (75 mg, 3 mmol) in 1,2-dimethoxyethane (10 mL) and a solution of sodium azide (80 mg, 1.2 mmol) in water (0.2 mL) with stirring. The initial yellow color produced upon addition of each aliquot of *N*-bromosuccinimide quickly disappeared. When all of the *N*-bromosuccinimide had reacted, the reaction mixture was stirred for 30 min at 25 °C, poured onto ice water (25 mL), and extracted with EtOAc (3 × 50 mL). Washing the EtOAc extract with cold water (2 × 5 mL), drying (Na_2SO_4), and removal of the solvent *in vacuo* gave a residue which was separated by silica gel column chromatography using chloroform–methanol (94:6, v/v) as eluent to yield a mixture of diastereomers **11**, **12**, and **13** (0.06 g, 55%) as a syrup. The mixture of diastereomers **11**–**13** could not be separated by flash silica gel column chromatography or PTLC: ^1H NMR ($CDCl_3$, mixture of diastereomers **11**–**13** in a ratio of 6:3.5:3 as determined from the integrals for the respective H-6 protons) δ 2.00, 2.02 and 2.04 (ratio 3:6:3.5) (three s, 3H total, CH_3), 2.20–2.76 (complex m, 2H total, H-2'), 3.80–4.04 (m, 2H total, H-5'), 4.21–4.40 (m, 1H total, H-4'), 5.12–5.40 (m, 1H total, H-3'), 5.44 (**12**), 5.60 (**11**), 5.78 (**13**) (ratio 3.5:6:3) (three s, 1H total, H-6), 5.88 (**12**), 6.18 (**13**), 6.40 (**11**) (ratio 3.5:3:6) (three dd, $J_{1',2''}$ = 9.6, $J_{1',2'}$ = 6.4 Hz, 1H total, H-1'), 8.54 and 8.60 (ratio 13:7) (two s, 1H total, NH); ^{13}C NMR ($CDCl_3$) δ 22.8 (**12**), 23.2 (**11**), and 27.6 (**13**) (CH_3), 36.19, 36.48, and 37.03 (C-2'), 52.2 and 52.7 (C-5), 61.9, 62.1, and 62.6 (C-5'), 73.8, 74.6, and 77.2 (C-6), 84.1–88.9 (C-4' and C-1'), 94.50, 94.64, and 94.83 (C-3', $J_{3',F}$ = 177.86, 176.41, and 177.9 Hz), 150.1 (C-2 *C*=O), 166.08, 166.17, and 166.70 (C-4 *C*=O). Anal. ($C_{10}H_{13}BrFN_5O_4$) C, H, N.

Partition Coefficients (*P*). The nucleoside test compound (**4**–**6**, **8**–**10**) was partitioned between equal volumes of presaturated 1-octanol and water. The concentration of the test compound in the water phase, before and after 1-octanol partitioning, was determined using the procedure (UV quantitation) reported previously.[41] Partition coefficients (*P*) were calculated as the ratio of the concentration in the octanol to the concentration in the water phase ($P = C_{1\text{-octanol/water}}$).

***In Vitro* Regeneration of the 5,6-Olefinic Bond.** Regeneration of FLT from the 5-halo-6-methoxy-5,6-dihydro-3'-fluoro-3'-deoxythymidines (**4**–**6**, **8**–**13**) was determined by incubating the test compound with glutathione (GSH, reduced). The test compound was incubated with GSH using a test compound:GSH molar ratio of 1:2 in phosphate buffer (pH = 7.4) at 37 °C for 30 min. The incubation sample was subjected to HPLC analysis using a C_{18} Radial-Pak Cartridge (8 mm i.d., 10 μm particle size) with UV detection at both 230 nm (5,6-dihydro absorption) and 265 nm (FLT absorption), using water–methanol (7:3, v/v) as eluent at a flow rate of 2 mL/min to quantitate the amount of 5,6-olefinic bond regeneration.

***In Vitro* Anti-HIV Assay.** The ability of the test compound to protect HIV-1 infected T4 lymphocytes (CEM cells) from cell death was determined using the reported procedure.[42] Small amounts of HIV are added to cells, and a complete cycle of virus reproduction is necessary to obtain the required cell killing. Agents that interact with virions, cells, or virus gene products to interfere with viral activities will protect cells from cytolysis. All compounds are compared with a positive (AZT-treated) control performed at the same time under identical conditions.

***In Vitro* Hepatitis B Virus Replication Inhibition (Anti-HBV) Assay.**[43] Chronically HBV-producing human liver cells (2.2.15) were seeded into 24-well tissue culture plates and grown to confluence. Test compounds were then added daily for a continuous 9 day period. Culture medium (changed daily during the treatment period) was collected and stored for analysis of extracellular (virion) HBV DNA after 0, 3, 6, and 9 days of treatment. Treated cells were lysed 24 h following day 9 of treatment for the analysis of intracellular HBV genomic forms. HBV DNA was then analyzed in a quantitative and qualitative manner for overall levels of HBV DNA (both extracellular and intracellular DNA) and the relative rate of HBV replication (intracellular DNA). **Toxicity assay:** Cells (2.2.15) were grown to confluence in 96-well flat bottomed tissue culture plates and treated with the test compound (in 0.2 mL culture medium/well) as described above in the anti-HBV assay. Four concentrations of each compound were assayed, each in triplicate cultures, in 3–10-fold steps. Untreated control cultures were maintained on each 96-well plate. On each 96-well plate, wells containing no cells were used to correct for light scattering. Toxicity was determined by the inhibition of the uptake of neutral red dye, as determined by absorbance at 510 nm relative to untreated cells, 24 h following day 9 of treatment.

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