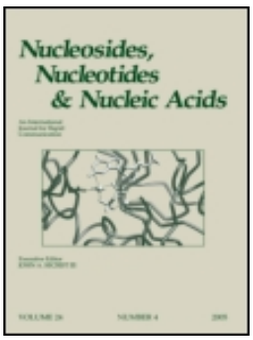

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SYNTHESIS, *IN VITRO* ANTI-HIV ACTIVITY, AND BIOLOGICAL STABILITY OF 5'-*O*-MYRISTOYL ANALOGUE DERIVATIVES OF 3'-FLUORO-2',3'-DIDEOXYTHYMIDINE (FLT) AS POTENTIAL BIFUNCTIONAL PRODRUGS OF FLT

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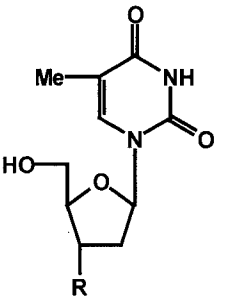
ABSTRACT: A group of 5'-*O*-myristoyl analogue derivatives of FLT **(2)** were evaluated as potential anti-HIV agents that were designed to serve as prodrugs to FLT. 3'-Fluoro-2',3'-dideoxy-5'-*O*-(12-methoxydodecanoyl)thymidine **(4)** (EC_{50} = 3.8 nM) and 3'-fluoro-2',3'-dideoxy-5'-*O*-(12-azidododecanoyl)thymidine **(8)** (EC_{50} = 2.8 nM) were the most effective anti-HIV-1 agents. There was a linear correlation between Log P and HPLC Log retention time for the 5'-*O*-FLT esters. The *in vitro* enzymatic hydrolysis half-life ($t_{1/2}$), among the group of esters **(3-8)** in porcine liver esterase, rat plasma and rat brain homogenate was longer for 3'-fluoro-2',3'-dideoxy-5'-*O*-(myristoyl)thymidine **(7)**, with $t_{1/2}$ values of 20.3, 4.6 and 17.5 min, respectively.

INTRODUCTION

Efforts to develop drugs for the treatment of human immunodeficiency virus (HIV) have concentrated on inhibitors of viral reverse transcriptase (RT), a key enzyme in the replicative cycle of viruses such as HIV and HBV. 2',3'-Dideoxynucleosides (ddNs), that are activated intracellularly by conversion to their active triphosphate form are competitive inhibitors, or alternate substrates (chain terminators) of HIV RT. There are currently five reverse transcriptase inhibitors approved for use in HIV disease. 2',3'-Dideoxynucleosides such as 2',3'-dideoxy-3'-thiacytidine (3TC, lamivudine, Epivir®),[1] 2',3'-dideoxycytidine (ddC, zalcitabine, Hivid®),[2] 3'-azido-2',3'-dideoxythymidine (AZT, **1**, zidovudine, Retrovir®),[3] 2',3'-didehydro-3'-deoxythymidine (d4T, stavudine,

Zerit®)[4] and 2',3'-dideoxyinosine (ddI, didanosine, Videx®)[5] are used clinically to treat HIV infection.



R = N_3 (AZT, 1)
R = F (FLT, 2)

3'-Fluoro-2',3'-dideoxythymidine (FLT) has been reported to be one of the most active RT inhibitors of HIV *in vitro* in various T-cells (EC_{50} = 1-10 nM). On the basis of cell culture data, FLT is a more potent inhibitor of HIV replication than AZT.[6] Although FLT was more effective than AZT in inhibiting HIV cytopathogenicity in CEM cells, it also proved to be considerably more cytotoxic than AZT toward uninfected lymphocytes. The selectivity index (SI) for AZT is much greater than that for FLT (10,000 *vs* 967). Clinical trials using FLT suggested that thrombocytopenia and anemia are the main limiting toxic effects, resulting in a narrow therapeutic window.[6-8] The clinical development of FLT has been discontinued. The observed side-effects for FLT has stimulated the search for closely related nucleoside analogs with increased antiviral activity and decreased cytotoxicity.[9]

AZT-resistant viruses are not cross resistant to FLT.[10] This absence of cross resistance suggests FLT may be a potential alternative to AZT as a single agent for the treatment of HIV infection[11,12] provided that a suitable dosage-form such as a FLT prodrug is used to decrease its cytotoxicity.

Several approaches have been used for the synthesis of FLT prodrugs which included the preparation of the 5'-hydrogen phosphonate,[13] -phosphate, -phosphoroamidate, -phosphorodiamidate, -*bis*(trichloroethyl)phosphate,[14] -ether phospholipid,[15] and 5,6-dihydro[16] prodrug derivatives of FLT.

Several studies have applied the 5'-*O*-fatty acyl ester prodrug approach to nucleosides.[17-21] These prodrugs are lipophilic, as indicated by their high partition

coefficients. The fatty acid moiety enhances drug penetration through membranes, and is rapidly cleaved from the nucleoside by any of a variety of enzymes present in the cell cytoplasm. The deacylated (parent) nucleoside can then be a substrate for phosphorylation that is needed to generate the active compound.

It was therefore of interest to design, synthesize and pharmacologically evaluate a new class of uracil nucleoside prodrugs that could act on two different molecular targets simultaneously. These targets include reverse transcriptase (RT) and myristoyl-CoA:protein *N*-myristoyltransferase (NMT) which catalyzes the post-translational myristoylation of viral proteins, for FLT and the myristic acid analogue, respectively.

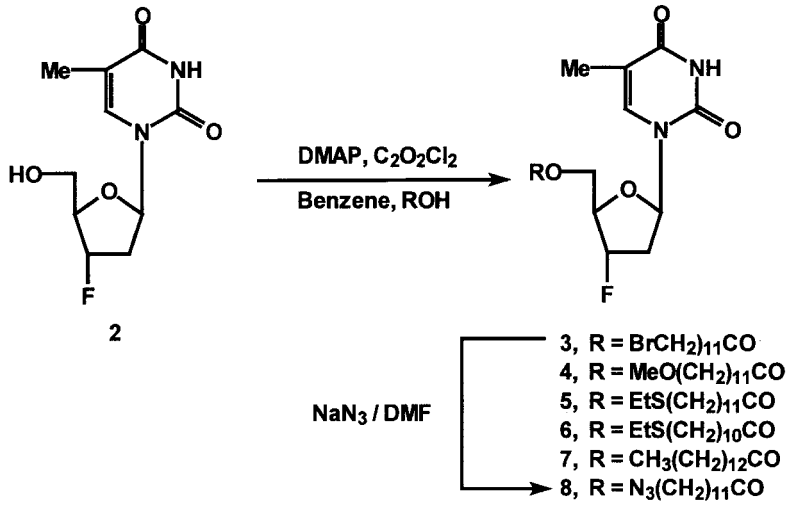
Protein *N*-myristoylation by viruses is catalyzed by myristoyl-CoA:protein *N*-myristoyltransferase (NMT) (E.C. 2.3.1.97) that results in the co-translational linkage of myristic acid (C14:0), via an amide bond, to NH_2-terminal glycine (Gly) residues of a variety of viral proteins following the removal of the initiator methionine residue by methionylpeptidase.[22] Recently, it was proposed that inhibition of myristoyl-CoA:protein *N*-myristoyltransferase (NMT) may be a more plausible mechanism for the antiviral activity exhibited by myristic acid analogues.[23,24] Myristoylation of retrovirus Gag polypeptides is essential for either the assembly or budding of virus particles at membranes, and for the release of preformed capsids. The antiviral activities exhibited by a variety of myristic acid analogues that possess different physicochemical properties and that contain a halogen, azide, oxygen, sulfur or aromatic substituent have been investigated previously.[25]

We now report the synthesis and evaluation of 5'-*O*-ester conjugates of FLT designed to act at the RT and NMT sites during retrovirus replication.

CHEMISTRY

Previously reported methods were used to synthesize 12-methoxydodecanoic acid,[26,27] 12-thioethyldodecanoic acid, 11-thioethylundecanoic acid[26] and 12-azidododecanoic acid.[28]

3'-Fluoro-2',3'-dideoxythymidine (FLT) was prepared using a literature procedure.[29] The 5'-OH functionality of FLT was esterified using a variety of



Scheme 1. General method for the synthesis of 5'-*O*-esters of FLT (**3-8**).

myristic acid analogues in the presence of oxalyl chloride and 4-(dimethylamino)pyridine (DMAP) to afford the corresponding esters **(3-8)** in 52-95% yields. Accordingly, the fatty acid (RCOOH) was reacted with oxalyl chloride in benzene prior to its reaction with FLT in the presence of approximately one equivalent of DMAP (Scheme 1).

Further reaction of 3'-fluoro-2',3'-dideoxy-5'-*O*-(12-bromododecanoyl)thymidine **(3)** with sodium azide at room temperature in dimethyl formamide gave the corresponding 12-azido analogue (**8**).

RESULTS AND DISCUSSION

Anti-HIV activity of 5'-*O*-myristoyl analogue derivatives of FLT (3-8).

The structures of the 5'-substituted FLT derivatives synthesized and their *in vitro* antiviral and cytotoxic activities, are listed in Table 1. The indicated values represent the mean values, obtained from at least three separate determinations. All of the 5'-*O*-myristoyl derivatives 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) with AZT and FLT included as reference compounds. It is interesting that these 5'-*O*-ester prodrugs **(3-8)** retained the replication inhibiting activity exhibited by the parent nucleoside when evaluated by *in vitro* assays for virus replication.

The FLT prodrugs, 3'-fluoro-2',3'-dideoxy-5'-*O*-(12-methoxydodecanoyl)thymidine **(4)** (EC_{50} = 3.8 nM) and 3'-fluoro-2',3'-dideoxy-5'-*O*-(12-azidododecanoyl)thymidine **(8)** (EC_{50} = 2.8 nM) were the most active anti-HIV-1 agents in this group. The ester **8** exhibited a potency comparable to FLT (EC_{50} = 2.5 nM) (Table 1).

All analogues **(3-8)** demonstrated lower toxicity (IC_{50} = 29-120 μM) than FLT (IC_{50} = 2.4 μM) (Table 1) in the CEM cell line. Compounds **3-8** also displayed a 2.8-35.4 higher selectivity than FLT. This lower toxicity and higher selectivity for the 5'-*O*-ester prodrugs **(3-8)** may be due to the sustained release of FLT upon hydrolysis of the prodrug and a lower intracellular concentration of FLT. In addition, intracellular release of the myristic acid analogue may contribute to this decreased cytotoxicity. The released molecules (myristic acid analogue and FLT) act by different mechanisms of action and myristic acid analogues are not associated with the cytotoxicity observed with FLT.[25] Myristic acid analogues are generally considered to be non toxic. For some cellular *N*-myristoylproteins, incorporation of these analogues leads to analog-specific and -dependent redistribution from membrane to cytosolic fractions. This dual level of selectivity, which encompasses selective incorporation and selective perturbation of function, probably accounts for their lack of cellular toxicity.[24] Therefore, lower toxicity and higher selectivity would be expected when myristic acid analogues are conjugated to FLT.

Some general conclusions pertaining to structure-anti HIV activity relationships for this category of compounds can be made on the basis of these results. There are three possible explanations pertaining to the mode of action and observed anti-HIV activity (EC_{50}) of these 5'-substituted compounds. (i) The most plausible explanation is that the fatty acid group is rapidly cleaved from FLT by any of a variety of hydrolytic enzymes present in the cell cytoplasm. The rate of intracellular hydrolysis would determine the amount of FLT released and ultimately the anti-HIV activity exhibited by the prodrug. (ii) The fatty acid released from the prodrug may produce synergistic or additive effects

with FLT. (iii) The rate of prodrug cellular uptake may be an important determinant of anti-HIV activity. Slow cellular uptake of the prodrug would result in a lower intracellular concentration of the prodrug and hydrolytically-released FLT that would translate into decreased anti-HIV activity (higher EC_{50}). A lower intracellular concentration of FLT arising from the prodrug, compared to FLT alone, may also be responsible for the lower toxicity and higher selectivity exhibited by these prodrug analogues.

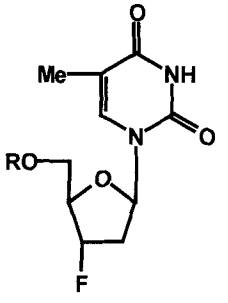
These results indicate that the chemical nature of the substituents on the fatty acid chain is a determinant of antiviral activity, since some of the esters **(4, 6, 8)** exhibited a potency comparable to FLT against HIV-1, whereas others **(3, 5, 7)** were less active than FLT (Table 1).

These results demonstrate the importance of a fluoro substituent at the 3'-position of FLT to achieve efficacy in the nanomolar range, since none of the 5'-*O*-myristoyl thymidine analogues described in an earlier study[30] exhibited anti-HIV activity in this range.

Partition coefficient (Log P) and HPLC retention time determinations.

The partition coefficients for some 5'-*O*-myristoyl analogues of FLT in a *n*-octanol/phosphate buffer (0.2 M, pH 7.4) system were determined at room temperature as an indicator of passive diffusion across cell membranes. All of the C-5' esters of FLT **(3-8)** are more lipophilic (Log P = 4.3 to > 5) than FLT (Log P = -0.30) as indicated in Table 2. Due to the high lipophilicity of several esters **(3-8)**, small changes in the concentration of the ester in the *n*-octanol phase (5 mL) were measurable only if a larger volume of buffer phase (10 mL) was used. In most cases only an approximate value was obtained. To obtain more reliable partition data, the relative lipophilicities of these compounds were also determined from their reverse-phase HPLC retention times, where more polar compounds have shorter retention times. All of the 3'-fluoro derivatives **(3-8)** showed a longer retention time and higher partition coefficient than AZT, FLT and dThd (see Table 2). The lower lipophilicity for 5'-*O*-myristoyl analogues of dThd reported previously by Parang *et al*,[30] relative to those for FLT described herein, is due to the presence of the 3'-hydroxy group in thymidine derivatives.

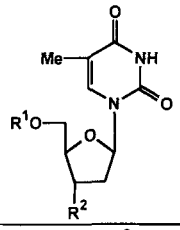
Table 1. Comparative potency and selectivity of 5'-*O*-myristoyl analogue derivatives of FLT (**3-8**) as inhibitors of HIV replication in T4 cells (CEM-SS cell line).



Compound	R	EC_{50}, nM[a]	IC_{50}, μM[b]	TI_{50}[c]
3	$Br(CH_2)_{11}CO$	25.0 ± 3.0	29.0 ± 3.0	1160
4	$MeO(CH_2)_{11}CO$	3.8 ± 1.5	79.0 ± 1.0	21051
5	$EtS(CH_2)_{11}CO$	16.0 ± 0.5	33.0 ± 0.0	2063
6	$EtS(CH_2)_{10}CO$	6.0 ± 1.8	42.5 ± 1.3	7036
7	$CH_3(CH_2)_{12}CO$	44.50 ± 4.8	120.0 ± 0.0	2697
8	$N_3(CH_2)_{11}CO$	2.8 ± 1.0	94.0 ± 6.0	34182
AZT	H	10.0	100.0	10000
FLT	H	2.5[d]	2.4[d]	967

[a]EC_{50} is defined as the 50% antiviral effective concentration, required to produce a 50% reduction in the cytopathic HIV effect in T4 lymphocytes (mean ± SE, n = 5). [b]IC_{50} is defined as the inhibitory concentration, required to reduce the number of viable untreated T4 lymphocytes by 50% (mean ± SE, n = 5). [c]TI_{50} is defined as the therapeutic index (IC_{50} / EC_{50}) (mean ± SE, n = 5). [d] FLT activity taken from reference # 9 (CEM cell line).

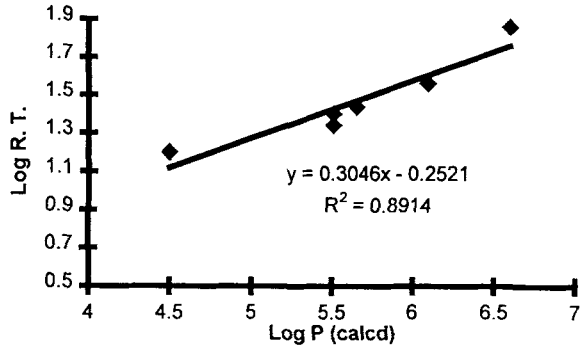
Table 2. Physical properties of 5'-*O*-myristoyl analogue derivatives of FLT (**3-8**).



No.	R^1	R^2	RT (min)[a]	Log P (exp)[b]	Log P (calcd)[c]	Log $D_{7.4}$ (calcd)[d]
3	$Br(CH_2)_{11}CO$	F	27.3	> 5	5.65	5.70
4	$MeO(CH_2)_{11}CO$	F	16.0	4.30	4.50	4.50
5	$CH_3CH_2S(CH_2)_{11}CO$	F	36.6	> 5	6.09	6.10
6	$CH_3CH_2S(CH_2)_{10}CO$	F	25.3	> 5	5.51	5.60
7	$CH_3(CH_2)_{12}CO$	F	72.4	> 5	6.60	6.60
8	$N_3(CH_2)_{11}CO$	F	21.8	> 5	5.51	5.50
AZT	H	N_3	4.3	0.11	0.06	0.06
FLT	H	F	4.1	-0.30	-0.52	-0.53
Dthd	H	OH	3.8	-1.19	-1.55	-1.25

[a]Retention time: Methanol/water (75/25, v/v), flow rate of 1 mL/min using reverse phase HPLC. [b]Partition coefficient in *n*-octanol and phosphate buffer at pH 7.4 (0.2 M). [c]Partition coefficient of the ester analogue calculated using the Prolog 5.1 program. [d]Distribution coefficient of the ester analogue at pH 7.4 calculated using the PrologD 2.0 prediction program.

PALLAS computational programs, PrologP 5.1 and PrologD 2.0, were used to calculate theoretical partition coefficients (Log P) and distribution coefficients (Log $D_{7.4}$) values, respectively for 5'-*O*-esters of FLT **(3-8)** in an *n*-octanol/water system. Calculated partition coefficients for these esters **(3-8)** extended over a broad range (Log P = 4.5 to 6.6). The calculated partition for these analogues at pH 7.4 (Log $D_{7.4}$) were similar to values calculated for pH 7 (Log P) (Table 2).



5'-O-esters of FLT

Figure 1. The correlation between Log retention time and lipophilicity (Log P) for 5'-*O*-esters of FLT.

Balzarini *et al.*[31] reported that there was a correlation (correlation coefficient > 0.97) between P and HPLC retention time values for 36 anti-HIV nucleoside analogues. Correlations between Log P and Log retention time for the FLT derivatives also show a linear relationship (Figure 1). This method may provide a useful tool to predict the partition coefficients of other ester analogues from their retention time using the same HPLC conditions.

According to Hansch and Fujita,[32] there should be an optimum Log P for achieving optimal biological activity. Although no general relationships were evident between optimum selectivity and potency with Log P values for 5'-*O*-fatty acyl derivatives of FLT, compounds (**3**, **5** and **7**) having a high partition coefficient (5.7-6.6) showed lower anti-HIV activity than other analogues. Compounds **4**, **6** and **8**, which were the most active and selective compounds, had Log P values in the 4.5-5.5 range. However, compounds **6** and **8**, that have the same partition coefficient (5.5), exhibited significantly different selectivities. Accordingly, it appears that there are several factors which determine apparent *in vitro* anti-HIV activity of the prodrug ester, that include partition coefficient, rate of cellular uptake and intracellular rate of hydrolysis of the 5'-*O*-ester.

Stability of prodrug esters toward esterases.

Each prodrug will be activated by ester cleavage according to its relative overall substrate capacity towards carboxyesterases. The usefulness of these FLT prodrugs will therefore depend not only on the stability of the prodrug prior to its passage across the cell membrane and into the central nervous system (CNS), but also upon its intracellular bioconversion to the parent compounds, especially in virus infected cells.

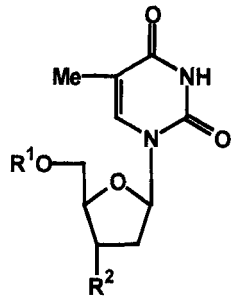
The half-lives ($t_{1/2}$) for hydrolysis of 5'-*O*-esters of FLT **(3-8)** were determined by *in vitro* incubation at 37 °C in several media including porcine liver esterase, rat plasma and rat brain homogenate. In these incubation studies, the decrease in concentration of the ester prodrug followed pseudo-first-order kinetics ($C = C_0e^{-kt}$, where C_0 represents the initial concentration of ester prodrug, and C is the concentration of ester at time t).

There are various factors which could contribute to the stability of these esters. Differences in their rates of ester hydrolysis are likely dependent upon the presence of heteroatoms in the myristoyl moiety and the type and/or concentration of the esterase in the medium. The kinetics for the bioconversion of ester prodrugs to the parent drug(s) appear to be dependent upon the presence of a heteroatom or halogen in the myristoyl moiety since compounds **(3-6, 8)** exhibited a shorter half-life than **7** in all media. This effect may be due, at least in part, to binding of the heteroatom **(4-6, 8)** to the active site of the enzyme. Therefore, the nature of the myristoyl moiety may play an important role in determining the rate and/or extent of enzymatic cleavage of the ester prodrug by the esterase.

The ester cleavage half-life was also dependent upon the type or concentration of esterase in the tissue or plasma. Most esters were rapidly hydrolyzed to FLT upon incubation with rat plasma, probably due to the very high hydrolytic capacity of the plasma (high activity or concentration of esterase)[33] (See Table 3). The half-life of **3** in rat plasma was not measurable due to rapid hydrolysis. Generally, these ester analogues were hydrolyzed less extensively by brain homogenate than by rat plasma or porcine liver esterase. These data show that esters showing a range of stabilities can be designed by selecting an appropriate heteroatom or halogen in the myristoyl moiety.

It is concluded that (i) the ester prodrug approach described could be used to improve the *in vitro* stability and biological properties with respect to anti-HIV activity. All of the

Table 3. Half-life for *in vitro* hydrolysis of 5'-*O*-myristoyl analogue derivatives of FLT **(3-8)** in the presence of porcine liver esterase, rat plasma, and rat brain homogenate.



No.	R^1	R^2	Porcine liver esterase, $t_{1/2}$ (min)[a]	Rat plasma, $t_{1/2}$ (min)[a]	Rat brain homogenate, $t_{1/2}$ (min)[a]
3	$Br(CH_2)_{11}CO$	F	1.9	NM[b]	ND[c]
4	$MeO(CH_2)_{11}CO$	F	2.2	0.8	13.9
5	$CH_3CH_2S(CH_2)_{11}CO$	F	3.8	1.0	ND
6	$CH_3CH_2S(CH_2)_{10}CO$	F	2.0	2.1	11.7
7	$CH_3(CH_2)_{12}CO$	F	20.3	4.6	17.5
8	$N_3(CH_2)_{11}CO$	F	4.4	0.8	21.9

[a]$T_{1/2}$ is the time required for 50% hydrolysis of the ester at 37 °C upon incubation with the specified esterase (mean, n = 3). In all cases, the regression equation obtained had r = 0.99. [b]NM = Not measurable (very rapid hydrolysis). [c]ND = Not determined.

esters **(3-8)** had lower toxicity and higher selectivity than FLT, (ii) the lipophilicities of these prodrugs are greater than that of the parent drug; which may permit better penetration of the drug through biological membranes, and (iii) hydrolysis of the ester analogues is influenced by the nature of the myristoyl moiety at the 5'-position. Although facile *in vitro* cleavage of the esters was observed, it may be possible to control the rate of ester hydrolysis *in vivo*, which would influence the blood half-life and toxicity, by selecting the appropriate myristoyl moiety.

EXPERIMENTAL SECTION

12-Bromododecanoic acid, myristic acid, *n*-octanol and all other reagents and chemicals were purchased from the Aldrich Chemical Co, unless noted otherwise. *n*-Octanol (99%), used for measurement of P values, was distilled before use. Phosphate buffer solution (0.2 M, pH = 7.4) was used for measurement of P values. All solvents used for HPLC analyses were of HPLC grade, purchased from Mallinckrodt and degassed before use. All solvents used were analytical grade. Porcine liver esterase (EC 3.1.1.1) suspension in 3.2 M $(NH_4)_2SO_4$ (200 units/mg protein) was purchased from the Sigma Chemical Co. 3'-Azido-2',3'-dideoxythymidine (AZT, **1**) and 3'-fluoro-2',3'-dideoxythymidine (FLT, **2**), prepared using the literature procedures[29,34] , were used as reference compounds in several experiments.

Thin layer chromatography (TLC) was performed using Whatman MK6F silica gel microslides (250 μm thickness). Quantitative and qualitative analyses of samples for all experiments described were performed using a high performance liquid chromatography (HPLC) system comprised of a Waters baseline 810 computer program operating on a 486/33 MHz computer, a Waters model 501 pump, Waters model U6K injector, Hewlett Packard 1040A photodiode-detector or Waters 486 variable wavelength detector and a HP 79994A workstation or a Waters system interface module. The UV detector was set to monitor absorbance at 265 nm. HPLC analyses for *in vitro* studies of the esters and measurement of retention times for the esters were performed using a Waters Radial-Pak C18 reverse phase cartridge column (8 mm ID × 10 cm length, 10 μm particle size) at 25 °C with methanol:water (75:25, v/v) as eluent for retention time measurements, and methanol:water or acetonitrile:water (70:30, v/v) as eluent for *in vitro* studies, at a flow rate of 1 mL/min. Melting points were determined with a Büchi capillary apparatus and are uncorrected. Nuclear magnetic resonance spectra (^{1}H NMR, ^{13}C NMR) were determined on a Bruker AM-300 spectrometer. The assignment of all exchangeable protons (O*H*, N*H*) 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. Quantitative ultraviolet (UV) absorption spectra were recorded

on a Shimadzu UV 160 spectrophotometer. Microanalyses, performed by the Chemistry Department, University of Alberta using an EA1108-Elemental Analyzer (Carlo Erba Instruments), were within ± 0.4% of theoretical values for all elements listed, unless otherwise indicated.

General Procedure for the 5'-*O*-esterification of FLT (3-8).

A solution of the fatty acid selected (1.3 mmol) and oxalyl chloride (0.25 g, 1.95 mmol) in anhydrous benzene (18 mL) was stirred at 25 °C for 1 h and the solvent from the yellow solution was removed *in vacuo*. The residual oil was dissolved in benzene (18 mL) and the solution was added dropwise to an ice-cold, stirred solution of FLT (0.32 g, 1.3 mmol) and 4-dimethylaminopyridine (DMAP) (0.23 g, 1.9 mmol) in anhydrous benzene (18 mL) under anhydrous conditions. This solution was stirred at ice bath temperature for 1 h and then heated under reflux for 3 h. The mixture was cooled to 25 °C and benzene (72 mL) was added. The organic solution was washed with saturated aqueous sodium carbonate (2 × 11 mL) and then with water (2 × 11 mL). The organic layer was dried (Na_2SO_4) and the solvent was removed *in vacuo*. The residue, consisting of one major product, was purified by silica gel chromatography using chloroform as eluent to yield the respective product **(3-8)**. The physical properties, NMR spectral data and microanalytical analyses for products **3-8** are listed below.

3'-Fluoro-2',3'-dideoxy-5'-*O*-(12-bromododecanoyl)thymidine (3).

Oil; yield (343 mg, 52%); ^{1}H NMR ($CDCl_3$) δ 1.20-1.50 (br m, 14H, methylene envelope), 1.58-1.70 (m, J = 7.3 Hz, 2H, CH_2CH$_2$CO), 1.86 (quintet, J = 6.8, J = 7.3 Hz, 2H, CH_2CH$_2$Br), 1.94 (d, $J_{5\text{-}CH_3,6}$ = 1.0 Hz, 3H, 5-CH_3), 2.10 (dddd, $J_{2'',F}$ = 35.5, J_{gem} = 14.5, $J_{2'',1'}$ = 8.9, $J_{2'',3'}$ = 5.5 Hz, 1H, H-2''), 2.35 (t, J = 7.3 Hz, 2H, CH_2CO), 2.69 (ddd, $J_{2',F}$ = 20.5, J_{gem} = 14.5, $J_{2',1'}$ = 5.7 Hz, 1H, H-2'), 3.42 (t, J = 6.8 Hz, 3H, CH_2Br), 4.27 and 4.40 (two dd, J_{gem} = 12.2, $J_{5',4'}$ = 4.3 Hz, 1H each, H-5'), 4.47 (dt, $J_{4',F}$ = 25.7, $J_{4',5'}$ = 4.3 Hz, 1H, H-4'), 5.18 (dd, $J_{3',F}$ = 52.5, $J_{3',2''}$ = 5.5 Hz, 1H, H-3'), 6.34 (dd, $J_{1',2''}$ = 8.9, $J_{1',2'}$ = 5.7 Hz, 1H, H-1'), 7.26 (d, $J_{6,5\text{-}CH_3}$ = 1.0 Hz, 1H, H-6),

9.14 (s, 1H, N-*H*); ^{13}C NMR ($CDCl_3$) δ 12.60 (5-*C*H_3), 24.77, 26.80, 28.09, 28.66, 28.78, 29.05, 29.13, 29.32 (methylene carbons), 34.08 (*C*H_2COO), 38.29 (C-2', $J_{2',F}$ = 21.7 Hz), 45.10 (*C*H_2Br), 63.23 (C-5', $J_{5',F}$ = 10.5 Hz), 82.43 (C-4', $J_{4',F}$ = 25.72 Hz), 85.37 (C-1'), 93.40 (C-3', $J_{3',F}$ = 179.3 Hz), 111.42 (C-5), 134.63 (C-6), 150.12 (C-2 *C*=O), 163.47 (C-4 *C*=O), 172.92 (*C*OO). Anal. calcd. for $C_{22}H_{34}BrFN_2O_5$: C 52.28, H 6.78, N 5.54 ; Found: C 52.49, H 7.02, N 5.43.

3'-Fluoro-2',3'-dideoxy-5'-*O*-(12-methoxydodecanoyl)thymidine (4).

Yield (598 mg, 58%); mp 56-57 °C; ^{1}H NMR ($CDCl_3$) δ 1.20-1.42 (br m, 14H, methylene envelope), 1.50-1.72 (m, 4H, C*H*$_2$CH$_2$O, C*H*$_2$CH$_2$CO), 1.94 (d, $J_{5\text{-}CH_3,6}$ = 1.0 Hz, 3H, 5-C*H*$_3$), 2.09 (dddd, $J_{2'',F}$ = 36.5, J_{gem} = 14.7, $J_{2'',1'}$ = 8.9, $J_{2'',3'}$ = 5.4 Hz, 1H, H-2''), 2.35 (t, *J* = 7.6 Hz, 2H, C*H*$_2$CO), 2.69 (ddd, $J_{2',F}$ = 20.8, J_{gem} = 14.7, $J_{2',1'}$ = 5.4 Hz, 1H, H-2'), 3.34 (s, 3H, OC*H*$_3$), 3.37 (t, *J* = 6.6 Hz, 2H, OC*H*$_2$CH$_2$), 4.37 and 4.41 (two dd, J_{gem} = 12.3, $J_{5',4'}$ = 4.1 Hz, 1H each, H-5'), 4.45 (dt, $J_{4',F}$ = 25.5, $J_{4',5'}$ = 4.1 Hz, 1H, H-4'), 5.18 (dd, $J_{3',F}$ = 53.4, $J_{3',2''}$ = 5.3 Hz, 1H, H-3'), 6.34 (dd, $J_{1',2''}$ = 8.9, $J_{1',2'}$ = 5.4 Hz, 1H, H-1'), 7.26 (d, $J_{6,5\text{-}CH_3}$ = 1.0 Hz, 1H, H-6), 9.60 (s, 1H, N-*H*); ^{13}C NMR ($CDCl_3$) δ 12.57 (5-*C*H_3), 24.78, 26.06, 29.05, 29.13, 29.32, 29.40, 29.46, 29.55 (methylene carbons), 34.08 (*C*H_2COO), 38.29 (C-2', $J_{2',F}$ = 21.2 Hz), 58.43 (OCH_3), 63.23 (C-5', $J_{5',F}$ = 9.8 Hz), 72.99 (OCH_2), 82.43 (C-4', $J_{4',F}$ = 26.7 Hz), 85.35 (C-1'), 93.41 (C-3', $J_{3',F}$ = 180.3 Hz), 111.42 (C-5), 134.60 (C-6), 150.18 (C-2 *C*=O), 163.52 (C-4 *C*=O), 172.94 (*C*OO). Anal. calcd. for $C_{23}H_{37}FN_2O_6$: C 60.51, H 8.17, N 6.14 ; Found: C 60.28, H 8.43, N 6.12

3'-Fluoro-2',3'-dideoxy-5'-*O*-(12-thioethyldodecanoyl)thymidine (5).

Yield (339, 53%); mp 82-83 °C; ^{1}H NMR ($CDCl_3$) δ 1.23-1.48 (br m, 14H, methylene envelope), 1.26 (t, *J* = 7.1 Hz, 3H, CH$_2$C*H*$_3$), 1.50-1.72 (m, 4H, C*H*$_2$CH$_2$S, C*H*$_2$CH$_2$CO), 1.94 (d, $J_{5\text{-}CH_3,6}$ = 1.3 Hz, 3H, 5-C*H*$_3$), 2.09 (dddd, $J_{2'',F}$ = 35.6, J_{gem} = 14.0, $J_{2'',1'}$ = 9.0, $J_{2'',3'}$ = 5.5 Hz, 1H, H-2''), 2.34 (t, *J* = 7.3 Hz, 2H, C*H*$_2$CO), 2.51 (t, *J*

= 7.3 Hz, 2H, CH_2CH_2S), 2.53 (q, J = 7.1 Hz, 2H, CH_3CH_2S), 2.68 (ddd, $J_{2',F}$ = 21.0, J_{gem} = 14.0, $J_{2',1'}$ = 5.6 Hz, 1H, H-2'), 4.26 and 4.39 (two dd, J_{gem} = 12.2, $J_{5',4'}$ = 4.0 Hz, 1H each, H-5'), 4.45 (dt, $J_{4',F}$ = 25.5, $J_{4',5'}$ = 4.0 Hz, 1H, H-4'), 5.17 (dd, $J_{3',F}$ = 52.5, $J_{3',2''}$ = 5.5 Hz, 1H, H-3'), 6.32 (dd, $J_{1',2''}$ = 9.0, $J_{1',2'}$ = 5.6 Hz, 1H, H-1'), 7.24 (d, $J_{6,5\text{-}CH_3}$ = 1.2 Hz, 1H, H-6), 8.97 (s, 1H, N-*H*); ^{13}C NMR ($CDCl_3$) δ 12.63 (5-*C*H_3), 14.79 (*C*H_3CH_2S), 24.82, 25.93, 28.90, 29.09, 29.19, 29.37, 29.45, 29.62, 31.68 (methylene carbons), 34.11 (*C*H_2COO), 38.33 (C-2', $J_{2',F}$ = 22.1 Hz), 63.26 (C-5', $J_{5',F}$ = 10.1 Hz), 82.46 (C-4', $J_{4',F}$ = 25.7 Hz), 85.39 (C-1'), 93.40 (C-3', $J_{3',F}$ = 179.3 Hz), 111.45 (C-5), 134.65 (C-6), 150.10 (C-2 *C*=O), 163.42 (C-4 *C*=O), 172.97 (*C*OO). Anal. calcd. for $C_{24}H_{39}FN_2O_5S$: C 59.23 , H 8.08, N 5.76; Found: C 59.52, H 8.37, N 5.62.

3'-Fluoro-2',3'-dideoxy-5'-*O*-(11-thioethylundecanoyl)thymidine (6).

Oil; yield (455 mg, 74 %); ^{1}H NMR ($CDCl_3$) δ 1.22-1.44 (br m, 12H, methylene envelope), 1.24 (t, J = 7.2 Hz, 3H, CH_3), 1.50-1.74 (m, 4H, CH_2CH_2S, CH_2CH_2CO), 1.93 (d, $J_{5\text{-}CH_3,6}$ = 1.3 Hz, 3H, 5-CH_3), 2.08 (dddd, $J_{2'',F}$ = 36.0, J_{gem} = 14.0, $J_{2'',1'}$ = 9.0, $J_{2'',3'}$ = 4.9 Hz, 1H, H-2''), 2.34 (t, J = 7.5 Hz, 2H, CH_2CO), 2.50 (t, J = 7.3 Hz, 2H, CH_2CH_2S), 2.51 (q, J = 7.2 Hz, 2H, CH_3CH_2S), 2.68 (ddd, $J_{2',F}$ = 20.5, J_{gem} = 14.0, $J_{2',1'}$ = 5.5 Hz, 1H, H-2'), 4.25 and 4.38 (two dd, J_{gem} = 12.0, $J_{5',4'}$ = 4.1 Hz, 1H each, H-5'), 4.45 (dt, $J_{4',F}$ = 25.5, $J_{4',5'}$ = 4.1 Hz, 1H, H-4'), 5.16 (dd, $J_{3',F}$ = 52.5, $J_{3',2''}$ = 4.9 Hz, 1H, H-3'), 6.31 (dd, $J_{1',2''}$ = 9.0, $J_{1',2'}$ = 5.5 Hz, 1H, H-1'), 7.24 (d, $J_{6,5\text{-}CH_3}$ = 1.3 Hz, 1H, H-6), 8.71 (s, 1H, N-*H*); ^{13}C NMR ($CDCl_3$) δ 12.56 (5-*C*H_3), 14.73 (*C*H_3CH_2S), 24.73, 25.84, 28.80, 29.00, 29.10, 29.23, 29.31, 29.53, 31.59 (methylene carbons), 34.02 (*C*H_2COO), 38.24 (C-2', $J_{2',F}$ = 22.0 Hz), 63.21 (C-5', $J_{5',F}$ = 10.8 Hz), 82.39 (C-4', $J_{4',F}$ = 26.9 Hz), 85.33 (C-1'), 93.40 (C-3', $J_{3',F}$ = 180.4 Hz), 111.39 (C-5), 134.62 (C-6), 150.25 (C-2 *C*=O), 163.67 (C-4 *C*=O), 172.91 (*C*OO). Anal. calcd. for $C_{23}H_{37}FN_2O_5S$: C 58.45, H 7.89, N 5.93; Found: C 58.58, H 8.14, N 5.90.

3'-Fluoro-2',3'-dideoxy-5'-*O*-(tetradecanoyl)thymidine (7).

Yield (561 mg, 95%); mp 62.5-64.0 °C; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J = 6.6 Hz, 3H,

C*H*$_3$), 1.20-1.52 (br m, 20H, methylene envelope), 1.63 (quintet, J = 7.5, J = 7.3 Hz, 2H, C*H*$_2$CH$_2$CO), 1.93 (d, $J_{5\text{-}CH_3,6}$ = 1.0 Hz, 3H, 5-C*H*$_3$), 2.10 (dddd, $J_{2'',F}$ = 36.6, J_{gem} = 14.5, $J_{2'',1'}$ = 8.9, $J_{2'',3'}$ = 4.6 Hz, 1H, H-2''), 2.34 (t, J = 7.5 Hz, 2H, C*H*$_2$CO), 2.69 (ddd, $J_{2',F}$ = 21.4, J_{gem} = 14.5, $J_{2',1'}$ = 5.5 Hz, 1H, H-2'), 4.27 and 4.38 (two dd, J_{gem} = 12.2, $J_{5',4'}$ = 4.0 Hz, 1H each, H-5'), 4.47 (dt, $J_{4',F}$ = 27.5, $J_{4',5'}$ = 4.0 Hz, 1H, H-4'), 5.18 (dd, $J_{3',F}$ = 53.1, $J_{3',2''}$ = 4.6 Hz, 1H, H-3'), 6.33 (dd, $J_{1',2''}$ = 8.9, $J_{1',2'}$ = 5.5 Hz, 1H, H-1'), 7.27 (d, $J_{6,5\text{-}CH_3}$ = 1.0 Hz, 1H, H-6), 9.45 (s, 1H, N-*H*); ^{13}C NMR ($CDCl_3$) δ 12.59 (5-*C*H$_3$), 14.09 (*C*H$_3$), 22.71, 24.81, 24.91, 29.17, 29.25, 29.27, 29.36, 29.46, 29.65, 31.94, 33.95 (methylene carbons), 34.18 (*C*H$_2$COO), 38.41 (C-2', $J_{2',F}$ = 22.0 Hz), 63.23 (C-5', $J_{5',F}$ = 9.9 Hz), 82.56 (C-4', $J_{4',F}$ = 26.4 Hz), 85.55 (C-1'), 93.39 (C-3', $J_{3',F}$ = 180.18 Hz), 111.39 (C-5), 134.74 (C-6), 150.10 (C-2 *C*=O), 163.63 (C-4 *C*=O), 172.87 (*C*OO); ^{19}F NMR ($CDCl_3$) (C_6F_6 external std) δ -13.04 (dddd, $J_{F,3'}$ = 53.1, $J_{F,2''}$ = 36.6, $J_{F,4'}$ =27.5, $J_{F,2'}$= 21.4 Hz, 1F, F-3'). Anal. calcd. for $C_{24}H_{39}FN_2O_5$: C 63.41, H 8.65, N 6.16; Found: C 63.71, H 8.70, N 6.16.

3'-Fluoro-2',3'-dideoxy-5'-*O*-(12-azidododecanoyl)thymidine (8).

General procedure; oil; yield (556 mg, 91%); Using an alternative method, a solution of 3'-fluoro-2',3'-dideoxy-5'-*O*-(12-bromomyristoyl)thymidine (**3**, 164 mg, 0.3 mmol) and sodium azide (28 mg, 0.4 mmol) in DMF (3.3 mL) were stirred for 6 h at 25 °C under a nitrogen atmosphere. The solvent was removed *in vacuo*, the residue was dissolved in water (5 mL) and extracted with chloroform (3 × 5 mL). After drying with sodium sulfate and removal of chloroform *in vacuo*, the residue consisting of one major product was purified by silica gel chromatography using methylene chloride/ethyl acetate (80:20, v/v) as eluent to yield **8** (120 mg, 80%) as a colorless foam. ^{1}H NMR ($CDCl_3$) δ 1.16-1.46 (br m, 14H, methylene envelope), 1.50-1.70 (m, 4H, C*H*$_2$CH$_2$CO, C*H*$_2$CH$_2$N$_3$) 1.94 (d, $J_{5\text{-}CH_3,6}$ = 1.2 Hz, 3H, 5-C*H*$_3$), 2.10 (dddd, $J_{2'',F}$ = 35.6, J_{gem} = 14.2, $J_{2'',1'}$ = 8.9, $J_{2'',3'}$ = 5.2 Hz, 1H, H-2''), 2.35 (t, J = 7.4 Hz, 2H, C*H*$_2$CO), 2.69 (ddd, $J_{2',F}$ = 21.1, J_{gem} = 14.2, $J_{2',1'}$ = 5.5 Hz, 1H, H-2'), 3.26 (t, J = 7.8 Hz, 3H, C*H*$_2$N$_3$), 4.27 and 4.40 (two dd, J_{gem} = 12.0, $J_{5',4'}$ = 3.9 Hz, 1H each, H-5'), 4.47 (dt, $J_{4',F}$ = 26.0, $J_{4',5'}$ = 3.9 Hz, 1H, H-4'),

5.18 (dd, $J_{3',F}$ = 53.5, $J_{3',2''}$ = 5.2 Hz, 1H, H-3'), 6.34 (dd, $J_{1',2''}$ = 8.9, $J_{1',2'}$ = 5.5 Hz, 1H, H-1'), 7.26 (d, $J_{6,5\text{-}CH_3}$ = 1.2 Hz, 1H, H-6), 9.00 (s, 1H, N-*H*); ^{13}C NMR ($CDCl_3$) δ 12.54 (5-CH_3), 24.73, 26.59, 27.23, 28.72, 29.00, 29.07, 29.26, 29.31 (methylene carbons), 34.02 (CH_2COO), 38.24 (C-2', $J_{2',F}$ = 21.7 Hz), 51.39 (CH_2N_3), 63.21 (C-5', $J_{5',F}$ = 21.7 Hz), 82.39 (C-4', $J_{4',F}$ = 26.92 Hz), 85.33 (C-1'), 93.40 (C-3', $J_{3',F}$ = 179.3 Hz), 111.38 (C-5), 134.63 (C-6), 150.29 (C-2 *C*=O), 163.72 (C-4 *C*=O), 172.91 (*C*OO). Anal. calcd. for $C_{24}H_{34}FN_5O_5$: C 56.52, H 7.32, N 14.98; Found: C 56.21, H 7.61, N 14.58.

***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 by the United States National Institutes of Health (NIH) testing program using the reported procedure.[35] 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* cytotoxic assay.**

The cytotoxicity of the test compounds (prodrug esters of FLT) against the T4 lymphocyte CEM cell line was determined by the United States National Institutes of Health (NIH) testing program using the reported procedure.[35] This assay determines the percentage of surviving uninfected cells exposed to the test compound relative to uninfected, unexposed controls using the tetrazolium salt (XTT) assay. XTT was added to the cell culture and samples were analyzed spectrophotometrically (λ = 450 nm) to quantitate formazan production.

Physicochemical calculations.

Determination of partition coefficients (Log P).

The test compound (four different concentrations, 0.15-0.96 mg/5 mL) was partitioned between presaturated *n*-octanol (5 mL) and phosphate buffer (10 mL, 0.2 M,

pH = 7.4) for 21 h at 25 °C using a mechanical shaker and the two phases were then separated. The concentration of test compound in the *n*-octanol phase, before and after phosphate buffer partitioning, was determined using the procedure (UV quantitation at 265 nm) reported previously.[36] Partition coefficients (P) were calculated as the ratio of the concentration in the *n*-octanol (moles/volume) to the concentration in the phosphate buffer phase (moles/volume) ($P = C_{n\text{-octanol}}/C_{water}$).

Calculation of physicochemical properties.

Physicochemical properties were estimated using the PALLAS computational program (PrologD 2.0 for windows, PrologP 5.1 for Windows 1995, Compudrug Chemistry Ltd, Hollàn Ernö utca 5., H-1136 Budapest, Hungary). The PrologP 5.1 program module was used to calculate Log P values for myristate esters in an *n*-octanol/water system. To confirm the validity of the Log P calculations, experimental data were compared with calculated data for ester prodrug **4** (Table 2). The PrologD 2.0 program module was used to predict the logarithm of the distribution coefficient, Log $D_{7.4}$ from the compound structure. To perform these calculations, the test compound structure is drawn graphically, after which the computational program PrologD automatically calculates *pKa*, Log P and Log $D_{7.4}$ by activating the *pK*alc, PrologP and PrologD modules of PALLAS.

Stability of esters in the presence of porcine liver esterase.

Incubation of the ester prodrug with porcine liver esterase (Sigma EC 3.1.1.1) was used to measure the prodrug half-life, which is dependent upon the rate of ester hydrolysis, using a previously reported procedure.[30]

Preparation of biological media (rat plasma and brain homogenate) for kinetic enzyme studies.

Male Sprague Dawley rats (250-280 g) were anesthetized by intraperitoneal injection of pentobarbital. Blood samples were obtained by heart puncture, and collected in heparinized tubes, from the anesthetized rats. The blood was centrifuged at 1500 × *g* for

15 min, and the plasma fraction was stored at 0-5 °C for no longer than 30 h prior to use in the *in vitro* experiments.

The entire brain was removed after incision of the skull, rinsed with 15 mL saline, dried on a filter paper, weighed on a watch glass and then homogenized in phosphate buffer (pH = 7.4) (brain:phosphate buffer; 1:5 w/v) using a con-Torque power unit for 10-20 min in a glass homogenizer with a radially-serated Teflon pestle. The homogenate was centrifuged at 1500 × *g* for 10 min, and the supernatant was used for the experimental studies. The temperature was maintained at 0-5 °C during the preparation and storage of the brain homogenate. These preparations were stored for no longer than 30 h prior to use.

Stability of esters in the presence of tissue preparations (rat plasma and brain homogenate).

Bioactivation experiments were performed at 37 °C in the presence of various enzymes preparations diluted with phosphate buffer. The experiments were initiated by adding an aliquot of a stock solution of the ester in ethanol 98% (60 μL, 9.04×10^{-3} M for plasma, 0.014 M for brain homogenate) to the enzyme preparations (1 mL, plasma:phosphate buffer, 1:9 v/v, pH = 7.4, or brain:phosphate buffer, 1:5 w/v) previously equilibrated at 37 °C in a shaker bath, and mixed thoroughly to generate a final concentration of 5.11×10^{-4} M for plasma and 7.87×10^{-4} M for brain homogenate. At appropriate times (2, 4, 6, 8, 10, 12 min for plasma, and 2, 4, 6, 8, 10, 20, 120, 240 min for brain homogenate), an aliquot of the reaction mixture (100 μL) was withdrawn, ice-cold methanol (200 μL) was added to stop the reaction, and the mixture was vortexed. The resultant mixtures were placed on ice for 10 min before centrifugation (1500 × *g*) for 5 min to remove precipitated proteins. The supernatant layers were filtered through a 0.45 μm filter, and a 200 μL aliquot was subjected to HPLC analysis. Time-dependent decreases in the concentration of the ester due to hydrolysis were used to calculate pseudo-first-order rate constants. Half-lives were calculated using the equation $t_{1/2}$ = 0.693/rate constant. It was not possible to determine the change in concentration of FLT released from the prodrugs upon incubation with brain homogenate and plasma by HPLC due to interfering peaks that precluded precise quantification.

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