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In vitro antiviral activities of myristic acid analogs against human immunodeficiency and hepatitis B viruses

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Abstract

A group of myristic acid analogs, designed as alternative substrates for *N*-myristoyltransferase (NMT), were evaluated against human immunodeficiency virus (HIV), hepatitis B virus (HBV) and duck hepatitis B virus (DHBV) in vitro. Antiviral potency was increased when S or O was substituted for $-CH_2-$ in myristic acid and selectivity was affected by the presence and position of the heteroatoms and phenyl groups. A correlation was established among anti-HIV activity, Log P and Log $D_{7.4}$ and between anti-HIV activity and carbonyl-heteroatom interatomic distances in the myristoyl analogs. 12-Thioethyldodecanoic acid **6** was moderately active ($EC_{50} = 9.37$ μM) against HIV-infected T4-lymphocytes (CEM-SS cell line), and it exhibited in vitro activity ($EC_{50} = 17.8$ μM) against HBV-producing 2.2.15 cell cultures derived from a human hepatoblastoma cell line (Hep G2). 12-Methoxydodecanoic acid **1** exhibited in vitro activity ($EC_{50} = 20–30$ μM) against hepatitis B in the HBV DNA-transfected 2.2.15 cell line. At a concentration of 10 μg/ml, none of the fatty acids significantly inhibited the replication of DHBV in infected hepatocytes. © 1997 Elsevier Science B.V.

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1. Introduction

Human immunodeficiency virus (HIV) and hepatitis B virus (HBV) are prominent infections which warrant the search for novel therapeutic

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targets against these viruses. Myristoyl CoA:protein *N*-myristoyltransferase (NMT) is an attractive target for antiviral therapy to inhibit HIV-1 replication in AIDS patients and HBV replication in individuals infected by HBV (Fig. 1). Protein *N*-myristylation in viruses is catalyzed by NMT (E.C. 2.3.1.97) and 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 (Paige et al., 1990). The reaction mechanism is highly ordered, a binary complex of NMT:myristoyl-CoA is formed initially that is followed by subsequent binding to a peptide substrate. Formation of a myristoyl-CoA.NMT.peptide ternary complex is then followed by transfer of myristate from CoA to the peptide (Heuckeroth et al., 1988; Rocque et al., 1993) (Fig. 1).

Covalent myristylation of eukaryotic viral proteins by retroviruses and hepadnaviruses has been observed. These myristylated proteins include matrix proteins such as $Pr160^{gag\text{-}pol}$, $Pr55^{gag}$, the capsid protein P17 derived from proteolytic processing of *gag* and negative factor (*nef*) proteins in HIV (Bryant et al., 1993), Pre-S1 protein in the membrane envelope of HBV (Persing et al., 1987), and surface (S) protein and presurface (S) protein in the envelope of the duck hepatitis B virus (DHBV) (Macrae et al., 1991). Addition of a tetradecanoyl group is essential for assembly/replication of HIV, Moloney murine leukemia virus and poliovirus, and for production of infectious virions in the case of HBV (Kishore et al., 1993). For example, myristylation of retrovirus *gag* polypeptides is essential for either the assembly or budding of virus particles at membranes and for the release of preformed capsids. Myristylation of the preS protein of DHBV is required for virion infectivity, but not for assembly or release (Marc et al., 1990).

Myristic acid analogs that are incorporated to produce *N*-acylproteins may function as antiviral agents by perturbing viral protein function (Langner et al., 1992). A number of saturated and aromatic fatty acids have therefore been tested to determine anti-HIV and anti-HBV activities. In vitro antiviral structure–activity relationships for this group of myristic acid analogs are now described.

2. Material and methods

2.1. Compounds

Previously reported methods were used to synthesize 12-methoxydodecanoic acid **1**, 11-methoxyundecanoic acid **2** (Gordon et al., 1991), 4-oxatetradecanoic acid **3**, 9-oxatetradecanoic acid **4**, 12-thioethyldodecanoic acid **6** (Kishore et al., 1991), 11-thioethylundecanoic acid **7** (Gordon et al., 1991), 9-thiatetradecanoic acid **8** (Kishore et al., 1991), 12-phenyldodecanoic acid **15** (Goodman et al., 1984; Eisenhut and Liefhold, 1988), 10-phenyldecanoic acid **16**, 11-phenoxyundecanoic acid **18** (Diekman et al., 1969), 12-azidododecanoic acid **20** (Adams et al., 1992) and 2-fluorotetradecanoic acid **23** (Paige et al., 1990). Methods for the synthesis and analysis of 4-oxa-12-chlorododecanoic acid **5**, 12-iodododecanoic acid **9**, 12-chlorododecanoic acid **11**, 12-fluorododecanoic acid **12**, 12-phenoxydodecanoic acid **17**, 11-(4-iodophenoxy)undecanoic acid **19**, 2-iodotetradecanoic acid **24** and 2-chlorotetradecanoic acid **25** have been described previously (Parang et al., 1996). Compounds **5**, **17** and **19** are novel fatty acids which were synthesized in our group (Parang et al., 1996). 12-Bromododecanoic acid **10**, 12-hydroxydodecanoic acid **13**, 11-aminoundecanoic acid **14**, 2-bromotetradecanoic acid **21** and myristic acid **22** were purchased from

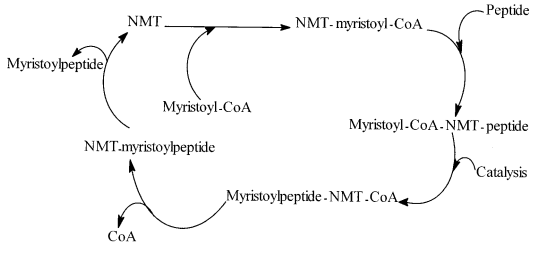

Fig. 1. Myristylation of viral peptides by Myristoyl-CoA: protein NMT.

the Aldrich Chemical Company. All other reagents and chemicals were obtained from Aldrich unless otherwise noted. 2′, 3′-Dideoxycytidine (ddC), 3′-azido-3′-deoxythymidine (AZT) and 2′,3′-dideoxy-3′-thiacytidine (3TC) were used as reference compounds in the antiviral assays employed.

2.2. Physicochemical properties

Physicochemical properties were estimated using the PALLAS computational program (PrologD 2.0 for Windows, pKalc 3.1 for Windows, PrologP 5.1 for Windows 1995. Compudrug Chemistry, Hollàn Ernöutca 5., H-1136 Budapest, Hungary). The pKalc 3.1, an add-on module of the PALLAS system, estimates acidity/basicity and calculates pK_a values. The validity of calculated data was established by comparing the experimental values of a few fatty acids with their pK_a values, as previously explained (Parang et al., 1996). The PrologP 5.1 program module was used to calculate the Log P values for myristic acid analogs in an octanol/water system. To confirm the validity of the Log P calculations, experimental data were compared with calculated data for selected fatty acids (Parang et al., 1996). The PrologD 2.0 program module was used to predict the logarithm of the distribution coefficient, Log $D_{7.4}$ from the compound structure. The distribution coefficient (D) considers the partitioning of all ionic species of a compound. In order to calculate the Log $D_{7.4}$ value for a test compound, both the logarithms of the partition coefficient (Log P) and the dissociation constant(s) (pK_a) are needed. 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 pKalc, PrologP and PrologD modules of PALLAS. Interatomic distances between the CO and terminal heteroatom moieties were measured after AM1 optimization using the Hyperchem Release 4.0 computational program.

2.3. In vitro anti-HIV assay

A number of the fatty acids (**9**–**14**, **17**–**19**) described have not been tested previously against HIV. Some of the fatty acids (**1**–**4**, **20**–**22**), for which in vitro anti-HIV test results have been reported (Bryant et al., 1989, 1991), were evaluated using assay procedures that are different from those used in this study. The ability of the test compound to protect HIV-1-infected T4-lymphocytes (CEM cells) was determined by the United States National Institutes of Health (NIH) testing service using the reported procedure (Weislow et al., 1989). The syncytium formation assay described by Nara et al. (1987) was used in the NIH anti-HIV screen for quantitation of the number of virus-producing cells in an infected H9 culture where 80–90% of these cells were producing virus. Typical concentrations of infectious virus were 3×10^5 syncytium-forming units (SFU)/ml for the III_b variant of HIV-1 and 5×10^5 SFU/ml for the RF variant.

Sensitivity to the lytic effects of HIV-1 infection was determined in preliminary experiments for each host cell line by titrations of cell-free virus or H9 cells chronically infected with HIV-1. In studies employing cell-free virus infections, the amount of virus added was such that the final multiplicity of infection (MOI; ratio of number of infectious virus particles/number of target cells) was the lowest MOI yielding 70% suppression of XTT formazan production in host cells at 7 days. In the cell-free virus assays the MOIs, which were established by syncytium formation analysis were 0.1 for CEM-SS.

1. All stock solutions were prepared in 100% dimethyl sulfoxide at the highest achievable concentration for each agent, then diluted 1:100 in cell culture medium before preparing serial half-log_{10} dilutions. T4-lymphocytes (CEM cell line) were added and after a brief interval HIV-1 was added, resulting in a 1:200 final dilution of the compound. Initial drug dilutions (1:200) resulted in a culture maximum concentration for dimethyl sulfoxide which had no apparent direct toxic effects on the cell lines used or the HIV-1 infection.

2. Cultures were incubated at 37°C in a 5% carbon dioxide atmosphere for 6 days.

3. The tetrazolium salt, XTT, was added to all wells and cultures were incubated to allow formazan color development by viable cells.

4. Individual wells were analyzed spectrophotometrically to quantitate formazan production and also viewed microscopically for detection of viable cells and confirmation of protective activity.

5. Drug-treated virus-infected cells were compared with drug-treated non-infected cells and with other appropriate controls (untreated infected and untreated non-infected cells, drug-containing wells without cells, etc.) on the same plate.

6. Data were reviewed in comparison with other tests done at the same time and a determination of activity was made.

In this assay, agents that interact with virions, cells, or virus gene products to interfere with viral activities will protect cells from cytolysis. Nine dilutions of the compounds were used for each test and different ranges of concentrations for each compound. The assay was repeated twice for most analogs, but five times for **6**, **9** and **10**. The data for all compounds were compared with a positive (AZT-treated) control performed at the same time under identical conditions.

2.4. In vitro anti-HBV assays

Two assay methods were used to determine activity against HBV.

2.4.1. Analysis of test compounds against HBV replication in cultures of 2.2.15 cells

A screening service was provided by the United States NIH (Korba and Milman, 1991). The protocol for assaying anti-HBV activity in cultures of 2.2.15 cells is briefly summarized as follows. Chronically HBV-producing human liver cells (Acs et al., 1987) 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 for 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).

2.4.1.1. Assay parameters. Both intracellular and extracellular HBV DNA were analyzed in order to: (i) allow for verification of compound efficacy; and (ii) provide potential data on the target site in the HBV replication pathway for the compound based on examination of viral replicative forms. The culture medium was changed daily during the treatment period to: (i) prevent the buildup of potentially toxic metabolites derived from the test compound; and (ii) provide an analysis of HBV virion production during discrete 24 h intervals which enables a quantitative comparison of any effect on virion production. The analysis of HBV DNA was performed using blot hybridization techniques (southern and slot blot) and ^{32}P-labelled HBV-specific probes. HBV DNA levels were measured by comparison to known amounts of HBV DNA standards applied to each nitrocellulose membrane (gel or slot blot). An AMBIS beta scanner, which measures the radioactive decay of the hybridized probes directly on the nitrocellulose membranes, was used for the quantitative analysis. Standard curves, generated by multiple analyses, were used to correlate CPM measurements made by the beta scanner with relative levels of target HBV DNA. The levels of HBV virion DNA released into the culture medium were analyzed by a slot blot hybridization procedure. HBV DNA levels were then compared to those at day 0 to determine the effect of drug treatment.

2.4.2. In vitro assay for screening drugs against HBV and DHBV

2.4.2.1. Ducks. Congenital fertilized Peking 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. Sera from newly hatched ducklings were screened for the presence of DHBV DNA by dot hybridization (Lee et al., 1989).

2.4.2.2. Cell cultures. 1) Primary duck hepatocytes: primary cultures of duck hepatocytes were prepared from 9–14-day-old DHBV ducklings using a modified method of Tuttleman et al. (1986). Cells were cultured in 60 mm cell culture dishes in 4 ml L-15 medium containing 5% fetal bovine serum, penicillin G sodium 10 IU/ml, streptomycin sulfate 10 μg/ml and nystatin 25 U/ml.

2) HepG2 cell culture: the hepatoblastoma cell line HepG2 was obtained from the American type cell culture collection (ATCC) (Rockville, MD). The monolayer cultures were grown in MEM supplemented with 750 mg/l sodium bicarbonate, 300 mg/l L-glutamine, 50 IU/ml penicillin G sodium, 10 μg/ml streptomycin sulfate and 10% fetal bovine serum. Those cell cultures which had no HBV DNA were treated as negative control.

3) 2.2.15 cell culture: The HBV-producing 2.2.15 cell cultures were obtained from Dr M.A. Sells. These cultures were derived from HepG2 cells that were transfected with a plasmid vector containing G418-resistance sequences and two head-to-tail dimers of the HBV genome (Sells et al., 1987). The cells were found to produce elevated level of HBeAg and HBsAg, secrete infectious virions into the culture medium and contain chromosomally integrated HBV DNA sequence, as well as relaxed circular, covalently closed incomplete episomal copies of the genome, (Acs et al., 1987; Sells et al., 1987, 1988). The 2.2.15 cells were grown in MEM containing 750 mg/l sodium bicarbonate, 300 mg/l L-glutamine, 380 mg/l geneticin sulfate and 10% fetal bovine serum, that were maintained in a 37°C humidified incubator under a 5% CO_2 atmosphere. Both HepG2 and 2.2.15 cells were seeded in six well culture dishes at 106 cells/dish (3 ml cell suspension) and the medium was changed every other day.

2.4.2.3. Drug treatment. The test compound was added to the hepatocyte cultures on day 2 and maintained in culture with media changed every second day until day 12. Cells were harvested at day 14. The test compound was screened at a 10 μM final concentration. The test compound was added to the 2.2.15 cell cultures when the cell monolayers were about 80% confluent, usually after about 4 days. The cultures were treated with the test compound every second day at 10 μg/ml for 12 days and cells were harvested on day 14.

2.4.2.4. Isolation of DNA from hepatocyte cultures. The hepatocytes were lysed with 1 ml lysis buffer containing 10 mM Tris–HCl (pH = 7.8), 5 mM EDTA (pH = 8.0), 150 mM NaCl and 1% SDS. The lysate was digested with 0.5 mg/ml Proteinase K at 42°C overnight and deproteinized by extraction with an equal volume of phenol saturated with 20 mM Tris–HCl (pH = 7.5), 0.5 mM EDTA and 0.1% 8-hydroxyquinoline followed by an extraction with chloroform. Concentrated NaCl (5 M) was added to the aqueous phase to yield a final 0.1 M NaCl concentration and the DNA was precipitated with 2 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 10 mM Tris–HCl (pH = 7.5) and 1 mM EDTA.

2.4.2.5. Isolation of cytoplasmic HBV core particles from 2.2.15 cells. The procedure reported by Staprans et al. (1991) was used with some modifications. Cell monolayers were washed with PBS and incubated at room temperature for 20 min with 1 ml lysis buffer (10 mM Tris–HCl, pH = 7.5, 1 mM EDTA pH = 8.0, 50 mM NaCl—8% Sucrose—0.25% Nonindet P-40) and then centrifuged for 3 min in an Eppendorf microcentrifuge at 4°C to remove debris and cell nuclei. To the supernatant was added $MgCl_2$ to give a final 6 mM concentration and treated for 45 min at 37°C with 20 μg/ml DNase 1 and 20 μg/ml RNase A. The viral particles contained in each 1 ml sample were precipitated by addition of 0.33 ml of 26% polyethylene glycol in 1.4 M NaCl, 0.025 M EDTA. After 30 min on ice, the pellets were resuspended in 0.2 ml of 10 mM Tris–HCl, 5 mM EDTA and 1% SDS (pH = 7.5) and the samples were incubated at 42°C overnight with 500 μg/ml final Proteinase K. The deproteinized and ethanol precipitation steps were the same as previously described.

2.4.2.6. Dot hybridization. DNA samples were applied to a nylon filter (Hybond-N, Amersham) using a Bio-Dot™ (Bio-Rad Laboratories) mi-

crofiltration apparatus. DNA on the filter was denatured with 0.5 M NaOH, 1.5 M NaCl at room temperature for 30 min and neutralized in 1 M Tris–HCl (pH = 8.0), 1.5 M NaCl. The filters were exposed to ultraviolet irradiation for 3 min (UV transilluminator, UVP, 254 nm, total exposure = 1.44 J/cm^2). Prior to hybridization, the filter was prehybridized for 2 h at 65°C in 5 × SSPE (20 SSPE: 3.6 M NaCl, 200 mM NaH_2PO_4, pH = 7.4, 20 mM EDTA), 5 × Denhardt's (50 × Denhardt's solution contains 10 g of Ficoll, 10 g of polyvinylpyrrolidone, 10 g of bovine serum albumin dissolved in water to a final volume of 1 l), 0.1% SDS, 100 μg/ml denatured salmon sperm.

DNA hybridization was initiated by adding recently prepared DHBV [^{32}P]DNA probe at 1 × 10^6 cpm/ml using the same prehybridization procedure overnight. Filters were washed twice in 1 × SSC (20 × SSC: 3 M NaCl, 0.3 M sodium citrate, pH = 7.0), 0.1% SDS at 65°C for 2 h and 1 × SSC at room temperature for 30 min with gentle, constant agitation. The filter was dried and autoradiographed at −70°C using X-ray film (Kodak X-OMAT AR) with an enhancer screen. After an autoradiographic image had been obtained, the filter was exposed in phosphoimaging screen for 1–2 h, and the samples were quantitated by Fujix BAS1000 (Fuji) and the percentage density of phosphoimaging units was calculated.

(−)-β-L-2′,3′-dideoxy-3′-thiacytidine (3TC, lamivudine) was used as the reference compound. Tests were repeated twice and the data for each test compound were compared with a positive and negative control performed at the same time under identical conditions. HepG2 cells and the culture medium, which do not hybridize to the HBV DNA probe, were used in all subsequent experiments as a negative control. Four dilutions of the test compound were used in the anti-HBV assay at different concentration ranges. A 10 μg/ml concentration of the myristic acid analog was employed for the anti-DHBV assay and each experiment was performed in triplicate.

(Sample (treated 2.2.15 sample) − negative Control (HepG2)) × 100/(untreated 2.2.15 positive control − negative Control (HepG2)) = % of replication or (treated hepatocyte sample − negative hepatocyte) × 100/(congenital untreated hepatocyte − negative hepatocyte) = % of replication.

2.5. Cytotoxicity assays

Cytotoxicity assays were performed using different cell lines and methods.

(1) A T4-lymphocyte CEM cell line was used according to the United States National Cancer Institute procedure (Weislow et al., 1989). 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 to quantitate formazan production. The experimental details were previously described in Section 2.3.

(2) The protocol for determining toxicity of test compounds in cultures of 2.2.15 cells can be briefly summarized as follows (Korba and Gerin, 1992). The 2.2.15 cells were grown to confluence in 96-well flat-bottomed tissue culture plates and treated with test compound (in 0.2 ml /culture medium/well) as described above. Four concentrations of each test compound were assayed, each in triplicate cultures, in three to ten-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 from the inhibition of neutral red dye uptake, as determined from the absorbance at 510 nm relative to untreated cells, at 24 h following day 9 of treatment.

(3) In vitro KB cell cytotoxicity was determined for a few compounds using the terazolium salt (MTT) method which involves conversion of MTT to colored formazan (Parang et al., 1996).

3. Results

3.1. Physicochemical properties (log P, log $D_{7.4}$ and pK_a)

These theoretical physicochemical properties were calculated to ascertain whether they correlate with and/or are determinants of anti-HIV activity. Partition (Log P = 3.3–6.9) and distribution (Log $D_{7.4}$ = −1.0–4.4) coefficients, which were calculated for the fatty acids investigated

Table 1
Anti-HIV activity and selected physicochemical properties of the myristic acid analogs

No.	Structure	LogP[a]	LogD$^{b}_{7.4}$	IC$^{c}_{50}$ (μM)	Concentration[d] (μM)				
					0.6	6.3	20.0	63.2	200.0
					Percent protection (%)[e]				
1	$MeO(CH_2)_{11}COOH$	3.88	1.4	>200	NA[f]	NA	32.0	10.8	18.0
2	$MeO(CH_2)_{10}COOH$	3.36	0.9	>200	NA	NA	0.9	6.2	18.4
3	$Me(CH_2)_9O(CH_2)_2COOH$	4.45	1.4	83.9	NA	NA	4.8	NA	NA
4	$Me(CH_2)_4O(CH_2)_7COOH$	3.88	1.4	89.1	1.3	1.4	13.4	37.3	9.2
5	$Cl(CH_2)_8O(CH_2)_2COOH$	3.29	0.3	>200	5.8	7.0	2.2	NA	39.2
6	$EtS(CH_2)_{11}COOH$	5.47	3.0	38.6	31.7	47.0	96.6	18.3	NA
7	$EtS(CH_2)_{10}COOH$	4.95	2.5	66.4	NA	11.2	14.9	33.2	NA
8	$Me(CH_2)_4S(CH_2)_7COOH$	4.95	2.5	60.0	4.2	11.2	5.8	4.2	NA
9	$I(CH_2)_{11}COOH$	5.34	2.9	110	13.2	29.5	45.3	70.5	NA
10	$Br(CH_2)_{11}COOH$	5.03	2.5	165	14.9	25.9	35.2	78.6	38.4
11	$Cl(CH_2)_{11}COOH$	4.82	2.3	92.5	NA	NA	14.8	5.6	NA
12	$F(CH_2)_{11}COOH$	4.29	1.8	>200	NA	0.8	10.4	48.6	21.5
13	$HO(CH_2)_{11}COOH$	3.26	1.4	>200	17.0	31.4	24.7	28.9	26.6
14	$H_2N(CH_2)_{10}COOH$	2.81	−1.0	>200	18.9	6.99	2.2	NA	3.1
15	$Ph(CH_2)_{11}COOH$	6.59	4.1	22.8	NA	6.4	15.3	6.8	NA
16	$Ph(CH_2)_9COOH$	5.56	2.5	108	4.3	1.0	10.9	24.3	NA
17	$PhO(CH_2)_{11}COOH$	6.14	3.7	63.6	1.5	NA	18.0	1.6	NA
18	$PhO(CH_2)_{10}COOH$	5.62	3.2	41.6	NA	0.8	9.4	1.6	NA
19	4-I-$PhO(CH_2)_{10}COOH$	6.89	4.4	29.9	NA	6.5	NA	NA	NA
20	$N_3(CH_2)_{11}COOH$	4.88	2.4	20	NA	12.2	2 6.6	7.3	NA
21	$Me(CH_2)_{11}CH(Br)COOH$	6.93	2.8	—	—	—	—	—	—
22	$Me(CH_2)_{12}COOH$	5.97	3.5	—	—	—	—	—	—
AZT	—	0.06	0.06	100	$EC^{h}_{50} = 0.01$	—	—	—	—
3TC[g]	—	−0.27	−0.57	>100	$EC_{50} = 0.07$	—	—	—	—

[a] Partition coefficient of the fatty acid calculated using the PrologP 5.1 program (Parang et al., 1996).
[b] Distribution coefficient of the fatty acid at pH 7.0 calculated using the PrologD 2.0 prediction program.
[c] IC_{50} is defined as the test compound concentration required to reduce the number of viable cells in untreated T4-lymphocytes by 50%.
[d] Concentration of the test compound that produces the percentage protection specified against HIV infected-T-lymphocytes.
[e] The percentage of surviving HIV-infected cells treated with the test compound (at the concentration indicated) relative to uninfected, untreated controls (the value is the average of 2–5 separate experiments).
[f] NA, no activity.
[g] See Schinazi et al. (1992).
[h] EC_{50} is defined as the test compound concentration required to afford 50% protection of T4-lymphocytes against HIV infection.

(Table 1), extended over a broad range (Parang et al., 1996). It was found that the calculated pK_a values for most myristic acid analogs investigated was 4.91, except for the 2-substituted and the 4-oxa-analogs. For example, 2-bromotetradecanoic acid **21** had a calculated pK_a of 3.06, whereas 4-oxatetradecanoic acid **3** and 12-chloro-4-oxadodecanoic acid **5** had a calculated pK_a value of 4.37.

The calculated interatomic distances between the CO and the ω-heteroatom were: 13.61Å (**2**), 9.84Å (**4**), 15.00Å (**5**), 15.16Å (**6**), 10.15Å (**8**), 15.47Å (**9**), 15.34Å (**10**), 15.18Å (**11**), 14.88Å (**12**), 14.90Å (**13**), 13.75Å (**14**), 14.86Å (**17**), 13.60Å (**18**), 19.42Å (**19**).

3.2. In vitro anti-HIV activities

The results from the in vitro anti-HIV screen are presented in Tables 1 and 2. Since most of the myristoyl analogs investigated exhibited weak anti-HIV activity, the EC_{50}, IC_{50} and selec-

tivity index could not be determined. Accordingly, inhibition of viral replication (% protection) was scored as a percentage reduction in the virus-induced cytopathic effect (CPE, Table 1). Cytotoxicity (IC_{50}) values for the test compounds and the test compound concentration required to provide the specified level of protection to infected T4-lymphocytes are presented in Table 1. Some of the important results for individual subclasses of the myrisotyl analogs investigated are summarized below.

3.2.1. Thioethers

The most active compound of the 20 test compounds evaluated in the acute viral propagation assay was 12-thioethyldodecanoic acid **6**, which produced a 97% reduction in the cytopathic effect of HIV at a 20 μM concentration (Table 1). The EC_{50} for 12-thioethyldodecanoic acid **6** was 9.37 μM, and its $IC5_0$ was 38.6 μM. This latter compound **6** is therefore moderately active when compared to the active reference nucleosides AZT and 3TC (Table 1 and 2).

3.2.2. 12-Halosubstituted fatty acids

12-Halododecanoic acids such as the 12-iodo-**9** and 12-bromo-**10** analogs were only weakly cytotoxic, while simultaneously increasing the percentage of surviving HIV-infected cells (70–79%) at a test compound concentration of 63–70 μM. Several test compounds such as 12-iodododecanoic acid **9**, 12-bromododecanoic acid **10** and 12-fluorododecanoic acid **12** produced 48–79% reduction in virus-induced cytopathicity at a concentration that was two to four-fold lower than the test drug IC_{50}.

Table 2
Comparative potency and selectivity for three fatty acids as inhibitors of HIV replication in T4 cells (CEM-SS cell line)

Compound	IC_{50}^{a} (μM)	EC_{50}^{b} (μM)	TI_{50}^{c}
6	38.6 ± 14.1	9.37 ± 7.1	3.0 ± 0.9
9	110 ± 0	30 ± 8.9	4.5 ± 2.9
10	165 ± 20.6	38 ± 10.7	4.5 ± 1.0
AZT	100	0.01	10 000
3TC[d]	>100	0.07	>1429

[a] IC_{50} is defined as the inhibitory concentration, required to reduce the number of viable cells in untreated T4-lymphocytes by 50%. (mean $\pm$ S.D., $n = 5$).
[b] 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 $\pm$ S.D., $n = 5$).
[c] TI_{50} is defined as the therapeutic index (IC_{50}/EC_{50}) (mean $\pm$ S.D., $n = 5$).
[d] See Schinazi et al., 1992 (CEM cell line).

3.2.3. Phenylsubstituted fatty acids

The in vitro data for a series of aromatic myristic acid analogs show that 12-phenyldodecanoic acid **15** is more cytotoxic than 10-phenyldecanoic acid **16** toward uninfected T4-lymphocytes. 12-Phenoxydodecanoic acid **17**, 11-phenoxyundecanoic acid **18** and 11-(4-iodophenoxy)undecanoic acid **19** were synthesized to determine the effect of oxygen substitution in conjunction with aromatic rings. These aromatic ether analogs (**17**, **18** and **19**) were generally less active than the corresponding aromatic analogs (**15** and **16**) and the alkyl ether analog **1** against HIV-infected T4-lymphocytes cells.

3.2.4. Other myristoyl analogs

A number of compounds (**1**, **2**, **5**, **12**, **13**, **14**) exhibited low cytotoxicity ($IC_{50} > 200$ μM, Table 1). Several compounds, including 12-thioethyldodecanoic acid **6**, 12-phenyldodecanoic acid **15**, 11-phenoxyundecanoic acid **18** and 11-(4-iodophenoxy)undecanoic acid **19** were substantially more cytotoxic ($IC_{50} = 23–42$ μM). These data show that all of the sulfur containing compounds are more cytotoxic than their oxygen substituted analogs (12-thioethyldodecanoic acid **6**, $IC_{50} = 38.6$ μM; 12-methoxydodecanoic acid **1**, $IC_{50} > 200$ μM). The combination of oxygen at the 4-position and chlorine at the 12-position in 12-chloro-4-oxadodecanoic acid **5** resulted in a lower cytotoxicity than observed for either 4-oxa-tetradecanoic acid **3** or 12-chlorododecanoic acid **11** against HIV infected T-cells. The myristoyl analogs investigated were, at most, moderately effective with respect to their ability to protect HIV-infected T4-lymphocytes.

3.3. In vitro anti-HBV activities

Three fatty acids (**1**, **6** and **21**) were evaluated against HBV-producing 2.2.15 cell cultures derived from a human hepatoblastoma cell line (Hep G2). The EC_{50} for selected compounds **1**, **6**, **21**, 2′, 3′-dideoxycytidine (ddC) and 2′,3′-dideoxy-3′-thiacytidine (3TC) (Xie et al., 1995) (compound number (EC_{50} for intracellular DNA)) in HBV 2.2.15 (HepG2 cell line) for 14 days was: **1** (81.8 μM), **6** (17.8 μM), **21** (62.2 μM), ddC (2.8 μM) and 3TC (0.076 μM) where each value represents the mean of duplicate samples. Among the fatty acids, 12-thioethyldodecanoic acid **6** showed the most potent activity. No cytotoxicity values were determined in this assay, since this method is used solely as an in vitro model for screening anti-hepatitis virus therapeutics. Intracellular DNA was assayed by dot hybridization and phosphodensitometry. Compound **1** was selected for further studies to gain a better understanding regarding its efficacy and toxicity since compound **1** has been reported previously to exhibit medium anti-HIV activity (Bryant et al., 1991, 1993).

12-Methoxydodecanoic acid **1**, which was also evaluated against chronically HBV-producing human liver cells (2.2.15), exhibited medium activity against HBV DNA replication in 2.2.15 cells (HBV virion extracellular DNA, HBV RI intracellular DNA) and it was less toxic than dideoxydidehydrocytidine (ddC) (Table 3). This latter assay, which serves as a model for chronic HBV infection in man, provided additional information regarding the activity and toxicity for compound **1**.

3.4. In vitro anti-DHBV activities

DHBV a member of the family Hepadnaviridae, has a similar virion structure and genome organization to that of human HBV. Thus, DHBV has been used extensively to screen potential drugs to control chronic HBV infection (Lin et al., 1996). A group of myristic acid analogs were also evaluated using a duck hepatocyte culture system infected with DHBV. At a test compound concentration of 10 μg/ml, none of fatty acids significantly inhibited the replication of DHBV-infected hepatocytes. Viral replication (the values are the average of two different experiments) was estimated from the amount of DHBV DNA present in the supernatant of the cell culture after incubation with the test compound (10 μg/ml). The data are expressed as a percentage of control values where the cell culture was not incubated with the test compound. The percentage of viral replication for selected compounds (compound number (percentage of viral replication)) was **1** (64.4%), **3** (49.1%), **4** (94.8%), **6** (62.1%), **7** (65.1%), **9** (68.9%), **10** (80.2%), **11** (78.9%), **21** (93.0%), **22** (78.9%), **23** (77.2%), **24** (89.2%), **25** (53.0%) and control (100.0%). The most potent compound in this group, 4-oxatetradecanoic acid **3**, inhibited DHBV-infected hepatocytes by 51%.

4. Discussion

4.1. Anti-HIV activity

The antiviral activity of additional myristic acid analogs containing halogen, oxygen, sulfur, azido and aromatic substituents have been investigated to determine the effect of physicochemical properties and their correlation with anti-HIV activity. First, these compounds show a large variation in the physicochemical parameter Log P (3.3–6.9). Second, the importance of the steric size of substituents for different aromatic (**15**–**19**) and halo (**9**–**12**) derivatives could help to identify the putative binding site for the ω-terminal of these fatty acids. Finally, the interatomic distance between the CO and terminal heteroatom, which varies in different compounds, can be used to explain relative activity in a series of myristoyl compounds. Studies involving substituent variation will help to confirm that the acyl-CoA binding site may possess a complex sensor that is sensitive to the distance between the carboxyl and the heteroatom moieties of the fatty acid, and the steric volume at the ω-terminus as proposed by Kishore et al. (1993).

The procedure employed in the United States National Institutes of Health antiviral assay to evaluate activity against human immunodeficiency

Table 3
Relative in vitro potency and selectivity of 12-methoxydodecanoic acid (**1**), **3TC**, and ddC as inhibitors of HBV DNA replication in 2.2.15 cell culture (HBVRI intracellular DNA, HBV virion extracellular DNA)

No.	CC_{50}^{a} (μM)	EC_{50}^{b} (μM)	EC_{90}^{c} (μM)	EC_{50}^{b} (μM)	EC_{90}^{c} (μM)	SI[d]	
		Extracellular DNA	Extracellular DNA	Intracellular DNA	Intracellular DNA	Extracellular DNA	Intracellular DNA
1	378	22 ± 1.8	60 ± 6.3	31 ± 3.4	90 ± 11	6.3	4.2
ddC[e]	252 ± 28	1.3 ± 0.1	6.8 ± 0.7	2.6 ± 0.3	13 ± 1.8	37	19
3TC[f]	1180	0.033 ± 0.004	0.188 ± 0.020	0.076 ± 0.008	0.293 ± 0.031	6280	4030

[a] CC_{50}, 50% cytotoxic concentration.
[b] EC_{50}, 50% effective concentration.
[c] EC_{90}, 90% effective concentration.
[d] Selectivity Index (SI: CC_{50}/EC90).
[e] ddC = 2′, 3′-dideoxycytidine.
[f] 2′, 3′-dideoxy-3′-thiacytidine (Xie et al., 1995).

virus (HIV) is designed to detect agents acting at any stage of the virus reproductive cycle. Agents that interact with virions, cells, or virus gene-products to interfere with viral activities will protect cells from cytolysis. This assay is suitable to determine the anti-HIV activity of compounds that act by different mechanisms of action. Although this assay does not discriminate between inhibition of virus binding/penetration and inhibition of intracellular replication, it has been reported that myristic acid analogs traverse the cell membrane and act as substrates for NMT. Recent metabolic labeling studies using tritiated oxatetradecanoic acids with an oxygen at C6, C11 and C13, using several cultured mammalian cell lines, indicated that these low molecular weight compounds readily traverse the cell membrane and are substrates for both acyl CoA synthetase and NMT (Johnson et al., 1990). Myristoyl analogs are selectively incorporated into distinct, yet overlapping subsets of cellular *N*-myristoylproteins. This selective analog-specific incorporation presumably occurs due to cooperative interactions between the NMT acyl-CoA peptide binding sites and the enzyme's ordered Bi Bi reaction mechanism.

Our expectation that some dramatic difference would be observed for structurally different myristoyl analogs was not realized, although certain fatty acids show a difference in cytotoxicity (IC_{50}) against T4-uninfected-lymphocytes. Of these, 12-thioethyldodecanoic acid **6**, 12-iododododecanoic acid **9** and 12-bromododecanoic acid **10** were moderately active against HIV-infected T4-lymphocytes. For compounds having moderate activity (**6**, **9** and **10**), the concentration that produced a 50% cytotoxic effect on uninfected cells was at least three to four-fold larger than its corresponding EC_{50} value (Table 2). 12-Thioethyldodecanoic acid **6**, 12-iododododecanoic acid **9**, and 12-bromododecanoic acid **10** had calculated partition coefficients (Log P) between 5.0–5.5 (Log $D_{7.4}$ between 2.5 and 3.0) (Fig. 2). There does appear to be a direct correlation between anti-HIV activity and Log P for these fatty acids, although the anti-HIV data suggest that the partition coefficient should be in the Log P 5.0–5.5 range for optimal anti-HIV activity, since myristoyl analogs with higher or lower partition coefficients exhibit reduced activity. Previous in vitro NMT assay data showed that the activities of NMT acyl-CoA substrates diminish with increasing polarity (Devadas et al., 1992), which is in agreement with our data, since myristoyl analogs with Log P less than 5.0 exhibit weak anti-HIV activities (Fig. 2). No relationship between pK_a and anti-HIV activity was evident for these myristoyl analogs, since their pK_a values were generally similar (4.9). This result could be due to the small inductive effect of an alkyl group, an effect that decreases rapidly with increasing distance from the carboxylate substituent. Thus, only atoms which are adjacent to the acidic center will exert a significant effect on the observed pK_a value (Perrin et al., 1981).

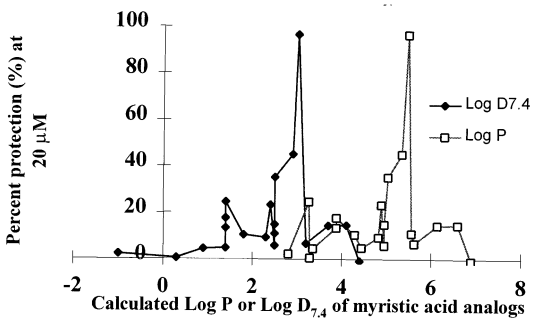

Fig. 2. Percent protection provided by myristic acid analogs against HIV infected T4-lymphocytes at 20 μM vs. calculated Log P or Log $D_{7.4}$.

It has been suggested that specific amino acid residues present in NMT bind to specific sections of the fatty acid backbone, that fatty acid acyl CoA may be rigidly bound by NMT, and that the acyl-CoA binding site is sensitive to the distance between the carboxyl and the ω-end of the fatty acid, and to the steric volume at the ω-terminus (Kishore et al., 1991). The interatomic distances between the CO and the ω-moieties for compounds **6**, **9** and **10**, all having an ω-heteroatom group, can be used to explain the activity exhibited by these compounds. For moderately active compounds (**6**, **9** and **10**), the distances between the CO and the heteroatom at the terminal end (sulfur, iodine and bromine) of the molecule are 15.16, 15.47 and 15.34 Å, respectively. In the case

of other myristoyl analogs, this distance is either shorter or longer. Changing the halogen to chlorine (**11**) from iodine (**9**) and bromine (**10**) resulted in a significant decrease in antiviral activity. In the case of other 12-halo analogs such as 12-chloro-(**11**) and 12-fluoro-(**12**), this distance is 15.18 and 14.88 Å, respectively. This interatomic distance is similar to that for **9** and **10** in the case of **11**. However, the larger steric volume for bromine, iodine and sulfur, relative to chlorine could be an important factor with respect to binding of the fatty acid analog to its binding site on the NMT enzyme. In addition, the lower partition coefficient for **11** may decrease uptake of the chloro analog.

To assess whether antiviral activity and cytotoxicity are affected by an aromatic ring or a heteroatom, a number of aromatic myristic acid analogs were synthesized. It was found that the anti-HIV activity of myristic acid analogs is not affected by oxygen substitution or placement of an iodine substituent at the para-position of a phenyl ring. All of these analogs exhibited very weak activity, without a change in cytotoxicity relative to the corresponding aromatic or ether analog, against HIV-infected T4-lymphocyte cells. Kishore et al. (1991) proposed that compounds having a terminal phenyl should not fit into the postulated conical receptor, and therefore should exhibit a lower activity than analogs having terminal methyl groups. Our in vitro anti-HIV data for the aromatic fatty acids **15**–**19** confirm this hypothesis.

A comparison of the anti-HIV activity for myristoyl analogs investigated in this study, excluding compounds **6**, **9** and **10**, with previously reported data warrants discussion. Recent studies have shown that some oxatetradecanoic acid analogs such as 12-methoxydodecanoic acid **1** inhibit replication of HIV-1-infected cells without accompanying cellular toxicity in both acutely and chronically infected $CD4^+$ H9 cells (Devadas et al., 1992). Metabolic labeling studies using tritiated 12-methoxydodecanoic acid **1** revealed that it was selectively incorporated into subsets of cellular *N*-myristoylproteins. Compound **1** was metabolized slowly, and only a small fraction of the drug entered the cell (Bryant et al., 1993). Compound **1** was potent as judged by its ability to produce a concentration-dependent decrease in reverse transcriptase activity, p24 antigen levels, and syncytium formation. For example, a 80–90% reduction in reverse transcriptase activity was achieved when a 20 μM test compound solution was added to serum-containing medium (Bryant et al., 1991, 1993). In contrast, our data indicate that compound **1** produced only a 32% reduction in virus replication (infected T4-lymphocyte in CEM-SS cell line) at a 20 μM test compound concentration. This result is in agreement with in vitro NMT assay data which showed that **1** is a poor NMT substrate (Kishore et al., 1991). 4-Oxatetradecanoic acid **3** inhibits HIV-1 assembly (Kishore et al., 1993). It has been reported that **3** produced a 50% reduction of HIV-I replication in acutely infected human T-lymphocyte cell lines at a concentration of 18 μM (Langner et al., 1992). In contrast to reported data, in this study a 20 μM concentration of **3** produced only a 4.8% inhibition of the CPE. It has been reported that 12-azidododecanoic acid **20**, like 12-methoxydodecanoic acid **1**, inhibits HIV-1 production by 60–70% in chronically infected T-lymphocyte cell lines at a concentration of 10–50 μM without appreciable associated cellular toxicity (Devadas et al., 1992). In spite of the previously reported data, the maximum protection observed in our in vitro assay against HIV-infected T4-lymphocytes at a 20 μM concentration of **20** was 26%. Results obtained in this study for **1**, **3**, and **20** do not indicate a similar high anti-HIV activity for these analogs. The differences in anti-HIV activity observed in this study, compared to literature data, could be due to fact that 100% dimethyl sulfoxide (DMSO) was used as solvent to dissolve the test compound rather than ethanol. This explanation is based on a report by Harper et al. (1996) that stock solutions of 2-hydroxymyristic acid prepared in DMSO, rather than ethanol, did not significantly inhibit VZV or HIV-1 replication, suggesting that the use of DMSO as a universal solvent for drug screening may result in false negative results for some compounds. Furthermore, the NIH method which measures the in vitro cytopathic effect of HIV-1 on suitable host cells, is different from other anti-HIV assays that

are used to analyze viral antigen p24 synthesis and reverse transcriptase (RT) activity. Myristic acid analogs are generally considered to be non-toxic, to readily traverse the cell membrane and to act as substrates for NMT. For some cellular *N*-myristoylproteins, incorporation of these analogs 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 (Devadas et al., 1992). The cytotoxicity could be avoided due to the stability of cellular myristoylated protein (McIlhinney, 1990). Some myristic acid analogs, such as 12-methoxydodecanoic acid **1** and 12-azidododecanoic acid **20**, inhibit HIV-1 replication in acutely and chronically infected human T-lymphocyte cell lines at doses which do not cause cellular toxicity (Langner et al., 1992). The in vitro cytotoxicity data acquired using a KB cell line (Parang et al., 1996) are in agreement with the reported data, since the concentration of the test compounds (**1**, **7**, **10** and **21**) that was cytotoxic to 50% of the cells (TD_{50}) was in the 45–47 μg/ml (150–204 μM) range. Our cytotoxicity assay results using the T4-lymphocyte CEM cell line ($IC_{50} > 200$ μM for **12**, **13** and **14**) and 2.2.15 cell line (CC_{50} for **1** = 378 μM) confirm these conclusions. Although compound **6** is only a moderately active anti-HIV agent, its antiviral activity is not believed to be the result of cytotoxicity since more cytotoxic compounds such as **15**, **19** and **20** do not exhibit comparable antiviral activity.

4.2. Anti-HBV activity

12-Methoxydodecanoic acid **1** was moderately active as an inhibitor of HBV DNA replication in 2.2.15 cell culture (HBV RI intracellular DNA, HBV virion extracellular DNA) compared to the reference compound ddC and is less toxic. However, the potency, selectivity index and cytotoxicity for **1** were much lower than for 3TC (Table 3). The EC50 values for compound **1** in these two assays are different, since the harvesting time, the duration of exposure, and the time at which cells were exposed to the test compound, differ. The myristic acid analogs **1**, **6** and **21** exhibit differential activity between DHBV and HBV. These results clearly indicate that evaluation of anti (D)HBV activity in only one cell culture system may not provide representative antiviral activity data for the drug. The reason for the observed differences between the two cell systems may be related to metabolic peculiarities, as documented for the HepG2.2.2.15 cells (Heijtink et al., 1993). Also, the genomic organization of hepadnavirus DNA appears to differ from one cell system to another. For example, no integration is observed for DHBV, whereas in HEPG2 2.2.15 cells HBV DNA shows two integration bands. It is not known whether this differential genomic organization influences the susceptibility of viral DNA synthesis upon exposure to antiviral drugs (Heijtink et al., 1993). HBV and DHBV, which belong to the hepadnavirus family, possess different N-terminal sequences for myristoylation. Marked differences have also been observed for intracellular metabolism of different compounds in DHBV-infected hepatocytes, and a human HIV cell line, that can effect its antiviral activity. The N-terminal sequence for *N*-myristoylated proteins for HBV is GGTSSLPA (Valenzuela et al., 1979), while that for DHBV is GQHPALSM (Macrae et al., 1991). Although myristoylation occurs at the terminal-amino glycine residue, other amino acids are also important in this pathway. More importantly, since these assays measure DNA synthesis, the nucleotide sequence for the HBV genome differs from that for the nucleotide sequence for DHBV. Consequently, DNA synthesis inhibition for these two viruses may be different for the same agent. Another explanation why DHBV production was not significantly affected, may be due to the fact that inhibition was determined in non-growing primary hepatocyte cultures, whereas inhibition of HBV was evaluated in cultures under conditions where cell division is expected.

Additional studies are required to make definitive conclusions regarding the anti-HBV effects exhibited by these myristoyl compounds. 12-Thioethyldodecanoic acid **6** showed the highest anti-HBV activity, compared to other fatty acids at a lower concentration (5 μM) against HBV

producing 2.2.15 cell cultures derived from Hep G2. Although the mechanism(s) by which these myristoyl analogs inhibit DNA synthesis to produce their anti-HBV effect is unclear, it may due at least in part to drug cytotoxicity on DNA. Infected cells are also more sensitive to inhibitors than uninfected cells. HBV replication is cell cycle-dependent which supports the concept of effect on viral DNA replication by these agents (Ozer et al., 1996).

Our assay results indicate there was no significant inhibition of DHBV replication at a myristic acid analog concentration of 10 μg/ml. Macrae et al. (1991) reported that myristoylation of DHBV envelope is essential for infectivity, but not for virus assembly. Myristoylation is most likely required as an early step in the life cycle involving the entry of uncoated virus particles. HBV particle formation can occur in the presence or absence of the myristoyl moiety. Even though myristoylation-null HBV mutants form virion particles, these particles are non-infectious (Macrae et al., 1991). In this study, compounds were tested only to detect a significant effect on DNA synthesis, but not virus infectivity. Therefore, these data cannot be used to define a relationship between myristylation and anti-(D)HBV activity.

In summary, we have shown that replacing one or more methylene groups by oxygen, sulfur, and/or an aromatic ring exerts a substantial effect on antiviral activity, and that selectivity with respect to the nature and position of the heteroatom and aromatic groups is observed. There appears to be a relationship among anti-HIV activity, Log P and Log $D_{7.4}$. In addition, there is a correlation between anti-HIV activity and interatomic distances between carbonyl and heteroatom end terminal moieties of the fatty acid chain. Finally, the myristic acid analogs investigated showed differential activity against DHBV and HBV.

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