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SYNTHESIS AND CHARACTERIZATION OF INHIBITORS OF MYRISTOYL-CoA:PROTEIN *N*-MYRISTOYLTRANSFERASE

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Abstract—Several substrate and product analogs were synthesized and tested as *in vitro* inhibitors of bovine brain *N*-myristoyl-CoA:protein *N*-myristoyltransferase (NMT; EC 2.3.1.97). At 40 μM, the acyl CoA analog, *S*-(2-ketopentadecyl)-CoA, completely inhibited NMT in the presence of 80 μM myristoyl CoA. Decreasing but marked inhibition was also observed with the acyl CoA analogs, *S*-(2-bromotetradecanoyl)-CoA and *S*-(3-(epoxymethylene)dodecanoyl)-CoA, and the multisubstrate derivative *N*-(2-*S*-CoA-tetradecanoyl)glycinamide in the presence of 40 μM myristoyl CoA. Inhibition was also observed with the non-coenzyme A myristoyl analog, 1-bromo-2-pentadecanone. All of the above compounds exhibited reversible competitive inhibition kinetics with respect to myristoyl CoA with K_i values of 0.11 to 24 μM. Two additional acyl CoA analogs, *S*-(*cis*-3-tetradecenoyl)-CoA and *S*-(3-tetradecynoyl)-CoA, functioned as alternative substrates for NMT.

Protein acylation, in the form of the addition of a myristic acid in amide linkage to the N-terminal glycine of certain cellular [1–4], oncogenic [5–7], and/or viral [8–17] proteins, is becoming an increasingly important target for drug development as more specific information regarding the importance of this modification is revealed. For example, N-myristoylation of the N-terminal glycine of the $Pr55^{gag}$ of human immunodeficiency virus (HIV-1) has been shown to be essential for stable membrane association, for proteolytic processing *in vivo*, and for subsequent development of the nascent virus particles to mature, infectious extracellular virions [18–21]. N-Myristoylation apparently plays a similar role for virus particle formation by Moloney murine leukemia virus [22, 23], poliovirus [24], and Mason–Pfizer monkey virus [25]. Myristate has also been identified in the *gag* polyprotein precursor of human T-cell lymphotropic virus types I and II [14], simian immunodeficiency virus [26], and capsid proteins in hepatitis B virus [13] where it may play an essential structural and/or functional role(s).

N-Myristoylation also plays an essential role in the membrane binding and transforming activity of the Rous sarcoma virus protein tyrosine kinase, $p60^{v\text{-}src}$ [27, 28]. If N-myristolyation is blocked, then cellular transformation by *src* is inhibited because subsequent membrane localization is blocked and proximity of the kinase to phosphorylation targets essential for transformation is possibly precluded [29, 30]. For more information on N-myristoylation, the reader is referred to several recent reviews [31–33].

A critical role for N-terminal myristate in these potentially pathological phenomena suggests that N-myristoylation may be an excellent target for the development of new drugs which could interfere with the resulting transformation and/or retroviral replication. One of the more obvious targets for blocking the N-myristoylation pathway is the enzyme which co-translationally transfers myristate from myristoyl CoA to the N-terminal glycine of nascent peptide substrates, myristoyl-CoA:protein *N*-myristoyltransferase (NMT; EC 2.3.1.97) [33]. In this paper, we describe the synthesis of a number of new compounds specifically designed as potential inhibitors of NMT. We also describe the results of testing of these compounds with respect to their *in vitro* inhibition of bovine brain NMT. These inhibitors represent prototypes for the design of potential chemotherapeutic drugs which may be used for inhibiting N-myristoylation-dependent abnormalities.

MATERIALS AND METHODS

1-*Bromo-2-pentadecanone* (2)

1-Bromo-2-pentadecanone (**2**) was prepared using a two-step procedure:

1-*Diazo-2-pentadecanone* (**1**). A solution of myristoyl chloride (20 mmol) in anhydrous ether (20 mL) was added dropwise to a cold (5°) solution of diazomethane (45.7 mmol) in anhydrous ether (160 mL), and the mixture was allowed to stand at room temperature overnight. A slow stream of nitrogen was bubbled through the mixture to reduce the volume to 75 mL; then the solution was flash evaporated to a dry yellow solid. Recrystallization from ether-hexanes afforded 4.5 g (89%) of pure crystalline diazoketone **1** as yellow flakes, m.p. 53–55° [Anal. Calc. for $C_{15}H_{28}N_2O$ (252.40): C,

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71.38%; H, 11.18%; N, 11.09%. Found: C, 71.18%; H, 10.90%; N, 11.12%]. Compound **1** was homogeneous on silica gel thin-layer chromatography (TLC) (chloroform; $R_f = 0.36$).

1-Bromo-2-pentadecanone (**2**). Excess 48% hydrobromic acid (32 mmol) was added to a solution of **1** (14.26 mmol) in ether (70 mL) with stirring at room temperature until the nitrogen evolution had ceased. The ether layer was separated and washed with 5% sodium bicarbonate and water and dried with Na_2SO_4. The solution was filtered and concentrated to dryness to give a white solid. Recrystallization from ether-hexanes afforded 3.88 g (89%) of pure crystalline **2** as white flakes, m.p. 58–59° [Anal. Calc. for $C_{15}H_{29}BrO$ (305.31): C, 59.01%; H, 9.57%; Br, 26.71%; O, 5.24%. Found: C, 59.02%; H, 9.74%; Br, 25.89%; O, 5.45%]. Compound **2** was homogeneous on silica gel TLC (chloroform; $R_f = 0.62$, iodine vapor).

S-*(2-Ketopentadecyl)-CoA* (**3**)

S-*(2-Ketopentadecyl)-CoA* (**3**). Coenzyme A sodium salt (268.1 mmol) was dissolved in freshly degassed deionized water. The solution was stirred under a nitrogen atmosphere and dithiothreitol (26 μmol) was added. The pH was adjusted to 8.5, freshly prepared **2** (326.2 μmol) in 95% ethanol (25 mL) was added, and the milky solution was stirred for 30 min. The turbid reaction mixture was filtered through celite and lyophilized to give 300 mg of crude **3**. This material was dissolved in water and purified by chromatography on Sephadex G-15 (3 × 56 cm) by elution with water. The UV absorbing product fractions were pooled and lyophilized to give 192 mg (79%) of **3** as a white amorphous solid, 220° (dec) [Anal. Calc. for $C_{36}H_{60}N_7O_{17}P_3SNa_4 \cdot 3H_2O$ (1133.91): N, 8.64%; P, 8.19%; S, 2.82%. Found: N, 8.74%; P, 7.80%; S, 2.97%]. There was one primary spot on silica gel TLC (*n*-propanol: methanol:water, 4:3:2; $R_f =$ 0.44).

S-*(2-Bromotetradecanoyl)-CoA* (**5**)

S-(2-Bromotetradecanoyl)-CoA (**5**) was prepared using a two-step procedure:

2-Bromotetradecanoyl chloride (**4**). Oxalyl chloride (48.9 mmol) was added to 2-bromotetradecanoic acid (32.6 mmol) in dry benzene (50 mL) containing a catalytic amount of dimethylformamide (3.2 mmol). The reaction mixture was stirred at room temperature for 5 hr, and the solution was decanted from a gummy material and concentrated to give 10.5 g of crude **4** as a light brown liquid. The crude product was distilled to give pure 8.4 g (79%) **4** as a colorless liquid, b.p. 120–123°/0.05 mm Hg. [Anal. Calc. for $C_{14}H_{26}BrClO$ (325.73): C, 51.62%; H, 8.05%; Br, Cl, 21.76%. Found: C, 51.44%; H, 7.91%; Br, Cl, 21.79% (total halogen as Cl)]. Compound **4** was homogeneous on silica gel TLC (chloroform; $R_f = 0.70$).

S-*(2-Bromotetradecanoyl)-CoA* (**5**). Coenzyme A sodium salt (484.4 μmol) was dissolved in a freshly degassed deionized water *t*-butanol mixture (35 mL, 1:1, v/v) and stirred under nitrogen, and then dithiothreitol (42.9 μmol) was added. The pH was adjusted to 8.5, and **4** (4.6 μmol) was added dropwise over 0.5 hr while the pH was maintained at 8.5 with 1 N sodium hydroxide. The milky solution was diluted with deionized water and lyophilized to give crude **5**. The crude product was washed twice with 95% ethanol, dissolved in water, and purified by elution from Sephadex G-25 (4 × 48 cm) in cold water. The UV absorbing product fractions were lyophilized to give 281 mg (51%) of **5** as a white amorphous solid, 230° (dec) [Anal. Calc. for $C_{35}H_{57}BrN_7O_{17}P_3SNa \cdot 3H_2O$ (1198.78): Br, 6.67%; N, 8.18%; P, 7.75%; S, 2.67%. Found: Br, 6.61%; N, 8.35%; P, 7.72%; S, 2.46%]. Compound **5** was homogeneous on silica TLC chromatography (*n*-propanol:methanol:water, 4:3:2; $R_f = 0.52$).

N-*(2-S-CoA-tetradecanoyl)glycinamide* (**7**)

N-(2-*S*-CoA-tetradecanoyl)glycinamide (**7**) was prepared by a two-step procedure:

N-*(2-Bromotetradecanoyl)glycinamide* (**6**). To a mixture of **4** (15.6 mmol), glycinamide (15.5 mmol) and methylene chloride (50 mL), was added dropwise at 0° a solution of *N,N*-diisopropylethylamine (31.1 mmol) in methylene chloride (25 mL). After stirring overnight at room temperature, water (30 mL) was added, the resulting colorless crystals were filtered and washed with water, and **6** was recrystallized from absolute ethanol. Yield 58%, m.p. 142–144° [Anal. Calc. for $C_{16}H_{31}BrN_2O_2$ (363.35): C, 52.89%; H, 8.60%; N, 7.71%. Found: C, 53.02%; H, 8.86%; N, 7.98%].

N-*(2-S-CoA-tetradecanoyl)glycinamide* (**7**). *N*-(2-*S*-CoA-tetradecanoyl)glycinamide (**7**) was prepared from **6** by a procedure analogous to that described for **5**.

S-*(3-(Epoxymethylene)dodecanoyl)-CoA* (**8**)

S-(3-(Epoxymethylene)dodecanoyl)-CoA was prepared from the corresponding fatty acid by a mixed anhydride mediated coupling [34] with coenzyme A. Experimental details of the preparation of **7** and **8** will be described elsewhere.*

N-*Myristoylglycinal diethylacetal* (**9**)

A mixture of diisopropylethylamine (1.3 mL, 7.5 mmol) and glycinal diethylacetal (1.0 g, 7.5 mmol) in dichloromethane (5 mL) was added slowly to a cooled (0°, ice bath) stirred solution of myristoyl chloride (1.85 g, 7.5 mmol) in dichloromethane (10 mL). The mixture was then allowed to come to room temperature overnight protected by a drying tube. The mixture was diluted with water (20 mL), and the organic phase was washed with aqueous sodium bicarbonate (1 M, 10 mL) and then dried over anhydrous sodium sulfate. Concentration and recrystallization from absolute ethanol afforded colorless crystals of the acetal (**9**), 860 mg, m.p. 69–70° (lit. 65–67° [35]), ^{1}H-NMR ($CDCl_3$): δ 5.84 (bt, 1H), 4.54 (t, 1H), 3.84–3.50 (m, 4H), 3.40 (t, 2H), 2.21 (t, 2H), 1.64–0.84 (m, 31H); IR ($CHCl_3$): 3320, 1645, 1560 cm^{-1}, MS: $M^+ = 343$. [Anal. Calc. $C_{20}H_{41}NO_3$: C, 69.92%; H, 12.03%; N, 4.08%.

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Found: C, 70.04%; H, 11.87%, N, 3.94%]. Compound **9** was homogeneous on silica TLC [chloroform, ethyl acetate, hexanes-ethyl acetate (4:1)]. Two additional crops of colorless crystals totaling 800 mg, m.p. 63–65°, could be obtained from the mother liquor.

N-*Myristoylglycinal* (**10**)

Compound **9** (0.35 mmol) in freshly distilled tetrahydrofuran (9 mL) containing 1 N hydrochloric acid (3 mL) was heated under nitrogen in a water bath to an external temperature of 45–50° for 1 hr. The solution was neutralized with anhydrous sodium carbonate, diluted with chloroform (20 mL), and washed with water. The aqueous phase was extracted with chloroform, the organic phases were combined and dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure to give a white solid. Recrystallization from absolute ethanol afforded **10** as a colorless solid (43 mg, 46%), m.p. 84–88° [^{1}H-NMR ($CDCl_3$): δ 9.61 (s, 1H), 6.24 (bs, 1H), 4.23 (d, 2H), 2.262 (t, 2H), 1.83 (m, 2H), 1.25 (m, 22H), 0.88 (t, 3H); IR ($CHCl_3$): 3429.8, 2927.2, 2854.6, 1729.3, 1668.1, 1508.4, 1220.0 cm^{-1}].

NMT assay

NMT activity was assayed using as the acyl acceptor an [^{125}I]-labeled 16 residue peptide corresponding to the N-terminal 15 residues of p60^{src} and including a C-terminal tyrosine amide to allow for radioiodination. Radioiodination and C18 reverse phase HPLC assay for NMT were performed as previously described except for the absence of dithiothreitol and the presence of 0.1% bovine serum albumin [36]. Under these assay conditions enzyme activity was linear with time and protein. Hyperbolic enzyme kinetics were evaluated by nonlinear regression analysis using the ENZFITTER program (Biosoft, Cambridge, U.K.). Inhibition kinetic data were performed in duplicate at 6 different substrate concentrations and at 2–3 different inhibitor concentrations and are representative of 2–3 experiments. These data were fit to appropriate inhibition models, and inhibition constants were determined using the weighted nonlinear least-squares curve-fitting program ENZYME-PC [37] or they were evaluated from Dixon plots [38]. Catalytic efficiences were determined from V_{max}/K_m ratios (expressed as a percentage of V_{max}/K_m of NMT for myristoyl CoA) which should be proportional to pseudo-second-order rate constants.

Purification of NMT

NMT was partially purified from bovine brain by a procedure similar to that described for the purification of yeast NMT [39]. Proteolytic enzyme inhibitors were purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN, and the Sigma Chemical Co., St. Louis, MO. All steps were carried out at 4°. Fresh or frozen (−70°) bovine brain (100 g) was homogenized in 300 mL of 50 mM Tris–HCl, pH 7.4, homogenization buffer containing 0.5 mM EDTA, 0.5 mM iodoacetamide, 50 μg/mL L-1- chloro -3-[4 -tosylamido]-7-amino-2-heptanone-HCl (TLCK), 20 μg/mL soybean trypsin inhibitor, 2 μg/mL leupeptin, 2 μg/mL aprotinin, 20 μg/mL (4-amidinophenyl)-methanesulfonyl fluoride, 28 μg/mL phenylmethanesulfonyl fluoride (PMSF), 1 μg/mL pepstatin A, 70 μg/mL L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone (TPCK) and 0.05% Triton X-100 in a Waring blender for 30 sec in three separate 10-sec bursts, filtered through cheesecloth, and centrifuged at 100,000 *g* for 90 min. NMT activity recovered in the 35–70% ammonium sulfate fraction was suspended and exhaustively dialyzed in buffer A (20 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.5 mM iodoacetamide, 40 μg/mL TLCK, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 20 μg/mL soybean trypsin inhibitor, 28 μg/mL PMSF, 1 μg/mL pepstatin A, and 70 μg/mL TPCK). The dialyzed ammonium sulfate fraction was added to a column of DEAE Sepharose Fast Flow (Pharmacia LKB Biotechnology, Piscataway, NJ), washed with about 30 mL of buffer A followed by buffer A containing 60 mM NaCl until the absorbance at 280 nm was near baseline. The enzyme was then eluted with 500 mL of buffer A containing 130 mM NaCl, the 280 nm absorbing fractions were pooled and concentrated by ultrafiltration with a YM10 membrane (Amicon, Beverly, MA), and the concentrated material was exhaustively dialyzed against buffer B (10 mM Tris–HCl, pH 7.4, 0.1 mM EDTA, plus the same concentration of proteinase inhibitors described for buffer A). The dialyzed DEAE Sepharose-treated enzyme was mixed with 10 mL of packed CoA-agarose resin (Type V AG-CoA, Pharmacia LKB) (previously washed with buffer B), mixed end-over-end for 15 min, and the resin transferred to a 1.5-cm diameter glass column. The resin slurry was packed, washed with buffer B until 280 nm absorbance was near baseline, and the enzyme was eluted with buffer B containing 0.5 M KCl. The 280 nm absorbing protein peak was concentrated to 6–10 mg protein/mL by ultrafiltration and stored at 5° in 0.02% sodium azide. Under these conditions, loss of NMT activity was minimal over a period of 4–6 months. This fraction represented a 30–50% recovery of NMT activity and a 35- to 40-fold purification over the original 100,000 *g* bovine brain homogenate fraction and was used for all assays described in this study.

RESULTS

Synthesis and testing of possible NMT inhibitors

NMT is a bisubstrate enzyme which catalyzes the transfer of a myristic acid from myristoyl CoA to the N-terminal glycine of a number of interesting cellular and viral proteins. To obtain inhibitors of this reaction, we have designed and chemically synthesized analogs of myristoyl CoA, the myristate moiety, and an enzyme multisubstrate or tetrahedral intermediate and examined their potential as inhibitors of NMT. Table 1 summarizes the effect of a 40 μM concentration of these compounds on the *in vitro* enzymatic activity of NMT partially purified from bovine brain. Depending on the conditions of the assay, dramatic effects could be demonstrated for several of these compounds. For example, in the presence of 40 μM myristoyl CoA, the acyl CoA analog, *S*-(2-ketopentadecyl)-CoA inhibited NMT activity completely. Inhibition was also observed

Table 1. Compounds tested for NMT inhibition

Compounds (40 μM)	NMT activity (%)
Control	100
S-(2-Ketopentadecyl)-CoA	0
S-(2-Bromotetradecanoyl)-CoA	9.3
1-Bromo-2-pentadecanone	42.5
N-(2-*S*-CoA-tetradecanoyl)glycinamide	55.6
S-(3-(Epoxymethylene)dodecanoyl)-CoA	68.8
S-(*cis*-3-Tetradecenoyl)-CoA	90
S-(3-Tetradecynoyl)-CoA	115

Synthetic compounds were tested for their abilities to inhibit the transfer of myristate from myristoyl CoA to a 16-residue peptide corresponding to the N-terminal sequence of $p60^{src}$. The NMT assay included 20 mM Tris–HCl, pH 7.4, 0.1% bovine serum albumin, 40 μM myristoyl CoA, 100 μM [^{125}I]-labeled *src* peptide (GSSKSKPKDPSQRRR[^{125}I]Y), and partially purified bovine brain NMT. The data presented are the averages of duplicate values which differed by less than 5% and are representative of two separate experiments. In the control assay without inhibitors, 100% corresponds to an NMT activity of 27 nmol/min/mL.

with the acyl CoA analogs, *S*-(2-bromotetradecanoyl)-CoA (91%), and *S*-(3-(epoxymethylene)dodecanoyl)-CoA (31%), the myristate analog, 1-bromo-2-pentadecanone (58%), and the multisubstrate analog, *N*-(2-*S*-CoA-tetradecanoyl)-glycinamide (44%). In contrast, no inhibition was seen with *N*-(2-bromotetradecanoyl)glycinamide (data not shown). In the presence of 80 μM myristoyl CoA, NMT was still totally inhibited by *S*-(2-ketopentadecyl)-CoA, whereas the inhibition by the other compounds was reduced dramatically: *S*-(2-bromotetradecanoyl)-CoA (75%), *S*-(3-(epoxymethylene)dodecanoyl)-CoA (20%), *N*-(2-*S*-CoA-tetradecanoyl)glycinamide (0%), and 1-bromo-2-pentadecanone (0%) (data not shown).

Two additional compounds tested as possible NMT inhibitors, *S*-(*cis*-3-tetradecenoyl)-CoA and *S*-(3-tetradecynoyl)-CoA, were found to serve as alternative substrates. When *S*-(3-tetradecynoyl)-CoA was substituted for myristoyl CoA, a prominent *N*-acylpeptide product was eluted from the C18 reverse phase column with a retention time of 8 min (compared with the *N*-myristoylpeptide elution of 12 min). Similarly, when *S*-(*cis*-3-tetradecenoyl)-CoA was assayed as an alternative co-substrate under the same conditions, an *N*-acylpeptide product with an elution time of 11.5 min was formed. Kinetic analysis under the standard assay conditions gave a K_m = 36 μM and a V_{max} = 17% for *S*-(*cis*-3-tetradecenoyl)-CoA and a K_m = 285 μM and a V_{max} = 58% for *S*-(3-tetradecynoyl)-CoA (compared to a K_m = 33 μM and a relative V_{max} = 100% for myristoyl CoA). Respective apparent second-order rate constants (V_{max}/K_m) of 16 and 7%, relative to that of myristoyl CoA, suggest that these substrates exhibit catalytic efficiencies considerably lower than the native myristoyl CoA substrate.

It has been reported recently that the N-myristoylation of $p60^{src}$ in Rous sarcoma virus transformed chick embryo fibroblasts and of $p17^{gag}$ in HIV and HTLV-1 infected human T-cells is blocked by *N*-myristoylglycinal diethylacetal [35, 40, 41]. We have synthesized *N*-myristoylglycinal diethylacetal and its corresponding aldehyde, *N*-myristoylglycinal, and tested them under our assay conditions as possible direct inhibitors of NMT. Both compounds were preincubated separately with the enzyme at 100 μM (*N*-myristoylglycinal diethylacetal) or 400 μM (*N*-myristoylglycinal) in the absence of myristoyl CoA and then were added to the standard assay mixture containing myristoyl CoA and acyl peptide acceptor, or both compounds were separately added directly into the assay mixture at 40 μM and reactions were started by the addition of enzyme. We found no direct inhibition (<5% deviation from noninhibited controls) of the enzyme by either approach by either of these compounds, suggesting that these compounds, at least under these assay conditions, do not block *N*-myristoylation by inhibition of NMT.

Characterization of NMT inhibitors

The two most potent inhibitors, *S*-(2-ketopentadecyl)-CoA and *S*-(2-bromotetradecanoyl)-CoA, were shown to inhibit NMT in a concentration-dependent manner exhibiting relative 50% inhibition levels at 0.2 and 2 μM concentrations, respectively, in the presence of 80 μM myristoyl CoA (Fig. 1).

The effects of several of the above inhibitors on NMT Michaelis–Menten kinetic parameters were determined and used to predict the most plausible model of inhibition and their corresponding inhibition constants (Table 2). All of the compounds tested appeared to exhibit their primary effects on the K_m of myristoyl CoA, indicating a competitive inhibition mechanism. It would appear that with K_i values from 0.11 to 24 μM, all of these inhibitors exhibit apparent affinities for the enzyme that are substantially greater than that of myristoyl CoA with a K_m = 33 μM.

All of the compounds were also routinely tested as potential irreversible inhibitors of NMT. In each

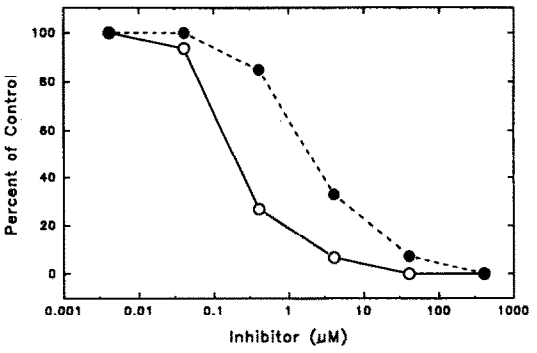

Fig. 1. Inhibition of NMT in the presence of increasing concentrations of *S*-(2-ketopentadecyl)-CoA (○) or *S*-(2-bromotetradecanoyl)-CoA (●). The NMT assay was carried out as described in Table 1 in the presence of 80 μM myristoyl CoA. The plotted data are the averages of duplicate determinations which varied by less than 5%. In the non-inhibited controls, 100% corresponds to an NMT activity of 4 nmol/min/mL.

case, the enzyme was preincubated with up to a 100 μM concentration of the compound in the absence of either substrate for 15–30 min at 30° or 37° and then diluted 20-fold into the complete assay mixture for determining NMT activity. Under these conditions, none of the compounds described above was found to inhibit NMT irreversibly.

DISCUSSION

The apparent dependency of a number of viral and oncprotein mediated disorders upon protein N-myristoylation is just beginning to be appreciated. For both oncproteins and viral proteins, if N-myristoylation is prevented by mutation, the subsequent cellular transformation and/or viral replication is blocked. This dependency represents a prime target for drugs designed to specifically inhibit N-myristoylation and thereby to arrest and perhaps reverse *N*-myristoylprotein-dependent diseases [33].

NMT functions as a key step in the N-myristoylation pathway and therefore could be susceptible to pharmacological manipulation [33]. NMT activities have been detected in a wide variety of eukaryotic species and tissues [36, 42–44]. Studies on enzyme activities from wheat germ, yeast, and mammals confirm a strong specificity for myristoyl CoA compared with the corresponding thioesters of other naturally occurring fatty acids. The fact that this relatively rare fatty acid [45] is incorporated with such invariant fidelity [31–33] attests to the specificity of the enzyme and, therefore, to its possible susceptibility as a target for the design of antitumor and antiviral agents.

We have synthesized a number of myristoyl CoA analogs which were found to function as competitive inhibitors of NMT. The most potent inhibitor, *S*-(2-ketopentadecyl)-CoA, is a structural isomer of myristoyl CoA containing a thioether rather than a thioester linkage. We anticipated that this structurally similar analog should bind to the enzyme myristoyl CoA binding site but the methylene group between the sulfur and carbonyl carbon would not serve as an expected electrophilic target during the acyl transfer. Indeed, this analog proved to be a potent competitive inhibitor of bovine brain NMT with respect to myristoyl CoA exhibiting an apparent $K_i = 0.11$ μM. When compared to the myristoyl CoA $K_m = 33$ μM, this result suggests that the inhibitor exhibits a markedly enhanced affinity for the enzyme relative to its native substrate. The apparent high-affinity binding of this compound to NMT is consistent with studies on the yeast and rat brain enzymes, indicating that acyl CoAs of various lengths are able to bind the myristoyl CoA binding site although without necessarily supporting acyl transfer [36, 39]. While the results of our work were in preparation, similar results were reported for this compound with rat brain NMT [46].

Two analogs originally designed as potential irreversible inhibitors containing electrophilic reactive groups were also found to be good inhibitors of NMT. With a halogen at position 2, it was anticipated that *S*-(2-bromotetradecanoyl)-CoA may alkylate an active site nucleophile(s) presumably involved in the acyl transfer. Similarly, the highly reactive oxirane in the *S*-(3-(epoxymethylene)dodecanoyl)-CoA analog may be reactive with an appropriately located nucleophile. However, neither of these compounds

Table 2. Characterization of NMT inhibitors

Inhibitor	Type of inhibition	K_i (μM)
S-(2-Ketopentadecyl)-CoA	Competitive	0.11
S-(2-Bromotetradecanoyl)-CoA	Competitive	2.3
N-(2-*S*-CoA-tetradecanoyl)glycinamide	Competitive	3.1
S-(3-(Epoxymethylene)dodecanoyl)-CoA	Competitive	19
1-Bromo-2-pentadecanone	Competitive	24

Compounds found to inhibit NMT (see Table 1) were characterized for their effects on Michaelis–Menten kinetic parameters, and their inhibition kinetics were used to predict the type of inhibition mechanism and corresponding inhibition constants.

proved to be an irreversible inhibitor of NMT. Instead, both inhibited NMT in a reversible competitive manner with K_i values of 2.3 and 19 μM, respectively, again indicating that they bind to NMT with affinities at least as high as myristoyl CoA. These results confirm that despite the high level of specificity for myristate in the acyl transfer reaction, the myristoyl CoA binding site allows a considerable flexibility in overall acyl structure. In particular, these two compounds indicate that modifications in both the 2- and 3-carbon positions of the fatty acyl chain are allowed without seriously compromising acyl CoA analog affinity for the enzyme.

Another compound, *N*-(2-*S*-CoA-tetradecanoyl)-glycinamide, was prepared as a potential multisubstrate or tetrahedral intermediate analog containing both the myristoyl and coenzyme A moieties in amide linkage with glycinamide. Since NMT is known to be sensitive to product inhibition with *N*-myristoylpeptides [36, 47], such a compound which incorporates an *N*-acyl glycinamide linkage resembling the *N*-acylpeptide product as well as structural features of myristoyl CoA may bind to the enzyme more tightly than any of the individual groups. With a $K_i = 3.1$ μM, this compound did prove to be a good inhibitor of NMT, again apparently binding to the enzyme at least as tightly as myristoyl CoA. To understand the structural characteristics contributing to this high affinity, it is interesting to compare this result with a similar compound, *N*-(2-bromotetradecanoyl)glycinamide, which showed no inhibition when tested under conditions similar to those described in Table 1. Both compounds retained the structural features of the *N*-myristoyl chain and the amide linked glycinamide. However, when a bromo group was substituted for the coenzyme A moiety on the acyl 2-carbon, no inhibition was observed. This comparison suggests that the coenzyme A moiety is an essential structural element in the binding of these particular inhibitors to NMT. However, since inhibition was also seen with the non-CoA analog, 1-bromo-2-pentadecanone, our results reiterate the enzyme requirement for an appropriate acyl moiety in addition to coenzyme A. Our data would indicate that, while not significantly interfering with inhibitor binding, the amide linked glycinamide makes no significant contribution to the overall inhibitor–enzyme interaction. Considering the sensitivity of the enzyme to *N*-myristoylpeptide product inhibition, however, we would anticipate that similar analogs containing amide linked *N*-myristoylation polypeptide consensus sequences might exhibit considerably enhanced affinity for the enzyme and, therefore, serve as highly specific inhibitors.

The lack of a positive contribution of the amide linked glycinamide to enzyme–inhibitor affinity is consistent with our failure to demonstrate *in vitro* inhibition of NMT using the *N*-myristoylglycinal diethylacetal analogs. *N*-Myristoylglycinal diethylacetal was prepared on the basis of the structure of myristate in amide linkage with an N-terminal glycine residue of N-myristoylated proteins [35]. It was reported that this compound inhibited the *in vivo* N-myristoylation of HTLV-I $p19^{gag}$ [35, 41], HIV $p17^{gag}$ [35, 41], and Rous sarcoma virus $p60^{src}$ [40]. It was also reported that concomitant with this anti-myristoylation effect, there was a corresponding inhibition of virus production in HIV-1-infected MT-4 cells [41] and an inhibition of morphological transformation of *v-src* infected chick embryo fibroblasts [40]. We have synthesized *N*-myristoylglycinal diethylacetal and examined its effect in our *in vitro* NMT assay. Since 80% of the *N*-myristoylglycinal diethylacetal taken up by chicken embryo fibroblasts was present as *N*-myristoylglycinal [40], we also synthesized and tested the free aldehyde as the possible reactive species. We found no apparent effect of these compounds on NMT activity under our assay conditions. While it is possible that poor compound solubility and/or the presence of albumin in our assay mixtures limited the availability of soluble compound to the enzyme, our results suggest that NMT may not be the specific target for inhibition of the N-myristoylation pathway by *N*-myristoylglycinal diethylacetal.

Two additional acyl CoA analogs, *S*-(*cis*-3-tetradecanoyl)-CoA and *S*-(3-tetradecynoyl)-CoA, were originally designed as possible NMT catalytic inhibitors. With β-γ-vinyl or β-γ-acetylene groups, we speculated that these compounds might be converted to Michael acceptors and be susceptible to alkylation by an appropriately positioned nucleophile [48]. In fact, neither of these compounds inhibited NMT but instead functioned as alternative substrates participating in the acyl transfer although with catalytic efficiencies less than myristoyl CoA. In addition, the reaction of *S*-(3-tetradecynoyl)-CoA with NMT produced an *N*-acylpeptide which eluted from the C18 reverse phase column at 8 min, indicating a product considerably less hydrophobic than the *N*-myristoylpeptide product which eluted at 12 min. These results suggest that unsaturated tetradecanoic fatty acyl analogs with markedly reduced hydrophobicities can still function as excellent substrates for bovine brain NMT. It has been reported that unsaturated fatty acid analogs function as efficient substrates for yeast NMT and that chain length rather than hydrophobicity determines the specificity of yeast NMT for fatty acyl substrates [49].

N-Myristoylation appears to be an excellent target for the development of chemotherapeutic strategies for inhibiting a number of pathogenic viruses [33]. One strategy for inhibiting N-myristoylation, the inhibition of *de novo* fatty acid synthesis with cerulenin, has been shown to block *N*-myristoylation in Moloney murine leukemia virus-infected cells [50]. A second strategy is to use NMT to incorporate heteroatom-substituted fatty acid analogs with altered physical–chemical properties (i.e. hydrophobicities) into selected viral proteins in order to alter their subcellular membrane targeting [49, 51, 52]. A final strategy for blocking N-myristoylation is the direct inhibition of NMT. To date, direct *in vitro* inhibition of NMT has been demonstrated only with glucosamine [53] and *S*-(2-ketopentadecyl)-CoA [46]. While another inhibitor of N-myristoylation, *N*-myristoylglycinal diethylacetal [35, 40, 41], was designed as a specific inhibitor of NMT, its direct effect on NMT has yet to be demonstrated. This report extends the list of

in vitro NMT inhibitors which may be useful as potential anti-myristoylation therapeutic agents.

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