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INHIBITION OF OCTAPEPTIDE *N*-MYRISTOYLATION BY ACYL AMINO ACIDS AND ACYL ALKANOLAMINES

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Several acyl amino acids and acyl alkanolamines were prepared and screened for their inhibition of octapeptide *N*-myristoylation and HIV-1 replication in MT-4 cells. Of the 62 acyl derivatives tested, *N*-myristoyl-*O*-caproyl-*L*-serine, *N*-myristoyl-*O*-caproyl-*D*-serine and *N*-decanoyl-*O*-myristoyl-*L*-serine were found to be uncompetitive inhibitors of *N*-myristoylation, but did not prevent HIV-induced cytopathicity in MT-4 cells. However, other acyl derivatives such as *N*-3-hydroxymyristoyl ethanolamine, *N*-3-hydroxymyristoyl-*D*-serine and *N*-myristoyl-*L*-cysteine, which did not inhibit *N*-myristoylation, suppressed the cytopathicity in the infected cells. The acyl derivatives described here may serve as lead compounds for antiviral agents.

Keywords: *N*-Myristoylation; human immunodeficiency virus; fatty acyl derivatives.

INTRODUCTION

Myristic acid is covalently linked via an amide bond to the amino-terminal glycine residues of several proteins encoded by the human immunodeficiency virus type 1 (HIV-1) genome.[1] For HIV-1 containing myristoylated proteins, prevention of protein myristoylation can lead to a failure of replication.[2,3] Furthermore, since myristoylation depends on host enzymes, we have assumed that the prevention of protein *N*-myristoylation by inhibitors may be little affected by mutations in the

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virus. We have focused our interest on the anti-HIV effect, due to inhibition of protein myristoylation, of 61 newly synthesized fatty acyl derivatives which are analogous to *N*-myristoyl glycine, the amino-terminal residue of the myristoylated protein.

Thus far inhibitors for protein *N*-myristoylation have been reported for only myristoyl-CoA:protein *N*-myristoyltransferase (NMT).[3,4] However, the myristoylation is a sequence of reactions catalyzed by acyl CoA synthetase (AS) and NMT.[5] If some inhibitors inhibit not only NMT but also AS, it would be expected that HIV production would be prevented by them. We selected the inhibitors using a screening system containing both AS and NMT. Using the *N*-terminal octapeptide, Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg-NH_2, as substrate (a sequence derived from the catalytic subunit of the cyclic AMP-dependent protein kinase of bovine cardiac muscle[6],) we determined the effects of various acyl derivatives on *N*-myristoylation. We also investigated the suppression of the cytopathicity in the HIV-1 infected cells.

MATERIALS AND METHODS

Materials

Saccharomyces cerevisiae strain JR153 was a kind gift from Peter Burgers (Washington University School of Medicine, St. Louis, MO). The peptide Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg-NH_2 was a kind gift from I. Umemura and T. Shibatani (Tanabe Seiyaku Co., Ltd., Japan). The peptide was >98% pure as determined by reverse-phase HPLC on a μ Bondasphere 15 μ C18-100A column (19 mm by 30 cm). *N*-Acetyl-*L*-cysteine was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and all other chemicals were of analytical grade.

Preparation of *N*-Acyl Amino Acids and *N*-Acyl Alkanolamines

The test compounds except for the previously described acyl derivatives[7] were synthesized as follows. The *N*-hydroxysuccinimide esters of fatty acids, prepared by coupling with *N*-hydroxysuccinimide, were reacted with amino acids and alkanolamines in aqueous solutions to afford the corresponding fatty acyl amino acids and alkanolamines, respectively.

The acyl amino acids or acyl alkanolamines and fatty acid vinyl esters were treated with *Alcaligenes* Lipase QL (Meito Sangyo Co. Ltd.) in diisopropyl ether to afford the corresponding *N*, *O*-diacyl amino acids and alkanolamines. *N*-Acyl amino methanol was obtained from the acyl amide in a solution of formaldehyde-water in the presence of potassium hydroxide.

Synthesis of *N*-Myristoyl Octapeptide Standard

N-Myristoyl octapeptide was synthesized by the method of Towler and Glaser.[5] Briefly, the synthesis of the *N*-myristoyl peptide was performed by reacting myristic acid anhydride with Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg-NH_2 in pyridine. 100 mg of myristic acid anhydride was dissolved in 3 ml of pyridine and the solution was added to 10 mg of the octapeptide. The reaction was allowed to proceed overnight with mixing. Pyridine was evaporated under vacuum, and the residue was extracted with petroleum ether (2 × 5 ml) and then redissolved in 2 ml methanol-water (1:1). The product was purified by reverse-phase HPLC on a Phase Sep S5 ODS2 column.

Enzymes and Virus

Pseudomonas AS was purchased from Sigma Chemical Co. (St. Louis, MO). *Saccharomyces* NMT was prepared by the method of Towler and Glaser.[5] Briefly, yeast cells were grown by shaking at 30°C in YPD medium (1% yeast extract, 2% peptone and 2% dextrose) and centrifuged at 7500× g for 30 min. Cells were broken up with 0.5 mm glass beads by mixing in a Vortex. Cellular debris was removed from the supernatant fluid by centrifugation at 1000× g for 10 min. The supernatant was then centrifuged at 12, 000× g for 30 min. The supernatant was removed, and the pellet was resuspended in extraction buffer (10 mM Tris·HCl, pH 7.4, 1 mM dithiothreitol, 0.1 mM EGTA). The suspension was used as the crude enzyme of NMT. Protein was determined by the method of Lowry *et al.*[8]

The LAV-1 strain of HIV-1 was obtained from the culture supernatant of MOLT-4 cells that had been persistently infected with LAV-1.[9]

Inhibitory Assay for *N*-Myristoylation

The inhibitory activity for *N*-myristoylation was assayed by modifying the method of Towler and Glaser[5] as follows: per assay tube, 2.5 μl of 20 mM myristic acid; 25 μl of 2× buffer (20 mM Tris·HCl, pH 7.4, 2 mM dithiothreitol, 10 mM $MgCl_2$ and 0.2 mM EGTA); 5 μl of 50 mM ATP; 2.5 μl of 20 mM lithium CoA; 10 μl of 1 mM Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg-NH_2; 15μl of AS (15 mU); and 10 μl of yeast cell extract (50 μg of protein: NMT). The reaction mixture was incubated with or without 1 μl of each test compound for 120 min at 30°C. The assay was terminated by the addition of 110 μl of methanol and 10 μl of 100% trichloroacetic acid. Precipitated protein was removed by centrifugation at 5000 × g for 5 min. Twenty microliters of the supernatant was analyzed by reverse-phase HPLC on a Phase Sep S5 ODS2 column (4.6 mm by 25 cm) using a mobile phase (37.5% acetonitrile-water, 0.1% trifluoroacetic acid and

TABLE I Inhibition of *N*-myristoylation by acyl amino acids

no.	*Test compound*			*N-myristoylation % inhibition*
	skeleton	*chain length*		
		N-acyl	*O-acyl*	*2 mM*
1	glycine	10	0	7
2		11	0	40
3		11 (undercylenic)	0	25
4		14	0	0
5		14 (3OH)[a]	0	40
6		14 (2OH)[b]	0	17
7	L-cysteine	2	0	0
8		14	0	0
9		14 (3OH)	0	16
10		14 (2OH)	0	23
11	D-cysteine	14	0	38
12		14 (3OH)	0	18
13		14 (2OH)	0	11
14	L-serine	18	0	14
15		14	0	0
16		14	2	0
17		14	6	74 (1.03)[c]
18		14	10	5
19		14	14	2
20		14	18	0
21		14 (3OH)	0	6
22		11	0	13
23		11 (undercylenic)	0	21
24		10	0	0
25		10	14	34 (0.50)
26	D-serine	14	0	0
27		14	2	0
28		14	6	39 (1.17)
29		14	10	21
30		14	14	35
31		14	18	0
32		14 (3OH)	0	6
33		14 (2OH)	0	24
34		11	0	18
35		11 (undecylenic)	0	23

[a]3-hydroxy. [b]2-hydroxy. [c]The figures in parentheses refer to K_i value (mM).

TABLE II Inhibition of *N*-myristoylation by alkanolamines

Test compound				
no.	*skeleton*	*chain length*		*N-myristoylation % inhibition 2 mM*
		N-acyl	*O-acyl*	
36	methanolamine	14	0	5
37		14 (3OH)[a]	0	0
38	ethanolamine	18	0	7
39		14	0	11
40		14	2	15
41		14	6	24
42		14	10	2
43		14	14	1
44		14	18	15
45		14	14 (3OH)	22
46		14 (3OH)	0	0
47		14 (3OH)	14	0
48		14 (2OH)[b]	0	0
49		10	0	0
50		10	14	0
51		6	0	2
52		6	14	8
53		2	0	0
54		2	14	13
55	propanolamine	14 (3OH)	0	6
56	isopropanolamine	14	0	0
57		14 (2OH)	0	9
58		14 (3OH)	0	12
59	2-aminopropanol	14 (3OH)	0	0
60	butanolamine	14 (3OH)	0	13
61	valinol	14	0	14
62		14 (3OH)	0	9

[a]3-hydroxy. [b]2-hydroxy.

0.05% triethylamine). Myristoyl-Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg-NH_2 was eluted at 24 min.

The percent inhibition was calculated from the *N*-myristoylation activities with and without each inhibitor.

Anti-HIV-1 Activity Assay

The HTLV-I–carrying cell line MT-4 was used. MT-4 cells were infected for 1h with HIV-1(LAV-1) at $TCID_{50}$(50% tissue culture infectious dose) of 0.001/cell. Then,

the cells were washed and resuspended at 1×10^5 cells/ml in RPMI-1640 medium. A 200 μl/well of the cell suspension was cultured for 5 days in a 96-well culture plate containing various concentrations (12 doses) of the test compounds. Control assays were performed, without compounds, with HIV-1-infected and –uninfected cultures. On day 5, the inhibitory concentration (IC) of the test sample to completely prevent the HIV-1-induced cytopathic effect and the cytotoxic concentration to reduce the viability of MT-4 cells were determined under an optical microscope.[9]

RESULTS AND DISCUSSION

Screening for Inhibition of Peptide *N*-Myristoylation

In view of the possibility of the antiviral action, through inhibition of not only NMT but also AS, we screened the prepared acyl derivatives for the inhibition of *N*-myristoylation of the octapeptide using myristic acid, CoA, and ATP as the substrates instead of myristoyl CoA. Of 62 derivatives tested, 7 derivatives (no. 2, 4, 11, 17, 25, 28, 30) exhibited an inhibitory activity above 30% (Table I). The relationship between inhibitory activity and the structure of the acyl derivatives was not clear, but the inhibitory activities were observed in the amino acid derivatives but not in the alkanolamine derivatives (Table II). The type and side-chain lengths of the fatty acyl group were not correlated with the inhibitory activities for *N*-myristoylation. It has been reported by Saermark and Bex[11] that myristoyl glycine inhibits 50% of NMT activity at 6 μg per ml, but this compound did not inhibit the myristoylation of the octapeptide, even at 2 mM, in our screening system. On the other hand, *N*-3-hydroxymyristoyl glycine showed a relatively strong inhibition. It does not appear that this inhibition resulted from the 3-hydroxymyristoyl group, because none of the 3-hydroxymyristoyl derivatives of the amino acids, except for glycine, and alkanolamines significantly inhibited the myristoylation. The NMT inhibition has been reported in 2-hydroxymyristic acid,[12] but none of the 2-hydroxymyristoyl derivatives exhibited any inhibition for myristylation, as shown in Table I and II. Among the diacyl derivatives, *N*-myristoyl-*O*-caproyl-*L*-serine (**17**), *N*-myristoyl-*O*-caproyl-*D*-serine (**28**) and *N*-decanoyl-*O*-myristoyl-*L*-serine (**25**) strongly inhibited *N*-myristoylation.

Kinetic Analysis of Inhibition

A kinetic analysis of the inhibition was carried out using *N*-myristoyl-*O*-caproyl-*L*-serine which is the most potent inhibitor found here. The rate of myristoylation was investigated by varying the concentrations of myristic acid and the inhibitor, as shown in Figure 1. The inhibition curves were parallel to each other at varying

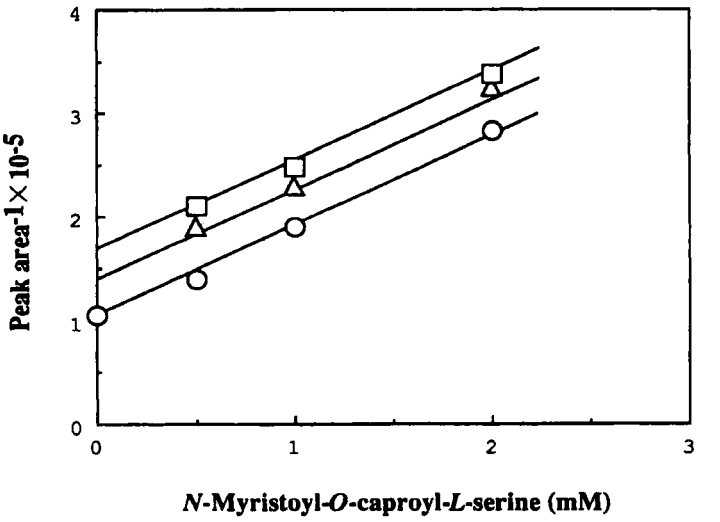

FIGURE 1 Inhibitory activity of *N*-myristoyl-*O*-caproyl-*L*-serine (**17**) against *N*-myristoylation. Initial velocities of *N*-myristoylation activity were determined in the absence or presence of (**17**) at various concentrations in increasing myristic acid concentrations (□:0.03, △:0.05, ○:1.0 mM) under the conditions described in MATERIALS AND METHODS.

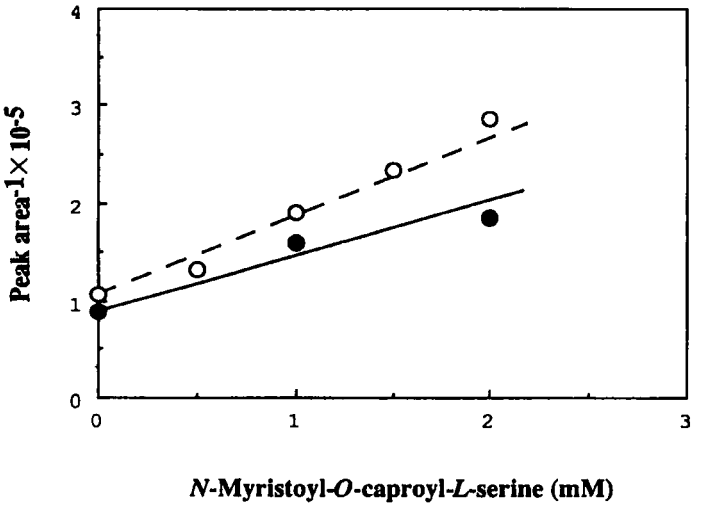

FIGURE 2 Comparison of inhibitory activities of *N*-myristoyl-*O*-caproyl-*L*-serine (**17**) against *N*-myristoylation using myristic acid (○:1.0 mM) and myristoyl-CoA(●:0.2 mM). The inhibition for *N*-myristoylation by (**17**) was measured at 1.0 mM myristic acid or 0.2 mM myristoyl-CoA with increasing concentrations of (**17**).

concentrations of myristic acid. This result suggests that the inhibition is uncompetitive. No inhibitor of NMT is known as an uncompetitive inhibitor, although a number of inhibitors have been reported as competitive or noncompetitive inhibitors.[6,10,11,13–16] We also found that both *N*-myristoyl-*O*-caproyl-*D*-serine and *N*-decanoyl-*O*-myristoyl-*L*-serine with relatively high inhibition activities were also uncompetitive inhibitors (data not shown). These findings suggested the possibility that these inhibitors either inhibit the peptide *N*-myristoylation with a novel mechanism or that the inhibition is against AS and not NMT. We investigated the latter possibility using myristoyl CoA instead of myristic acid. As shown in Figure 2, inhibition by *N*-myristoyl-*O*-caproyl-*L*-serine was also observed with myristoyl CoA as the substrate, but to a weaker degree than that with myristic acid. This result supports the view that the inhibition of octapeptide *N*-myristoylation may result from inhibition of both NMT and AS. The kinetics of inhibition for each of these two enzymes are separately under investigation.

Prevention of Cytopathicity in HIV-infected Cells

Since several acyl compounds inhibited the myristoylation of octapeptide, they were examined for anti-HIV activity. Several *N*-hydroxymyristoyl derivatives suppressed the replication of HIV-1 (Table III), but none of them were inhibitors for myristoylation of the octapeptide (Table I and II). This apparent dissociation between the suppression of replication and the inhibition of myristoylation would need further investigation at or below a concentration of 10^{-5} M of inhibitors. The microbial origin of AS and NMT used in the myristoylation assay might be another reason for the dissociation from the suppression of cytopathicity examined with animal cells. The effective compounds except for *N*-acetyl-*L*-cysteine were slightly toxic toward MT-4 cells.

Among the thiol derivatives, *N*-acetyl-*L*-cysteine, which is a known antiviral compound,[17–23] showed suppression of denaturation for MT-4 cells at 31.3 μg per ml. Since the suppression activities for other cysteine derivatives were similar to that of *N*-acetyl-*L*-cysteine, the antiviral action with the cysteine derivatives seems to depend on an antioxidation action by the thiol group rather than inhibition of *N*-myristoylation of virus proteins. However, hydroxymyristoylserine and alkanolamine derivatives were also effective suppressors. The suppression seems to depend on the hydroxymyristoyl groups because hydroxymyristic acid also suppressed the replication of the varicella-zoster virus.[24–25] The hydroxymyristoyl group suppresses the replication of HIV-1. With *N*-acyl seryl-lysine derivatives, powerful inhibition of NMT was reported by Devadas *et al.*[14] A screening of novel *N*-acyl serine derivatives for NMT inhibition is worthy of further study.

TABLE III Inhibitory effects of *N*-myristoyl derivatives on HIV-induced cytopathicity in MT-4 cells

no.	Test compound	*N-myristoylation* % inhibition 2 mM	*inhibitory concentration*[a] (μg/ml)	*cytotoxic concentration*[b] (μg/ml)
8	*N*-myristoyl-*L*-cysteine	0	31.3	62.5
9	*N*-3-hydroxymyristoyl-*L*-cysteine	16	62.5	125
12	*N*-3-hydroxymyristoyl-*D*-cysteine	18	62.5	125
13	*N*-2-hydroxymyristoyl-*D*-cysteine	11	62.5	62.5
32	*N*-3-hydroxymyristoyl-*D*-serine	6	31.3	62.5
46	*N*-3-hydroxymyristoylethanolamine	0	31.3	62.5
57	*N*-2-hydroxymyristoylisopropanolamine	9	62.5	125
7	*N*-acetyl-*L*-cysteine	0	31.3	> 1000

[a]Minimum concentration for the complete inhibition of HIV-induced cytopathicity in MT-4 cells. [b]Minimum concentration for appearance of MT-4 cell toxicity.

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