Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 2055–2059, March 1991
Cell Biology

Incorporation of 12-methoxydodecanoate into the human immunodeficiency virus 1 gag polyprotein precursor inhibits its proteolytic processing and virus production in a chronically infected human lymphoid cell line

(protein N-myristoylation/AIDS/fatty acid analogs)

MARTIN L. BRYANT*, LEE RATNER†‡, ROBERT J. DURONIO§, NANDINI S. KISHORE¶, BALEKUDRU DEVADAS||, STEVEN P. ADAMS||, AND JEFFREY I. GORDON†§

Departments of *Pediatrics, †Medicine, ‡Molecular Microbiology, and §Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, MO 63110; ¶Molecular and Cell Biology Department, G. D. Searle, St. Louis, MO 63198; and ||Corporate Research Department, Monsanto Company, St. Louis, MO 63198

*Communicated by Howard A. Schneiderman**, November 21, 1990 (received for review September 4, 1990)*

ABSTRACT **Covalent linkage of myristate (tetradecanoate; 14:0) to the NH_2-terminal glycine residue of the human immunodeficiency virus 1 (HIV-1) 55-kDa gag polyprotein precursor (Pr55^{gag}) is necessary for its proteolytic processing and viral assembly. We have shown recently that several analogs of myristate in which a methylene group is replaced by a single oxygen or sulfur atom are substrates for *Saccharomyces cerevisiae* and mammalian myristoyl-CoA:protein *N*-myristoyltransferase (EC 2.3.1.97; NMT) despite their reduced hydrophobicity. Some inhibit HIV-1 replication in acutely infected CD4$^+$ H9 cells without accompanying cellular toxicity. To examine the mechanism of their antiviral effects, we performed labeling studies with two analogs, 12-methoxydodecanoate (13-oxamyristate; 13-OxaMyr) and 5-octyloxypentanoate (6-oxamyristate; 6-OxaMyr), the former being much more effective than the latter in blocking virus production. [^{3}H]Myristate and [^{3}H]13-OxaMyr were incorporated into Pr55^{gag} with comparable efficiency when it was coexpressed with *S. cerevisiae* NMT in *Escherichia coli*. [^{3}H]6-OxaMyr was not incorporated, even though its substrate properties *in vitro* were similar to those of 13-OxaMyr and myristate. [^{3}H]13-OxaMyr, but not [^{3}H]6-OxaMyr, was also efficiently incorporated into HIV-1 Pr55^{gag} and nef (negative factor) in chronically infected H9 cells. Analog incorporation produced a redistribution of Pr55^{gag} from membrane to cytosolic fractions and markedly decreased its proteolytic processing by viral protease. 13-OxaMyr and 3′-azido-3′-deoxythymidine (AZT) act synergistically to reduce virus production in acutely infected H9 cells. Unlike AZT, the analog is able to inhibit virus production (up to 70%) in chronically infected H9 cells. Moreover, the inhibitory effect lasts 6–8 days. These results suggest that (*i*) its mechanism of action is distinct from that of AZT and involves a late step in virus assembly; (*ii*) the analog may allow reduction in the dose of AZT required to affect viral replication; and (*iii*) combinations of analog and HIV-1 protease inhibitors may have synergistic effects on the processing of Pr55^{gag}.**

Myristic acid (14:0) is covalently linked via an amide bond to the NH_2-terminal glycine residues of several proteins encoded by the human immunodeficiency virus 1 (HIV-1) genome—the 55-kDa gag polyprotein precursor (Pr55^{gag}), the 180-kDa gag-pol fusion protein, and a 27-kDa protein termed "negative factor" (nef), whose precise role in regulating virus replication remains uncertain (1, 2). Attachment of this rare fatty acid to Pr55^{gag} is required for HIV-1 replication: transfection of HeLa, COS, or Jurkat cells with a functional provirus clone of HIV-1 containing a Gly-2 → Ala mutation in Pr55^{gag} eliminates virus production (3, 4). The nonmyristoylated mutant protein appears to undergo redistribution from the membrane to the cytosolic fraction (4). Blockade of myristoylation is also associated with a dramatic reduction in the rate of proteolytic processing of the polyprotein precursor by viral protease (4).

We have shown (5) that heteroatom-substituted analogs of myristate inhibit HIV-1 replication in acutely infected CD4$^+$ H9 cells. In these analogs, a methylene group is replaced by a single oxygen or sulfur atom. Such replacements produce a reduction in hydrophobicity comparable to the loss of two to four methylene groups without significant alterations in chain length or stereochemistry (6). Metabolic labeling studies (7) using several cultured cell lines indicated that these analogs enter mammalian cells and are substrates for acyl-CoA synthetase and myristoyl-CoA:protein *N*-myristoyltransferase (NMT). Analog incorporation is very selective: only a subset of cellular N-myristoylated proteins incorporate a given analog, and a given protein may incorporate one but not another analog depending on the site of heteroatom substitution (7). This probably reflects the cooperative interactions that occur between the acyl-CoA and peptide binding sites of NMT: *in vitro* studies with purified *Saccharomyces cerevisiae* NMT indicate that binding of analog-CoA species can produce changes in the catalytic efficiencies (V_m/K_m) of some but not all octapeptide substrates (6, 8). These studies (7) also revealed that analog incorporation had very selective effects on protein targeting. Only a small subset of analog-substituted proteins underwent redistribution from the membrane to cytosolic fractions. An even smaller subset of proteins displayed analog-specific redistribution—i.e., one analog affected targeting while another analog, containing an oxygen-for-methylene substitution at a different position, produced no detectable change in protein compartmentalization (7).

Exploiting cellular NMT activity to deliver analogs of myristate with altered physicochemical properties provides an opportunity to examine (*in vivo*) structural features of the acyl moiety that affects the functional properties of individual

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: HIV-1, human immunodeficiency virus 1; NMT, myristoyl-CoA:protein *N*-myristoyltransferase (EC 2.3.1.97); AZT, 3′-azido-3′-deoxythymidine; 13-OxaMyr, 13-oxamyristate (12-methoxydodecanoate); 6-OxaMyr, 6-oxamyristate (5-octyloxypentanoic acid); RT, reverse transcriptase; Pr55^{gag}, 55-kDa gag polyprotein precursor; nef, negative factor.
**Deceased December 5, 1990.

N-myristoylated proteins. In this report, we used radiolabeled analogs to begin to define the mechanism of their inhibitory effects on HIV-1 assembly in acutely and chronically infected lymphoid cell lines. The results are consistent with the notion that these compounds function in a manner that is distinct from 3′-azido-3′-deoxythymidine (AZT).

MATERIALS AND METHODS

Vectors for Expression of gag, gag-pol, or nef in *Escherichia coli*. pGG1 contains a functional clone of HIV-1 (HXB2gpt2 in ref. 9). pGA1 was produced by mutagenesis of the codon encoding Gly-2 in the gag gene of pGG1 to a codon-encoding alanine (4). Oligonucleotide-directed mutagenesis was used to introduce a new *Nco* I restriction site at the initiator methionine (ATG) codon contained in the 5.3-kilobase (kb) *Sac* I fragment of pGG1 or pGA1. pMGG1 and pMGA1 are gag expression vectors created by subcloning the *Nco* I–*Eco*RI "subfragments" from each of the above plasmids into pMON5840 (10). pMGPG1 and pMGPA1 are gag-pol expression vectors with codons encoding Gly-2 and Ala-1, respectively. They were constructed by subcloning the *Nco* I–*Nco* I fragments from pGG1 and pGA1 into pMON5840.

The polymerase chain reaction was used to introduce simultaneously a new *Nco* I site at the initiator methionine (ATG) codon and a unique *Hin*dIII site 3′ to the termination codon of the nef gene, in p2/3MBNG1 (11) and in p2/3MBNA1 (which contains a Gly-2 → Ala mutation). The 627-base-pair (bp) *Nco* I–*Hin*dIII fragment from each plasmid was subcloned into pMON5840, yielding the nef expression vectors pMNG1 and pMNA1, respectively.

Metabolic Labeling Studies in Human Lymphoid Cells. Uninfected $CD4^+$ H9 cells or those chronically producing HIV-1 (H9IIIB; ref. 9) were grown in RPMI 1640 medium containing 10% (vol/vol) fetal bovine serum, 100 units of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM glutamine. Cells (10^7) were "starved" overnight in RPMI 1640/5% delipidated fetal bovine serum and then incubated for 8 hr with fresh medium supplemented with 1 mCi (1 Ci = 37 GBq) of [^{3}H]myristate or tritiated analog (final specific activity, 32 Ci/mmol) per ml and 5 mM pyruvate. Cells were washed twice with phosphate-buffered saline (PBS) at 4°C, scraped into ice-cold PBS, pelleted by centrifugation at 250 × *g* for 10 min, and lysed in radioimmunoprecipitation assay buffer (4). For immunoblot analyses, 50 μg of reduced and denatured lysate protein was separated by SDS/PAGE (12) and electroblotted onto nitrocellulose membranes. Virus-specific proteins were identified by sequential incubation of the blot with (*i*) pooled sera of AIDS patients or a monospecific rabbit polyclonal anti-nef serum; (*ii*) biotin-conjugated goat anti-human (or anti-rabbit) IgG; (*iii*) avidin-conjugated horseradish peroxidase (HRP); and (*iv*) the HRP substrate 4-chloro-1-naphthol.

Chronic Virus Replication Assay. H9IIIB cells were dispensed at 2×10^5 cells per ml of RPMI 1640 medium into each well of 48-well culture plates. An equal volume of serum-free RPMI 1640 medium with or without analog (or AZT) was immediately added. After 48 hr, the cells were washed with serum-free medium to remove residual virus, refed with complete medium with or without analog, and maintained in culture for an additional 48 hr. The cell culture supernatant was subsequently collected, filtered through a 0.22-μm Millipore filter, and assayed for reverse transcriptase activity (13) or p24 virus antigen (Du Pont, ELISA).

Toxicity Studies. Cell viability was measured at the end of the treatment period in both acute (4) and chronic replication assays by (*i*) metabolic labeling studies with [^{3}H]leucine or [^{3}H]thymidine (5) and (*ii*) determination of the number of viable cells based on trypan blue exclusion (5).

RESULTS

Heteroatom-Containing Analogs of Myristate Serve as Alternative Substrates for *S. cerevisiae* NMT and Are Linked to the gag and nef Proteins of HIV-1 Expressed in *E. coli*. HIV-1 $Pr55^{gag}$ is efficiently N-myristoylated when expressed in *S. cerevisiae* (14). Therefore, we first compared the substrate properties of myristate with the oxygen-substituted analogs in a coupled *in vitro* assay (6) that contained purified *S. cerevisiae* NMT (8) and a radiolabeled octapeptide (Gly-Ala-Arg-[^{3}H]Ala-Ser-Val-Leu-Ser-NH_2) representing residues 2–9 of $Pr55^{gag}$. The results (Table 1) indicated that when converted to CoA thioesters, the three fatty acids have quite similar kinetic properties.

A second and more "physiologic" assay for analog incorporation involved the use of a dual plasmid expression system (Fig. 1*A*) that allows us to recreate this eukaryotic protein modification in *E. coli*, a bacterium that contains no endogenous NMT activity (10). *S. cerevisiae* NMT can be efficiently synthesized (≈1% of total *E. coli* proteins) when its gene is placed under the control of the isopropyl β-D-thiogalactoside-inducible *tac* promoter in the plasmid pBB131 (10). Moreover, the fatty acid and peptide substrate specificities of *E. coli*-derived *S. cerevisiae* NMT are indistinguishable from those of the authentic yeast enzyme (8, 10). A second plasmid containing the nalidixic acid-inducible *recA* promoter can be used to direct production of a protein that is a known or potential substrate for the enzyme. Sequential induction of each promoter (NMT first) allows N-myristoylation to occur "*in vivo*" (10). When NMT and either wild-type (Gly-2) gag-pol (pMGPG1) or gag (pGG1) sequences were coexpressed in *E. coli*, a prominent radiolabeled 41-kDa band was recovered from [^{3}H]myristate-labeled lysates by using anti-HIV-1 serum (Fig. 1*B*, lanes 1 and 3). Since synthesis of the 180-kDa gag-pol polyprotein in eukaryotic cells occurs by an inefficient translational frameshifting mechanism (15), we did not expect to find a labeled product of this size in *E. coli*. However, only trace quantities of the intact [^{3}H]myristate-labeled $Pr55^{gag}$ polypeptide were noted after immunoprecipitation of *E. coli* lysates even though this species could be easily detected in immunoblots (Western blots) of the same lysates (compare Fig. 1*B Left* and *Right*). [Previous studies in *E. coli* (16, 17) and *S. cerevisiae* (14) indicated that $Pr55^{gag}$ undergoes proteolytic processing to a 41-kDa form even in the absence of HIV protease.]

The [^{3}H]myristate 41-kDa labeled band was not present in lysates prepared from (*i*) strains producing NMT and mutant (Ala-2-containing) gag-pol (pMGPA1) or gag (pGA1) polypeptides and (*ii*) strains that contained pBB131 and pMON5840, the expression vector lacking HIV-1 sequences (Fig. 1*B*, lanes 2, 6, and 9). Western blot analysis revealed

Table 1. *In vitro* characterization of fatty acid analogs using an octapeptide (GARASVLS-NH_2) derived from the NH_2 terminus of HIV-1 $Pr55^{gag}$

Fatty acid	Peptide K_m	Peptide V_{max}	Peptide V_{max}/K_m	Fatty acid K_m	Fatty acid V_{max}
$CH_3(CH_2)_{12}COOH$	9	100	12	2	100
$CH_3O(CH_2)_{11}COOH$	8	100	12	1	370
$CH_3(CH_2)_7O(CH_2)_4COOH$	14	118	8	2	99

As in previous reports (e.g., ref. 6), the apparent peptide K_m and V_{max} was first determined by using saturating concentrations of analog (15 μM). The acyl-CoA K_m and V_{max} was then measured by using the peptide substrate (specific activity, 0.8 Ci/mmol) at its K_m. The kinetic properties of each analog CoA were determined in parallel with myristoyl-CoA. All experiments were performed three times, and the data were averaged. Direct measurements of acyl-CoA production by *Pseudomonas* acyl-CoA synthetase (6) indicate that each of the three tritiated fatty acids has similar substrate properties (efficiency of conversion to their CoA thioesters = 60–70%).

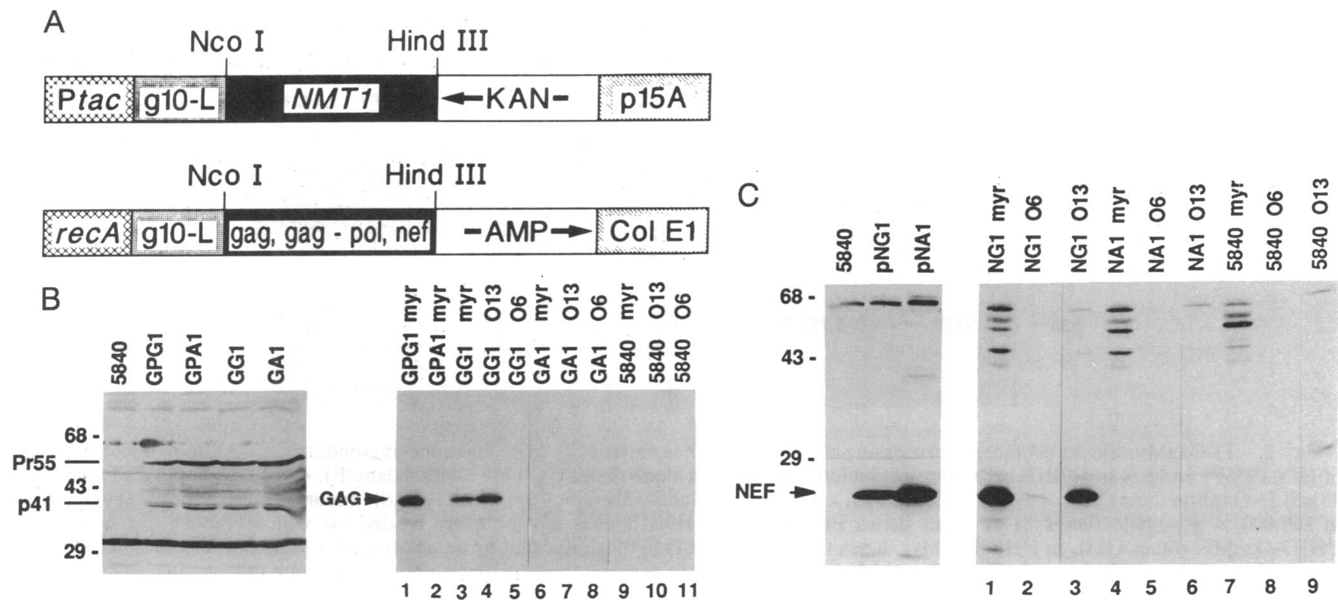

FIG. 1. Coexpression of *S. cerevisiae* NMT and HIV-1 gag, gag-pol, or nef in *E. coli*. Dual plasmid expression system. (*B*) Coexpression of NMT and HIV gag, or gag-pol, in midlogarithmic phase *E. coli* metabolically labeled (9) with [^{3}H]myristic acid (myr) or tritiated analog (ref. 7; final specific activity = 32 Ci/mmol; 100 μCi/ml of culture). (*B Left*) Immunodetection of HIV gag in the *E. coli* lysates (50 μg of total protein) after reduction, denaturation, and electrophoresis through 7.5–20% polyacrylamide gels. Anti-HIV-1 sera were used to probe the Western blot. (*B Right*) Immunoprecipitation of the [^{3}H]myristic acid- or tritiated analog-labeled HIV-1 gag protein with the same antiserum. (*C*) Coexpression of NMT and HIV-1 nef in *E. coli* (50 μg of total protein) labeled with [^{3}H]myristate or tritiated analog and detected by immunoblot (*Left*) or immunoprecipitation (*Right*) with rabbit anti-nef sera. O6, 6-OxaMyr; O13, 13-OxaMyr; g10-L, bacteriophage T7 gene *10* leader.

that the steady-state levels of the 55- and 41-kDa wild-type and mutant gag polypeptides were identical in all *E. coli* gag transformants and that neither species was present in bacteria containing pBB131 and pMON5840 (Fig. 1*B Left*). Control experiments also established that strains containing wild-type gag or gag-pol recombinant plasmids alone or either of the plasmids with pMON5839 (pBB131 without the *NMT1* insert) failed to incorporate [^{3}H]myristate (data not shown).

Parallel studies revealed that tritiated 12-methoxydodecanoate [13-oxamyristate (13-OxaMyr)] was as efficiently incorporated into the wild-type gag as myristate (compare lanes 3 and 4 in Fig. 1*B*). Incorporation was blocked with the Gly-2 → Ala mutation and required simultaneous expression of both NMT and gag (or gag-pol) (Fig. 1*B*, lanes 7 and 10). Interestingly, tritiated 5-octyloxypentanoate [6-oxamyristate (6-OxaMyr)] produced no detectable labeling of the wild-type gag proteins (Fig. 1*B*, lanes 5, 8, and 11). This finding, together with the *in vitro* data presented in Table 1 and the fact that treatment with the radiolabeled analog had no effect on the steady-state levels of the HIV-1 proteins in *E. coli* (as determined by Western blots), suggested that the efficiency of import of [^{3}H]6-OxaMyr by the *E. coli* fatty acid transporter (18) may be poor. Subsequent analyses showed that after a 30-min incubation period, uptake of [^{3}H]6-OxaMyr into logarithmically growing *E. coli* strain JM101 is 1/10th that of [^{3}H]13-OxaMyr, allowing us to postulate that the position of heteroatom substitution may effect interactions with this inner membrane-associated protein (18).

Similar labeling patterns were seen with nef (Fig. 1*C*). Myristate and 13-OxaMyr were incorporated into this protein with comparable efficiency (compare lanes 1 and 3 in Fig. 1*C Right*). Even though the wild-type (Gly-2) and mutant (Ala-2) nef proteins achieved comparable steady-state levels (Fig. 1*C Left*), only the Gly-2-containing species was labeled (Fig. 1*C Right*, compare lane 1 with 4 and 3 with 6). Incorporation of [^{3}H]myristate or the analog required simultaneous expression of NMT and nef (Fig. 1*C Right*, lanes 1 and 7); [^{3}H]6-OxaMyr labeled this protein poorly (lanes 2 and 5).

13-OxaMyr Inhibits Proteolytic Processing of HIV-1 Pr55^{gag} in Chronically Infected Human Lymphoid Cells. H9/IIIB cells chronically produce HIV-1. Labeling studies indicated that [^{3}H]myristate-labeled gag proteins (55 and 41 kDa) are almost equally distributed between cytosolic (S100) and membrane (P100) fractions prepared (4) from these cells (cytosolic/membrane ratio = 1; Fig. 2*B*). The p17 matrix antigen of HIV-1 contains the NH_2-terminal myristoyl moiety and is cleaved by the virus-specific protease from Pr55^{gag} at the plasma membrane during virus assembly. Fig. 2*B* shows that this [^{3}H]myristate-labeled protein is almost exclusively associated with the P100 fraction. The additional 27- and 25-kDa labeled bands seen in these lanes represent incorporation of myristate into nef species (these were immunoprecipitated with a monospecific rabbit anti-nef serum) (Fig. 2*C*). Detection of two N-myristoylated forms of nef is not surprising because the H9/IIIB isolate is a mixture of at least three viral strains and produces several nef species including the intact 27-kDa protein as well as a species that is truncated at the COOH terminus (refs. 9, 11, and 12 and our unpublished observations).

13-OxaMyr was incorporated into the 27- and 25-kDa nef proteins but did not affect their predominant cytosolic distribution (Fig. 2*C*). By contrast, 13-OxaMyr incorporation into Pr55^{gag} was associated with a redistribution from the membrane to the cytosolic fraction (cytosolic/membrane ratio = 3; Fig. 2*B*). Moreover, there was a marked reduction in the intensity of labeling of p17 (compare the myristate- and 13-OxaMyr-labeled pellet fractions in Fig. 2*B*), suggesting that the analog inhibited proteolytic processing of Pr55^{gag}. Incubation of H9/IIIB cells with [^{3}H]6-OxaMyr for 8 hr resulted in very poor labeling of nef (Fig. 2*C*) and virtually undetectable labeling of the gag proteins (Fig. 2*B*).

To examine the specificity of the analog's effect on proteolytic processing, we incubated H9/IIIB cells for 48 hr with 40 μM 13-OxaMyr, 40 μM decanoate (10:0) (the hydrophobicities of 13-OxaMyr and decanoate are equivalent; ref. 6), 0.1% ethanol (the solvent used to dissolve the analog), or 5 μM AZT. Western blots of total cellular proteins were then probed with a mouse monoclonal anti-p24 antiserum (4). Pr55^{gag} and its specific cleavage product p24 were readily identified in each of the lysates (Fig. 2*A*). A marked decrease

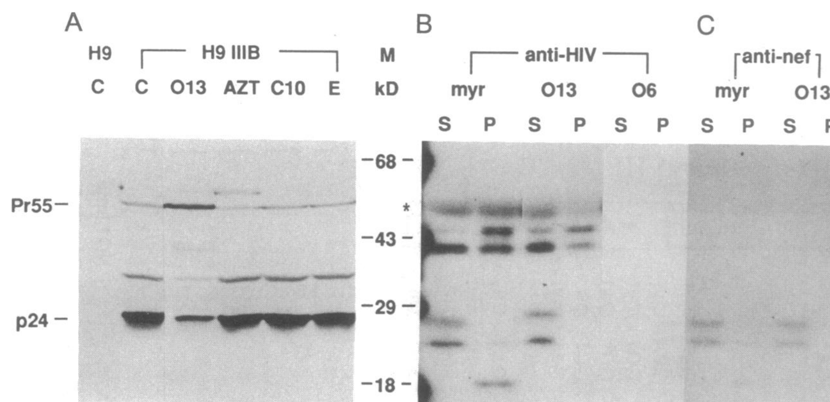

FIG. 2. 13-OxaMyr blocks proteolytic processing of HIV-1 Pr55^{gag} in chronically infected human lymphoid cells. (*A*) Immunoblot detection of HIV Pr55^{gag} and p24 in H9IIIB cells after incubation with medium alone (lanes C), 0.1% ethanol (lane E), 40 μM decanoic acid (lane C10), 40 μM 13-OxaMyr (lane O13), or 5 μM AZT (lane AZT). A monoclonal anti-p24 antibody (4) was used. (*B*) Immunoprecipitation of HIV-1 proteins in 100,000 × *g* soluble (lanes S) or pellet (lanes P) fractions of H9IIIB cells metabolically labeled (4) with [^{3}H]myristate (lanes myr), [^{3}H]13-OxaMyr (lanes O13), or [^{3}H]6-OxaMyr (lanes O6). The intact Pr55^{gag} is indicated by an asterisk. (*C*) Immunoprecipitation of HIV nef species from the same fractions as in *B*. The results shown in *A–C* are representative of those obtained in three independent experiments.

in proteolytic processing of Pr55^{gag} to p24 was noted only with analog treatment.

[^{3}H]13-OxaMyr Reduces the Concentration of AZT Required to Inhibit HIV-1 Replication in an Acute Replication Assay. 13-OxaMyr produces a dose-dependent reduction in HIV-1 production in acutely infected CD4$^+$ H9 cells without accompanying cellular toxicity: the ≈90% reduction in cell-free reverse transcriptase (RT) and p24 antigen levels achieved with 20–40 μM analog is comparable to that produced by 5 μM AZT (5). We examined the combined effect of 13-OxaMyr and AZT because (*i*) the data presented in Fig. 2 suggested that 13-OxaMyr may affect a step that occurs late in virus replication (i.e., assembly at the membrane and/or proteolytic processing of gag polyprotein precursors), and (*ii*) AZT blocks proviral DNA synthesis by inhibiting RT—an early step in virus infectivity. We determined in preliminary experiments (data not shown) that 0.03 μM AZT produced a 50% reduction in virus-associated RT activity. At concentrations where neither analog nor AZT alone produced significant (>10%) reductions in virus production, the combination of the two compounds produced a moderate but reproducible synergistic effect (0.01 or 0.02 μM AZT plus 0.1 or 1.0 μM analog in Fig. 3*A*). When the concentration of analog exceeded 10 μM or when AZT was added at 0.05 μM or more, the advantage of combining drugs was obscured by the significant effect of each alone.

[^{3}H]13-OxaMyr Inhibits Chronic HIV-1 Production in H9/IIIB Cells. Chronically infected cells in human hosts could play a significant role in HIV-1 persistence and latency. Virus production from such cells would not be affected by treatment with AZT. However, compounds that disrupt late steps in virus replication could decrease virus load and interrupt the replicative cycle. Therefore, the effects of 40 μM 13-OxaMyr, 40 μM decanoate, 5 μM AZT, and 0.1% ethanol were compared in chronically infected cells. A 60% reduction in p24 antigen and RT activity was documented 2 days after treatment with analog: AZT, decanoate, or ethanol had no effect on these measures of virus production (Fig. 3*B*). Furthermore, analog treatment had no demonstrable toxic effects on the host H9 cells as measured by cell number and protein synthesis (Fig. 3*B*).

The long-term effect of this analog (50 μM) on virus production was tested by extending the treatment period to 12 days (Fig. 3*C*). RT activity was reduced ≈70% by day 6 of treatment and was maintained at this level until removal of 13-OxaMyr from the culture medium. Analog withdrawal resulted in a progressive rise in RT activity. However, no increases were noted for 48 hr, and levels had not fully returned to pretreatment or control (0.1% ethanol) values 8 days later (Fig. 3*C*). (Note that the number of viable cells in each of the test groups was comparable at the end of the test period.) The long-lived antiviral effect suggests that the analog is able to avoid metabolic processing and/or that the intracellular levels of drug that are required to inhibit HIV-1 replication may be very low.

DISCUSSION

We have explored the mechanism by which myristic acid analogs can selectively inhibit HIV-1 replication and assembly. A dual plasmid expression system that reconstitutes protein N-myristoylation in *E. coli* was initially used to determine whether two fatty acid analogs—differing only in the site of the oxygen-for-methylene substitution—could be converted to their CoA thioesters and incorporated into HIV-1 Pr55^{gag} and nef proteins. [^{3}H]13-OxaMyr worked as well as myristate in this assay system and also was incorporated into these two proteins in H9 cells. This analog causes Pr55^{gag} to redistribute from membrane to cytosolic fractions and markedly reduces its proteolytic processing by viral protease. These results have implications not only about the function of the myristoyl moiety in Pr55^{gag} but also about potential therapeutic strategies for the treatment of AIDS.

The metabolic labeling studies carried out in HIV-1-infected H9 cells confirmed our earlier hypothesis (5) that 13-OxaMyr serves as an alternative substrate for human lymphocyte acyl-CoA synthetase and NMT. The poor incorporation of [^{3}H]6-OxaMyr into gag and nef was part of a general failure to label H9 cellular proteins compared with [^{3}H]myristate and [^{3}H]13-OxaMyr (data not shown). Studies with a murine myocyte cell line (BC_3H1) and rat fibroblasts have indicated that 6-OxaMyr is incorporated into a much smaller subset of cellular proteins than is 13-OxaMyr (7). This may reflect differences in their uptake, conversion to acyl-CoAs, susceptibility to metabolic processing, and/or interactions with NMT.

The two observed consequences of incorporating 13-OxaMyr into Pr55^{gag} (redistribution from membrane to cytosolic fractions and inhibition of proteolytic processing) may be interrelated. Gly-2 → Ala mutagenesis of Pr55^{gag} blocks N-myristoylation and proteolytic processing in HeLa cells (4). However, the Myr$^+$-Pr55^{gag} can be processed by purified HIV protease *in vitro* (4). The myristoyl moiety may be required for intermolecular association of the larger gag-pol precursor and autocatalytic release/activation of viral pro-

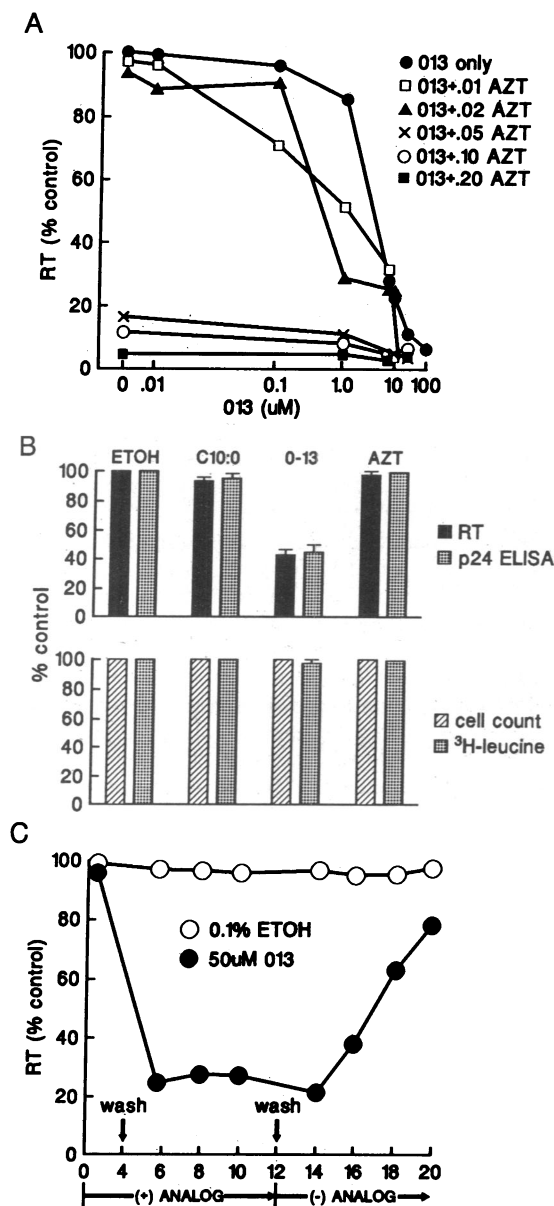

FIG. 3. Effect of 13-OxaMyr (O13) on the propagation of HIV-1 in acute and chronic replication assays. (*A*) Combined effect of 13-OxaMyr and AZT on virus replication in an acute infectivity assay (5). HIV-1-infected H9 cells were treated with 13-OxaMyr, AZT, or both for 10 days (5), and virus replication was measured by RT activity and compared to untreated or ethanol (0.1%)-treated infected cells. The SEM at each point varied no more than 10% (n = 2 experiments, each assay done in triplicate). (*B*) The effect of 40 μM 13-OxaMyr (bars O-13), 5 μM AZT, 40 μM decanoate (bars C10:0), and 0.1% ethanol on virus production and cell viability in chronically infected H9/IIIB cells is expressed as a percentage of untreated virus-producing cells. (*C*) The long-term effect of 50 μM 13-OxaMyr on HIV-1 replication in chronically infected H9IIIB cells. The virus-producing cells were treated with 50 μM 13-OxaMyr (O13) or 0.1% ethanol for 48 hr, washed to remove residual virus, and refed every other day with medium containing either 13-OxaMyr or ethanol. On day 12, the cells were washed and refed with medium alone.

tease (19) or it may exert its effects by stabilizing association with the plasma membrane where such protein–protein interactions could become more likely, and autocatalytic cleavage, more efficient. Although the precise mechanism is unclear, out data suggest that treatment of H9 cells with combinations of HIV protease inhibitors and heteroatom-containing analogs may produce even greater (synergistic?) inhibition of gag polyprotein precursor processing. Additional experiments with radiolabeled analogs containing other structural perturbations may provide further insights about the physical-chemical features of the acyl chain that are required for targeting and processing of HIV-1 gag proteins.

The results obtained in the chronic virus replication assay are consistent with the hypothesis that 13-OxaMyr affects a late step in the virus life-cycle. However, it is premature to conclude that the analog exerts its antiviral effects only as a result of its incorporation into gag, gag-pol, or nef. It may perturb the biological function of other cellular N-myristoylated proteins that could modulate the course of viral infection. For example, the NH_2-terminal region of the src-like protein tyrosine kinase, $p56^{lck}$, interacts with the cytoplasmic domain of the cell-surface glycoproteins CD4/CD8 on the inner aspect of the plasma membrane (20, 21). Such interactions appear to be important in T-lymphocyte proliferation and activation (21). Analog incorporation into $p56^{lck}$ may disrupt these events. Our results do raise the possibility that combination chemotherapy with these analogs and AZT not only may allow reduction in the dose of AZT, but also may provide a therapeutic strategy that is useful for both acute and chronic aspects of viral infection.

This work was supported by grants from the National Institutes of Health, the Monsanto Company, and the American Foundation for AIDS Research.

1. Veronese, F. D., Copeland, T. D., Oroszlan, S., Gallo, R. C. & Sarngadharan, M. G. (1988) *J. Virol.* **62,** 795–801.
2. Guy, B., Kieny, M. P., Riviere, Y., Lepeuch, C., Dott, K., Girard, M., Montagnier, L. & Lecocq, J. P. (1987) *Nature (London)* **330,** 266–269.
3. Gottinger, H. G., Sodroski, J. G. & Haseltine, W. A. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 5781–5785.
4. Bryant, M. L. & Ratner, L. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 523–527.
5. Bryant, M. L., Heuckeroth, R. O., Kimata, J. T., Ratner, L. & Gordon, J. I. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 8655–8659.
6. Heuckeroth, R. O., Glaser, L. & Gordon, J. I. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8795–8799.
7. Johnson, D. R., Cox, A. D., Solski, P. A., Devadas, B., Adams, S. P., Leimgruber, R. M., Heuckeroth, R. O., Buss, J. E. & Gordon, J. I. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 8511–8515.
8. Rudnick, D. A., McWherter, C. A., Adams, S. P., Ropson, I. J., Duronio, R. J. & Gordon, J. I. (1990) *J. Biol. Chem.* **265,** 13370–13378.
9. Fisher, A. G., Ratner, L., Mitsuya, H., Marselle, L. M., Harper, M. E., Broder, S., Gallo, R. C. & Wong-Staal, F. (1986) *Science* **233,** 655–659.
10. Duronio, R. J., Jackson-Machelski, E., Heuckeroth, R. O., Olins, P. O., Devine, C. S., Yonemoto, W., Slice, L. W., Taylor, S. S. & Gordon, J. I. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 1506–1510.
11. Ratner, L., Fisher, A., Jagodzinski, L. L., Mitsuya, H., Liou, R.-S., Gallo, R. C. & Wong-Staal, F. (1987) *AIDS Res. Hum. Retroviruses* **3,** 57–69.
12. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
13. Poiesz, B. J., Ruscetti, F. W., Gazdar, A. F., Bunn, P. A., Minna, J. D. & Gallo, R. C. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 7415–7419.
14. Bathurst, I. C., Chester, N., Gibson, H. L., Dennis, A. F., Steimer, K. S. & Barr, P. (1989) *J. Virol.* **63,** 3176–3179.
15. Jacks, T., Power, M. D., Masiarz, F. R., Luciw, P. A., Barr, P. J. & Varmus, H. E. (1988) *Nature (London)* **331,** 280–283.
16. Debouck, C., Gorniale, J. G., Strickler, J. E., Meek, T. D., Metcalf, B. W. & Rosenberg, M. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 8903–8906.
17. Komiyama, N., Hattori, N., Inoue, J.-I., Sakuma, S., Kurimura, T. & Yoshida, M. (1989) *AIDS Res. Hum. Retroviruses* **5,** 411–419.
18. Nunn, W. D., Colburn, R. W. & Black, P. N. (1986) *J. Biol. Chem.* **261,** 167–171.
19. Navia, M. A., Fitzgerald, P. M. D., McKeever, B. M., Leu, C., Heimbach, J. L., Herber, W. K., Sigal, I. S., Darke, P. L. & Springer, J. P. (1989) *Nature (London)* **337,** 615–620.
20. Veillette, A., Bookman, M. A., Horak, E. M., Samelson, L. E. & Bolen, J. B. (1989) *Nature (London)* **338,** 257–259.
21. Turner, J. M., Brodsky, M. H., Irving, B. A., Levin, S. O., Perlmutter, R. M. & Littman, D. R. (1990) *Cell* **60,** 755–765.