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NOTES

DMP 323, a Nonpeptide Cyclic Urea Inhibitor of Human Immunodeficiency Virus (HIV) Protease, Specifically and Persistently Blocks Intracellular Processing of HIV *gag* Polyprotein

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DMP 323, a C-2-symmetrical cyclic urea, is representative of a new class of inhibitors of human immunodeficiency virus protease. In this study, we correlate the potent antiviral activity of DMP 323 in acute infections with antiprotease activity assessed by monitoring the inhibition of the processing of viral *gag* precursor polyprotein from chronically infected lymphoid and monocytoid cell lines. Electron microscopic examination confirmed that the inhibition of *gag* processing was associated with the production of immature viral particles. Reduction of DMP 323 in the environment of unprocessed *gag* viral particles did not result in the resumption of *gag* processing for at least 72 h.

DMP 323, a novel C-2-symmetrical cyclic urea, represents a new class of nonpeptide human immunodeficiency virus (HIV) protease inhibitors with oral bioavailability in rats and dogs (21). We present here results of studies which demonstrate that the previously described potent antiviral effect of DMP 323 in acute infections (25) correlates with an inhibitory effect on viral *gag* polyprotein processing. This inhibition leads to the production of immature virus from both human lymphocyte cell lines (H9 and Molt4) and monocyte/macrophage cell lines (U937 [1]) persistently infected with HIV, an important finding relative to the roles that these two cell types play in the pathogenesis of HIV disease (4, 5, 8, 17). Additionally, we present evidence that the virus produced from chronically infected cells in the presence of DMP 323 remains predominantly immature, containing unprocessed *gag* p55 or p47, for at least 72 h after the inhibitor concentration falls below the IC_{50} (concentration of compound that inhibited viral replication by 50%).

Protease inhibitors. All inhibitors tested were synthesized at The DuPont Merck Pharmaceutical Co. Q8111 was synthesized as the chemical equivalent of Ro-31-8959, a potent HIV protease inhibitor (31) currently in clinical trials. Q8024 is a linear C-2-symmetrical diol (12). Compounds were dissolved in dimethyl sulfoxide and further diluted in test media to a final concentration of ≤1.0% dimethyl sulfoxide for the treatment of cells.

Effect in acute infections and toxicity in MT2 cells. The anti-HIV activity in acute infections (23, 27) of MT2 cells was determined by a p24 assay or by measurement of viral RNA. MT2 cells (10^5) were treated with serial dilutions of compounds for 1.5 h at 37°C, inoculated with strain HIV-1_{RF} (3×10^5 PFU) in a 96-well plate, and incubated at 37°C for 3 days. Viral replication was monitored by either a p24 enzyme-linked immunosorbent assay (ELISA) (DuPont NEN p24 ELISA kit) with serial dilutions of virus pelleted from supernatant fluid or by an RNA hybridization assay performed directly with crude cell lysates (2, 25). The compound concentration required to inhibit viral replication in MT2 cells by 90% relative to that in untreated infected control cells (IC_{90}) was determined. Cytotoxicity for treated uninfected cells was assessed by use of an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] dye uptake assay as described by Mosman (24); the compound concentration which reduced the signal 50% compared with that in untreated cells (TC_{50}) was determined. Table 1 contains IC_{90}s and TC_{50}s for DMP 323 and, for comparison, Q8024 and Q8111. All three compounds exhibited potent antiviral activity. Both the p24 ELISA with serial dilutions of pelleted extracellular virus and the RNA hybridization assay with crude cell lysates yielded similar IC_{90}s. Q8024 exhibited comparable antiviral activity and similar cytotoxicity relative to DMP 323, while Q8111 was approximately six times more potent in antiviral activity but about four times more toxic than DMP 323.

Analysis of virion-associated *gag* proteins from treated, chronically infected cells by RIP. The ability of DMP 323, Q8024, and Q8111 to inhibit viral *gag* p55 polyprotein processing was assessed by treatment of chronically infected cells (Molt4 cells infected with strain HIV-1_{RF}, U937 cells infected with strain HIV-1_{RF}, and H9 cells infected with strain HIV-2_{ROD}) with inhibitors. Chronically infected cultures of Molt4 or U937 cells expressing HIV-1_{RF} or H9 cells expressing HIV-2_{ROD} were established essentially as described previously (13, 14, 22). Viral polyprotein processing in overnight-treated cells was assessed by radioimmunoprecipitation (RIP) of cell-associated viral proteins and cell-free virus (10, 15) by use of an antibody (16) to viral p24 and sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by fluorography (20). Bands corresponding in molecular weight to *gag* p55, *gag* p41,

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TABLE 1. Inhibition of viral RNA, antigen, and polyprotein processing in cell cultures by HIV protease inhibitors

Drug	Mol wt	Parameter, μM,[a] as determined by the indicated method (cell type; virus strain; assay)					
		TC_{50} (MT2; none; MTT toxicity)	IC_{90}				
			RNA hybridization (MT2; HIV-1_{RF}; acute infection)	p24 ELISA (MT2; HIV-1_{RF}; acute infection)	RIP (Molt4; HIV-1_{RF}; chronic infection)	RIP (U937; HIV-1_{RF}; chronic infection)	RIP (H9; HIV-2_{ROD}; chronic infection)
DMP 323	567	61.5 (10.4)	0.057 (0.028)	0.05	0.084 (0.03)	0.07	0.02
Q8024	767	65	0.054 (0.059)	0.052	0.09		
Q8111	671	16	0.009 (0.003)	0.003	0.012	0.012	0.012

[a] Numbers in parentheses are standard deviations.

and *gag* p24 were quantitated by densitometry, and the quantity of a compound required to reduce p24 density 90% with a concomitant increase in precursor quantity (RIP IC_{90}), relative to the value for untreated controls, was determined.

The fluorograms of extracellular virus (Fig. 1) were compared. The patterns for Molt4 cells infected with strain HIV-1_{RF} (Fig. 1A) and H9 cells infected with strain HIV-2_{ROD} (Fig. 1B) were found to be essentially identical. Increasing the concentration of the compound resulted in a decrease in the level of immunoprecipitated material corresponding to p24 and an increase in the intensity of bands corresponding to processing intermediates p40 (13) and p47 (22) and full-length *gag* p55. Surprisingly, the immunoprecipitation of DMP 323-treated U937 cells infected with strain HIV-1_{RF} (Fig. 1C) showed the expected decrease in the level of p24 but the accumulation of only the p40 intermediate, without the appearance of full-length *gag* p55. The accumulation in U937 cells (or the virus expressed from these cells) of this intermediate species only has been observed with all other HIV protease inhibitors that we have tested to date (data not shown). The basis of this apparent difference in precursor species that accumulate or are detected in different cell types is presently unexplained. Western blots (immunoblots) of cell-associated HIV *gag*-related polyproteins from protease inhibitor-treated, persistently infected (with HIV_{IIIB}) U937 cells, tested with antibody to viral p24 (18), distinctly show *gag* p55 and p24. Plots of the quantitation of expressed viral p55 or p40 and p24 by densitometer scanning (and IC_{90}s) are shown beside the appropriate fluorograms in Fig. 1D to F; the DMP 323 densitometer values plotted are averages from five RIP assays. Table 1 shows the RIP IC_{90}s for the decrease in the level of immunoprecipitated p24 for DMP 323 and other protease inhibitors, Q8024 and Q8111; the values are the same within experimental error. These results indicate that DMP 323 can inhibit polyprotein processing of both HIV type 1 and HIV type 2 and is effective in both lymphoid cells and monocytoid cells. More importantly, these RIP IC_{90}s for the inhibition of viral polyprotein processing are comparable to the IC_{90}s obtained for the inhibition of virus production in an acute infection (Table 1), suggesting that inhibition of the viral protease is the basis for the antiviral effect.

Effects of HIV protease inhibition on virion morphology. To confirm whether DMP 323 treatment resulted in the production of structurally immature particles, electron microscopic studies were performed on chronically infected cultures treated with the compound for 4 days. These conditions of compound concentration (8.8 μM) relative to antiviral potency (IC_{90}, 0.07 μM) and treatment time are similar to those used to assess the effects of a peptidic hydroxyethylene isostere inhibitor of HIV protease on chronically infected cells (32). Samples of treated HIV-1_{RF}-infected U937 cells and cells plus concentrated extracellular virus from HIV-1_{RF}-infected Molt4 cells were prepared for electron microscopy with glutataldehyde-formaldehyde fixative (3%:2%) and postfixed with OsO4 containing $K_3Fe(CN)_6$ in 0.1 M phosphate buffer (pH 7.4). Uranyl acetate and lead citrate were used to stain 600-Å (60-nm) sections in Epon-Araldite resin (35).

In untreated control samples (Fig. 2A and C), viral particles were observed to have the condensed conical cores typical of mature virus (7). In contrast, viral particles released from DMP 323-treated Molt4 cells infected with strain HIV-1_{RF} (Fig. 2B) had a morphology consistent with that reported previously for immature virus (32). Immature viral particles were very apparent in the high-magnification micrograph of DMP 323-treated U937 cells infected with strain HIV-1_{RF} (Fig. 2D). The virus in this case was present within intracellular vacuoles and tuboreticular inclusions (4, 7, 33). A significant number of the enveloped particles resembled incompletely assembled immature virions with envelope projections and a darkly stained double-layer ribonucleoprotein shell directly attached to the inner layer of the lipid bilayer, as described by Schatzl et al. (32).

Fate of immature virions produced in the presence of DMP 323. In a clinical setting, levels of drugs in plasma and/or tissue will fluctuate, depending on the pharmacokinetic parameters of the specific agent under study. Between doses of the compound, the virus may thus be exposed to compound levels lower than that required for complete inhibition of HIV replication. If immature virions were to mature efficiently and become infectious (11) during periods of low compound concentrations, a relative lack of efficacy might result. Thus, it is important to address concerns about the stability of immature virions when the inhibitor is removed or its concentration is reduced.

We investigated the stability, as determined by RIP analysis, of extracellular HIV-1_{RF} produced in Molt4 cells in the presence and absence of a 4-day treatment with 1 μg of DMP 323 per ml (1.76 μM). The cell-free virus was pelleted from the culture fluid after the 4-day treatment, labeling, and an overnight chase (to maximize the amount of labeled virus present). Viral pellets were resuspended in 500 μl of RPMI medium with 1% fetal calf serum but without added inhibitor. Resuspended virus was incubated at 37°C. Samples were taken at 0, 2, 3, 4, 24, 48, and 72 h of incubation and frozen at −80°C for subsequent RIP analysis.

To determine the concentration of inhibitor remaining after the viral pelleting and resuspension procedure, triplicate samples of Molt4 cells infected with strain HIV-1_{RF} were treated identically with 1 μg of [^{14}C]DMP 323 per ml (0.086 μCi). [^{14}C]DMP 323 was synthesized by DuPont New England Nuclear Research Products Division, Boston, Mass. The specific activity was 48.4 mCi/mmol. After isolation of viral particles, the radioactivity present was determined by liquid scintillation counting and compared with a standard curve for

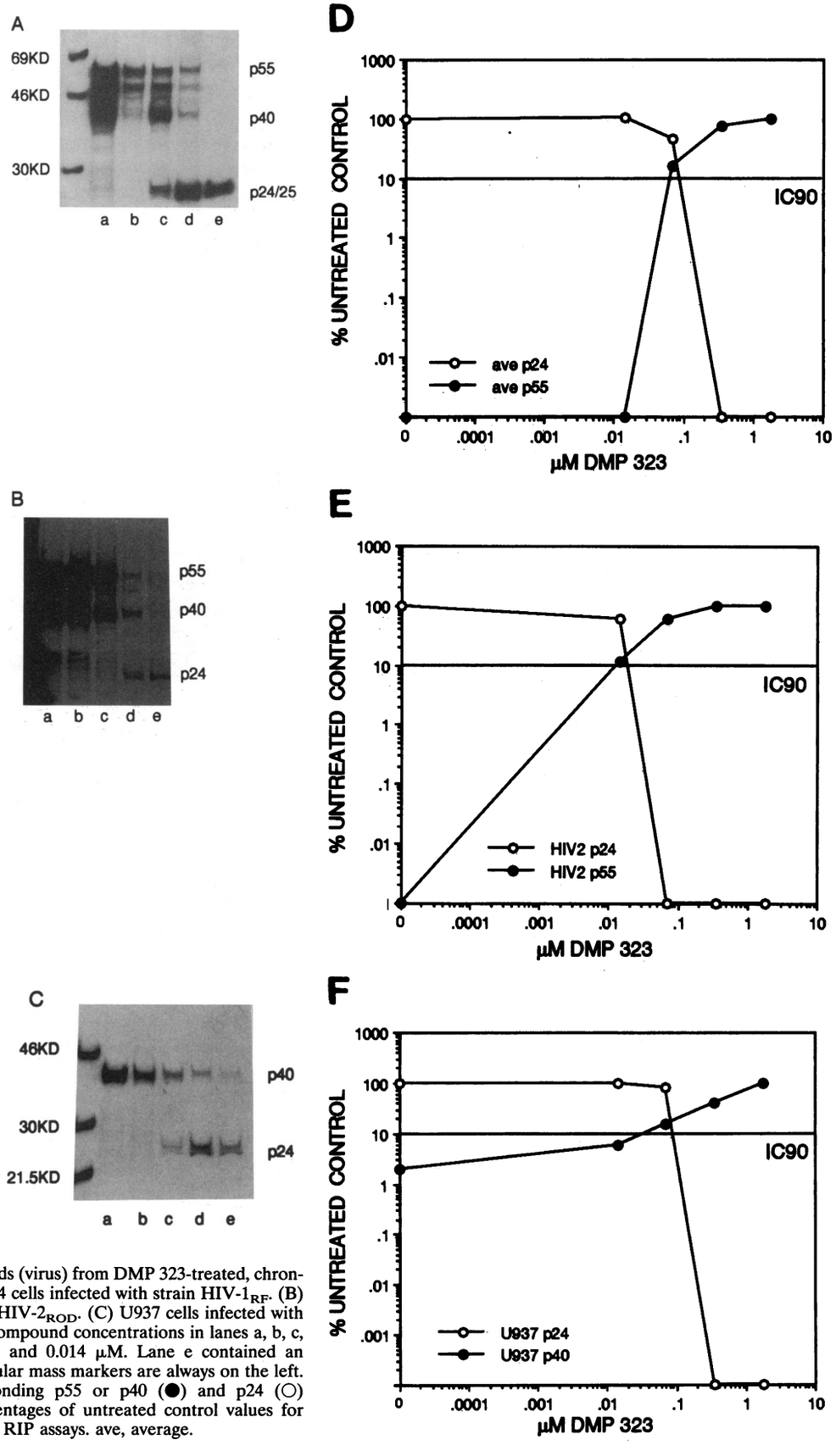

FIG. 1. RIP of culture fluids (virus) from DMP 323-treated, chronically infected cells. (A) Molt4 cells infected with strain HIV-1_{RF}. (B) H9 cells infected with strain HIV-2_{ROD}. (C) U937 cells infected with strain HIV-1_{RF}. Respective compound concentrations in lanes a, b, c, and d were 1.76, 0.35, 0.07, and 0.014 μM. Lane e contained an untreated control. ^{14}C molecular mass markers are always on the left. (D to F) Plots of corresponding p55 or p40 (●) and p24 (○) densitometer values as percentages of untreated control values for concentrations a to e used in RIP assays. ave, average.

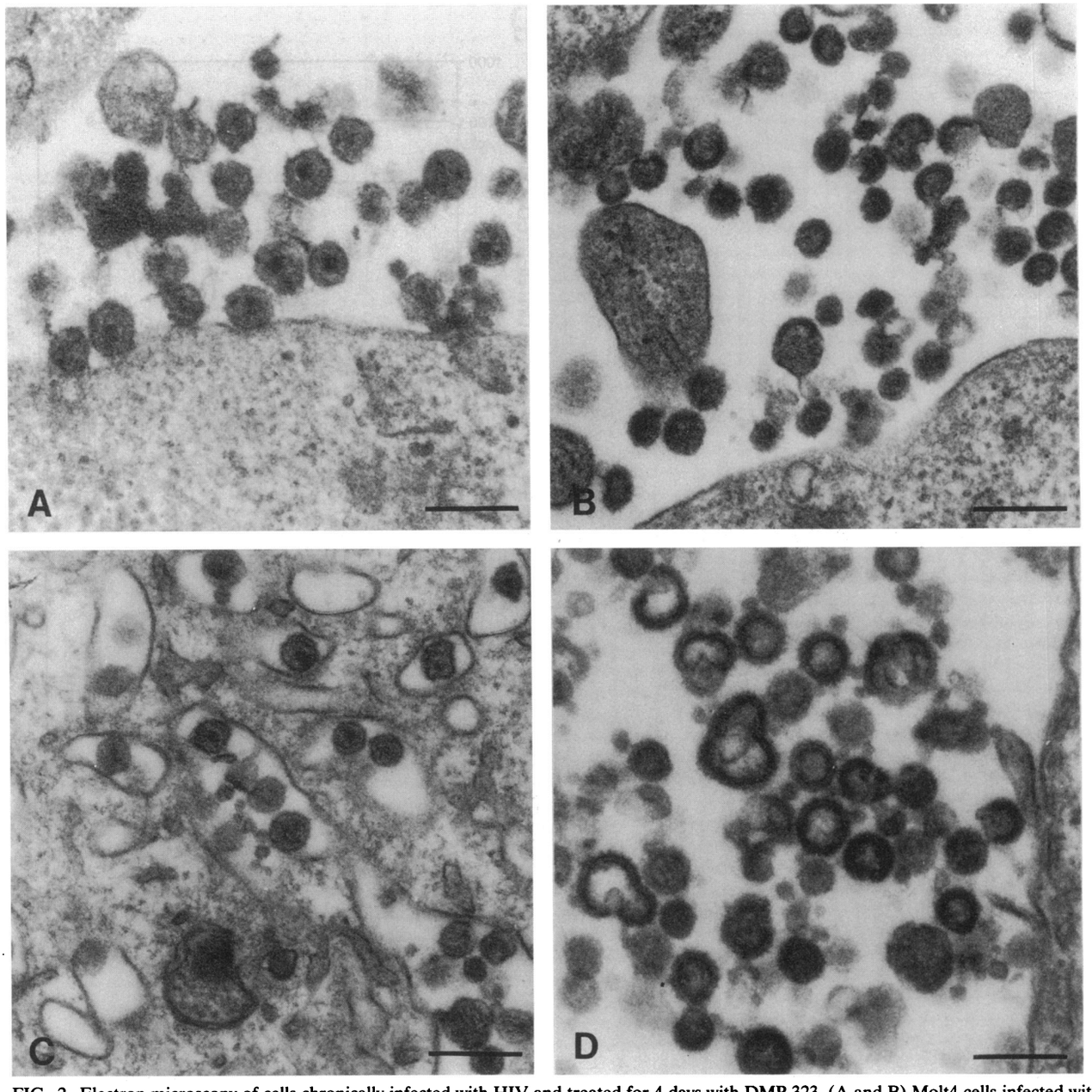

FIG. 2. Electron microscopy of cells chronically infected with HIV and treated for 4 days with DMP 323. (A and B) Molt4 cells infected with strain HIV-1_{RF}. (C and D) U937 cells infected with strain HIV-1_{RF}. (A and C) Untreated controls. (B and D) Samples treated with 8.8 μM DMP 323. Bars, 200 nm.

untreated, pelleted virus that had been resuspended in 500 μl of RPMI medium with 1% fetal calf serum, and spiked with known concentrations of [^{14}C]DMP 323. The concentration of DMP 323 remaining after this process and final viral resuspension was determined to be 9 ng/ml (0.016 μM).

In experiments such as those shown in Fig. 1A or D, a DMP 323 concentration of 0.016 μM would inhibit *gag* processing no more than 30% (IC_{30}) and would correspond to an IC_{40} for the inhibition of virus production in an acute infection, as measured by p24 or viral RNA assays (data not shown). Fluorograms presented in Fig. 3 show the results of such experiments and can be directly compared with Fig. 1A and Table 1.

As shown in Fig. 3A, the samples of DMP 323-treated virus contained predominantly unprocessed viral *gag* precursors throughout the 72-h incubation period, despite the fact that the concentration of DMP 323 had been reduced below the IC_{50} for the inhibition of *gag* processing. Material corresponding to p24 appeared at 24 h and increased slightly in amount by 48 h but reflected only 1 to 2% of the total *gag* radioactivity present in the RIP samples. Additional experiments showed no subsequent changes in the *gag* RIP pattern over 7 days (data not shown). The amount of p24-containing mature virus obtained from untreated samples remained unchanged during incubation at 37°C throughout the time period examined. In a

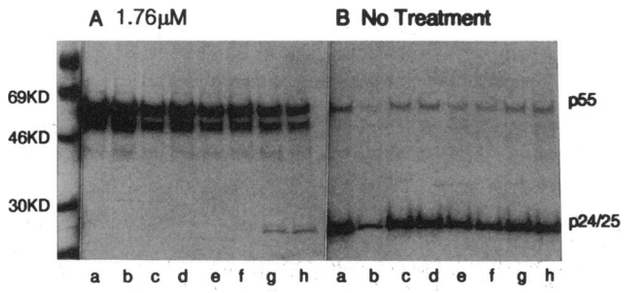

FIG. 3. Time course determined by RIP of *gag* processing in extracellular virus produced from Molt4 cells chronically infected with strain HIV-1_{RF} after a 4-day treatment with 1 μg of DMP 323 per ml (1.76 μM). (A) Samples treated with 1.76 μM DMP 323. (B) Untreated controls. Lanes a, RIP of unconcentrated infected culture fluid; b to h, 0-, 2-, 3-, 4-, 24-, 48-, and 72-h concentrated samples. ^{14}C molecular mass markers are on the left.

comparison with the RIP data in Fig. 1A, the level of *gag* processing to p40 and p24 in Fig. 1A was more extensive at 0.014 or 0.071 μM than that in Fig. 3 at 0.016 μM. This comparison indicates that once *gag* polyprotein has been incorporated into immature virions in the presence of DMP 323, it is largely resistant to further processing, even when inhibitor levels are reduced below those at which significant processing is observed in infected cells.

An assessment of the infectivity of virions produced in protease inhibitor-treated cultures under conditions in which any residual inhibitor present is further diluted in the infectivity assay and with comparable amounts of virus, based on the intensity of the RIP profile (13, 22, 32; unpublished results), has shown that the immature viral particles produced in the presence of a protease inhibitor have significantly lower infectivity titers than similar untreated viral preparations.

The reasons for this persistence as it relates to processing and infectivity are under investigation. Although inhibitors such as DMP 323 bind the enzyme with a high affinity, inhibition is of rapid onset and likely is rapidly reversible (6). One possibility is that normal polyprotein processing and virus assembly may have a defined timing and sequence; alterations in this sequence of events may not allow subsequent processing to occur efficiently (13, 14, 19).

The correlation of the sequence of events beginning with the inhibition of HIV protease and resulting in the inhibition of *gag* processing and the production of immature, noninfectious viral particles was demonstrated in both lymphoid and monocytoid cells. The demonstration of compound efficacy in both cell types is important in the design of a therapeutic strategy. Recent evidence suggests that lymphoid organs, $CD4^+$ lymphocytes, and monocytes/macrophages in particular serve as long-lived reservoirs of viral replication throughout the course of HIV disease (3–5, 8, 26, 28). Thus, these organs and cells may act as "Trojan horses", carrying infectious virus throughout the body (including the brain) (9, 17, 29, 30). These results suggest that DMP 323, as a potent and persistent inhibitor of HIV protease in cells, may have the potential to reduce the load of infectious virus substantially and hence the spread of virus within the host. This information also supports the development of protease inhibitors, alone or in combination with the current reverse transcriptase inhibitors, for therapeutic intervention in HIV disease.

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