JOURNAL OF VIROLOGY, Apr. 1997, p. 3023–3030 Vol. 71, No. 4
0022-538X/97/$04.00+0

Chemical Barriers to Human Immunodeficiency Virus Type 1 (HIV-1) Infection: Retrovirucidal Activity of UC781, a Thiocarboxanilide Nonnucleoside Inhibitor of HIV-1 Reverse Transcriptase

GADI BORKOW, JOHN BARNARD, THUY MINH NGUYEN, ANTOINETTA BELMONTE, MARK A. WAINBERG, AND MICHAEL A. PARNIAK*

Lady Davis Institute for Medical Research and McGill University AIDS Centre, Sir Mortimer B. Davis-Jewish General Hospital, Montreal, Quebec H3T 1E2, Canada

Received 22 October 1996/Accepted 8 January 1997

UC781, a thiocarboxanilide nonnucleoside inhibitor of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT), inhibited RT DNA polymerase activity in vitro with marked potency. Significant inhibition was noted at a 1:1 molar ratio of UC871 to RT, characteristic of a tight-binding inhibitor. Infectivity of the HIV-1_{IIIB} laboratory strain was eliminated in a concentration-dependent manner following short exposure of isolated virion particles to UC781. Neither nevirapine nor certain other carboxanilide nonnucleoside inhibitors were effective in this manner. Endogenous reverse transcription in UC781-treated virus particles was markedly reduced. Treatment of chronically HIV-1-infected H9 cells with UC781 did not alter virus production, but the infectivity of the virus produced by the cells during drug exposure was markedly reduced. Moreover, the infectivity of nascent virus produced by the UC781-treated H9 cells after removal of exogenous drug was dramatically attenuated. Similarly, pretreatment of peripheral blood lymphocytes isolated from HIV-infected patients abolished the infectivity of virus produced by these cells after removal of exogenous drug, as measured by coculture experiments with uninfected cord blood mononuclear cells, indicating the utility of UC781 against a variety of clinical HIV samples. Importantly, preincubation of uninfected MT2 cells with UC781 rendered these cells refractory to subsequent HIV infection in the absence of extracellular drug, an effect that persisted for several days following removal of exogenous drug. These unique properties of UC781 indicate that this nonnucleoside inhibitor may have considerable promise for use in retrovirucidal formulations to minimize the spread of HIV from infected to noninfected individuals.

Current clinical treatments for human immunodeficiency (HIV) infection involve antiviral agents such as 3′-azido-2′,3′-dideoxythymidine (AZT), an inhibitor of the viral reverse transcriptase (RT), and viral protease inhibitors (4, 10, 16). These drugs are designed to be used in patients already infected with HIV. However, there is a crucial need to identify prophylactic strategies that may minimize the spread of HIV from infected individuals to noninfected individuals. Such strategies include the development of anti-HIV vaccines. Unfortunately, despite considerable effort and expense, the vaccine strategy has so far been unsuccessful.

Heterosexual contact is the primary mode of transmission of HIV infection worldwide (14). Thus, retrovirucidal spermicides or vaginal microbicides could minimize the spread of HIV from infected to noninfected individuals. Accordingly, the World Health Organization has established a research priority for the development of effective anti-HIV vaginal microbicides (20). Potential retrovirucides or vaginal microbicides include inhibitors of HIV-CD4 receptor interaction (1, 15, 17) and virus envelope-disrupting agents (18, 21).

Ideally, a retrovirucidal agent should fulfill several requirements. First, it should act directly on the virus. Dideoxynucleoside antivirals such as AZT require cellular metabolic activation and are therefore of little use in this respect. Second, a retrovirucide should act at replication steps prior to integration of proviral DNA into the infected host cell genome. Although protease inhibitors prevent maturation of newly produced virion particles, they are ineffective against preexisting mature infectious HIV. Third, a retrovirucide should be absorbable by uninfected cells, in order to provide a barrier to infection by residual active virus. Finally, a retrovirucide should be effective at noncytotoxic concentrations readily attainable in vivo.

In this report, we describe the antiviral properties of *N*-[4-chloro-3-(3-methyl-2-butenyloxy)phenyl]-2-methyl-3-furancarbothioamide (UC781), one of the thiocarboxanilide nonnucleoside inhibitors (NNI) of HIV type 1 (HIV-1) RT developed by Uniroyal Chemical (UC) (3). We show that HIV infectivity is effectively eliminated following short exposure of isolated virus to UC781 and that incubation of uninfected cells with UC781 renders these cells refractory to subsequent HIV infection. Interestingly, exposure to UC781 of chronically infected H9 cells as well as peripheral blood lymphocytes (PBL) isolated from infected individuals results in the appearance of noninfectious nascent virus. Furthermore, UC781 inhibits endogenous reverse transcription (ERT) in HIV-1 virions, an important factor in sexual transmission of HIV-1 infection (27, 28). UC781 has considerable promise for use in retrovirucidal as well as systemic antiviral formulations.

MATERIALS AND METHODS

Reagents. The HIV_{IIIB} laboratory strain of HIV-1, courtesy of R. C. Gallo, was obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases. The $CD4^+$ MT2 cell line was obtained from the American Type Culture Collection. The

* Corresponding author. Mailing address: Lady Davis Institute for Medical Research, 3755 Cote Ste-Catherine Rd., Montreal, Quebec H3T 1E2, Canada. Phone: (514) 340-8260. Fax: (514) 340-7502. E-mail: mparniak@ldi.jgh.mcgill.ca.

carboxanilide NNI UC84, UC38, and UC781 were provided by A. W. Harrison and W. G. Brouwer, UC Ltd. Research Laboratories (Guelph, Ontario, Canada). Nevirapine was provided by Boehringer-Ingelheim. H9 cells chronically infected with HIV (HIV_{IIIB}) were established in our laboratories. Cord blood mononuclear cells (CBMC) were isolated by Ficoll-Hypaque centrifugation from cord blood obtained from the Department of Obstetrics, Sir Mortimer B. Davis-Jewish General Hospital. PBL cells were isolated by Ficoll-Hypaque centrifugation from blood obtained from HIV-1-infected patients through the Department of Infectious Diseases at Sir Mortimer B. Davis-Jewish General Hospital. All patients were being treated with the protease inhibitor saquinavir, and some of the patients had been previously treated with AZT. However, none had undergone NNI therapy. RPMI 1640 cell culture medium and heat-inactivated fetal bovine serum (FBS) were obtained from Canadian Life Technologies/GIBCO, Toronto, Ontario, Canada. Centriprep-100 concentrating devices were from Amicon (Beverly, Mass.). Purified recombinant p51/p66 RT was prepared by a rapid method that we have recently described (7). HIV-1 RT RNA-dependent DNA polymerase (RDDP) and DNA-dependent DNA polymerase (DDDP) activities were measured as previously described (5, 6).

Cell culture and virus replication. All cells were cultured in RPMI 1640 containing 10% FBS. HIV-1 stocks were propagated by subculture in $CD4^+$ MT-2 cells; aliquots of the cell-free culture supernatants were generally used as viral inocula. In some experiments, virus was further concentrated from these cell-free supernatants by ultracentrifugation. CBMC were prestimulated with phytohemagglutinin prior to use in infection experiments, as previously described (22). Stock solutions of NNI were prepared in dimethyl sulfoxide (DMSO). Aliquots of the NNI stock solutions were added to culture media immediately before use. The final concentration of DMSO in these working solutions was 1% or less. Control experiments showed that these concentrations of DMSO had no effect either on virus infectivity or on cell viability. Virus production was assessed by measurement of RT activity and viral p24 antigen levels in the culture supernatants. Cytopathic effects of HIV infection of $CD4^+$ MT2 cells were also analyzed by microscopic assessment of syncytium formation as previously described (23, 26). The latter data were obtained by analysis of duplicate samples by two independent observers. Cytotoxicity assays were carried out according to the method of Hirabayashi et al. (9).

Preincubation of isolated HIV-1 with NNI. HIV-1 (50% tissue culture infective dose [$TCID_{50}$] = 5×10^4) was incubated in the absence or in the presence of various concentrations of NNI in a total volume of 1 ml of RPMI 1640 without FBS for 2 h at 37°C with gentle agitation every 15 min. After incubation, drug-free RPMI 1640 was added to a final volume of 15 ml, and the diluted sample was concentrated to a final volume of 0.7 ml with a Centriprep-100 centrifugal concentrator, according to the manufacturer's directions. The virus was diluted with drug-free media to 10 ml and again concentrated to a final volume of 0.7 ml. Then 0.5 ml of this concentrated virus was added to 0.5 ml of phytohemagglutinin-activated CBMC (4×10^6 cells) in RPMI 1640–10% FBS and incubated for 2 h at 37°C with occasional gentle mixing. The residual NNI concentration at this stage was about 0.2% of that in the initial virus-NNI incubation. The HIV-CBMC incubation mix was diluted with the addition of 10 ml of drug-free RPMI 1640, and residual HIV was removed by pelleting the cells at $300 \times g$ for 10 min, followed by removal of the supernatant and resuspension of the cells in 2 ml of RPMI 1640–10% FBS containing interleukin-2 (10 U/ml). The entire sample was plated into a single well of a 24-well dish. After 4 days of culture, 1 ml of medium was removed and replaced with 1 ml of fresh medium. On day 7, culture supernatants were isolated and HIV production was assessed by the measurement of RT activity and p24 antigen levels in these cell-free supernatants.

Incubation of chronically HIV-1-infected H9 cells with NNI. Chronically infected H9 cells (5×10^5 cells) were incubated with various concentrations of NNI in a total volume of 1 ml of RPMI 1640–10% FBS for 18 h at 37°C. The cells were then separated from the culture supernatants by centrifugation at $300 \times g$ for 10 min. The virus particles were isolated from the culture supernatants and from residual NNI by using Centriprep-100 centrifugal concentrating devices and the dilution-concentration protocol described above, with one additional dilution-concentration of the virus suspension prior to use for infection, in order to ensure extensive removal of all residual exogenous drug. Control experiments showed that the level of residual drug in these concentrated samples was below that required to significantly affect infectivity of virus that had not been previously exposed to drug. The pelleted H9 cells were washed by suspension in 10 ml of RPMI 1640 followed by centrifugation at $300 \times g$ for 10 min. The cell pellet was resuspended in 4 ml of RPMI 1640–10% FBS and used in coculture experiments as described below.

Aliquots of the final concentrated HIV suspension were added to MT2 cells (3×10^5 cells/ml). Virus infectivity was assessed 6 days postinfection by analysis of syncytium formation and measurement of RT activity and p24 antigen in the MT2 cell culture supernatants. Similarly, aliquots of the drug-treated H9 cells were added to MT2 cells (3×10^5 cells/ml) to give a final MT2/H9 cell ratio of 30:1. Cells were cocultured for 4 days, and HIV infection of MT2 cells was scored by assessing syncytium formation and by analysis of RT activity in the culture supernatants.

Incubation of PBL cells with NNI. PBL cells (2×10^6 cells) isolated from blood of HIV-1-infected patients were incubated with medium, 10 μM nevirapine, or 10 μM UC781 in a total volume of 1 ml for 2 h at 37°C. Excess drug was then removed by pelleting the cells by centrifugation at $300 \times g$ for 10 min and removal of the medium. The cell pellet was washed by suspension in 10 ml of medium followed by centrifugation. This washing step was repeated twice. The final cell pellet was resuspended in 1 ml of medium and then cocultured with 1 ml of activated CBMC (2×10^6 cells). The culture medium was changed every 2 days, and fresh activated CBMC (2×10^6 cells) were added once per week. HIV-1 production was monitored by measurement of p24 antigen levels in cell-free culture supernatants.

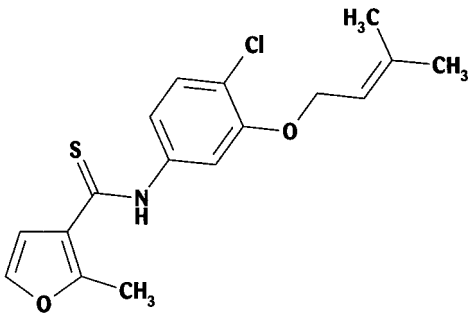

FIG. 1. Structure of UC781.

Effect of preincubation of uninfected MT2 cells with NNI on subsequent HIV infection. Uninfected MT2 cells (2×10^5) were incubated in the absence or the presence of various concentrations of NNI in RPMI 1640–10% FBS at 37°C for various time periods ranging from 10 min to 18 h. After incubation with the drug, the cells were pelleted by centrifugation and washed twice by resuspension in 10 ml of medium followed by centrifugation in order to ensure removal of residual exogenous drug. The final cell pellet was resuspended with 1 ml of medium, and an appropriate inoculum of HIV was added ($TCID_{50} = 5 \times 10^4$). Cells and virus were then incubated at 37°C, and the culture medium was changed every 2 to 3 days. Viral infection was scored by assessing the extent of syncytium formation, viral p24, or RT activity in cell-free culture supernatants at various times thereafter.

ERT assay. ERT was measured essentially as described elsewhere (27). Briefly, HIV-1 particles (50 μl; 7.5 ng of p24) were incubated with different concentrations of UC781 for 1 h at 37°C and then mixed with 50 μl of ERT reaction mix (final concentrations: 10 mM Tris, pH 7.4; 150 mM NaCl; 1 mM $MgCl_2$; 50 μM [each] dATP, dCTP, dGTP, and dTTP; 150 μCi of [α-^{32}P]dCTP). After 3 h at 37°C, reactions were stopped by the addition of 10 mM EDTA, 1% sodium dodecyl sulfate, and proteinase K (50 μg/ml [final concentration]) and heated for 1 h at 60°C. DNA was extracted with phenol-chloroform and precipitated with ethanol. The DNA pellet was dissolved in 98% formamide containing 10 mM EDTA, 0.025% xylene cyanol FF, and 0.025% bromophenol blue and then subjected to denaturing gel electrophoresis. Resolved products were visualized by autoradiography.

RESULTS

During our characterizations of the UC series of NNI (5, 6), UC781 (Fig. 1) was found to be an exceptionally potent inhibitor of HIV-1 RT. UC781 inhibited RT RDDP activity with a 50% inhibitory concentration (IC_{50}) of 0.2 nM (Table 1), a concentration similar to that of the p51-p66 RT heterodimer in the in vitro assays (approximately 0.5 nM p51-p66 heterodimer). Decreased activity at these levels of inhibitor to target is characteristic of tight-binding inhibitors (24, 25). Subsequent detailed in vitro enzyme kinetic analyses of UC781 inhibition of HIV-1 RT have confirmed that UC781 is indeed a rapid tight-binding inhibitor (3a). Thus, UC781 once bound to RT does not rapidly dissociate from it.

The high affinity and apparent tight binding of UC781 to HIV-1 RT prompted us to examine the effect of preincubation of HIV virion particles with UC781. We compared this with UC84 and UC38, structurally similar carboxanilide inhibitors which have decreased in vitro inhibitory potency compared to UC781, and nevirapine, which has a 50% effective concentration of 40 nM against HIV-1 (8, 13). None of these act as tight-binding inhibitors of HIV-1 RT (3a). As seen in Fig. 2, virus was rendered noninfectious after a 2-h incubation with UC781. This viral inactivation was concentration dependent,

TABLE 1. Antiviral activity of selected UC NNI of HIV-1 RT

Compound	IC_{50} (nM)[a] (mean ± SD)		EC_{50} (nM)[b] (mean ± SD)	CC_{50} (μM)[c]	TI_{50}[d]
	RDDP	DDDP			
UC781	0.2 ± 0.1	1 ± 0.3	8 ± 3	>30	>3,000
UC84	200 ± 30	1,600 ± 200	240 ± 70	>40	>150
UC38	30 ± 10	600 ± 50	80 ± 15	>40	>500

[a] IC_{50}s were determined with purified p51-p66 RT by using [^{3}H]dGTP and poly(rC)-oligo(dG) or poly(dC)-oligo(dG) as the template-primer for the measurement of RDDP and DDDP activities, respectively, as previously described (5, 6).

[b] EC_{50}, 50% effective concentration. Inhibition of HIV-1 replication in MT2 cells was determined by assessing syncytium formation, RT activity, and viral p24 antigen production, as described elsewhere (22).

[c] The 50% cytotoxic concentration (CC_{50}) was determined by [^{3}H]thymidine incorporation, cell growth kinetics, and trypan blue exclusion as described elsewhere (9, 26).

[d] TI_{50}, 50% effective in vitro therapeutic index (ratio of CC_{50}/EC_{50}).

with essentially complete inactivation noted with 0.5 μM UC781. No viral inactivation was noted with nevirapine (Fig. 2) or with UC38 or UC84 (data not shown), even at concentrations above 10 μM.

HIV-infected-patient seminal and vaginal fluids contain virus-infected cells in addition to virus particles (11). Indeed, HIV-infected cells may be a more significant source for viral transmission than free virus in seminal fluids (11, 19). We therefore examined the effect of UC781 on the infectivity of HIV produced from chronically infected H9 cells. These cells were incubated with UC84 or UC781 for 18 h, exogenous drug was removed, and the cells were cocultured with uninfected MT2 cells, as described in Materials and Methods. Neither UC84 nor UC781 had any effect on the amount of HIV-1 particles produced by these cells, as determined by viral p24 antigen levels (Fig. 3A). However, HIV-1 produced by the H9 cells during 18 h of exposure to UC781 was significantly attenuated in infectivity compared to that from no-drug controls or cells treated with UC84 (Fig. 3B). Similar effects were noted in experiments using PBL isolated from HIV-1-infected patients. In these experiments, PBL were incubated in the absence or the presence of NNI for 18 h, then exogenous drug was removed, and the infected PBL were cocultured with uninfected CBMC in the absence of added drug. As with the chronically infected H9 cells, UC781 abolished the infectivity of HIV-1 produced by the patient PBL. Figure 4 illustrates representative experiments from three different patients. PBL from eight different HIV-1-infected patients have been tested in this manner. In all cases, UC781 was markedly effective, with no viral infectivity noted even after 30 days of coculture with uninfected CBMC. In contrast, neither nevirapine (Fig. 4) nor UC84 (data not shown) was able to significantly delay viral infectivity.

A recent study indicated that ERT in HIV-1 particles in seminal plasma facilitates transmission of the virus, since virus infectivity increases significantly upon initiation of ERT (27). Exposure of HIV-1 virion particles to UC781 dramatically inhibited ERT (Fig. 5).

Inactivation of HIV-1 produced from the infected PBL or chronically infected H9 cells likely resulted in part from penetration of UC781 into virions after virus budding from the cells. Importantly, virus produced by the chronically infected H9 cells following removal of UC781 was also attenuated in infectivity, as determined in coculture experiments with MT2 cells (Fig. 3C). This effect persisted for at least 4 days following removal of the extracellular drug. These data indicate that UC781 may also be incorporated into the nascent virion during assembly and budding and imply a prolonged intracellular bioavailability for this inhibitor.

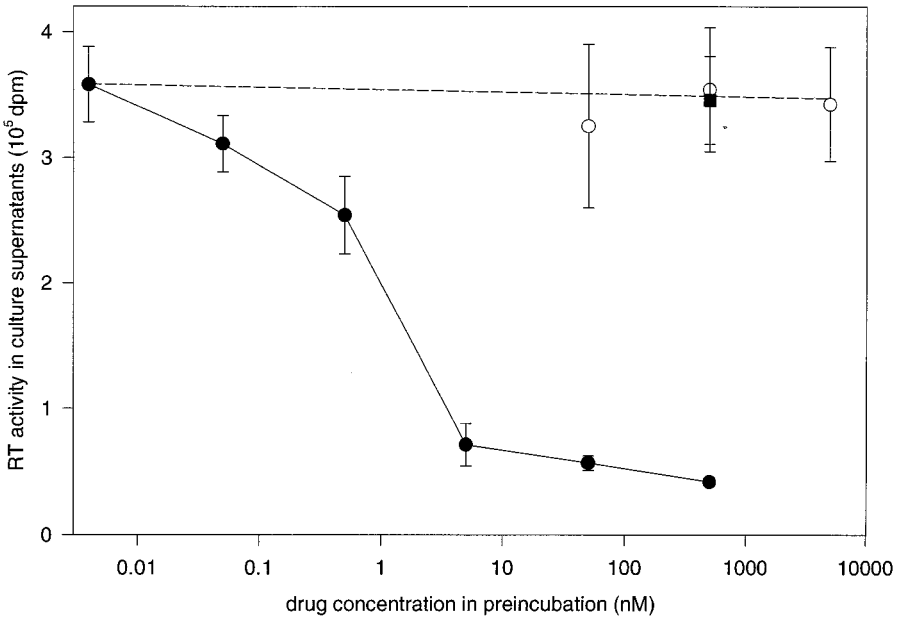

FIG. 2. Effect of preincubation of isolated HIV-1 with NNI on subsequent viral infectivity. Isolated HIV-1 was incubated with the indicated concentrations of UC781 (●) or nevirapine (○) for 2 h, and then residual drug was removed as described in Materials and Methods. Identical viral inocula (with respect to viral p24 levels) were then added to phytohemagglutinin-activated CBMC. Viral infectivity was assessed by measurement of RT activity in cell-free culture supernatants after 7 days of incubation of cells and virus. ■, a residual drug effect control in which 0.5 μM UC781 was carried through the dilution and concentration steps as for pretreated virus and then added to culture supernatants to test for effect on viral infectivity and possible inhibition of RT activity in the in vitro assay. The results are averages ± standard deviations of triplicate determinations from three separate experiments.

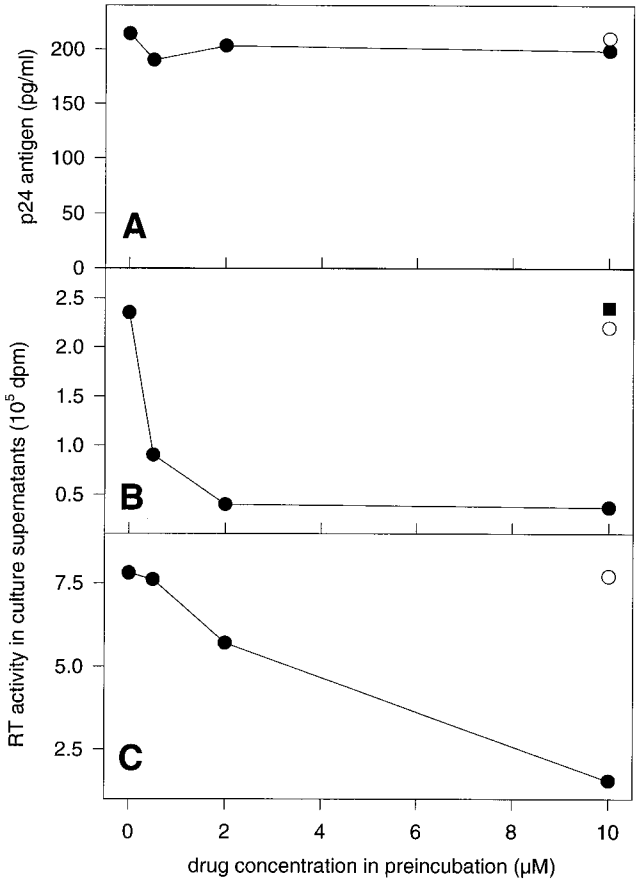

FIG. 3. Effect of pretreatment of chronically infected H9 cells with UC781 or UC84 on virus particle release from H9 cells during drug exposure as assessed by measurement of HIV-1 p24 levels (A), infectivity of isolated virus produced by H9 cells during drug treatment as assessed by ability to subsequently infect MT2 cells in the absence of drug (B), and ability of UC781-pretreated chronically infected H9 cells to subsequently infect MT2 cells in coculture experiments carried out in the absence of drug (C). The data illustrated for panels B and C were obtained 4 days after exposure of MT2 cells to virus or H9 cells, respectively. ○, UC84; ●, UC781. (B) ■, a residual drug effect control in which 10 μM UC781 was carried through the dilution and concentration steps as for pretreated virus and then added to culture supernatants to test for effect on viral infectivity and possible inhibition of RT activity in the in vitro assay.

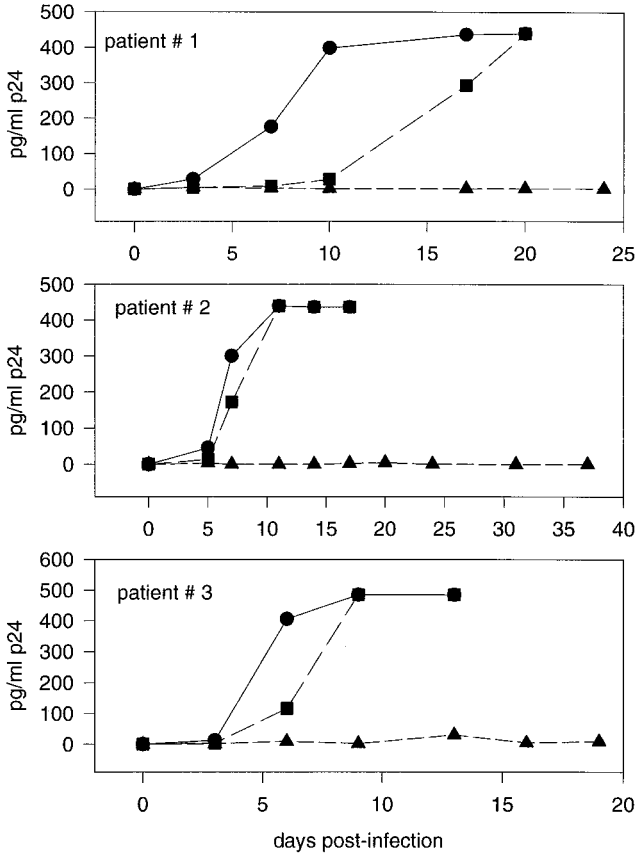

FIG. 4. Effect of UC781 pretreatment of PBL from HIV-1-infected patients on the subsequent virus infectivity of CBMC. Isolated PBL cells (2×10^6) were incubated for 2 h with medium only (●), 10 μM nevirapine (■), or 10 μM UC781 (▲), and then residual drug was removed as described in Materials and Methods. The cells were then cocultured with 2×10^6 CBMC, and p24 production was monitored.

Given the latter possibility, we examined whether exposure of uninfected MT2 cells to UC781 would render these cells refractory to HIV-1 infection subsequent to removal of the drug. Cells were incubated in the absence or the presence of NNI for 18 h, separated from exogenous drug, and then exposed to virus. HIV-1 infection of UC781-pretreated MT2 cells was dramatically attenuated, as assessed by measurement of syncytium formation, p24 production, and/or RT activity in cell-free supernatants (Fig. 6A). These data also show that under our assay conditions, we find an excellent correlation among these measurements of HIV-1 replication. Neither UC84 nor nevirapine was able to inhibit HIV-1 infection under these conditions (data not shown). The protective effect of UC781 was concentration dependent (Fig. 6). Interestingly, MT2 cells exposed to higher concentrations of UC781 were highly resistant to HIV-1 infection, with no apparent viral cytopathicity observed even when an additional virus inoculum was added several days following the first (Fig. 6B). Similarly, incubation of uninfected cells with UC781 at concentrations of 2 μM or above rendered these cells refractory to infection by increased viral loads at least up to 2.5×10^5 $TCID_{50}$ (data not shown).

The protective effect of UC781 was further examined by treating uninfected MT2 cells with UC781 and then culturing the cells for various periods in the absence of the inhibitor prior to addition of an HIV-1 inoculum. When HIV-1 was added immediately following removal of exogenous drug, the appearance of viral cytopathicity was substantially delayed compared to untreated controls (Fig. 7A). The time to appearance of viral cytopathic effects decreased with increasing time between removal of exogenous UC781 and addition of the HIV-1 inoculum. Nevertheless, significant delays in the appearance of viral cytopathicity were noted even in cells cultured for 4 days following removal of UC781 prior to exposure to HIV-1. Under the conditions used in the experiments illustrated in Fig. 7A, UC781 showed an intracellular antiviral protective effect with a half-life of 5.5 days. In contrast, neither UC84 nor nevirapine exhibited such an antiviral protective effect (data not shown).

Finally, the experiments illustrated in Fig. 6 and 7A involved exposure of MT2 cells to UC781 for 18 h. Significant UC781 antiviral protective effects were noted with as little as 10 min of exposure of uninfected cells to the drug (Fig. 7B). These data imply that cellular UC781 uptake is rapid, whereas subsequent efflux may occur at a somewhat lower rate. More detailed cell uptake-efflux studies are required to address this possibility.

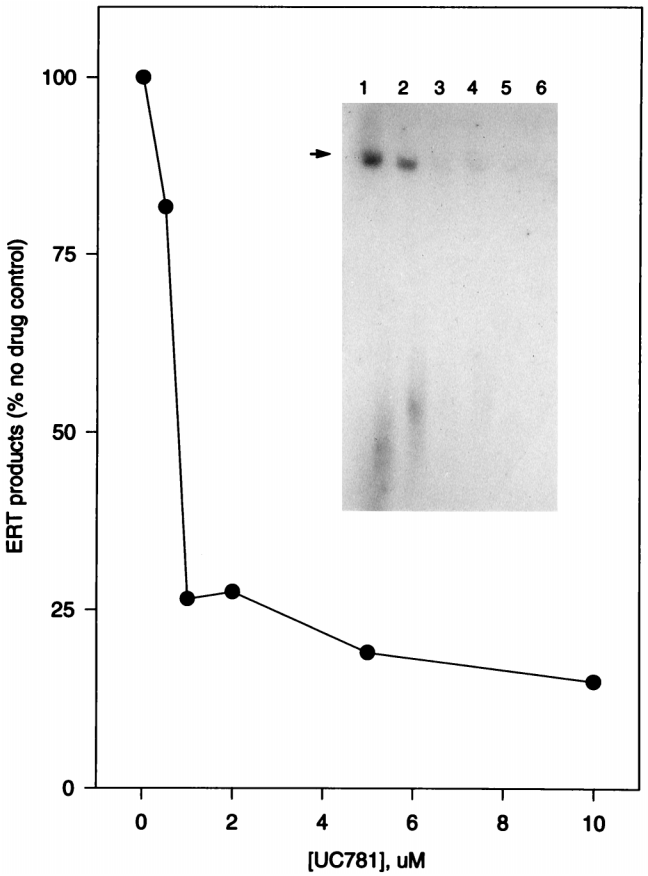

FIG. 5. UC781 inhibition of ERT. The main figure illustrates the densitometric analysis of the autoradiogram (inset) of ERT products synthesized in isolated virions of HIV-1. The arrow in the inset indicates the full-length 9.7-kb proviral DNA product. Lanes 1 to 6 correspond, respectively, to 0, 0.5, 1, 2, 5, and 10 μM UC781 in the virion preincubation.

DISCUSSION

Almost all of the effort in the development of antivirals against HIV has concerned drugs that may be effective in patients already infected with HIV, in order to delay disease progression and prolong the life spans of these individuals. Very little work has addressed development of drugs that might prevent the transmission of HIV to noninfected individuals. Nonetheless, there is a critical need to develop the latter, perhaps in the form of a vaginal microbicide effective against HIV, to control the spread of the disease. The World Health Organization has recently established a research priority for the development of effective vaginal microbicides in this respect (20).

So far, compounds considered as potential retrovirucides or vaginal microbicides include inhibitors of HIV-CD4 receptor interaction, such as dextran sulfate (1, 15) and modified β-lactoglobulin (17), and virus envelope-disrupting agents, such as nonoxynol-9 (18, 21).

NNI of HIV RT are also potential retrovirucides or vaginal microbicides (19). Typically, NNI are hydrophobic molecules and readily traverse membrane barriers such as that surrounding the HIV core. However, this property would also facilitate NNI efflux from the virion, in the absence of sufficient extravirion levels of the NNI. In order for NNI to be effective retrovirucides, they should possess some property that will prolong inhibitor residence within the virion. UC781 binds to HIV-1 RT with very high affinity and acts as a tight-binding inhibitor (3a). Thus, when UC781 penetrates the HIV-1 membrane envelope and capsid cores and binds to RT, the drug does not readily dissociate from the enzyme and is therefore trapped within the virion. This property imparts to UC781 the potential as a topical barrier to the transmission of HIV. Indeed, exposure of isolated HIV-1 to UC781 readily inactivates the virus. This viral inactivation is not noted with other NNI, such as nevirapine or UC84, neither of which is a tight-binding inhibitor of RT. We suggest that only NNI that act as tight-binding inhibitors of RT may be effective in retrovirucide or vaginal microbicide formulations.

Perhaps more importantly, exposure of uninfected cells to UC781 renders these cells refractory to subsequent HIV-1 infection, even in the absence of a continued extracellular presence of the drug. The window of efficacy of UC781 in our experimental conditions is a complex function of the resident intracellular lifetime of UC781 and the amount and stability of HIV in the extracellular culture and of the intracellular subviral ribonucleoprotein complex. The intracellular half-life of UC781 as a chemical barrier to HIV-1 infection in MT2 cells in vitro is about 5.5 days in cells pretreated with 10 μM UC781. The protective effect of UC781 is unlikely to be due to residual exogenous drug, since the cells were extensively washed after drug pretreatment. In addition, the cell culture medium was changed every 2 to 3 days following drug pretreatment, which would further reduce any possible residual drug.

UC781 may provide anti-HIV benefits in a microbicidal formulation in several ways. First, UC781 inactivates free virus. Evidently, the drug readily permeates the viral envelope and the matrix and core shells of HIV virions and binds tightly to the viral RT. HIV-1 infectivity would thereby be reduced due to inhibition of ERT in the free virion, a function shown to enhance virus infectivity during sexual contact (27, 28), as well as diminished reverse transcription following virus entry into the cell. Second, UC781 attenuates the infectivity of nascent HIV-1 produced by infected cells after exposure to the drug. Third, UC781 is effective against patient HIV-1 isolates. Finally, UC781 is readily taken up and remains for extended periods within uninfected HIV-susceptible cells, thereby inhibiting infection due to subsequent exposure to HIV. Only short times of exposure to UC781 were necessary to produce this protective effect. This is an important consideration for the potential use of UC781 in a vaginal microbicidal formulation. Thus, UC781 may serve as an effective retrovirucide or vaginal microbicide to minimize the sexual transmission of HIV-1 from infected to noninfected individuals. An important advantage of the use of NNI in this manner is that viral resistance would not develop since the drug would be used primarily by noninfected individuals.

It is likely that combinations of UC781 with compounds that inhibit virus-cell interaction, such as modified β-lactoglobulin (17), and compounds that disrupt the viral envelope membrane, such as nonoxynol-9 (18, 21), would provide a safe and effective vaginal retrovirucidal formulation. Such a formulation would work at three levels. Intact viruses that escape the disrupting effects of spermicidal surfactants would be prevented from virus-cell fusion by the anionic macromolecule component of the formulation. Those viruses that managed to fuse with the cell would be inhibited in carrying out reverse transcription by intracellular UC781. It is very possible that such a formulation would provide a synergistic inhibition of viral transmission.

UC781 also has excellent potential as a systemic antiviral agent in individuals already infected with HIV, since the drug functions as a classical NNI, suppressing concomitant HIV

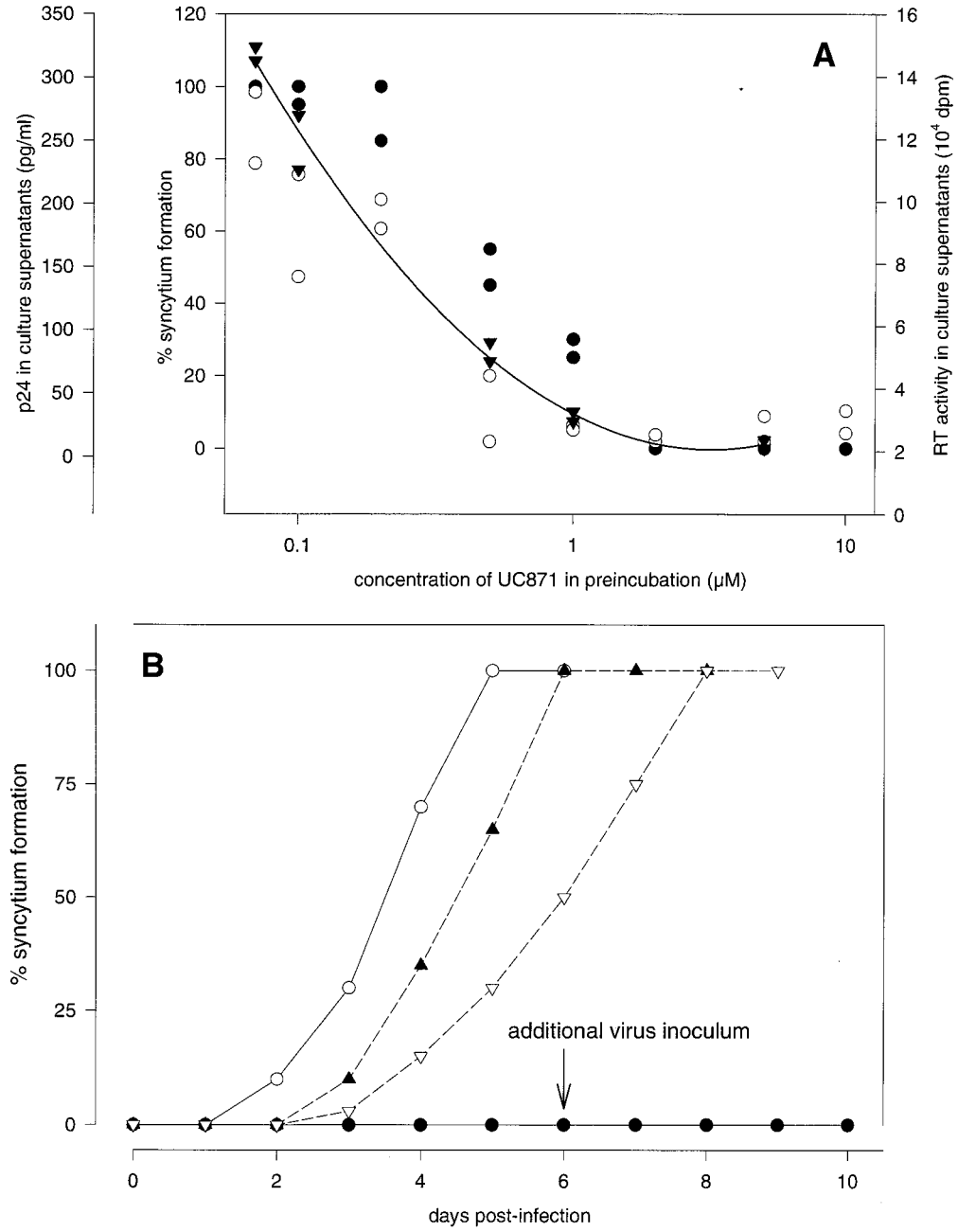

FIG. 6. Effect of pretreatment of uninfected MT-2 cells with various concentrations of UC781 on the susceptibility of these cells to subsequent HIV infection in the absence of exogenous drug. (A) Uninfected MT2 cells were incubated for 18 h with various concentrations of UC781 in 1 ml of medium, and then the cells were pelleted by centrifugation and washed twice by resuspension in 10 ml of medium followed by centrifugation. The cells were exposed to identical inocula of HIV-1 and cultured in the absence of added drug. Four days after removal of UC781, the extent of HIV-1 infection was assessed by measurement of syncytium formation (●), viral p24 antigen levels (▼), and RT activity (○) in cell-free culture supernatants. (B) Concentration dependence of the UC781 antiviral protective effect. Uninfected MT2 cells were incubated for 18 h in the absence (○) or the presence of 0.5 μM (▲), 1 μM (▽), or 2 μM (●) UC781. Residual exogenous drug was removed, and the cells were immediately exposed to identical inocula of HIV and cultured in the absence of added drug. The arrow indicates the addition of another aliquot of HIV-1 equivalent to that added to the cells at day 0. Viral cytopathicity was scored by microscopic assessment of syncytium formation as described in Materials and Methods. Datum points are the averages from duplicate samples.

infection of uninfected cells as well as diminishing infectivity of virus produced by already-infected cells. Importantly, we have shown this not only with the HIV-1_{IIIB} laboratory strain and H9 cell line but also with primary viral isolates (PBL isolated from HIV-infected patients). Pharmacokinetic studies have shown that UC series antivirals maintain high bioavailability (2, 12). Importantly, recent studies have shown that UC781 is active against a large number of viral strains that are resistant to other NNI (3). In addition, we have found that the time required for the development of HIV-1 resistance to UC781 is significantly prolonged relative to that for most other NNI (3b).

In conclusion, the UC series of NNI of HIV RT continues to provide compounds with unique antiviral properties. We have

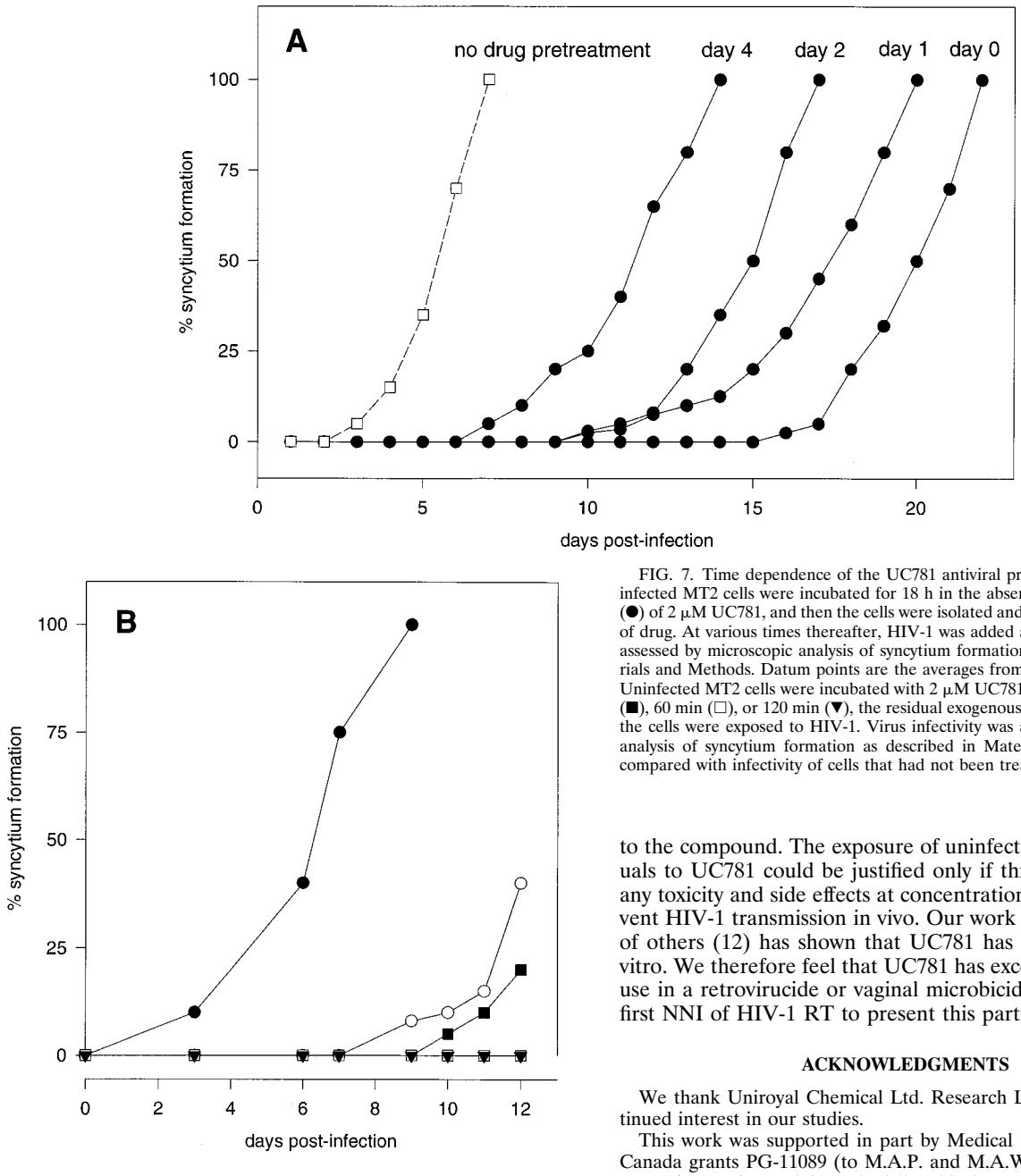

FIG. 7. Time dependence of the UC781 antiviral protective effect. (A) Uninfected MT2 cells were incubated for 18 h in the absence (○) or the presence (●) of 2 μM UC781, and then the cells were isolated and cultured in the absence of drug. At various times thereafter, HIV-1 was added and virus infectivity was assessed by microscopic analysis of syncytium formation as described in Materials and Methods. Datum points are the averages from duplicate samples. (B) Uninfected MT2 cells were incubated with 2 μM UC781 for 10 min (○), 30 min (■), 60 min (□), or 120 min (▼), the residual exogenous drug was removed, and the cells were exposed to HIV-1. Virus infectivity was assessed by microscopic analysis of syncytium formation as described in Materials and Methods and compared with infectivity of cells that had not been treated with the drug (●).

previously shown that structurally similar UC NNI interact with different mechanistic forms of RT (5) and that appropriate combinations of UC series antivirals act synergistically to inhibit HIV-1 replication (6). Continued development of this class of NNI has resulted in UC781, which we have now shown provides an effective chemical barrier to HIV-1 infection. The present work involved virus-to-T-cell and T-cell-to-T-cell transmission of HIV-1. For use in a vaginal microbicidal formulation, UC781 must also be effective in preventing viral infection of and transmission to epithelial cells. As UC781 readily penetrates cells, this is a likely possibility. We are presently carrying out studies to test the efficacy of UC781 in this respect. Repeated use of UC781 as a microbicidal agent would result in prolonged chronic exposure of sexually active women to the compound. The exposure of uninfected healthy individuals to UC781 could be justified only if this compound lacks any toxicity and side effects at concentrations sufficient to prevent HIV-1 transmission in vivo. Our work (Table 1) and that of others (12) has shown that UC781 has low cytotoxicity in vitro. We therefore feel that UC781 has excellent potential for use in a retrovirucide or vaginal microbicide formulation, the first NNI of HIV-1 RT to present this particular property.

ACKNOWLEDGMENTS

We thank Uniroyal Chemical Ltd. Research Laboratories for continued interest in our studies.

This work was supported in part by Medical Research Council of Canada grants PG-11089 (to M.A.P. and M.A.W.) and UI-13039 (to M.A.P.). G.B. is a recipient of a fellowship from the Medical Research Council of Canada. M.A.P. is a Chercheur-boursier senior (Career Research Scholar) of the Fonds de la Recherche en Santé du Québec.

REFERENCES

1. **Baba, M., R. Pauwels, J. Balzarini, J. Arnout, J. Desmyter, and E. De Clercq.** 1988. Mechanism of the inhibitory effect of dextran sulfate and heparin on the replication of human immunodeficiency virus *in vitro*. Proc. Natl. Acad. Sci. USA **85:**6132–6136.
2. **Bader, J. P., J. B. McMahon, R. J. Schultz, V. L. Narayanan, J. B. Pierce, O. S. Weislow, C. F. Midelfort, S. F. Stinson, and M. R. Boyd.** 1991. Oxathiin carboxanilide, a potent inhibitor of human immunodeficiency virus reproduction. Proc. Natl. Acad. Sci. USA **88:**6740–6744.
3. **Balzarini, J., W. G. Brouwer, D. C. Dao, E. M. Osika, and E. Declercq.** 1996. Identification of novel thiocarboxanilide derivatives that suppress a variety of drug-resistant mutant human immunodeficiency virus type 1 strains at a potency similar to that for wild-type virus. Antimicrob. Agents Chemother. **40:**1454–1466.

3a. **Barnard, J., G. Borkow, and M. A. Parniak.** Submitted for publication.

3b. **Borkow, G., N. Kaushik, and M. A. Parniak.** Unpublished data.

4. **Connolly, K. J., and S. M. Hammer.** 1992. Antiretroviral therapy: reverse transcriptase inhibition. Antimicrob. Agents Chemother. **36:**245–254.
5. **Fletcher, R. S., K. Syed, S. Mithani, G. I. Dmitrienko, and M. A. Parniak.** 1995. Carboxanilide derivative nonnucleoside inhibitors of HIV-1 reverse transcriptase interact with different mechanistic forms of the enzyme. Biochemistry **34:**4036–4042.
6. **Fletcher, R. S., D. Arion, G. Borkow, M. A. Wainberg, G. I. Dmitrienko, and M. A. Parniak.** 1995. Synergistic inhibition of HIV-1 reverse transcriptase DNA polymerase activity and virus replication *in vitro* by combinations of carboxanilide nonnucleoside compounds. Biochemistry **34:**10106–10112.
7. **Fletcher, R. S., G. Holleschak, E. Nagy, D. Arion, G. Borkow, Z. Gu, M. A. Wainberg, G. I. Dmitrienko, and M. A. Parniak.** 1996. Single step purification of HIV-1 recombinant wild type and mutant reverse transcriptase. Protein Expr. Purif. **7:**27–32.
8. **Grob, P. M., J. C. Wu, K. A. Cohen, R. H. Ingraham, C.-K. Shih, K. D. Hargrave, T. L. Mctague, and V. J. Merluzzi.** 1992. Nonnucleoside inhibitors of HIV-1 reverse transcriptase: nevirapine as a prototype drug. AIDS Res. Hum. Retroviruses **8:**145–152.
9. **Hirabayashi, K., S. Iwata, M. Ito, S. Shigeta, T. Narui, T. Mori, and S. Shibata.** 1989. Inhibiting effect of a lichen polysaccharide sulfate, GE-3-S, on the replication of human immunodeficiency virus (HIV) *in vitro*. Chem. Pharm. Bull. **37:**2410–2412.
10. **Johnson, V. A.** 1996. Combination therapy for HIV-1 infection. Overview: preclinical and clinical analysis of antiretroviral combinations. Antivir. Res. **29:**35–39.
11. **Levy, J. A.** 1993. Pathogenesis of human immunodeficiency virus infection. Microbiol. Rev. **57:**183–289.
12. **McMahon, J. B., Jr., R. W. Buckheit, J. R. Gulakowski, M. J. Currens, D. T. Vistica, R. H. Shoemaker, S. F. Stinson, J. D. Russell, J. P. Bader, V. L. Narayanan, R. J. Schultz, W. G. Brouwer, E. E. Felauer, and M. R. Boyd.** 1996. Biological and biochemical anti-human immunodeficiency virus activity of UC38, a new nonnucleoside reverse transcriptase inhibitor. J. Pharmacol. Exp. Ther. **276:**298–305.
13. **Merluzzi, V. J., K. D. Hargrave, M. Labadia, K. Grozinger, M. T. Skoog, J. C. Wu, C.-K. Shih, K. Eckner, S. Hattox, J. Adams, A. S. Rosehthal, R. Faanes, R. J. Eckner, R. A. Koup, and J. L. Sullivan.** 1990. Inhibition of HIV-1 replication by a nonnucleoside reverse transcriptase inhibitor. Science **250:**1411–1413.
14. **Merson, M. H.** 1993. Slowing the spread of HIV: agenda for the 1990s. Science **260:**1266–1268.
15. **Mitsuya, H., D. J. Looney, S. Kuno, R. Ueno, F. Wong-Staal, and S. Broder.** 1988. Dextran sulfate suppression of viruses in the HIV family: inhibition of virion binding to CD4+ cells. Science **240:**646–649.
16. **Moyle, G., and B. Gazzard.** 1996. Current knowledge and future prospects for the use of HIV protease inhibitors. Drugs **51:**701–712.
17. **Neurath, A. R., S. B. Jiang, N. Strick, K. Lin, Y. Y. Li, and A. K. Debnath.** 1996. Bovine β-lactoglobulin modified by 3-hydroxyphthalic anhydride blocks the CD4 cell receptor for HIV. Nat. Med. **2:**230–234.
18. **Niruthisard, S., R. E. Roddy, and S. Chutivongse.** 1991. The effects of frequent nonoxynol-9 use on the vaginal and cervical mucosa. Sex. Transm. Dis. **18:**176–179.
19. **Pauwels, R., and E. De Clercq.** 1996. Development of vaginal microbicides for the prevention of heterosexual transmission of HIV. J. Acquired Immun. Defic. Syndromes Hum. Retrovirol. **11:**211–221.
20. **Potts, M.** 1994. The urgent need for a vaginal microbicide in the prevention of HIV transmission. Am. J. Public Health **84:**890–891.
21. **Roddy, R. E., M. Cordero, and J. A. Fortney.** 1993. A dosing study of nonoxynol-9 and genital irritation. Int. J. STD AIDS **4:**165–170.
22. **Rooke, R., M. Tremblay, and M. A. Wainberg.** 1990. AZT (zidovudine) may act post-integrationally to inhibit generation of HIV-1 progeny virus in chronically-infected cells. Virology **176:**205–215.
23. **Rooke, R., M. A. Parniak, M. Tremblay, H. Soudeyns, X. Li, Q. Gao, X.-J. Yao, and M. A. Wainberg.** 1991. Biological comparison of wild-type and zidovudine-resistant isolates of human immunodeficiency virus type 1 from the same subjects: susceptibility and resistance to other drugs. Antimicrob. Agents Chemother. **35:**988–991.
24. **Szedlacsek, S. E., and R. G. Duggleby.** 1995. Kinetics of slow and tight-binding inhibitors. Methods Enzymol. **249:**144–180.
25. **Williams, J. W., and J. F. Morrison.** 1979. The kinetics of reversible tight-binding inhibition. Methods Enzymol. **63:**437–467.
26. **Yao, X.-J., M. A. Wainberg, and M. A. Parniak.** 1992. Mechanism of inhibition of HIV-1 infection *in vitro* by purified extract of *Prunella vulgaris*. Virology **187:**56–62.
27. **Zhang, H., G. Dornadula, and R. J. Pomerantz.** 1996. Endogenous reverse transcription of human immunodeficiency virus type 1 in physiological microenvironments: an important stage for viral infection of nondividing cells. J. Virol. **70:**2809–2824.
28. **Zhang, H., Y. Zhang, T. P. Spicer, L. Z. Abbott, M. Abbott, and B. J. Poiesz.** 1993. Reverse transcription takes place within extracellular HIV-1 virions: potential biological significance. AIDS Res. Hum. Retroviruses **9:**1287–1296.