The Thiocarboxanilide Nonnucleoside Inhibitor UC781 Restores Antiviral Activity of 3′-Azido-3′-Deoxythymidine (AZT) against AZT-Resistant Human Immunodeficiency Virus Type 1

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The conversion of viral genomic RNA into double-stranded DNA is an essential step in the replication of human immunodeficiency virus type 1 (HIV-1). This conversion is a multistep process that is catalyzed entirely by the viral enzyme reverse transcriptase (RT). RT therefore provides an important target for the development of anti-HIV chemotherapeutics (13).

RT inhibitors can be grouped into two major classes. Dideoxynucleosides (ddNs) such as 3′-azido-3′-deoxythymidine (AZT) and 2′,3′-dideoxy-3′-thiacytidine are substrate-like analogs and inhibit the virus by competing with the natural dideoxynucleoside triphosphate (dNTP) substrate for binding to the catalytic site of RT (minor mechanism) and, once they are incorporated into the nascent DNA chain, by terminating continued viral DNA synthesis due to the lack of a 3′ hydroxyl moiety (major mechanism) (19). The second class comprises the nonnucleoside RT inhibitors (NNRTIs), a structurally diverse assortment of compounds including nevirapine (24, 30), TIBO (34), the pyridinones (18), and the carboxanilides and thiocarboxanilides (3–7, 9, 28). NNRTIs act by binding to a site on RT distinct from the catalytic site (14, 23, 37).

Since ddNs and NNRTIs interact with different sites on RT, they may bind in a mutually nonexclusive manner; that is, both ddNs and NNRTIs may simultaneously bind to the enzyme. Accordingly, combinations of ddNs plus NNRTIs have the potential to act synergistically to inhibit HIV-1 replication. Although some investigators have reported synergy with combinations of ddNs plus NNRTIs, in inhibiting wild-type (wt) HIV replication (28, 41), others have found this inhibition to be additive only (3, 4). Few studies have addressed the inhibitory potential of antiviral agent-resistant HIV by combinations of ddNs plus NNRTIs, although in one report, a loss of synergistic response to combinations of AZT plus other drugs was noted against AZT-resistant HIV (11). These studies are often complicated by the significant differences in the inhibitory potencies of the compounds used in combination.

AZT and N-[4-chloro-3-(3-methyl-2-butenyloxy)phenyl]-2-methyl-3-furanecarbothioamide (UC781) have very similar antiviral potencies (ca. 5 nM), thereby providing a useful system to test whether combinations of ddNs plus NNRTIs can synergistically inhibit the replication of both wild-type and drug-resistant HIV-1. Synergy of inhibition by a combination of a ddN plus an NNRTI is unlikely when the combination is used against an HIV strain resistant to one of the drugs in the combination.

In the present study, we examined the inhibitory potencies of AZT alone, UC781 alone, and a 1:1 molar combination of AZT plus UC781 against wt HIV-1, UC781-resistant HIV-1, and AZT-resistant HIV-1. We found that a 1:1 molar combination of AZT plus UC781 showed very good synergy in inhibiting the replication of an AZT-resistant clinical isolate of HIV-1, implying that UC781 “restored” the antiviral activity of AZT against this virus. We also note that the time to the development of HIV resistance to a 1:1 molar combination of AZT plus UC781 is significantly delayed compared to that for either drug alone.

MATERIALS AND METHODS

Virus strains. The wt HIV-1 strain used in this study was the HIV-IIIb laboratory strain of HIV-1 obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (courtesy of R. C. Gallo). The AZT-resistant variant (strain 691A) was a viral clinical isolate from an AIDS patient treated with AZT monotherapy (35) and possessed the K70R and T215Y mutations. The UC781-resistant variant was developed during the present studies in our laboratories by in vitro methods that we have described previously (16, 17). The UC781-resistant virus possessed multiple mutations including K103T, V106A, and Y181C.

Other reagents. The CD4+/MT-2 cell line was purchased from the American Type Culture Collection (Rockville, Md.). RPMI 1640 cell culture medium and heat-inactivated fetal bovine serum were obtained from Canadian Life Technol-
Table 1. Inhibition of wt and drug-resistant HIV-1 variants by UC781, AZT, and a 1:1 molar ratio combination of UC781 and AZT

<table>
<thead>
<tr>
<th>HIV-IIIb variant</th>
<th>EC50 (nM)*</th>
<th>AZT</th>
<th>UC781</th>
<th>AZT plus UC781</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>5.3 ± 1.25</td>
<td>10.4 ± 5</td>
<td>2.8 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>UC781 resistant</td>
<td>6.6 ± 3.3</td>
<td>16,600 ± 2,300*</td>
<td>11.0 ± 4.6</td>
<td></td>
</tr>
<tr>
<td>AZT resistant</td>
<td>&gt;2,500*</td>
<td>13.1 ± 4</td>
<td>3.8 ± 0.8</td>
<td></td>
</tr>
</tbody>
</table>

* EC50 was assessed by measuring p24 antigen levels in culture supernatants and by assessment of syncytium formation. The values are the mean ± standard deviations from at least three separate experiments, each of which was carried out in triplicate.

RESULTS

Drug sensitivity of AZT-resistant and UC781-resistant HIV-1. Both the AZT-resistant clinical variant (strain 691A) and the in vitro-selected UC781-resistant HIV-1 strains were highly resistant to drug combinations (Table 1). Inhibition of wt HIV by the combination of AZT plus UC781 was additive (Table 2), confirming previous observations (3, 4). Similarly, UC781-resistant HIV was inhibited by AZT equally well as replication of wt virus (Table 1); thus, cross-resistance was not observed.

AZT and UC781 have similar antiviral potencies. Wild-type HIV-1 was inhibited equally well by AZT, UC781, and a 1:1 molar combination of AZT plus UC781 (Table 1). Inhibition of wt HIV by the combination of AZT plus UC781 was additive (Table 2), confirming previous observations (3, 4). Similarly, UC781-resistant HIV was inhibited by AZT equally well as wt virus was, but UC781-resistant HIV was insensitive to UC781 alone (Table 1). As expected, a 1:1 molar combination of AZT plus UC781 at any given nominal concentration was less effective at inhibiting replication of this HIV strain than AZT alone at the same nominal concentration.

Very different results were noted with AZT-resistant viral

Table 2. CIs for inhibition of wt and drug-resistant HIV-1 variants by AZT-NRTI combinations

<table>
<thead>
<tr>
<th>Virus</th>
<th>Drug combination Ratio</th>
<th>CI at the following effective concn:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50%</td>
</tr>
<tr>
<td>wt UC781-AZT</td>
<td>1:1</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>UC781 resistant</td>
<td>UC781-AZT</td>
<td>1:1</td>
</tr>
<tr>
<td>AZT resistant</td>
<td>UC781-AZT (691A clinical isolate)</td>
<td>1:1</td>
</tr>
<tr>
<td>Nevirapine-AZT</td>
<td>Nevirapine-AZT</td>
<td>1:1</td>
</tr>
<tr>
<td>T180-AZT</td>
<td>T180-AZT</td>
<td>1:1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10:1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10:1</td>
</tr>
</tbody>
</table>

* CIs were calculated by the method of Chou and Talalay (10). The values reported are the means ± standard deviation from at least three independent experiments conducted with duplicate samples. Values without standard deviations are the averages from two independent experiments conducted in duplicate.
strain 691A. This strain was highly resistant to AZT but was as sensitive to UC781 as wt HIV was (Table 1). However, the antiviral activity of a 1:1 molar combination of AZT plus UC781 against the 691A virus strain was significantly enhanced compared to that noted with similar nominal concentrations of UC781 alone (Fig. 1). The combination of AZT plus UC781 showed high-level synergy in inhibiting the replication of the AZT-resistant virus (Table 2). This implies (i) that AZT is functioning against AZT-resistant virus replication under these conditions and (ii) that UC781 therefore must restore antiviral activity to AZT against AZT-resistant HIV-1. Little or no synergy in the inhibition of AZT-resistant HIV-1 by combinations of AZT with other NNRTIs such as nevirapine and TSAO was noted (Table 2).

**UC781 inhibits RT-catalyzed pyrophosphorolysis.** The antiviral efficacy of ddN inhibitors such as AZT is due primarily to chain termination of the nascent viral DNA (19). We have recently found that HIV-1 RT containing mutations associated with AZT resistance has an increased sensitivity to PP\(_i\) (1). This results both in decreased binding of AZT triphosphate and in an increased pyrophosphorolytic cleavage of the 3’-terminal chain-terminating nucleotide. UC781 was a potent inhibitor both of DNA synthesis and of pyrophosphorolysis in vitro catalyzed by wt RT or by the RT with the D67N, K70R, T215F, and K219Q mutations (Fig. 2). The extent of inhibition of these reactions is readily discerned by the intensity of the starting 18-nt [\(^{32}\)P]prPBS primer (denoted as RTPBS\(_{18}\)) in Fig. 2. This UC781-mediated inhibition of RT pyrophosphorolysis presumably allows AZT to regain chain-terminating activity against AZT-resistant virus in the presence of the non-nucleoside inhibitor and enables AZT to contribute to the overall inhibition of AZT-resistant HIV-1 exposed to combinations of AZT plus UC781.

**In vitro development of resistance to combinations of AZT plus UC781.** As seen in Fig. 3, HIV-1 readily develops high-level resistance to either AZT or UC781 alone. The AZT-resistant virus showed the K70R and T215Y mutations, whereas the UC781-resistant virus possessed K103T, V016A, and Y181C mutations. In contrast, resistance to 1:1 molar combinations of AZT plus UC781 develops in vitro much more slowly and to a much reduced extent than resistance to either drug alone. Sequencing of the proviral DNA produced following infection with HIV partially resistant to combinations of AZT plus UC781 revealed the following three mutations in the same clone: K103N, V118I, and Y181S. None of the regular mutations conferring AZT resistance (M41L, K70R, and T215Y) were noted in these viruses; this, however, may be due to the fact that only low-level resistance to combinations of AZT plus UC781 has so far been achieved.

**DISCUSSION**

AZT has widespread clinical use in the treatment of HIV-infected and AIDS patients. Unfortunately, prolonged exposures to antiviral agents inevitably leads to the emergence of drug-resistant viruses (26, 29). This is particularly a problem in individuals who have received antiviral monotherapy, as was the case for AZT (12, 27, 32, 33, 38). Indeed, because of the initial treatment with AZT monotherapy, AZT-resistant HIV has become more prevalent, such that newly infected individuals may be infected with AZT-resistant strains of HIV. This, of course, presents considerable drawbacks for the continued use of AZT in antiviral therapy.

UC781 is a tightly binding inhibitor of RT (6), a property unique among the NNRTIs that have so far been described. This tight binding may indicate a somewhat different mode of
interaction with the NNRTI binding pocket, consistent with the observation that UC781 has excellent activity against HIV-1 mutants resistant to other NNRTIs in vitro (4, 5, 9). The AZT-resistant virus was not cross resistant to UC781 and vice versa. This is not surprising, because the mutations which confer resistance to each of these two drugs occur in different regions of RT (5, 9, 22, 26).

While some investigators have noted that combinations of AZT plus NNRTIs act synergistically to inhibit replication of wild-type (drug-sensitive) HIV-1 (28, 41), others have found this inhibition to be additive only (3, 4). Our data support the latter observation (Table 2). Importantly, we found that combinations of AZT plus UC781 showed high-level synergy in inhibiting the replication of AZT-resistant HIV-1 (Fig. 1; Table 2), implying that UC781 was somehow restoring the ability of AZT to act against AZT-resistant virus. To our knowledge, this is the first reported example of the restoration of AZT sensitivity to an AZT-resistant virus by use of a combination of AZT plus an NNRTI. It is important, however, that our results were obtained with an AZT-resistant clinical isolate possessing the K70R and T215Y mutations. Similar data have been obtained with recombinant HIV containing the D67N, K70R, T215F, and K219Q mutations (unpublished data). However, we have not yet tested whether UC781 is able to restore the activity of AZT against a range of AZT-resistant mutant HIV-1, such as those with only the T215Y mutation or the M41L plus T215Y mutations. These studies are in progress.

The phenotypic mechanism of resistance to ddNIs such as 2',3'-dideoxy-3'-thiacytidine, dideoxynosine etc., generally involves a decreased ability of the RT to bind to the inhibitor (20, 40). AZT resistance is unusual in that decreased binding does not appear to be the major factor in the resistance mechanism. Indeed, RT containing mutations associated with AZT resistance is as sensitive as wt RT to inhibition by AZT triphosphate in standard in vitro enzyme assays (25, 39). However, we have recently found that AZT resistance results in large part from RT-catalyzed pyrophosphorolytic removal of chain-terminating AZT after its incorporation into the nascent DNA strand (1). While wt and AZT-resistant HIV strains may show similar rates of incorporation of chain-terminating AZT, the AZT-resistant viral RT is more effective in subsequently removing it. The pyrophosphorolytic removal of the terminal AZT allows continuation of forward viral DNA synthesis. We found that UC781 was a potent inhibitor of in vitro pyrophosphorolysis carried out by both wt and AZT-resistant RT (Fig. 2). We propose that it is the inhibition of this activity by UC781 that allows AZT to again function as a chain terminator with AZT-resistant virus.

The rapid emergence of resistant HIV mutants represents a formidable challenge to the development of anti-HIV drugs (31). The time to the development of HIV resistance in vitro to UC781 alone is significantly delayed compared to the time to the development of HIV resistance to other carboxanilide NNRTIs such as UC84 and UC38 (8). This is not due to a decreased “fitness” of UC781-resistant HIV, since this resistant virus replicates as well as wt HIV (data not shown). The delayed resistance may be due to the need for multiple mutations in RT to achieve high-level resistance to UC781 (5, 9). High-level resistance to AZT also requires multiple mutations in HIV RT (22, 26). The development of in vitro viral resistance to a 1:1 molar combination of AZT plus UC781 was significantly attenuated both in rate and in extent compared to those for either drug alone (Fig. 3).

The delayed development of resistance to combinations of AZT plus UC781 may be due to the fact that high-level resistance to each of AZT and UC781 requires multiple mutations in HIV-1 RT (5, 9, 22, 26). However, it is interesting that none of the common mutations associated with AZT resistance appear in virus with partial resistance to combinations of AZT plus UC781. The V118I mutation noted in these virus is so far unreported. Site-specific mutagenesis experiments are necessary to confirm the role of this mutation in resistance to the combination of AZT plus UC781. It is possible that the numerous mutations required for resistance to the combination might have a detrimental effect on RT activity, possibly resulting in a virus with a decreased ability to replicate. Our inability to generate high-level viral resistance in vitro in the extended time frame of our experiments may be consistent with this possibility. Since resistance to AZT seems to develop more quickly in vitro than resistance to UC781 and since UC781 acts to restore the activity of AZT against AZT-resistant virus, it is possible that resistance to both drugs may not readily develop in the same virus strain without a concomitant reduction in replication capacity. We are using site-specific mutagenesis to test this hypothesis.

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