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Viral Resistance to the Thiazolo-Iso-Indolinones, a New Class of Nonnucleoside Inhibitors of Human Immunodeficiency Virus Type 1 Reverse Transcriptase

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Thiazolo-iso-indolinone derivatives with high specificity toward the reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1) were identified. The most potent compound, BM +51.0836, inhibited HIV-1 RT at a 50% inhibitory concentration of 90 nM in vitro. In cell culture assays, similar 50% inhibitory concentrations were obtained with high specificity for HIV-1. These substances were equally active against a zidovudine-resistant isolate. No antiviral effect was observed with an HIV-2 isolate. HIV-1 isolates resistant to the thiazolo-iso-indolinones were generated in cell culture, and the nucleotide sequences of the respective RT genes were analyzed subsequently. Comparison of the deduced amino acid sequences with the wild-type sequence showed an amino acid change at position 181 (Tyr to Cys). Substitutions of amino acid Lys-101 and Lys-103 as well as Tyr-181 and/or Tyr-188 by site-directed mutagenesis led to resistance against the thiazolo-iso-indolinones. A chimeric HIV-2 RT, substituted with amino acids at positions 179 to 190 from HIV-1, acquired only partial susceptibility to BM +51.0836.

Human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) is the target of nonnucleoside RT inhibitors, including O-TIBO (13), nevirapine (BI-RG-587) (9), and the pyridinones (12). These compounds, although structurally nonrelated, show high specificity for HIV-1 RT but have no effect on HIV-2 RT or any other DNA or RNA polymerase. Nunberg et al. (12) reported the isolation in cell culture of HIV-1 mutants that were resistant to HIV-1-specific pyridinone inhibitors because of mutations of amino acid Lys-103 to Asn and Tyr-181 to Cys of the RT gene. Pyridinone-resistant viruses, although susceptible to nucleoside analogs, showed cross-resistance to O-TIBO and nevirapine. Shih et al. (20) found that the tyrosine residues at positions 181 and 188, flanking the putative catalytic site of the enzyme, are responsible for the susceptibility of the enzyme to nevirapine. HIV-2 RT, which lacks two aromatic amino acids at positions 181 and 188, is completely resistant to the nonnucleoside inhibitors. On the other hand, a chimeric HIV-2 RT protein, in which amino acids at positions 176 to 190 were replaced by the respective HIV-1 sequence, acquired susceptibility to O-TIBO and nevirapine (20). The findings of cross-resistance of HIV-1 mutants and the chimeric HIV-2–HIV-1 enzyme to different classes of nonnucleoside RT inhibitors suggest that these substances may all share a similar binding site on the RT of HIV-1.

Here we report the identification of selective nonnucleoside inhibitors of HIV-1 RT in vitro by a nonradioactive RT assay (enzyme-linked immunosorbent assay [ELISA]) with biotin- and digoxigenin-labeled nucleoside triphosphates. A powerful substance with chiral structure and high specificity for HIV-1 was identified; this substance was BM 21.1298, a thiazolo-iso-indolinone (Fig. 1a). Investigations with several derivatives of BM 21.1298 led to the detection of BM +51.0836 [(*R*)-(+)-9*b*-(3,*S*-dimethylphenyl)-2,3-dihydrothiazolo[*2,3-a*]isoindol-5(*9bH*)-on; Fig. 1b], a substance with 10-fold lower 50% inhibitory concentrations (IC_{50}s) than the lead compound BM 21.1298 and higher antiviral activity than O-TIBO and nevirapine (BI-RG-587).

MATERIALS AND METHODS

Compounds. The thiazolo-iso-indolinones were synthesized by Boehringer Mannheim GmbH. A detailed description of the synthesis will be published elsewhere. Zidovudine (AZT; Wellcome), nevirapine (BI-RG-587; Boehringer Ingelheim), and O-TIBO (Janssen) were also synthesized by the chemical department of Boehringer Mannheim GmbH. The inhibitors were dissolved in dimethyl sulfoxide, and the mixture was added to the RT assay mixture (the dimethyl sulfoxide concentration was less than 5%). For cell culture assays, stock solutions were prepared by dissolving the inhibitors in 50% dimethyl sulfoxide–50% culture medium. In the cell culture supernatant, the dimethyl sulfoxide concentration was less than 1%.

Nonradioactive HIV-1 RT assay. Selective inhibitors of HIV-1 RT were identified by using a previously published high-efficiency screening system (4). The assay contains purified HIV-1 RT (11) expressed in *Escherichia coli*, an in vitro transcript of the HIV-1 long terminal repeat (21), and the primer-binding site as the template and as well as an 18-mer oligonucleotide as the primer. Inhibition of RT was determined by a nonradioactive RT assay (ELISA) with biotin- and digoxigenin-labeled nucleoside triphosphates (4). In brief, 1 μg of HIV-1 template RNA (1,080 nucleotides) and 20 ng of 18-mer deoxyoligonucleotide primer (complementary to the primer-binding site on the template) were annealed by heating them to 90°C and slowly cooling them to 37°C. Primers and templates were added to the reaction

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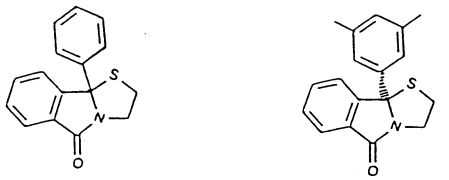

BM 21.1298(rac) BM +51.0836

a b

FIG. 1. Chemical structures of the nonnucleoside inhibitors BM 21.1298 (rac), the lead compound of the thiazolo-iso-indolinones (a), and the derivative BM +51.0836 (b).

mixture (40 ng of HIV-1 RT [p66/p51] expressed in *E. coli*). Nucleotides and buffers were used according to the instructions provided with the nonradioactive RT kit (Boehringer Mannheim) and were incubated in a streptavidin-coated microtiter plate for 60 min at 37°C with the different inhibitors. Plates were washed five times with 0.1% Tween 20 in phosphate-buffered saline (PBS). After washing, 100 μl of antidigoxigenin-peroxidase conjugate (150 mU/ml diluted with 100 mM Tris-HCl (pH 7.7) and 150 mM NaCl) was added to each well, and the plate was incubated for 30 min at room temperature; this was followed by five washes (see above). A total of 100 μl of 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) solution (1 g of ABTS per liter in ABTS buffer) was delivered to each well and the plate was subsequently incubated for 10 min at room temperature. Color development was measured in an ELISA reader at 405 nm.

Viruses. The original stocks of the HIV-1 strain human T-cell leukemia virus strain IIIB [HTLV(IIIB)] were kindly provided by M. Popovic and R. C. Gallo. Patient-derived strains HIV-1 D79, HIV-2 D194, HIV-1 D148/86, and HIV-1 D148/88 (HIV-1 D148/88 exhibited reduced susceptibility to AZT, with mutations at amino acid positions 67, 70, 215, and 219 of the RT gene) (16) were obtained from H. Ruebsamen-Waigmann, Georg-Speyer-Haus, Frankfurt, Federal Republic of Germany. Virus stocks were prepared by freezing clarified supernatants of virus producer cells at −70°C. Determination of the virus titer was done by endpoint dilution. Virus stocks of HTLV(IIIB) were prepared from chronically infected H9 cells and were used for infection of MT-2 and peripheral blood mononuclear cells (PBMCs). HIV-1 D79, HIV-1 D148/86, HIV-1 D148/88, and HIV-2 D194 were propagated in PBMCs. The titers of the different virus strains varied between 5,000 to 10,000 tissue culture infectious doses per ml.

HIV cell culture growth and testing of antiviral compounds. HIV infectivity studies were performed in MT-2 T-lymphoid cells (6), PBMCs, and human blood-derived monocytes/macrophages (M/M). In brief, MT-2 cells were infected with HTLV(IIIB) at a multiplicity of infection of 0.01. Syncytium formation and cell viability determined by a test with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) (14) were determined 7 days after infection. The supernatants of the cells were discarded, 100 μl of a solution of 0.7 mg of MTT per ml (in PBS) was added, and the cells were incubated for 4 h at 37°C. After solubilization of the dye with 200 μl of isopropanol–0.04 N HCl and thorough mixing, the A_{600} was measured and compared with that at A_{690}. Reduction of MTT to formazan by cellular dehydrogenases correlates with the number of viable cells. The reduction of cytopathic effect by antiviral substances were calculated and IC_{50}s were determined.

Primary PBMCs and M/M were isolated from buffy coats from seronegative donors by the Ficoll-Hypaque technique. Phytohemagglutinin-stimulated (1%) PBMCs were infected with the different HIV strains at a multiplicity of infection of 0.1 in RPMI 1640 medium supplemented with 20% fetal calf serum and 2 mM glutamine. The level of HIV infection was determined 7 days after infection by measuring the amount of p24 antigen in the supernatants of infected cells by using a commercially available ELISA (DuPont). Blood-derived human macrophages were separated from PBMCs by adherence to plastic. A total of 5×10^6 PBMCs were seeded in 24-well plates in RPMI 1640 medium (supplemented with 10% human type AB serum, 1% nonessential amino acids, 1 mM pyruvate, and 0.5% vitamins), and after 5 days, nonadherent cells were removed by five wash steps. Cells were infected with HIV-1 D79 at a multiplicity of infection of 0.1. Twenty-one days after virus infection, antigen p24 expression was measured by an ELISA (DuPont).

Isolation of resistant variants. MT-2 cells (3×10^5 cells per ml) were infected with cell-free virus at a low multiplicity (multiplicity of infection, <0.01) for 1 h. After infection, BM 21.1298, O-TIBO, and nevirapine were added to the culture, each at a final concentration of 40 nM. The amount of the added drugs was gradually increased (once a week) until each was present at a final concentration of 40,000 nM at 8 weeks. For each passage, cultures were infected by using the supernatant from the preceding passage. Cellular DNA from MT-2 cells infected with drug-resistant virus or wild-type HTLV(IIIB) was extracted by previously published procedures (17). RT genes (amino acids at positions 88 to 228) from resistant viruses were amplified by polymerase chain reaction (PCR) with the primers: sense 5'-GGAAGTTCAAT TAGGATT-3'; antisense 3'-GTAGTCTTTCTTGGAGGTA AGGAA-5'. Both strands of the resulting 420-bp fragment were sequenced by cycle sequencing (Perkin-Elmer Cetus) by using the 5'-[^{32}P]ATP-labeled PCR amplification primer.

Construction of HIV-1 RT mutants. Starting from pRT_{66} (11), which contains the RT gene of HIV-1 (5), mutants were created by site-directed mutagenesis by PCR. Reactions were carried out by standard protocols. Prior to enzymatic digestion, DNA fragments were purified from agarose gels. Restriction enzymes, *Taq* polymerase, T4 DNA ligase, calf intestinal phosphatase, and buffers were obtained from Boehringer Mannheim and New England BioLabs. The PCR primer for the Lys-101 to Ala mutation were 5'-GGCGCC CAGTACTAAATGGAGAAAATTAGTAGATTTCA-3' (sense) and 5'-CGCAGGAGTACTGTTACTGATTTTTTC TTTTTTGCTAACCCTGCGGG-3' (antisense). The PCR primers for the Lys-103 to Asn mutation were the same as above for the sense strand and 5'-GAGCCAGAGTACTG TTACTGATTTATTCTTTTTTAACC-3' for the antisense strand.

The amplified products were cut with *Sca*I, resulting in a 119-bp fragment. Wild-type pRT_{66} was cut with *Sca*I to remove the original *Sca*I fragment. The 4- and 2.2-kb *Sca*I fragments of pRT_{66} were religated. The resulting shortened pRT_{66} (missing the 119-bp segment) was partially digested with *Sca*I, dephosphorylated with calf intestinal phosphatase, and ligated with either one of the PCR fragments, leading to the pRT_{66} Lys-101 to Ala mutation or the pRT_{66} Lys-103 to Asn mutation. PCR primers for Tyr-181 to Cys were 5'-ATGCGTCCGGCGTAGAGGATCC-3' (sense) and 5'-GTCGCAGATCCTACATACAAATCATCCATGTAT TGACAGATAA-3' (antisense). The PCR primers for the

TABLE 1. Inhibition of different HIV-1 strains by RT inhibitors

RT inhibitor	IC_{50} (μM)[a]					
	HTLV(IIIB) (MT-2)	HTLV(IIIB) (PBMCs)	HIV-1 D79 (PBMCs)	HIV-1 D79 (M/M)	HIV-1 D148/86 (PBMCs)	HIV-1 D148/88 (PBMCs)
BM 21.1298	0.680 ± 0.09	0.536 ± 0.20	0.058 ± 0.002	0.083 ± 0.006	0.643 ± 0.17	0.640 ± 0.38
BM +51.0836	0.055 ± 0.02	0.016 ± 0.005	0.009 ± 0.006	0.016 ± 0.006	0.009 ± 0.002	0.008 ± 0.003
O-TIBO	0.214 ± 0.06	0.137 ± 0.04	0.057 ± 0.04	0.039 ± 0.02	0.257 ± 0.17	0.357 ± 0.20
BI-RG-587	0.171 ± 0.05	0.162 ± 0.06	0.051 ± 0.02	0.026 ± 0.005	0.422 ± 0.06	0.368 ± 0.27
AZT	0.024 ± 0.01	0.026 ± 0.015	0.022 ± 0.01	0.027 ± 0.004	0.020 ± 0.003	0.566 ± 0.23

[a] IC_{50}s are the 50% inhibitory concentrations measured by the MTT test in MT-2 cell culture and by p24 capture ELISA in PBMCs and human blood-derived M/M. Virus strain HIV-1 D148/88 showed reduced susceptibility to AZT. For assay conditions and virus strains, see text. Means ± standard deviations were calculated from the results of three independent assays.

Tyr-181 to Leu mutation were the same as above for the sense strand and 5′-GCTGCAGATCCTACAAGCAAATC ATCCATGTA-3′ for the antisense strand. The PCR primers for the double mutation Tyr-181 to Ile/Tyr-188 to Leu were the same as above for the sense strand and 5′-GTCAG ATCCTACCAACAAATCATCCATGTATTGAATGAT AAC-3′ for the antisense strand. By using the restriction sites close to both ends of the amplified products, PCR fragments were cut with *Eco*RV and *Xho*I, resulting in 138-bp fragments carrying the mutation. pRT_{66} was digested with *Eco*RV and *Hin*dIII, giving 5- and 1,250-bp fragments. The latter was digested with *Xho*I, resulting in DNA segments of 138 bp (containing the wild-type tyrosines that had to be removed) and 1,112 bp. Ligation of the PCR fragments, the 5-kb vector fragment, and the 1,112-bp fragment led to the pRT_{66} Tyr-181 to Cys mutation, the pRT_{66} Tyr-188 to Leu mutation, and the pRT_{66} Tyr-181 to Ile/Tyr-188 to Leu mutation, respectively. The region adjacent to each of the mutations was sequenced by the chain termination method (18) to confirm that the appropriate mutation had been introduced.

Construction of chimeric HIV-2–HIV-1 RT. Chimeric HIV-2 RT substituted with amino acids at positions 179 to 190 from HIV-1 were constructed from plasmid $pRT2_{66}$ (10). For this purpose, the following four synthetic DNA oligomers were used: oligomer 1, 5′-CGTTATCTACCAATACA TGGATGATTTGTATGTAGGATCCGACAGGGCGGG TT-3′; oligomer 2, 5′-TAGAGCATGACAAAGTAGTCCT GCAACTAAAAGAACTTCTGAATGGC-3′; oligomer 3, 5′-GTCCTGTCGGATCCTACATACAAATCATCCATGTAT TGGTAGATAACGACGT-3′; oligomer 4, 5′-CTAGGCCAT TCAGAAGTTCTTTTAGTTGCAGGACTACTTTGTCAT GCTCTAAACCC-3′.

Oligomers 2 and 4 were phosphorylated in separate 20-μl reaction mixtures containing 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, 300 μmol ATP, 1 μM DNA, and 2 U of T4 polynucleotide kinase (Boehringer Mannheim). After incubation at 37°C for 1 h, the reaction mixtures were combined and the kinase was inactivated by heating at 60°C for 10 min. Equal amounts of the two remaining oligomers were added, and the oligomers were annealed by heating this mixture to 90°C and then slowly cooling it to 25°C. Oligomers were joined in a 100-μl reaction mixture containing 50 mM Tris-HCl (pH 7.8), 20 mM $MgCl_2$, 5 mM dithiothreitol, 300 μM ATP, 200 nM DNA, and 10 U of T4 DNA ligase (Boehringer Mannheim). After incubation for 4 h at 16°C, the ligated product was purified on a native 10% polyacrylamide gel. One picomole of the purified insert and 0.25 pmol of *Aat*II-*Avr*II-digested plasmid $pRT2_{66}$ were incubated with T4 DNA ligase in 25 μl under the conditions described above. A total of 5 μl of this reaction mixture was used directly for transformation of *E. coli* TG1. Transformants harboring the plasmid DNA were screened for the insert by using *Eco*RV restriction analysis of minilysate plasmid DNA. To confirm the mutation, both strands of the HIV-2–HIV-1 RT hybrid gene were sequenced.

Enzyme purification. Proteins were expressed in *E. coli* CGSC 6662 cells. Prior to protein purification, cells containing the pRT_{66} subunit were mixed with cells harboring the pRT_{51} subunit so that the resulting heterodimers had mutations only in the 66-kDa subunit of the RT. Proteins were purified (10, 11) by using four chromatographic steps (DEAE-Sephacel, single-stranded DNA cellulose, Sephadex G-50, Fractogel EMD TMAE-650).

RESULTS

Antiviral activity of the thiazolo-iso-indolinones in cell culture assays. In MT-2 cells, BM +51.0836 (Fig. 1b) inhibited HIV-1 replication at an IC_{50} of 55 nM, as determined by inhibition of virus-induced cytopathic effect by using the tetrazolium salt (MTT) metabolic assay (Table 1) and checked visually by giant cell formation. Of all the compounds tested, only AZT had a lower IC_{50} than BM +51.0836 (Table 1). In assays with PBMCs and M/M, IC_{50}s were determined by p24 production. For all compounds used, the IC_{50}s for HTLV(IIIB) were higher than those for HIV-1 D79, although the relative antiviral activities of the different compounds were comparable. With IC_{50}s of 9 nM (HIV-1 D79) and 16 nM [HTLV(IIIB)] in assays with PBMCs and 16 nM in assays with M/M, BM +51.0836 showed higher antiviral activity against HIV-1 than any other compound tested. In assays with an HIV-2 isolate (HIV-2 D194), only very weak antiviral activity was obtained in PBMCs with the nonnucleoside inhibitors, while assays with HIV-2 D194 in M/M showed no activity against the nonnucleoside inhibitors (data not shown). Assays were also performed with an HIV-1 strain with 10- to 100-fold reduced susceptibility to AZT (16). The thiazolo-iso-indolinones as well as O-TIBO and nevirapine (BI-RG-587) were found to be equally effective against AZT-susceptible and AZT-resistant HIV-1 isolates (Table 1). Again, BM +51.0836 had an IC_{50} lower than those of O-TIBO and nevirapine. No cytotoxic effects on MT-2 cells, PBMCs, and M/M (measured by the MTT test) were observed with BM 21.1298 or BM +51.0836 when used in the medium at concentrations of up to 50 μM (data not shown).

Selection and characterization of thiazolo-iso-indolinone-resistant viruses. To investigate the mechanism of resistance, DNAs from MT-2 cells infected with drug-resistant HIV-1

TABLE 2. Inhibition of wild-type and substituted RT enzymes by nonnucleoside inhibitors

RT inhibitor	IC_{50} (μM)[a]							
	HIV-1 wild type	HIV-1 K101A	HIV-1 K103N	HIV-1 Y181C	HIV-1 Y188L	HIV-1 Y1811 Y188L	HIV-2 wild type	HIV-2 179-190 HIV-1
BM 21.1298	1.41 ± 0.97	55.0 ± 8.3	88.0 ± 10.5	44.0 ± 12.5	140.0 ± 20.5	>200	>200	50.7 ± 9.1
BM +51.0836	0.09 ± 0.02	4.8 ± 0.17	10.6 ± 0.6	5.2 ± 0.8	6.7 ± 1.4	9.8 ± 1.7	>200	4.5 ± 0.81
O-TIBO	0.16 ± 0.08	15.0 ± 4.2	36.5 ± 5.5	3.7 ± 0.28	>200	>200	>200	17.9 ± 3.1
BI-RG-587	0.47 ± 0.13	5.1 ± 0.17	12.2 ± 2.1	14.8 ± 0.9	>200	>200	>200	56.0 ± 7.7
AZT-triphosphate	0.47 ± 0.05	0.3 ± 0.07	0.4 ± 0.19	0.5 ± 0.1	0.5 ± 0.12	0.3 ± 0.03	1.7 ± 0.11	1.2 ± 0.05

[a] IC_{50}s are 50% inhibitory concentrations in the nonradioactive RT inhibition assay (see text). Means ± standard deviations were calculated from the results of three independent assays.

were extracted (see Materials and Methods) and a part of the RT region (amino acids at positions 88 to 228) was amplified by PCR. The resulting 420-bp DNA fragment was sequenced by the PCR cycle sequencing technique from both directions. For BM 21.1298-, O-TIBO-, and nevirapine-resistant virus isolates, only one nucleotide exchange (Tyr-181 to Cys [TAT to TGT]) could be detected.

Antiviral activity of the thiazolo-iso-indolinones in vitro. The anti-HIV-1 activity of BM 21.1298, a racemic mixture, as well as those of several derivatives and their purified optical isomers were confirmed in a nonradioactive RT assay with purified RTs from HIV-1 and HIV-2. From the different thiazolo-iso-indolinone derivatives, the enantiomer BM +51.0836 showed the greatest antiviral activity against HIV-1 RT. None of the different compounds tested showed activity against HIV-2 RT. To compare the antiviral activities of the thiazolo-iso-indolinones with those of other published RT inhibitors, assays were also performed with O-TIBO, nevirapine, and AZT-triphosphate. BM +51.0836 had a lower IC_{50} compared with those of AZT, O-TIBO, and nevirapine (Table 2).

Expression of mutated and chimeric HIV RTs. To investigate the appearance of cross-resistance of the thiazolo-iso-indolinones to O-TIBO and nevirapine, we used the pRT_{66} plasmid (11), containing the p66 subunit of HIV-1 RT, to introduce specific substitutions in the basic lysine tract at positions 101 and 103 and positions 181 and 188, respectively. All constructs showed similar enzymatic activities when compared with those of wild-type HIV-1 RT and no change in AZT susceptibility (data not shown). In agreement with the results from the cell culture screening, cross-resistance of the thiazolo-iso-indolinones to O-TIBO and nevirapine arose (Table 2). Nevertheless, in the case of mutated enzymes (Tyr-181 to Ile/Tyr-188 to Leu and Tyr-188 to Leu, respectively), the IC_{50} of the dose for BM +51.0836 was lower than those for O-TIBO and nevirapine. On the other hand, the substitution Tyr-181 to Cys resulted in an enzyme that was similarly resistant to BM +51.0836, O-TIBO, and nevirapine. Interestingly, substitution of Lys-101 to Ala led to 10-fold resistance to nevirapine, greater than 50-fold resistance to BM +51.0836, and 100-fold resistance to O-TIBO. Substitution of Lys-103 to Asn seemed to produce a twofold stronger effect than substitution at position 101 for all tested nonnucleoside inhibitors. In another approach, a chimeric HIV-2 RT substituted with amino acids at positions 179 to 190 from HIV-1 RT were constructed. In the RT assay with the chimeric enzyme, BM +51.0836 had the lowest IC_{50} (4.5 μM) (Table 2). Nevirapine and TIBO, with IC_{50}s of 56.0 and 17.9 μM, respectively, showed lower inhibitory effects to the chimeric enzyme. However, complete susceptibility to the chimeric enzyme did not occur for any of the added HIV-1-specific compounds. In comparison with HIV-1 RT, IC_{50}s were 50- to 100-fold higher for the chimeric HIV-2–HIV-1 enzyme.

DISCUSSION

In this report, we described a novel class of HIV-1 RT-specific inhibitors with high levels of antiviral activity. In our hands, the most potent derivative of the thiazolo-iso-indolinone (BM +51.0836) achieved lower IC_{50}s than those of the published nonnucleoside inhibitors O-TIBO and nevirapine on the molecular level as well as in assays with different cell culture systems (MT-2 cell line, PBMCs, and M/M). Thiazolo-iso-indolinones were found to be equally effective against an AZT-resistant strain of HIV-1. No antiviral activity for the thiazolo-iso-indolinones was observed when we used cloned and expressed HIV-2 RT or HIV-2 infection assays in cell culture. Kohlstaedt et al. (7) have recently published a crystal structure of HIV-1 RT complexed with nevirapine. In that study, HIV-1 RT subunit p66 was structurally divided into four parts, termed "thumb," "connection," "palm," and "fingers." Nevirapine binds in a deep pocket that lies between the β-sheet of the "palm" and the base of the "thumb." This pocket does not exist in p51, and accordingly, nevirapine does not bind to the smaller subunit. The inhibitor interacts with the side chains of Tyr-181 and Tyr-188 overlapping the highly conserved Met Asp Asp (amino acids at positions 184 to 186) motif (2). In analogy to the reports of resistance to other nonnucleoside inhibitors (1, 3, 8, 12, 15, 19, 20) and our own results, the mechanism of viral resistance to the pyridinones, the TIBO compounds, and the thiazolo-iso-indolinones should arise from the same interactions as described above for nevirapine. According to the model of HIV-1 RT of Kohlstaedt et al. (7), the basic residues at amino acids at positions 101 to 104 also lie in the "palm" and could therefore hypothetically bind to the nonnucleoside inhibitors. A common structural finding for TIBO, nevirapine, the pyridinones, and the thiazolo-iso-indolinones is aromatic ring systems and a heterocycle with =O or =S extracyclic substituents. Binding of amino acids at position 181 or 188 to the inhibitors is thought to occur via "electronic stacking," while the electronegative heterocycle could bind to the basic lysine residues at amino acids at positions 101 to 104. Destruction of the basic lysine tract could therefore hamper this interaction, resulting in a change in the susceptibilities of the nonnucleoside inhibitors to the mutated enzyme. Because of structural differences within the compounds, mutations at amino acid residues at positions 101 to 104 could have different effects on the resistance of virus to the

different compounds. In our hands, substitution of Lys-101 to Ala led to 10-fold higher IC_{50}s of nevirapine and 100-fold higher IC_{50}s of O-TIBO. Substitution of Lys-103 to Asn showed nearly the same effect as the substitution at the amino acid residue at position 101. Substitution of Tyr to Leu at position 188 resulted in a complete loss of susceptibility to O-TIBO and nevirapine, while the susceptibility of this mutated enzyme to BM +51.0836 was only partially reduced. On the other hand, substitution of Tyr-181 to Cys led to 30- to 50-fold greater resistance to all tested nonnucleoside inhibitors.

In contrast to the data of Shih et al. (20), our data demonstrate that the substitution of the region at positions 179 to 190 of HIV-2 RT by the respective HIV-1 sequence does not lead to the complete susceptibility of the chimeric enzyme to O-TIBO, nevirapine, and BM +51.0836. With regard to wild-type HIV-1 RT, there is still a 50- to 100-fold greater IC_{50} of the chimeric RT for the tested nonnucleoside inhibitors. Thiazolo-iso-indolinone-, nevirapine-, and O-TIBO-resistant HIV-1 mutants with 100-fold greater IC_{50}s were generated after eight passages in MT-2 cells. Genetic analysis of the amplified DNA fragments showed a mutation always in the same position of the RT gene (since we have not sequenced the whole RT genes, we cannot exclude additional mutations). In our experiments, codon 181 was changed from TAT to TGT, resulting in the previously described amino acid substitution Tyr to Cys. In contrast, the amino acid at position 188 and the lysine residues at positions 101 to 104 remained unchanged, indicating that mutation of the amino acid at position 181 is the first and most important event in the development of viral resistance to the nonnucleoside inhibitors which were included in our experiments.

Thiazolo-iso-indolinones do not act differently from other nonnucleoside inhibitors with regard to viral resistance, and therefore, their clinical use is limited. However, the high level of inhibitory activities of the compounds described here make the thiazolo-iso-indolinone derivatives useful in solving the puzzling mechanisms leading to viral resistance against nonnucleoside inhibitors.

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