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SPECIFIC HIV-1 REVERSE TRANSCRIPTASE INHIBITORS

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1. DIFFERENT CLASSES OF SPECIFIC HIV-1 REVERSE TRANSCRIPTASE INHIBITORS

Independently from each other, four chemically distinct classes of molecules were identified as specific HIV-1 inhibitors. From a group of acyclic uridine analogues, HEPT, 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine, emerged as a specific inhibitor of HIV-1 replication *in vitro*:[1] unlike the dideoxynucleoside triphosphates, the triphosphate of HEPT did not prove inhibitory to the HIV-1 reverse transcriptase (RT).[1] The discovery of the tetrahydroimidazo [4, 5, 1-jk][1, 4]-benzodiazepin-2(1H)-one and -thione (TIBO) derivatives, as specific HIV-1 inhibitors resulted from a rational, high-flux cellular-based screening program and subsequent lead optimization.[2] Mechanism of action studies then indicated the HIV-1 RT as the plausible target for the antiviral action of these compounds.[2] The discovery of the anti-HIV-1 activity of the dipyridodiazepinones originated from a large screening effort for HIV-1 RT inhibitors. The compounds subsequently proved effective against HIV-1 replication in cell culture.[3] More recently, some pyridinone derivatives have been reported to be HIV-1 specific inhibitors.[4]

2. THE SPECIFIC HIV-1 INHIBITORS ARE TARGETED AT THE HIV-1 REVERSE TRANSCRIPTASE

Whereas the mechansim of action of dipyridodiazepinones was obvious from the beginning since these compounds had been found by screening for HIV-1 reverse transcriptase inhibitors, the mode of action of the HEPT and TIBO derivatives was unsettled at the time that their antiviral activity was discovered.

Further experiments with the HEPT and TIBO derivatives then pointed to a reverse transcriptase-associated process as the likely target for their anti-HIV-action. From so-called time of addition (TOA) experiments, which pinpoint the ultimate time post-infection at which the drugs have to be added to be effective,[2] it was ascertained that the TIBO and HEPT derivatives had to interact with a process coinciding with

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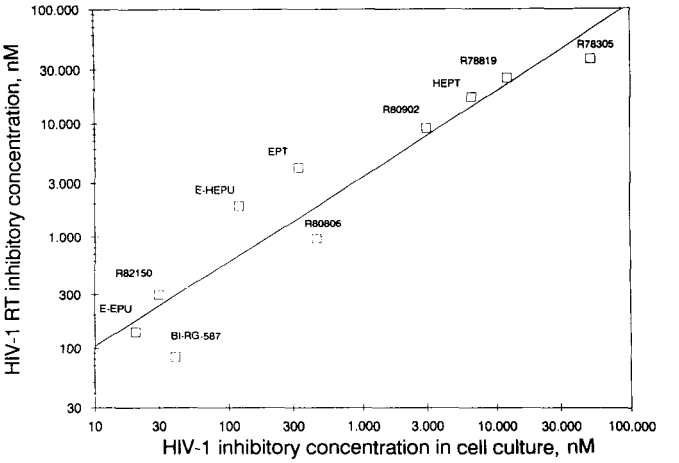

FIGURE 1 Correlation between inhibitory effects of a series of specific HIV-1 RT inhibitors on virus replication in cell culture and HIV-1 recombinant reverse transcriptase activity. The specific HIV-1 RT inhibitors were the TIBO derivatives R82150,[2] R78305,[2] R78819,[2] R80902,[2] R80806, the HEPT derivatives HEPT,[1] EPT,[6] E-HEPU,[6] E-EPU[6] and the dipyridodiazepinone BI-RG-587.[3] The correlation coefficient (R square) was 0.9 as estimated by the equation $y = ax^b$ (Power transformation, Enzfitter, Elsevier, Biosoft).

or occurring at the same time as the reverse transcription. For the TIBO and HEPT derivatives the time of addition could be delayed by 2–3 h compared to AZT and DDC, since dideoxynucleosides need extra time to be phosphorylated to their active triphosphate form.

Compatible with the TOA results were the results of follow-up experiments where the compounds were examined by a PCR (polymerase chain reaction) assay for their inhibitory effect on proviral DNA formation in acutely HIV-1-infected T cells. TIBO and HEPT were shown to inhibit HIV-1 proviral DNA formation in a dose-dependent manner.[5]

Convincing evidence for an RT-targeted action of the TIBO and HEPT derivatives stemmed from the close correlation between the inhibitory effects of these compounds on HIV-1 replication in cell culture and their ihibitory effects on RT activity in enzymatic assays. The more potent the TIBO compound was in inhibiting HIV-1 replication the higher also its inhibitory activity against HIV-1 recombinant reverse transcriptase.[2] As shown in Figure 1 this correlation can be extended to the HEPT derivatives [HEPT,[1] 1-ethoxymethyl-6-(phenylthio)thymine (EPT),[6] 5-ethyl-1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)uracil (E-HEPU)[6] and 5-ethyl-1-ethoxymethyl-6-(phenylthio)uracil (E-EPU)[6]] and the dipyridodiazepinone prototype BI-RG-587.[3]

3. CHARACTERISTICS OF THE SPECIFIC HIV-1 REVERSE TRANSCRIPTASE INHIBITORS

Detailed analysis of the characteristics of HIV-1 RT inhibition by the TIBO derivatives[5] revealed three specific properties of the novel non-nucleoside RT inhibitors (*i*) specificity for the reverse transcriptase of HIV-1, (*ii*) preference for poly(C).oligo(dG)$_{12-18}$ as the

TABLE I
Specificity of RT inhibition by novel non-nucleoside HIV-1 RT inhibitors

Enzyme	IC_{50}^{a} (μM)		
	R82150	E-EPU	BI-RG-587
HIV-1 reverse transcriptase	0.34	0.14	0.084
HIV-2 reverse transcriptase	> 300	> 500	> 10
DNA polymerase α	> 175	–	> 24
β	> 175	–	> 24
γ	> 175	–	> 24

[a]Concentrations of R82150,[5] E-EPU[6] and BI-RG-587[3,12] that inhibit HIV-1 RT activity by 50%. Poly(C).oligo(dG) and gapped calf thymus DNA were the template/primers used to assess RT and DNA polymerase activity, respectively.

template/primer, (*iii*) selective inhibition of the RNA dependent DNA polymerization (RDDP) function of HIV-1 RT.

3.1. *Specificity for the Reverse Transcriptase of HIV-1*

The "classical" RT inhibitors, such as AZT 5′-triphosphate and other dideoxynucleoside 5′-triphosphates, are equally effective against HIV-1 RT and HIV-2 RT,[7,8] despite the partial sequence homology (40–60%) between the two reverse transcriptases.[9] In contrast with the "classical" RT inhibitors, HEPT, TIBO, and BI-RG-587 are active only against the reverse transcriptase of HIV-1 (Table I). They are inactive against the reverse transcriptases of HIV-2, avian myelobastosis virus (AMV) and Moloney murine leukemia virus (MLV).[1-3,5,6] Dipyridodiazepinones have also been proved inactive against the reverse transcriptases of simian immunodeficiency virus (SIV) and feline leukemia virus (FLV). In addition to the reverse transcriptases from retroviruses other than HIV-1, human DNA polymerases α, β and γ are also refractory to inhibition by TIBO and TIBO-like compounds.

3.2. *Template Preference for RT Inhibition*

When poly(C).oligo(dG) is used as the template/primer, the 50% inhibitory concentration (IC_{50}) of TIBO R82150 for HIV-1 RT is 17-fold lower than the IC_{50} obtained with poly(A).oligo(dT) as the template/primer (0.34 and 5.9 μM, respectively) (Table II). A similar template preference for HIV-1 RT inhibition was noted with the HEPT derivatives. E-EPU[6] displays a 2-fold greater preference, and E-EBU-dM [5-ethyl-1-ethoxymethyl-6-(3, 5-dimethylbenzyl)uracil][10] a 4-fold greater preference, for poly(C) than for poly(A), as template. However, it cannot be ruled out that this

TABLE II
HIV-1 RT inhibition as measured with different template/primers

Template/primer	IC_{50}^{a} (μM)					
	R82150	E-EPU	E-EBU-dM	BI-RG-587	AZT-TP	ddGTP
Poly(A).oligo(dT)	5.9	0.27	0.16	0.100	0.05	> 5
Poly(C).oligo(dG)	0.34	0.14	0.036	0.084	> 5	0.04

[a]Concentrations of R82150,[5] E-EPU,[6] E-EBU-dM,[10] BI-RG-587,[3] AZT-TP[5] and ddGTP[5] that inhibit HIV-1 RT activity by 50%.

differential preference may at least partially be explained by the different reaction conditions used in these assays.

From Table II it is also clear that the dideoxynucleotides AZT-TP and ddGTP inhibit RT activity, i.e. by chain termination, only with the complementary homopolymer (poly(A) or poly(C), respectively) as template. This contrasts with the non-nucleoside RT inhibitors which are effective with different homopolymer templates.

3.3. *Selective Inhibition of RNA-Dependent/DNA Polymerization*

The HIV-1 reverse transcriptase calayzes different enzymatic reactions, including RNA-dependent DNA synthesis and DNA-dependent DNA synthesis. In addition, the ribonuclease H (RNase H) component of the HIV-1 reverse transcriptase catalyzes the hydrolysis of the RNA strand of the RNA.DNA hybrid. Whereas BI-RG-587 has been reported to cause partially inhibition of the HIV-1 RNase H activity,[3] no such inhibition was noted with the TIBO R82150.[5] R82150 causes a selective inhibition of the RNA-dependent DNA polymerization function of HIV-1 RT. It is at least 30-fold more potent in inhibiting the DNA polymerization directed by poly(C) as template than by poly(dC).[5]

4. KINETICS OF RT INHIBITION BY THE SPECIFIC HIV-1 REVERSE TRANSCRIPTASE INHIBITORS

Detailed kinetic studies have been performed with the novel non-nucleoside RT inhibitors. The TIBO derivative R82150 was shown to inhibit HIV-1 RT in a reversible way. This inhibitory effect was not influenced by the addition of excess dithiotreitol to the reaction mixture, which argues against the formation of a covalent disulfide bond between RT and R82150.[5] Inhibition of HIV-1 RT by R82150 was uncompetitive with respect to the template/primer poly(C).oligo(dG) and noncompetitive with respect to the natural substrates dGTP and dTTP (Figure 2).[5] Inhibition of HIV-1 RT by the HEPT congener E-EPU was noncompetitive with respect to dGTP but competitive with respect to dTTP.[6] Also, inhibition of HIV-1 RT by the dipyridodiazepinone BI-RG-587 proved noncompetitive with respect to dGTP.[3]

5. TOWARD A MODEL FOR REVERSE TRANSCRIPTASE INHIBITION BY THE SPECIFIC HIV-1 INHIBITORS

The observation that TIBO R82150 is uncompetitive with respect to the template/primer poly(C).oligo(dG) is indicative of an ordered binding of the inhibitor, which may thus be assumed to bind the enzyme after the template/primer has been bound. Likewise, the natural substrate is supposed to bind after the template/primer has been bound.[11]

Photoaffinity labelling studies with an azido-substituted BI-RG-587 have pointed to a non-substrate binding site for the dipyridodiazepinone since the natural substrate dGTP is unable to protect the enzyme from being labelled. In contrast, the TIBO R82150 was able to protect the enzyme from photoinactivation, and this suggests that TIBO and dipyridodiazepinone share a common binding site at the HIV-1 reverse

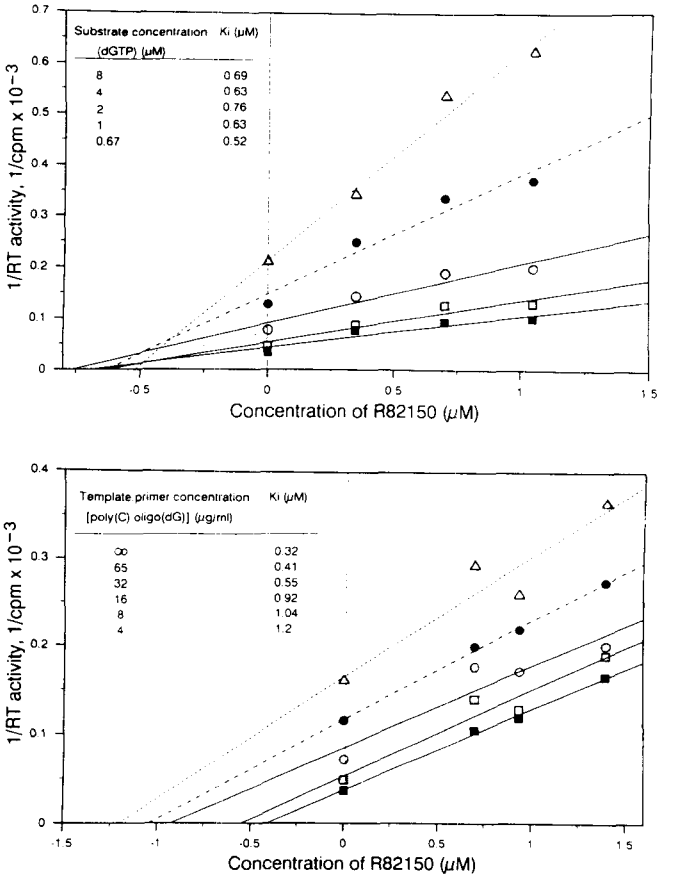

FIGURE 2 Kinetic studies with HIV-1 recombinant RT carried out under steady-state conditions. Data are presented as Dixon transformation plots. Upper panel: varying concentration of dGTP: 8 μM (■); 4 μM (□); 2 μM (○); 1 μM (●); and 0.67 μM (△). The noncompetitive mode of inhibition with regard to the substrate is indicated by the inset showing the apparent K_i values. Lower panel: varying concentration of template/primer poly(C).oligo(dG)$_{12-18}$: 65 μg/ml (■); 32 μg/ml (□); 16 μg/ml (○); 8 μg/ml (●); 4 μg/ml (△). The uncompetitive mode of inhibition is indicated by the inset showing the apparent K_i values.

transcriptase.[12] These findings are also in agreement with the fact that R82150 and BI-RG-587 inhibit the enzyme noncompetitively with respect to the natural substrate dGTP.[3,5]

Although the HEPT congeners may be postulated to interact with the same ("TIBO") site at HIV-1 RT as R82150 and BI-RG-587, the fact that the HEPT derivatives, i.e., E-EPU, are competitive with some of the natural substrates (i.e. dTTP) suggests that the site at which they bind somehow modulates the substrate binding site.

Analysis of the aminoacids involved in the binding of the specific HIV-1 RT inhibitors by affinity labelling and site-directed mutagenesis will be required to unequivocally localize the binding site of these compounds. A hypothetical model of RT inhibition by TIBO and TIBO-related compounds is shown in Figure 3. The

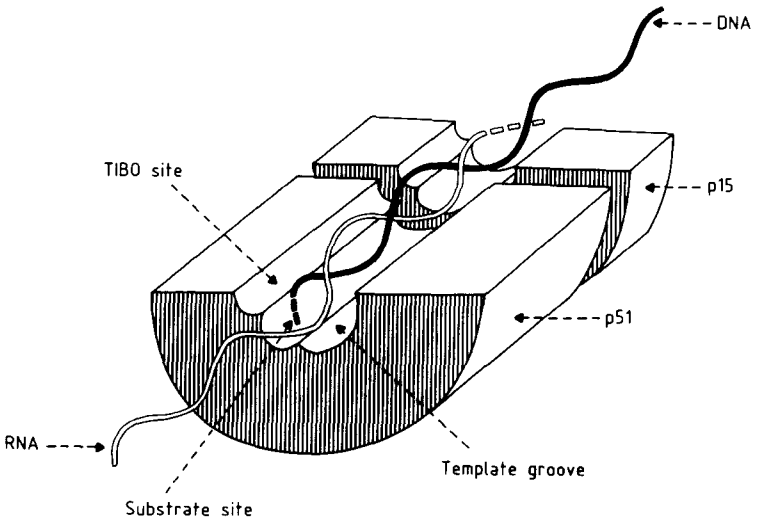

FIGURE 3 Hypothetical model of RT inhibition by TIBO derivatives. Both the p51 polymerase domain and the p15 RNase H domain are depicted with the RNA · DNA hybrid bound to a central cleft within the enzyme. Also indicated are the substrate binding site and the putative TIBO binding site. The TIBO site is assumed to be functionally, and perhaps spatially linked, to the substrate binding site. The RNA template directs the incorporation of complementary nucleotides into the DNA strand (p51 domain), after which the RNA template is degraded by RNase H (p15 domain), putatively located at 1.5 helical turn away from the DNA polymerization.

model displays monomeric HIV-1 RT, composed of the 51 kD DNA polymerase domain and the 15 kD RNase H domain. Recent studies[13] estimate the distance between the two active sites at 1.5 helical turn. In analogy with the structure of the Klenow fragment of DNA polymerase I,[14] the enzyme is assumed to completely surround the template during the polymerization process. Binding of the template in its groove would enable the substrates (dNTPs) and TIBO derivatives to occupy their respective binding sites. In Figure 3 the TIBO site is depicted as if it were functionally and perhaps also spatially associated with the substrate binding site.

Studies are in progress to assess the functional and structural role of the second p51 molecule of heterodimeric HIV-1 RT, which represents the form in which the enzyme is present in the virion and the infected cell.[15,16] Depending on the presence of one or two template binding sites in the heterodimer, two structural models could be envisaged. According to the first model, a central tunnel through the heterodimer might bind one template/primer and promote processive DNA polymerization.[17] A second model suggests the presence of two template/primer binding sites that are faced to one another.

Aminoacid alignments of the HIV-1 RT and HIV-2 RT should allow to clarify why HIV-2 RT is not susceptible to inhibition by the TIBO derivatives. Elucidation of the molecular determinants underlying the interaction of this class of HIV-1 RT inhibitors with their target enzyme should not only help in the rational design of novel anti-HIV agents but also increase our insight in the reverse transcription process.

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