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Resistance mutations selected in vivo under therapy with anti-HIV drug HBY 097 differ from resistance pattern selected in vitro

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Abstract

The quinoxaline derivative HBY 097, an orally active nonnucleoside inhibitor of HIV-1 reverse transcriptase (NNRTI), showed an efficient suppression of viral load in a dose-escalating phase I study with mean trough concentrations increasing from 137–1299 ug/l [Rübsamen-Waigmann et al., Lancet 349:1517]. Half-maximal inhibitory concentrations (IC_{50}) for viruses grown from the patients at entry of the study were 0.1–3 nM, except for one patient who had a virus with reduced susceptibility to HBY 097 at entry (IC_{50}: 160 nM). During therapy, only two patients developed a virus with a moderately increased IC_{50} (2.2 and 15 nM). This reduced susceptibility was associated with the known NNRTI-resistance mutation K ⇒ N at position 103, in contrast to resistance selection in vitro, which had yielded predominant mutations at positions 179 and 190. The Tyr mutation at position 181, inducing high resistance for other NNRTIs, was never observed. The resistant virus at study entry ($IC_{50} = 160$ nM) had a mutation at position 103 as well, combined with an AZT resistance mutation (K ⇒ R) at position 70, suggesting that nucleoside-resistance mutations may help increasing resistance to HBY 097. This is in line with our in vitro selection studies, where resistance mutations at the 'nucleoside sites' 74 and 75 increased the resistance phenotype of NNRTI mutations. Our findings highlight the crucial importance of IC_{50} determinations from cultured virus for determination

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of phenotypic resistance development during therapy and demonstrate that in vivo resistance development cannot be predicted from in vitro selection.

Keywords: HIV; Reverse transcriptase; NNRTI quinoxaline; Viral load; Resistance; Mutations

1. Introduction

Nonnucleoside inhibitors of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (NNRTIs) have been shown to be potent inhibitors of HIV-1 replication in vitro (De Clercq, 1993, 1996). Although these inhibitors belong to different chemical classes, they are all believed to bind to a common hydrophobic pocket of the p66 subunit of the enzyme (Kohlstaedt et al., 1992; Tong et al. 1993; Jacobo-Molina et al., 1993; Kleim et al., 1996; Riess et al., 1996). In spite of very good tolerability the use of NNRTIs has been limited in the past due to rapid emergence of drug-resistant variants of HIV in vivo when NNRTIs were used as monotherapy (Saag et al., 1993; Richman et al., 1994; Havlir et al., 1995, 1996). Mutations selected for by these drugs usually occur in segments of the reverse transcriptase composed of amino acids 98-108, 179-190 and 230-236 (Larder, 1992; Balzarini et al., 1993; Saag et al., 1993; Richman et al., 1994). The most common mutations for NNRTIs are the substitutions of cysteine for tyrosine at position 181 (Havlir et al. 1996) and of asparagine for lysine at position 103. Mutations at Tyr 181 can lead to 1000-fold resistance against some compounds.

Due to the potency of a combined antiviral effect but also because of interactions of resistance mutations (Larder, 1992), the combination of NNRTIs with nucleoside analogues leads to markedly reduced emergence of resistance in vitro (Balzarini et al., 1993) and in vivo (Staszewski et al. 1995). In fact, combination of a first generation inhibitor, nevirapine, with two additional drugs, zidovudine and didanosine has recently been shown to suppress viral load below detection limits in about 50% of the patients and has led to the approval of nevirapine as the first NNRTI (Montaner et al., 1998). This suggests that highly potent NNRTIs, when combined with 1 or 2 anti HIV-drugs with a different mechanism of action should be of high value for the treatment of HIV-1 infections (for review, see De Clercq (1998)).

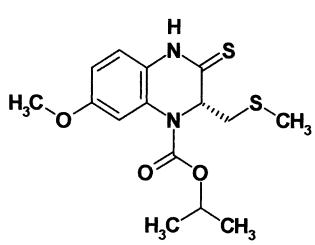

Fig. 1. The nonnucleoside inhibitor of HIV-1 reverse transcriptase of the quinoxaline class, HBY 097.

We have previously reported on a novel class of nonnucleosidic inhibitors of HIV reverse transcriptase, the quinoxaline derivatives (Kleim et al., 1993, 1994, 1995, 1996; Riess et al., 1996), from which HBY 097 [(*S*)-4-isopropoxycarbonyl-6-methoxy-3-methylthiomethyl-3,4-dihydroquinoxaline-2 (1H)-thione (Fig. 1)] was chosen for clinical development. HBY 097 inhibits a variety of HIV-1 laboratory and field isolates (including AZT-resistant strains) at an IC_{50} 0.1–3.3 nM (Kleim et al., 1995), which makes it about 100 × more potent than nevirapine (IC_{50}: 10–300 nM) [Murphy and Montaner, 1996]). Furthermore, in contrast to mutations at position 181 found with other NNRTIs, for HBY 097 a predominant resistance mutation was observed at position 190 (G190 ⇒ E) in vitro, which was shown to be associated with a less active viral reverse transcriptase (Kleim et al., 1995, 1996; Riess et al., 1996). This finding suggested that under therapy resistance mutations might less easily develop against this NNRTI.

In a recent phase I study (Rübsamen-Waigmann et al., 1997) plasma viral load was suppressed by a mean of 1.4 log during

administration of 3×125 to 3×1000 mg HBY 097 over a period of 7–14 days. Here, we describe the phenotypic and genotypic analysis of the viruses before and after therapy. Because of the known selection of variants in culture which also occurs in the absence of drugs (von Briesen et al., 1987; Fenyö et al., 1988; Rübsamen-Waigmann et al., 1991, 1994; Moutouh et al., 1996), the genotype in the RT region was determined in the virus population cultured from the patient's blood as well as directly from uncultured virus of the plasma of the patients.

2. Materials and methods

Each dose-step was planned to include nine subjects (6 on active drug and 3 on placebo). Subjects received 1 dose on day 1 and continued on a three-dose daily regimen from days 2–13 with a single dose on the final day of treatment. The HIV-positive patients were asymptomatic or mildly symptomatic, with CD4 cell counts between 200 and 500/ul. Two groups received 750 and 1000 mg t.i.d., respectively, for 14 days, followed by a washout period of 7 days. The patients were randomized to active drug and placebo. Blood for virus determinations was drawn on day 0 and 14 and, additionally, on day 21.

Isolation of HIV and phenotypic analysis was carried out by recovering HIV from patient peripheral blood mononunclear cells (PBMC) by co-cultivation with phytohemagglutinin-stimulated cord blood lymphocytes, which were obtained from the Department of Obstetrics, Jewish General Hospital, Montreal (Von Briesen et al., 1987; Salomon et al., 1994). Reverse transcriptase activity and p24 antigen levels in clarified culture supernatant were determined every 2–4 days to follow the course of viral replication. Fifty percent inhibitory concentrations (IC_{50}) for viruses isolated at the time of study entry and thereafter were calculated on the basis of p24 antigen assays, using the AIDS Clinical Trials Group-Department of Defence (ACTG-DOD) consensus protocol, with the exception that cord blood mononuclear cells were used instead of adult PBMCs as viral targets (Japour et al., 1993). Syncytium-inducing (SI) versus non-syncytium-inducing (NSI) phenotypes were determined using MT-2 cells that were inoculated with viruses initially isolated from the patients' PBMC as described (Montaner et al., 1993).

2.1. Determination of viral load

Viral load was determined in plasma samples using the PCR-kit from Roche according to the instructions of the Manufacturer. Detection limit for the Roche PCR method was 240 genome equivalents.

2.2. Determination of nucleotide sequences

Sequence information on the viral variants was either obtained from plasma virions or from culture supernatants. Viral RNA was isolated from 140 ul of plasma or culture supernatant using the QIAamp viral RNA kit (Qiagen, Hilden). After reverse transcription for 1 h at 37°C (2.5 U Superscript RT (Gibco-BRL), using random hexamer primers (50 ng/ml), 0.5 mM dNTPs in a buffer containing 40 mM Tris. HCl, 100 mM KCl, 5 mM $MgCl_2$, 0.2% Tween 20 and RNAse (0.8 U/μl) half of this cDNA was used in a nested PCR for amplification of a 650 bp pol fragment containing all known resistance mutations for RT inhibitors. PCR conditions were 5 min 95°C and 45 (outer) or 40 (inner) cycles of 94°C 30 s, 60°C 30 s, 72°C 30 s, followed by 5 min 72°C with 2.5 U Taq polymerase (Perkin Elmer Cetus) in the buffer supplied with this enzyme containing 0.2 μM dNTPs and 5 pmol of each of the respective primers. Outer primers were BRT5′(5′CCATTAGTCCTATTGAAACTACTGTACCAGT-3′) and H1p3519 r (5′-TCCTCTAAGGAGTTTACA-3′), inner primers were H1p2198d (CAATGGCCATTGACAGAAG-3′) and H1p2837r (5′-GATGGAGTTCATAACCCA-3′). This latter primer was biotinylated in order to allow purification of the PCR fragments with Dynabeads M280 Streptavidin (Dynal, Hamburg).

After denaturation direct sequencing of the solid-phase bound strand was performed using the

inner non-biotinylated PCR primer and fluoro dATP (Boehringer Mannheim) as label. Sequences were evaluated on an A.L.F. automated sequencer (Pharmacia, Freiburg). The relative amount of mutated resistance codons was determined in the fluorogram by comparing the heights of mutant and wild-type peaks in the corresponding positions (Larder et al., 1993; Albert et al., 1994).

Sequence determination on virus from culture supernatants was done in a similar way. HIV strains isolated from patients were transferred to fresh cultures of human PBL and grown to maximal antigen values which were determined by a p24 ELISA (Innogenetics Belgium) In a first PCR reaction 10 pmol/50 μl of primers JA99 and RIT137 (Albert et al., 1994) were used. Conditions for amplification reactions were 3 min at 94°C, 30 s at 60°C and 60 s at 72°C. An aliquot of the first PCR was used for a second amplification with primers JA100 (Albert et al., 1994) and RIT138 (Kleim et al., 1996; Riess et al., 1996) using the same protocol as above, omitting the initial denaturation step. Single-stranded DNA obtained using streptavidine-coated magnetic beads (Dynal) served as a template for the dideoxy chain termination sequencing reaction. 5′-fluoresceinated oligonucleotides RT1SEQ2F and RT8KF (Kleim et al., 1996; Riess et al., 1996) allowed the determination of HIV-1 sequences corresponding to RT amino acids 35-242.

3. Results

Plasma viral load was suppressed in both dose groups receiving 750 or 1000 mg HBY 097, 3 × daily, for 14 days, by about −1.4 logs in most patients (Rübsamen-Waigmann et al., 1997). In one patient of the 750 mg group the virus was undetectable after treatment. The placebo-treated patients had no significant change of viral load (Table 1).

3.1. Biological phenotype of the viruses

HIV was cultured from PBMC at day 0 and 14 and the phenotype of the virus was analyzed (Table 1). As expected from the CD4 counts, most patients isolates showed the non-syncytium-inducing (NSI) phenotype with medium to low replication, also termed c/d (von Briesen et al., 1990) or slow/low (Fenyö et al., 1988). No switch in phenotype was observed after treatment. Interestingly, among the five patients on active drug in the 1000 mg study, one patient (no. 2) showed longer times for virus isolation after treatment and in 1 patient virus culture had become negative after taking HBY 097 for 2 weeks (patient no. 7). In the 750 mg study, a prolonged culture time after therapy was seen for one patient (no. 4) as well. These results are very well in line with the reduced viral genome equivalents in the plasma.

3.2. Resistance phenotype and genotype

IC_{50} values at the start of therapy were between 0.1 and 3 nM, except for patient no. 6 in the 750 mg group, who had a highly resistant virus at entry (IC_{50}: 160 nM) (Table 1) and did not achieve viral suppression. Except for patient no. 2 in the 750 mg group and patient no. 6 in the 1000 mg group, no signs of development of resistant virus after therapy could be observed over this period of treatment. The isolates from these patients showed a moderate elevation in IC_{50} (15 and 2.2 nM, respectively) and correlated with a partial or full mutation at position 103 of the reverse transcriptase (Table 2).

While a predominant mutation in positions 190 and 179 would have been expected based on the in vitro resistance selection data (Kleim et al., 1994) we only observed a mutation at position 103. Most surprisingly, the patient with the highly resistant virus at study entry carried a genotype with this same mutation ($K \rightarrow N$) at position 103 and an AZT-resistance mutation in position 70 ($K \Rightarrow R$) (Table 2).

4. Discussion

The data presented here demonstrate that HBY 097, when tested over a period of 2 weeks, was able to suppress HIV viral load in the patients by about 1.4 logs with the exception of one individ-

Table 1
Biological phenotype and IC_{50} of viral isolates from the phase 1 clinical trial of HBY 097 and reduction in viral load

Dose	Patient no.	Phenotype[a] (week at which culture was positive)		IC_{50} day 0 (nM)	IC_{50} day 14 (nM)	Δlog viral load day 14	Δlog viral load day 21
		Day 0	Day 14				
750 mg t.i.d	2	c/d, NSI (2)	NSI (2)	0.4	15	−0.97	+0.41
	3	c/d, NSI (2)	NSI (2)	0.6	0.5	−1.71	−0.46
	4	c/d, NSI (2)	NSI (3)	3	N.D.	−1.63	+0.16
	6	c/d, NSI (2)	NSI (2)	160	N.D.	−0 054	+0.13
	7	N.D. (negative, 4)	N.D. (negative, 4)	N.D.	N.D.	< −2.4	< −2.4
Placebo	1	c/d, NSI (2)	NSI (2)	3	4	−0.028	+0.022
	5	c/d, NSI (2)	NSI (2)	0.7	3	+0.24	+0.8
	8	c/d, NSI (1)	NSI (2)	2	3	+0.27	+0.3
1000 mg t.i.d.	2	c/d, SI (1)	SI (3)	0.35	0.3	−1.34	+0.25
	3	c/d, NSI (2)	NSI (2)	0.1	0.2	−1.61	−1.1
	5	c/d, N.D. (negative, 4)	N.D. (negative, 4)	N.D.	N.D.	−1.35	+0.06
	6	c/d, NSI (1)	NSI, very low yield	0.2	2.2	−1.09	−0.02
	7	c/d, NSI (2)	N.D. (negative, 4)	0.25	N.D.	−1.48	−0.54
Placebo	1	N.D. (negative, 4)	N.D. (negative, 4)	N.D.	N.D.	+0.4	+0.5
	4	SI (1)	SI (1)	0.6	0.4	+1.4	+1.0
	8	NSI (1)	NSI (1)	0.3	0.4	+0.2	+0.15

[a] NSI phenotype with 2–3 weeks of cultivation is equivalent to c/d defined in von Briesen et al. (1990), SI phenotype with 1 week of cultivation corresponds to biological phenotype a (von Briesen et al., 1990). N.D., not determined.

Table 2
Resistance genotype of HIV before and after HBY 097 therapy

Dosage of HBY 097	Patient no.	Genotype in plasma day 0, condon[a]	Genotype in plasma day 14, condon[a]	Genotype in cell culture supernatant from p1 cell culture day 0 of study	Genotype in cell culture supernatant from p1 cell culture day 14 of study
750 mg t.i.d.	2	wt	K 103 N mixed with wt	wt	I.P.
	3	wt	wt	179: wt[4]	wt
	4	wt	98, 179: wt[2]	98, 179: wt[5]	98, wt[6]
	6	K 70 R K 103 N	6g: wt, K 70 R K 103 N	K 70 R K 103 N	I.P.
	7[b]	No product in triple nested PCR	No product in triple nested PCR	N.P.	N.P.
Placebo	1	wt	179: wt[3]	wt	I.P.
	5	A 98 G mixed with wt, T 215 Y	A 98 G mixed with wt	I.P.	T 215 Y
	8	wt	wt	wt	wt
1000 mg t.i.d.	2	T 215 Y	T 215 Y	T 215 Y	T 215 N
	3	wt	No PCR-product	wt	wt
	5	wt	No PCR-product	N.P.	N.P.
	6	wt	K 103 N	wt	K 103 N
	7	wt	No PCR-product	wt	N.P.
Placebo	1	wt	wt	N.P.	N.P.
	4	wt	wt	wt	wt
	8	wt	wt	wt	wt

[a] Only modifications from the wild-type sequence of HIV-1 reverse transcriptase are indicated; the position analyzed corresponded to all known mutations induced by NNRTIs and nucleoside analogues.

[b] Because of negative culture and PCR, the serology of this patient was repeated and found positive for HIV-1. (1) codon 179, GTT and ATT; (2) codon 98: TCA, codon 179 GTC 6: codon 98: TCA; (3) codon 179: ATT; (4) codon 179: GTG; (5) codon 98: TCA, codon 179 GTC; (6) codon 98:TCA.

ual, who had a mutant virus at study entry displaying an IC_{50} of 160 nM for HBY 097 (patient 6 in the 750 mg group).

For all anti-HIV-drugs, the quantitative suppression of viral load in patients differs between drugs, but is transient upon monotherapy. Comparisons between drugs, therefore, are only meaningful when studies with similar durations of treatment are analyzed. Comparison of our results to other studies with similarly short treatment times, yields a favorable profile for HBY 097. It is more active in short-term therapy than zidovudine (Glaxo Wellcome) and comparable to the NNRTIs nevirapine (Boehringer Ingelheim) and the pyridinone L 697661 (Merck), as well as the protease inhibitors ABT-538 (Ritonavir, Abbott) or L 735524 (Indinavir, Merck) (de Jong et al., 1995; Havlir et al., 1995; Kappes et al. 1995; Loveday et al., 1995; Wei et al., 1995; Havlir et al., 1996; Perelson et al., 1996).

Apart from primary suppression of the viral load, the speed at which resistant mutants arise in treated patients is another important parameter for judging a new drug. For nevirapine, development of resistance was seen as early as 7 days after the start of treatment, and 100% of the patients had resistant virus by 2–4 weeks (Wei et al., 1995). In contrast to these results, only one patient in each of the 14-day groups with HBY 097 had signs of development of less sensitive virus. These patients (no. 2 in the 750 mg study and no. 6 in the 1000 mg study) had a virus population with 40 × and 10 × reduced sensitivity, respectively, on day 14 (Table 1) and a slightly less efficient suppression of viral load compared to the other patients in the study. However, even in these patients, suppression of viral load was significant due to sufficient trough levels of the drug. It is noteworthy that the K 103 N mutation observed with HBY 097 is also seen with efavirenz (DMP 266) (Bacheler et al., 1997), which was recently approved for clinical use. No correlation could be observed in our study between initial viral load and the probability for development of a less sensitive virus (data not shown).

In our previous in vitro studies, the mutation at position 103 did not seem to contribute very much to resistance using the NM laboratory strain for starting selection (Kleim et al., 1996; Riess et al., 1996). In vitro, HBY 097 and other quinoxalines were found to select for G190⇒E RT mutants, which are highly resistant to inhibition by various NNRTIs and display decreased polymerase activities (Kleim et al., 1995, 1996; Riess et al., 1996). Alternatively—and depending on the selection process—multiple mutants evolved, resembling resistance development to protease inhibitors (Riess et al., 1996). During these experiments, mutations at RT codon 103 were observed only at low drug concentrations and/or as part of multiply mutant phenotypes (Kleim et al., 1993, 1995; Kleim et al. 1997). In agreement with the in vivo results, shown here, the K103⇒N mutation alone conferred only limited resistance to HBY 097.

However, in contrast to the in vitro selection for mutations in positions 179 and 190 in laboratory strains, the data from the present study demonstrate that these mutations are not selected in the patients during short term treatment, but that a mutation K⇒N occurs at position 103. Most likely, the virus is able to avoid the unfavorable 190 mutation in the in vivo setting where multiple variants are available.

Apart from the statistical selection process, the genetic background of the virus also affects the resultant phenotype after introduction of mutations into RT. A molecular clone of HIV-1 LAI in which we inserted the pol gene derived from a field strain with three most important AZT-resistance mutations, was only 5 × less sensitive towards AZT (unpublished). HIV-1 LAI, in contrast, shows an about 100-fold reduced sensitivity when the same mutations are present. The parental field isolate, however, was 100-fold less sensitive towards AZT (Biesert et al., 1991). Obviously, viral genetic structures other than the pol gene may have an important influence on the resistance phenotype. A very similar result was obtained with a recombinant virus containing the entire RT gene of a d4T (stavudine)-resistant, post-therapy virus. The recombinant clone was found to be sensitive to the drug (Lin et al., 1994).

A comparable phenomenon (Rose et al., 1996) was also observed when resistance against protease inhibitors was investigated. An identical

set of genetic changes produced a defective phenotype in the NL4-3 genetic background, but a replication-competent virus in the RF background, which was cross-resistant to four protease inhibitors. In this case, a genetic difference outside the known protease resistance mutations (Ile at position 10 in the RF strain) allowed the RF strain to grow.

These results, together with the data in the present study underline the crucial importance of IC_{50} determinations on cultured early-passage viruses from drug-treated patients. They also demonstrate that the analysis of genetical changes alone is insufficient to predict the resistance phenotype, particularly in cases, where a different viral background is being used for the analysis.

This conclusion is further corroborated by the data of patient no. 6 from the 750 mg cohort; most likely due to previous therapy, his virus contained an RT K103⇒N genotype at study entry, which was associated with a highly resistant phenotype ($IC_{50} = 160$ nM, Tables 1 and 2). Compared to the other patients in our study who developed a mutation at position 103 during therapy, this virus was much less sensitive to HBY 097 ($IC_{50} = 160$ nM versus 2.2 and 15 nM). This could either be due to the genetic background in this particular viral population as discussed above or to the additional AZT mutation (K70R). We believe that the latter is a likely explanation, because in our previous in vitro studies (Kleim et al., 1996; Riess et al., 1996) an influence of resistance mutations at the 'nucleoside analogue' site was shown to affect sensitivity to NNRTIs. Further work is needed to unequivocally prove this hypothesis.

In summary, apart from demonstrating the safety of HBY 097 (on short-term therapy), and in vivo suppression of viral load by HBY 097, there is also an indication that development of resistance might be somewhat slower than with other NNRTIs and that resistance due to the K 103 N mutation alone is moderate. Together with the nanomolar IC_{50} values of this drug, these data suggest that HBY 097 and related compounds may represent a highly promising second generation class of NNRTIs.

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