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COMMENTARY

Suppression of Resistance to Drugs Targeted to Human Immunodeficiency Virus Reverse Transcriptase by Combination Therapy

*Jan Balzarini**

REGA INSTITUTE FOR MEDICAL RESEARCH, KATHOLIEKE UNIVERSITEIT LEUVEN, B-3000 LEUVEN, BELGIUM

ABSTRACT. There are currently thirteen drugs approved for the treatment of human immunodeficiency virus (HIV)-infected individuals. Seven of them are targeted against the virus-encoded reverse transcriptase (RT). Appearance of drug-resistant virus strains under the selective pressure of anti-HIV chemotherapy rapidly occurs as a consequence of the low fidelity of the RT-catalyzed DNA polymerisation reaction and the massive viral turnover. Resistance-associated mutations appear in the RT of virus strains that are under selective pressure of both nucleoside RT inhibitors (NRTIs) and non-nucleoside RT inhibitors (NNRTIs). A variety of these mutations cause cross-resistance to several other NRTIs or NNRTIs and consequently may hamper the effectiveness of the other drugs. Other RT mutations are quite specific and selective in their drug-resistance spectrum and do not influence the potency of the majority of other available drugs. Moreover, drug-specific mutations are identified that are able to restore drug sensitivity again when concomitantly present with other drug-specific mutations. Combination therapy has proven to be able to markedly suppress virus replication (and subsequent appearance of drug resistance) for a relatively long time period. However, in a number of cases, multiple drug combination therapy results in the appearance of a different mutation spectrum than is expected to emerge under monotherapy. Also, it has been shown that drugs that alter cellular deoxynucleotide pools not only are able to potentiate the antiviral efficacy of some RT inhibitors, but also may influence the resistance spectrum of certain anti-HIV drugs. All available information argues for the use of a rational combination of different anti-HIV inhibitors with different resistance spectra to suppress virus replication efficiently and to delay the emergence of drug-resistant virus as long as possible, but it also indicates that there is a strong need for additional drugs to further optimize and improve the efficacy of long-term HIV treatment. BIOCHEM PHARMACOL **58**;1:1–27, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. HIV resistance; combination therapy; reverse transcriptase (RT); AIDS; nucleoside RT inhibitors (NRTIs); non-nucleoside RT inhibitors (NNRTIs)

There are currently six NRTIs†, three NNRTIs, and four protease inhibitors officially approved for the treatment of HIV-infected individuals (Table 1). Although the clinical efficacy of the individual drugs varies depending on the nature and the molecular target of the drugs, the compounds show a significant, but limited and transient, beneficial effect on inhibition of virus replication when administered as single drugs. Indeed, failure of long-term efficacy of these drugs is often associated with the appearance of dose-limiting side-effects or, more importantly, with the emergence of drug-resistant virus strains. Both RT inhibitors and protease inhibitors relatively easily select for virus strains that show a reduced susceptibility for the particular drugs. Moreover, the mutations that appear in the target (RT and protease) enzymes frequently (but not always) result in a decreased sensitivity to other RT and

* Correspondence: Dr. Jan Balzarini, Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium. Tel. 32-16-33-73-52; FAX 32-16-33-73-40; E-mail: jan.balzarini@rega.kuleuven.ac.be

† *Abbreviations*: NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; RT, reverse transcriptase; SIV, simian immunodeficiency virus; HIV, human immunodeficiency virus; AZT, azidothymidine (zidovudine); ddC, 2′,3′-dideoxycytidine (zalcitabine); ddI, 2′,3′-dideoxyinosine (didanosine); d4T, 2′,3′-didehydro-2′,3′-dideoxythymidine (stavudine); 3TC, 2′,3′-dideoxy-3′-thiacytidine (lamivudine); ABC, abacavir (ziagen); PMEA, 9-(2-phosphonylmethoxyethyl)adenine; PMPA, 9-(2-phosphonylmethoxypropyl)adenine; PMEApp, PMEA diphosphate; PMPApp, PMPA diphosphate; PFA, phosphonoformic acid; PBL, peripheral blood lymphocyte cells; dNTP, 2′-deoxynucleotide 5′-triphosphate; TIBO, tetrahydroimidazobenzodiazepinone; PETT, phenylethylthiazolylthiourea; BHAP, bisheteroarylpiperazine; HEPT, hydroxyethoxymethylphenylthiothymine; ANP, acyclic nucleoside phosphonate; MDR, multidrug resistance; PBMC, peripheral blood mononuclear cells; HU, hydroxyurea; ddATP, 2′,3′-dideoxyadenosine 5′-triphosphate; HGPRT, hypoxanthine/guanine phosphoribosyltransferase; dThd, 2′-deoxythymidine; dCyd, 2′-deoxycytidine; FLT, 3′-fluoro-2′,3′-dideoxythymidine; TK, thymidine kinase; d4TTP, d4T 5′-triphosphate; ddA, 2′,3′-dideoxyadenosine; ddDAP, 2′,3′-dideoxy-2,6-diaminopurineriboside; ddG, 2′,3′-dideoxyguanosine; FddA, 2′-fluoro-2′,3′-dideoxy-9-β-D-arabinofuranosyladenine; IMP-D, inosinate dehydrogenase; EICAR, 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide; IMP, inosine 5′-monophosphate, inosinate; XMP, xanthosine 5′-monophosphate, xanthosinate; GMP, guanosine 5′-monophosphate, guanosinate; THU, tetrahydrouridine; and dTHU, 2′-deoxytetrahydrouridine.

TABLE 1. Overview of drugs that have been officially approved for the treatment of HIV infections or that are currently the subject of advanced clinical trials

Name	Commercial name	Manufacturer
NRTIs		
AZT, zidovudine*	Retrovir	Glaxo Wellcome
ddC, zalcitabine*	Hivid	Hoffmann La Roche
ddI, didanosine*	Videx	Bristol-Myers Squibb
d4T, stavudine*	Zerit	Bristol-Myers Squibb
3TC, lamivudine*	Epivir	Glaxo Wellcome
AZT + 3TC*	Combivir	Glaxo Wellcome
1592U89 (ABC, Abacavir)	Ziagen	Glaxo Wellcome
FddaraA		National Cancer Institute (NIH)
(−)FTC		Triangle Pharmaceuticals
ANPs		
PMEA (adefovir)†	Preveon	Gilead Sciences
PMPA (tenofovir)‡		Gilead Sciences
NNRTIs		
BI-RG-587, nevirapine*	Viramune	Boehringer Ingelheim
BHAP U-90152, delavirdine*	Rescriptor	Pharmacia & Upjohn
DMP 266, efavirenz	Sustiva	Dupont Merck Pharmaceuticals
MKC-442, emivirine		Triangle Pharmaceuticals
GW420867X		Glaxo Wellcome
Protease inhibitors		
Saquinavir (Ro-31-8959)*	Invirase, Fortovase	Hoffmann La Roche
Ritonavir (ABT-538)*	Norvir	Abbott Laboratories
Indinavir (MK-639)*	Crixivan	Merck & Co.
Nelfinavir (AG-1343)*	Viracept	Agouron Pharmaceuticals
Tiprenavir (PNU-140690)		Pharmacia Upjohn
Amprenavir (141W94)	Agenerase	Glaxo Wellcome
ABT-378		Abbott Laboratories

*Drugs that have been officially approved by July 1, 1998.
†In clinical trials as its bis(POM)PMEA derivative (adefovir dipivoxil).
‡In clinical trials as its bis(POC)PMPA derivative (tenofovir disoproxyl fumarate).

protease inhibitors leading to cross-resistance. In addition, long-term side-effects that emerge during therapy may further limit the extended use of these compounds at sufficiently high doses in prolonged treatment schedules.

Combination therapy has several advantages over monotherapy: (i) it may allow the administration of lower doses of the individual drugs, resulting in a lower risk of appearance of severe toxic side-effects; and (ii) it may increase the efficacy of the therapeutic agents due to synergistic effects, in particular when the individual compounds are targeted to different sites of the virus replication or of the target enzyme. However, it is not a general rule that the combination of drugs necessarily results in an increased beneficial outcome of the therapy. There exist numerous examples showing antagonistic activity of certain drug combinations, increased toxicity, metabolic interference, or even accelerated drug resistance development. Thus, it is clear that drug combinations should not be performed blindly, but rationally designed not only to avoid an unfavorable or incompatible outcome of a particular drug combination, but also to allow an optimal exploitation of potential favorable interactions between drugs in terms of lower toxicity, higher efficacy, and/or more adequate resistance suppression.

Strategies for a rational combination therapy are urgently needed in view of the increasing number of drugs available for the treatment of HIV. Indeed, assuming paired drug combination therapy, given the 11 individual anti-HIV drugs currently available on the market, 55 different combined drug treatments are possible, and assuming a triple drug combination therapy, not less than 165 potential different drug cocktails can be designed. In this respect, a number of questions arise. Do we have rationales available to promote or to prefer one drug cocktail over the other? How confident are we that our *in vitro* data sufficiently predict the *in vivo* outcome of a given drug combination? What are the requirements that a drug needs to fulfill to be included in a rationally designed drug cocktail? Is there still a need for additional drugs acting at the "classical" targets, or do we need to develop novel drugs targeted to other sites of the replication cycle of the virus or even at cellular factors that may help to further compromise the virus replication? And, if an efficient drug cocktail has been found, can drug combinations eventually eradicate the virus from the body? In this commentary, focus will be placed on the underlying molecular and structural mechanisms of resistance development of HIV against RT inhibitors, and in particular on combinatorial modalities to increase the potency of RT inhibitors and/or to suppress or delay emergence of HIV resistance against RT inhibitors.

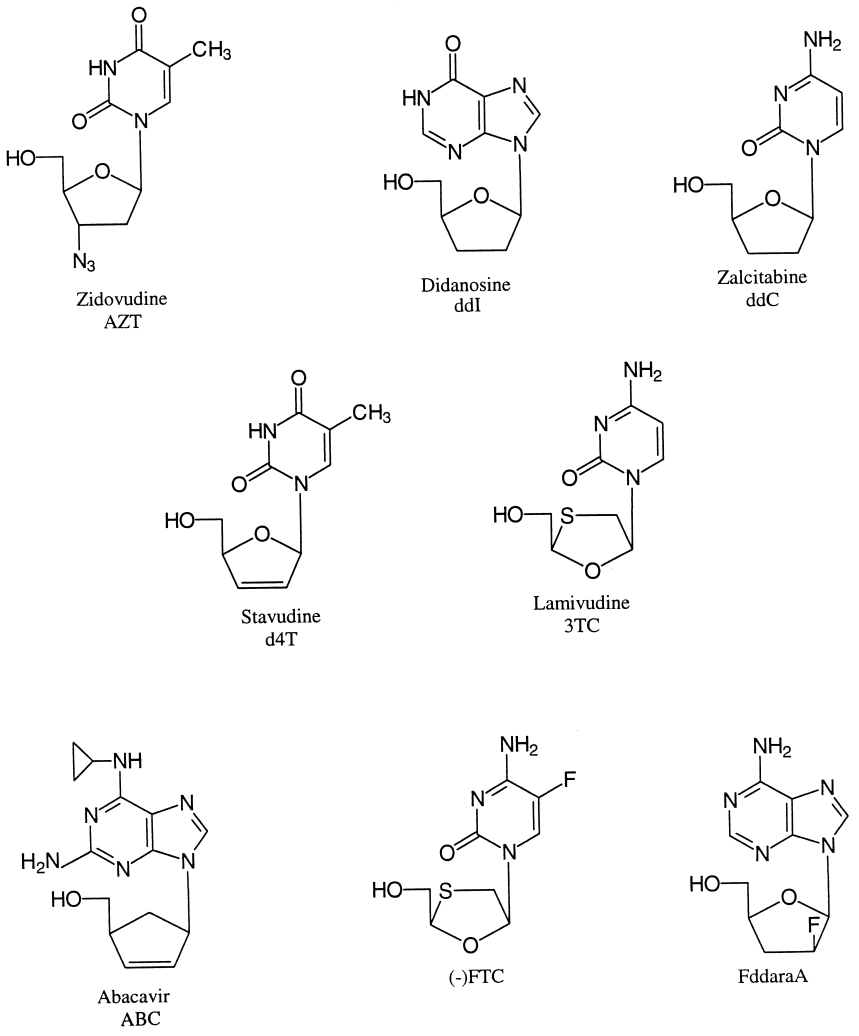

FIG. 1. Structural formulae of nucleoside RT inhibitors (NRTIs).

MECHANISM OF ANTIVIRAL ACTION OF NRTIs, ANPs, PFA, AND NNRTIs

Both pyrimidine and purine NRTIs act as RT inhibitors after intracellular conversion (phosphorylation) to their 5′-triphosphate derivatives by cellular enzymes ([1, 2] and references therein). They have to compete with the dNTP pools both for recognition by the RT as an alternative substrate, and for their eventual incorporation into the viral DNA chain. Due to the lack of a free 3′-OH group in the sugar part of the NRTIs, incorporation of the drugs into the growing viral DNA chain will necessarily result in DNA chain termination, which is believed to be the principal mechanism of antiviral action of NRTIs such as the 2′,3′-dideoxynucleosides AZT, ddC, ddI, d4T, 3TC, and ABC (Fig. 1).

Two ANPs, PMEA and PMPA, represent members of another class of potent and selective inhibitors of HIV RT. In these compounds, the sugar moiety is replaced by an aliphatic 2-hydroxyethyl (PMEA) or 2-hydroxypropyl (PMPA) group, and the phosphoric acid is replaced by an isopolar phosphonomethylether group linked to the 2-hydroxyl function of the acyclic chain (Fig. 2). The compounds are resistant to catabolic degradation such as dephosphorylation, but are phosphorylated to the corresponding diphosphates by cellular enzymes. The latter metabolites (i.e. PMEApp and PMPApp) are potent inhibitors of the retroviral RT and act upon incorporation into the growing viral DNA chain as DNA chain terminators ([1, 2] and references therein). Thus, their mechanism of antiretroviral action is virtually similar to that of NRTIs such as AZT and ABC.

Foscarnet (phosphonoformic acid, PFA), a pyrophosphate analogue inhibitor of RT, contains a phosphonate group linked to the carbon of the carboxylic acid group of formic acid (Fig. 3). PFA affords its inhibitory action against RT by interacting with the pyrophosphate site close to the nucleotide binding site of the enzyme. It is a noncompetitive inhibitor of HIV RT with respect to the natural substrates and an uncompetitive inhibitor against the template/primer ([1] and references therein).

The NNRTIs (Fig. 4) clearly act through an entirely different mechanism of action. These compounds need not

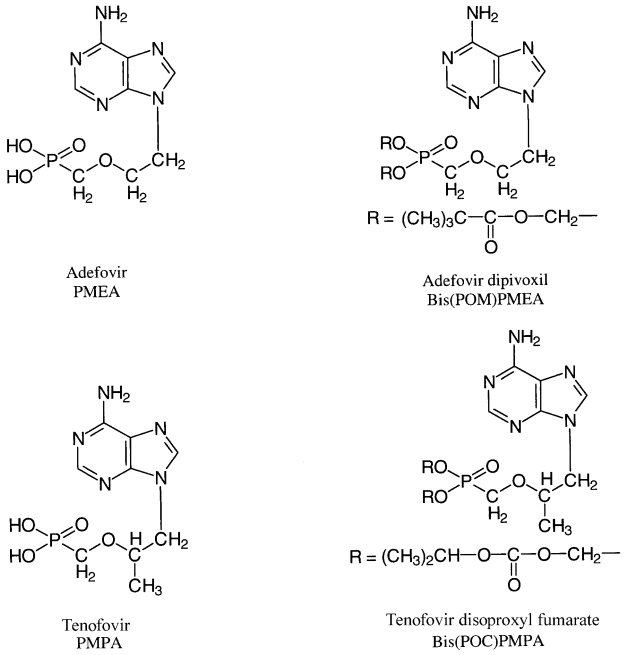

FIG. 2. Structural formulae of acyclic nucleoside phosphonates (ANPs).

be metabolised to inhibit the HIV-1 RT, but directly interact with a lipophilic pocket in the RT that is distinct from the substrate-active site. As a consequence, the NNRTIs are noncompetitive inhibitors of the HIV-1 RT with respect to both substrate and template/primer ([1, 2] and references therein). Crystal structures of RT/NNRTI complexes revealed that, upon binding of an NNRTI with the HIV-1 RT, the template/primer undergoes a repositioning in the protein, leading to a displacement of the binding groove by approximately 2 Å away from the active binding site. This, in turn, results in a markedly decreased enzyme activity in the presence of the NNRTI. Thus, NNRTIs inhibit the HIV-1 RT enzyme due to distortion of the polymerase-active site, a conclusion that has been supported by both structural [3] and kinetic [4] studies.

COMBINATION OF NRTIs AND NNRTIs IN HIV-1-INFECTED CELL CULTURES

It is now taken for granted that treatment of AIDS will eventually be based on the combination of three or more anti-HIV compounds. Numerous combination experiments have been performed between NRTIs and NRTIs, NRTIs and NNRTIs, and NNRTIs and NNRTIs ([5, 6] and references therein) for several reasons: (i) NRTIs like AZT and d4T, ddC and 3TC, ddA and ddI, ABC, and PMEA and PMPA follow different metabolic pathways for activation and conversion to their phosphorylated active metabolites, (ii) NRTIs and NNRTIs interfere with different sites on the RT (substrate binding site and lipophilic pocket at 10–15 Å distance from the substrate binding site), (iii) most NRTIs select for different resistance mutations that do not necessarily result in (marked) cross-resistance, and (iv) NRTI-resistant HIV-1 strains keep full sensitivity to NNRTIs and vice versa.

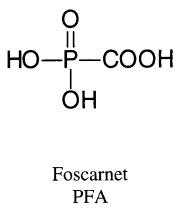

FIG. 3. Structural formula of phosphonoformic acid (PFA, foscarnet).

Antiviral antagonism has not been detected for virtually any drug combination performed between NRTIs and NNRTIs. Instead, abundant reports have been published on additive, subsynergistic, and synergistic activities found between combinations of NRTIs, ANPs, and NNRTIs, or between combinations within the NRTI or NNRTI class of drugs. These data were usually obtained in short-term cultivation experiments (i.e. 4–5 days for MT-4, CEM, MT-2, and Molt/4 cells or 10–12 days for PBLs). However, pronounced synergy, subsynergy, or just additivity for certain well-defined drug combinations has been observed by different investigators, but seems to depend upon the nature of the cell lines used to perform the experiments, or on the concentrations at which the drugs were combined, or even on the method of calculation and interpretation of the data obtained. Therefore, it is unclear and even questionable whether most of these *in vitro* observations can ever be realistically translated to and explored in the *in vivo* (patient) situation. An interesting example of synergy, however, has been reported for the combination of two different NNRTIs (UC-84 and UC-38) that belong to the same structural class of (thio)carboxanilides [7, 8]. The molecular basis of the synergy is thought to be the result of the preferential interaction of the compounds with different RT mechanistic forms. UC-84 preferentially binds to the free RT enzyme and the binary RT-primer/template complex, whereas UC-38 binds preferentially to the RT-primer/template-dNTP ternary complex. The synergy was observed for inhibition of both HIV-1 replication in MT-4 and cord blood mononuclear cells, and HIV-1 RT activity. Such differential mechanism-based inhibitory properties of NNRTIs against the HIV-1 RT should be further explored in combination therapy because they represent a scientifically based rationale for drug combination.

More interesting and more important, however, is the search for RT inhibitors that, when combined, suppress virus replication to a significantly higher extent than the single drugs do in long-term drug exposure experiments. For example, it was found that combinations of NNRTIs with an NRTI such as 3TC afford a markedly more pronounced suppression of virus replication in cell culture than the individual drugs do, even at $>$ 10-fold higher concentrations [9, 10]. Indeed, for these drug combinations, the concentrations of the individual drugs could be lowered by $\geq$ 25- to 50-fold to suppress virus breakthrough to an

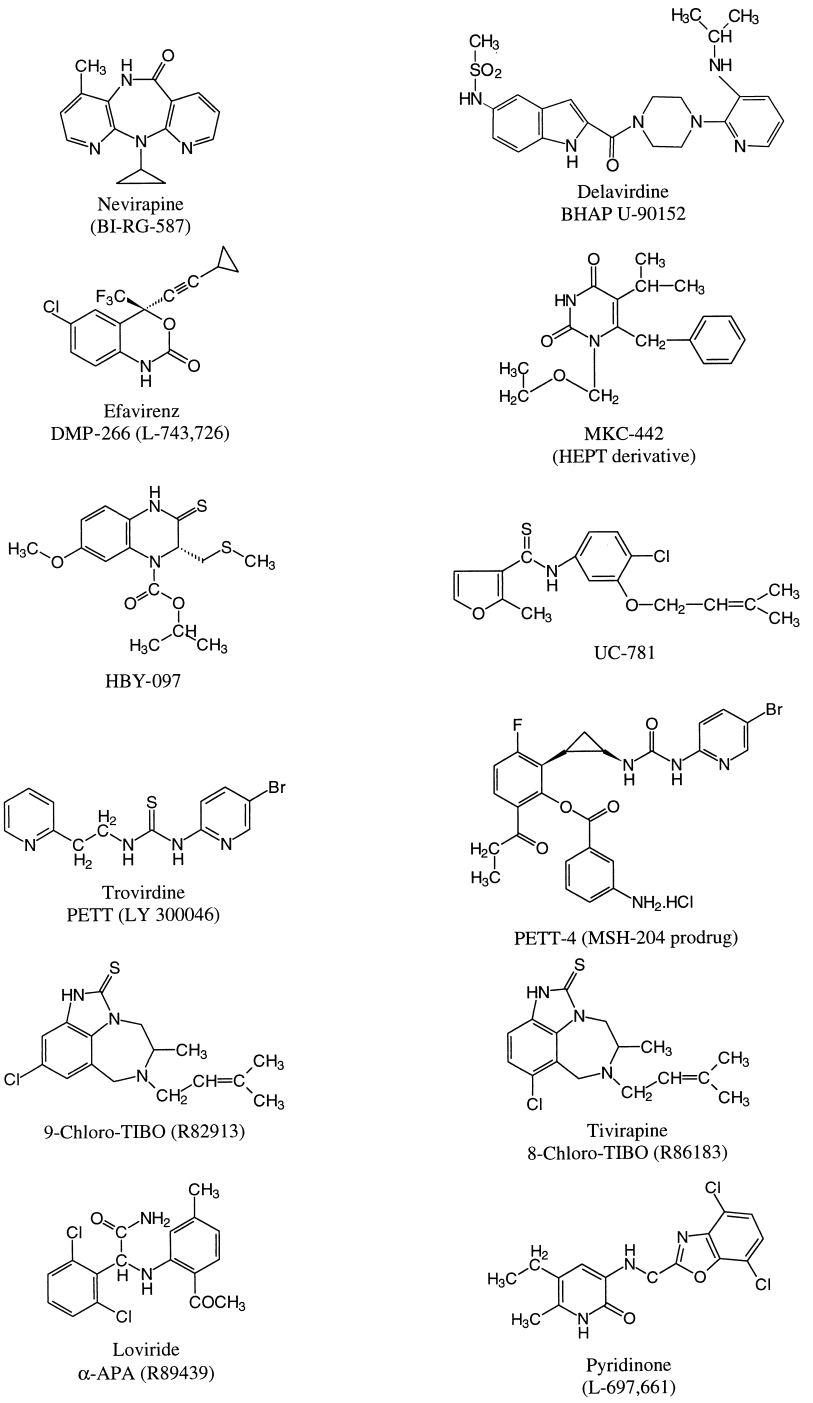

FIG. 4. Structural formulae of non-nucleoside RT inhibitors (NNRTIs).

extent equal to that obtained if the individual drugs were used as single compounds. It should be clear, however, that the durability of this pronounced virus suppression in cell culture remains to be assessed in drug-treated patients, may differ markedly from one drug combination to another, and may not necessarily be predicted from the *in vitro* experiments.

However, clinical data now increasingly emerge showing that inclusion of an NNRTI (i.e. nevirapine, delavirdine, or efavirenz) in a (combined) NRTI treatment schedule is able to delay the onset of resistant virus breakthrough and more strongly suppress the viral load in drug-treated patients. As has also been observed with protease inhibitors [11], the higher the NNRTI dose, the more pronounced the suppression and delay in drug resistance development [12, 13]. Therefore, there should nowadays be a consensus that

one should avoid giving the opportunity to the virus to accumulate resistance mutations in its RT genome. Such accumulation of mutations would make the virus easily resistant to the single and multiple drug combinations. This would occur when the drugs are applied at concentrations that are too low to suppress the virus replication efficiently. Thus, antiretroviral treatment of HIV-1-infected individuals should be performed not only with carefully selected drug combinations but also at the highest attainable drug doses to delay breakthrough of virus and to suppress virus replication as much as possible. It has been observed in cell cultures that the higher the NNRTI drug concentration, the more the virus breakthrough can be delayed, and at certain sufficiently high drug concentrations, the virus-infected cell cultures could be cleared (sterilized) of virus [14, 15]. Thus, for each individual drug, a knockout concentration can be determined in virus-infected cell cultures. Those NNRTIs that show the lowest virus knockout concentrations *in vitro* should therefore be considered as the preferential candidate compounds to be included in a double, triple, or quadruple drug combination therapy with NRTIs and protease inhibitors. To achieve this goal, the second-generation NNRTIs [i.e. UC-781, HBY-097 (nowadays replaced by GW867), efavirenz, PETT-4] that achieve virus knockout in cell culture at much lower concentrations than the first-generation NNRTIs (i.e. nevirapine, 9-chloro-TIBO, loviride, delavirdine) are likely better drug candidates to be part of future combination treatment modalities than the first-generation NNRTIs.

STRUCTURE OF HIV-1 RT

Crystals of a ternary complex that consists of a complex between the HIV-1 RT p66/p51 heterodimer and the NNRTI nevirapine, or between the HIV-1 RT p66/p51 heterodimer, a double-stranded DNA template/primer, and an antigen-binding fragment of an anti-RT antibody allowed resolution of the structure of HIV-1 RT [16–18]. The polymerase domain of p66 can be anatomically compared with a right hand and consists of four subdomains, namely the fingers, the palm, the thumb, and the connection domains. The N-terminal 440 amino acids of p66 constitute the polymerase domain, and the C-terminal 120 amino acids comprise the RNase H domain, which is present in the p66, but not in the p51, subunit. The overall folding of the subdomains is similar in p66 and p51, but the spatial arrangements of these subdomains differ markedly. Resolution of a subsequent HIV-1 RT structure showed that highly conserved amino acid regions in the p66 fingers and palm domains, together with two α-helices of the thumb domain, act as a clamp to position the template/primer relative to the polymerase active site. The heart of the active (substrate-binding) site of p66 consists of the catalytically essential Asp-110, Asp-185, and Asp-186 triad, which is further surrounded with several highly conserved amino acids [16–19].

The elucidation of the structure of unliganded HIV-1 RT and the co-crystallisations of HIV-1 RT with a variety of NNRTIs have provided valuable insights into the structure of RT, and have revealed non-nucleoside binding located in a pocket between the β-sheet comprising β4, β7, and β8, as well as the β-sheet comprising β9, β10, and β11 in the p66 palm domain [20–32]. This site is approximately 10–15 Å from the polymerase-active site represented by the Asp-110, Asp-185, and Asp-186 triad. Structural studies of seven RT-NNRTI complexes showed the volumes of the NNRTI-binding pockets ranging between 620 and 720 $Å^3$, of which the inhibitors occupy from 220 to 320 $Å^3$ [26]. Both mutational and crystallographic studies showed that the NNRTIs share a common binding site in the RT. It has been suggested that most of the NNRTIs can adopt a conformation in which the compound assumes a "butterfly" shape, consisting of two wing sections (one wing being proximal and the other distal from the polymerase active site). The wings of the molecules usually contain significant π-electron systems that can interfere efficiently with amino acid functional groups of the binding pocket. Many NNRTIs also form hydrogen bonds with the peptide main chain tuning the NNRTI-binding pocket. The internal surface of the NNRTI-specific pocket is mainly composed of hydrophobic amino acid residues with few hydrophilic amino acids in the vicinity of the drug. An almost general rule is that upon binding of NNRTIs to the lipophilic NNRTI-specific pocket in the p66 subunit, the functional groups of the highly conserved amino acid residues Tyr 181 and Tyr 188 are substantially reoriented and closely mimic the conformations of the equivalent side chains observed in the (inactive) p51 subunit of the enzyme [26]. Aromatic stacking interactions between aromatic rings of the NNRTI drugs and protein residues Tyr 181, Tyr 188, Trp 229, and Tyr 318 of the lipophilic pocket; electrostatic forces (especially significant for Lys 101, Lys 103, and Glu 138); van der Waals interactions with Leu 100, Val 106, Val 179, Tyr 181, Gly 190, Trp 229, Leu 234, and Tyr 318; and, last but not least, hydrogen bonding between the NNRTI drugs and the main chain (carbonyl/imino) peptide bonds (i.e. main –CO–NH–peptide chain between Lys 101 and Lys 102 or between Lys 103 and Lys 104), all contribute to the interaction and binding efficacies of the drugs in the NNRTI-specific pocket.

Crystallographic studies have given insights as to how mutated RTs become resistant to drugs [29, 32–34]. Some mutations (i.e. Tyr 181 Cys for 8-chloro-TIBO or Tyr 188 Leu for the quinoxaline HBY 097) have been shown to result in a loss of contact between the enzyme and the inhibitor at the particular amino acid sites. Steric hindrance between the Met 184 Ile/Val mutant and the oxathiolane ring of lamivudine (3TC) may explain the dramatic loss of sensitivity of the mutated enzyme for 3TC. More importantly, lower binding rates of NNRTIs caused by changes at the entrance of the pocket have been observed in Lys 103 Asn-mutated enzymes. This predomi-

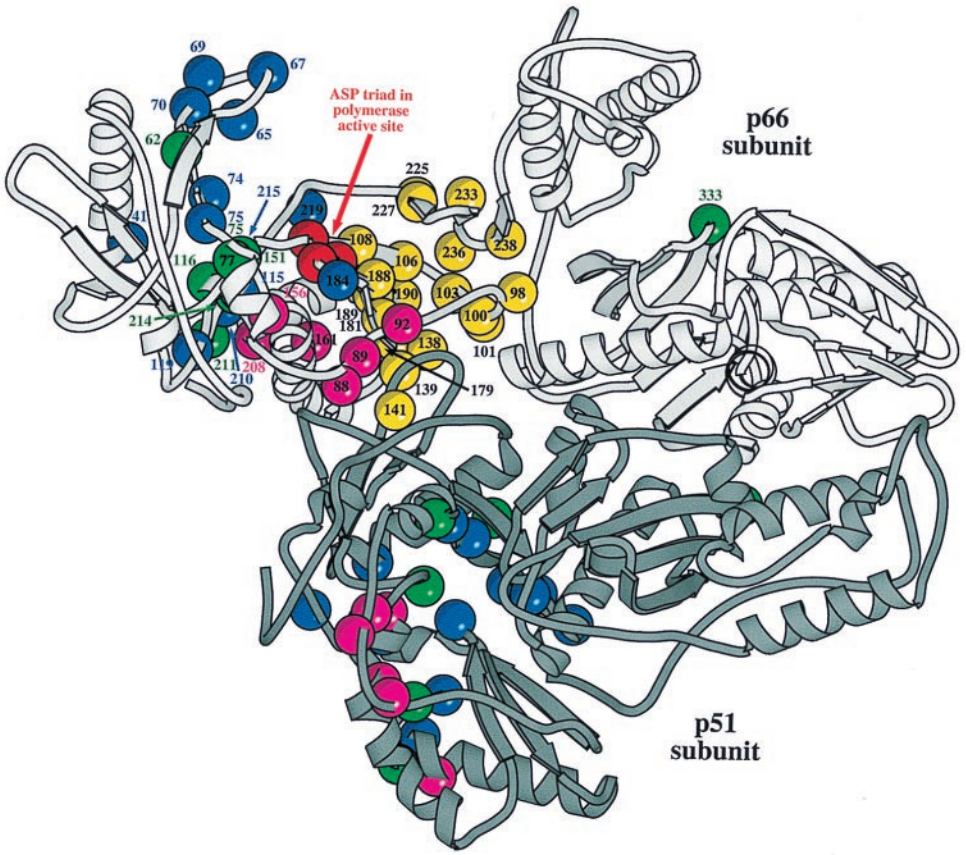

FIG. 5. Structure of the p66/p51 heterodimer RT. The aspartic acid triad in the substrate binding site is represented in red. The amino-acid mutations characteristic of NRTIs and ANPs are shown in blue, of PFA in magenta, and of NNRTIs in yellow. The multidrug NRTI-resistance amino-acid mutations are shown in green. Note that the NNRTI-characteristic 138, 139, and 141 amino acids in the fingers domain of p66 do not play a role in the formation of the NNRTI-specific lipophilic pocket. Instead, their corresponding 138, 139, and 141 amino acids in p51 participate in the formation of this pocket. This picture is presented courtesy of Dr. R. Esnouf, Rega Institute (published with his permission).

nant mutation, which appears to emerge with most clinically used NNRTIs [13, 35, 36], is thought to alter the structure of RT in such a way that it reduces the ability of the pocket mouth to open up and to "swallow" the incoming drug.

A number of amino acids that constitute the interior walls of the NNRTI-specific pocket are highly conserved for all HIV-1 strains, but markedly differ from their counterparts in closely related viruses such as HIV-2 or SIV. This most likely explains why the NNRTIs are highly virus (HIV-1)-specific and lack any significant activity against other related lentiviruses or oncoviruses. Although, in a few cases, marginal anti-HIV-2 activity has been observed for some NNRTIs (i.e. PETT, BHAP, HEPT) ([37] and unpublished data), replacement of a few key amino acids (i.e. Tyr 181 and Tyr 188) in HIV-1 RT by their counterparts from HIV-2 (i.e. Ile 181 and Leu 188) is already sufficient to afford a marked resistance of this double mutated HIV-1 RT against NNRTIs and, vice versa, replacement of Ile 181 and Leu 188 in HIV-2 RT by their Tyr 181 and Tyr 188 homologues of HIV-1 is sufficient to give a $>$ 1000-fold increased sensitivity of the double mutated HIV-2 RT against the NNRTIs [38–40]. Both mutant (hybrid) HIV-1 and HIV-2 RTs are perfectly viable enzymes. These findings are most intriguing and still leave—thus far—questions unanswered on the potential structural and/or functional role, if any, that the NNRTI-specific pocket may play in HIV-1 RT and why the primary amino acid sequence at several sites of the NNRTI-specific pocket of HIV-1 is so conserved in all known HIV-1 strains, but consistently differs from those of the other closely related lentiviruses such as HIV-2 or SIV.

RESISTANCE DEVELOPMENT OF HIV-1 AGAINST NRTIs, ANPs, PFA, AND NNRTIs

Exposure of HIV-1-infected cell cultures to NRTIs, ANPs, NNRTIs, or PFA eventually results in the appearance of mutations in the HIV-1 RT. These mutations—with a very few exceptions—are highly characteristic for either NRTIs, ANPs, PFA, or NNRTIs (for overviews, see Refs. 41–49). The distribution of the NRTI-, ANP-, PFA-, and NNRTI-specific resistance mutations in the HIV-1 RT is depicted in Fig. 5.

TABLE 2. NRTI- and ANP-specific resistance mutations in the HIV-1 RT

Amino acid number	Amino acid mutation	Codon mutation	NRTI that may select for the mutation	References
41	Met → Leu	ATG → TTG/CTG	AZT	50–52
65	Lys → Arg	AAA → AGA	ddI, DXG, PMEA, PMPA, ABC, ddC	53–62
67	Asp → Asn	GAC → AAC	AZT	50–52
69	Thr → Asp	ACT → GAT	ddC	63
70	Lys → Arg	AAA → AGA	AZT	50–52
	Lys → Glu	AAA → GAA	PMEA	64, 65
74	Leu → Val	TTA → GTA	ddI, ABC, DXG, ddC	56, 60–62, 66
75*	Val → Thr	GTA → ACA	ddC, d4T, ddI	67, 68
115	Tyr → Phe	TAT → TTT	ABC	60, 61
119	Pro → Ser	CCC → TCC	FddaraA	69
184	Met → Val	ATG → GTG/GTA	ddC, ddI, L-FddC, (−)FTC, 3TC, ABC	70–75
184	Met → Ile	ATG → ATA	3TC	72, 73, 75–77
184	Met → Thr	ATG → ACG	3TC	76, 78
210	Leu → Trp	TTG → TGG	AZT	79–82
215	Thr → Tyr	ACC → TAC	AZT	50–52, 83–85
215	Thr → Phe	ACC → TTC	AZT	50–52
215†	Thr → Cys	TTC → TGC	ddC	18
219	Lys → Gln	AAA → CAA	AZT	50–52
219	Lys → Glu	AAA → GAA	AZT	50–52

*Val 75 mutations to 75-Met/Ser/Ala have also been observed to occur in patients under NRTI treatment, resulting in ≥ 5-fold increased EC_{50} values for d4T [86].
†Arises in Thr 215 Tyr background.

Resistance Development of HIV-1 against NRTIs and ANPs

Among the NRTIs (Fig. 5, Table 2), AZT (zidovudine) predominantly leads to the appearance of Met 41 Leu, Asp 67 Asn, Lys 70 Arg, Leu 210 Trp, Thr 215 Tyr/Phe, and/or Lys 219 Gln amino acid changes, which, when properly combined, result in a 100- to 200-fold decreased sensitivity to AZT. Resistance to ddC (zalcitabine) is due to the appearance of the Lys 65 Arg and Thr 69 Asp amino acid changes, and ddI (didanosine) usually leads to the Leu 74 Val mutation. For d4T (stavudine), resistance development *in vitro* is conferred by the Val 75 Thr mutation, whereas 3TC (lamivudine) consistently selects for a Met 184 Val/Ile mutation *in vitro* and *in vivo*. The Lys 65 Arg mutation that may appear under ddC (and ddI) drug pressure also appears upon (*in vitro*) exposure of HIV to PMEA (adefovir), an ANP. However, the Lys 65 Arg mutation has not been observed thus far in PMEA-treated patients, but instead the Lys 70 Glu mutation has been found to emerge in patients, although at low incidence. ABC, a carbovir-monophosphate prodrug, predominantly selects for Met 184 Val, but, in addition, also for the Lys 65 Arg, Leu 74 Val, and Tyr 115 Phe amino acid changes, which, when all combined, only result in a 10-fold decreased activity of the drug against the mutant virus. It is clear that some of the NRTI-characteristic mutations result in (partial) cross-resistance to other (but not all) NRTIs, whereas other NRTI-characteristic mutations are rather specific for the particular nucleoside or nucleotide analogues against which they were selected (i.e. the AZT-resistance mutations).

Interestingly, under sequential NRTI monotherapy or combination NRTI treatment (i.e. AZT + ddI or AZT + ddC), multidrug resistant HIV-1 strains emerge containing a variety of amino acid changes (i.e. Ala 62 Val, Val 75 Ile, Phe 77 Leu, Phe 116 Tyr, and Gln 151 Met) in their RT (Fig. 5, Table 3). These mutations confer cross-resistance to all NRTIs, albeit to a variable extent. The Gln 151 Met mutation is the predominant marker mutation among the MDR mutations. The appearance of NRTI MDR strains is still rather rare (in most populations usually < 5% occurrence). In the majority of cases, the classical mutations for AZT, ddI, or ddC resistance are not found in these isolates, leading to the speculation that both resistance pathways (the classical NRTI resistance pattern and the NRTI MDR pattern) are incompatible and mutually exclusive. However, recently, several patients have been found that simultaneously harbor NRTI MDR-(i.e. Gln 151 Met) and AZT-(i.e. Met 41 Leu, Asp 67 Asn, and Thr 215 Tyr) specific mutations [95]. It could be demonstrated that these mutations were located on the same genome of the virus. Moreover, when generated by site-directed mutagenesis, it was shown that the Gln 151 Met/Leu and Thr 215 Tyr/Phe amino acid mutations could exist concomitantly in HIV-1 RT [87, 96–99]. Also, recombinant virus strains harboring the Gln 151 Met + Thr 215 Tyr/Phe mutations could be generated [96–99]. There are now two possible explanations for the concomitant presence of AZT-specific and NRTI MDR mutations in one virus particle: (i) it is possible for HIV-1 to acquire NRTI MDR mutations and the AZT-associated resistance profile simultaneously or sequentially in its RT, or (ii) the emergence of these types of virus strains is possible only if we imagine recombination events

TABLE 3. Multidrug (NRTI)-specific resistance mutations in the HIV-1 RT

Amino acid number	Amino acid mutation	Codon mutation	NRTI combination	References
62	Ala → Val	GCC → GTC	AZT + ddI/ddC	87, 88
69*	Thr → Ser Ser-Ser*	6 bp insert	AZT + ddI/ddC	89–91
75	Val → Ile	GTA → ATA	AZT + ddI/ddC	87, 88
77	Phe → Leu	TTC → CTC	AZT + ddI/ddC	87, 88
116	Phe → Tyr	TTT → TAT	AZT + ddI/ddC	87, 88
151	Gln → Met	CAG → ATG	AZT + ddI/ddC	87, 88, 92
211	Arg → Lys	AGG → AAG	AZT + 3TC	93, 94
214	Leu → Phe	CTT → TTT	AZT + 3TC	93, 94
333	Gly → Asp	GGC → ?	AZT + 3TC	93, 94
333	Gly → Glu	GGC → ?	AZT + 3TC	94

*Insert/duplication of Ser-Ser occurs between Thr 69 Ser and Lys 70 and is observed upon ddI, AZT + ddI, or AZT + ddC treatment of HIV-1-infected individuals that were exposed previously to AZT treatment.

between NRTI MDR and AZT-mutated virus strains. Further research is needed to clarify these issues. However, it should be mentioned that acquisition of multiple resistance mutations by recombination events has been shown before to occur in the HIV protease gene and may now increasingly emerge in drug-treated and drug-naïve HIV-infected individuals, which may further complicate and compromise future treatment modalities.

Very recently, an entirely different NRTI MDR has also been observed in a number of patients that were pretreated with AZT and exposed to ddI, ddC, and/or d4T. Insertion of two Ser residues or a serine and a valine/alanine/glycine/threonine residue between Thr 69 Ser and Lys 70 had occurred in these virus strains [86, 89–91]. When the two-amino-acid insertion represents the sole change in HIV-1 RT, a moderate increase of NRTI resistance is noted, but when combined with Leu 210 Trp and Thr 215 Tyr, a pronounced NRTI MDR is observed. The potential sequential events that may lead to the two-amino-acid insertions may be visualized as follows: Ser 68 – Thr 69 – Lys 70 → Ser 68 – **69 Ser** – Lys 70 → Ser 68 – **69 Ser – (Ser – Ser)** – Lys 70 → Ser 68 – **69 Ser – (Ser/Val/Ala – Ser/Gly/Thr)** – Lys 70. The exact mechanism by which the duplication/insertion occurs is presently unknown. It is clear that such mutant virus strains display a multi-NRTI drug resistance profile, whereas NNRTI sensitivity is unaffected in these virus strains.

Resistance Development of HIV-1 against PFA

PFA, a pyrophosphate analogue that also inhibits HIV RT, selects for a variety of resistance mutations, including Trp 88 Gly/Ser (located on the β5a strand), Glu 89 Gly/Lys, Leu 92 Ile, Ser 156 Ala, Glu 161 Leu (located in the αE helix), and His 208 Tyr (located on helix αF) mutations (Fig. 5, Table 4). They are located close to the template strand of the template/primer and rather far away from the putative pyrophosphate binding site, suggesting that the mechanism by which HIV becomes resistant to PFA is indirect, by an altered interaction of the mutant enzyme with the template strand distorting the geometry of the polymerase active site and thereby decreasing PFA binding.

Resistance Development of HIV-1 against NNRTIs

The amino acid changes that occur under NNRTI drug pressure in the HIV-1 RT are clearly different from those observed to occur in the presence of NRTIs (Fig. 5, Table 5). A cluster of mutations consists of Ala 98 Gly, Leu 100 Ile, Lys 101 Glu, Lys 103 Asn, Val 106 Ala, and Val 108 Ile, located in the β-sheet comprising β5b and β6. Another set of mutations is represented by Val 179 Asn, Tyr 181 Cys, Tyr 188 Cys/His, and Gly 190 Ala/Glu, located in the β-sheet comprising β9 and β10. In addition, Glu 138 Lys (located in the loop between β7 and β8 of the p51 subunit), Pro 236 Leu (located at β13/β14), and Pro 225 His and Phe 227 Leu (located at β13) were also found to appear under treatment with certain NNRTIs (Table 5). All these mutations conferring resistance to NNRTIs are very well clustered and are part of a lipophilic pocket in HIV-1 RT (Fig. 5). A characteristic property of these mutations is that a single amino acid change may result in a degree of resistance to one or more (first-generation) NNRTIs that is usually much more pronounced than that observed for single NRTI-specific mutations. Also, pronounced cross-resistance to a variety of NNRTIs against a

TABLE 4. Foscarnet (PFA)-specific resistance mutations in the HIV-1 RT

Amino acid number	Amino acid mutation	Codon mutation	Reference
88	Trp → Gly	TGG → GGG	100, 101
88	Trp → Ser	TGG → TCG	100, 102
89	Glu → Gly	GAA → GGA	103
89	Glu → Lys	GAA → AAA	101
92	Leu → Ile	TTA → ATA	101
156	Ser → Ala	TCA → GCA	101
161	Gln → Leu	CAA → CTA	100
208	His → Tyr	CAT → TAT	100

TABLE 5. NNRTI-specific resistance mutations in the HIV-1 RT

Amino acid number	Amino acid mutation	Codon mutation	NNRTIs that may select for the mutation	References
74*	Leu → Val	TTA → GTA	QUIN	104, 105
	Leu → Ile	TTA → ATA	QUIN	104
75*	Val → Ile	GTA → ATA	QUIN	104, 105
	Val → Leu	GTA → TTA	QUIN	104
98	Ala → Gly	GCA → GGA	PYR, NEV	106, 107
100	Leu → Ile	TTA → ATA	BHAP, DMP 266, PYR, NEV, 9-Cl-TIBO, TCA	13, 106, 108–122
101	Lys → Gln	AAA → CAA	PETT	123, 124
101	Lys → Glu	AAA → GAA	TIBO, DMP 266, PYR, TCA†	106, 113, 119, 120, 125–128
101	Lys → Ile	AAA → ATA	TCA‡	120
103	Lys → Asn	AAA → AAC	BHAP, TIBO, DMP 266, PYR, α-APA, HEPT, NEV, TCA	13, 106, 108, 113, 114, 120, 125, 127, 129–134
103	Lys → Thr	AAA → ACA	BHAP, TCA	120, 129
103	Lys → Gln	AAA → CAA	PYR, PETT	123, 124, 131
103	Lys → Arg	AAA → AGA	HEPT, PETT	123, 124, 135
106	Val → Ala	GTA → GCA	HEPT, NEV, QUIN, TCA§, BHAP	107–109, 114, 115, 119, 121, 135, 136
106	Val → Ile	GTA → ATA	QUIN, α-APA	132, 137
106	Val → Leu	GTA → TTA	QUIN	138
108	Val → Ile	GTA → ATA	DMP 266, PYR, α-APA, HEPT, NEV, TIBO, PETT	13, 106, 107, 111, 112, 123, 124, 132, 135
135	Ile → Met/Thr/Leu	ATA → ?	DMP 266	13
138	Glu → Lys	GAG → AAG	HEPT, TIBO‖, TSAO, TCA	117, 120–122, 139–143
138	Glu → Ala	GAG → GCG	TSAO¶	144
139	Thr → Ile	ACA → ATA	Calanolide A	110
141	Gly → Glu	GGG → GAG	TCA**	120
179	Val → Asp	GTT → GAT	DMP 266, PYR, TIBO, PETT††, TCA	106, 111, 112, 120, 123, 124, 145
179	Val → Glu	GTT → GAG	PYR, DMP 266	106, 111, 112
181	Tyr → Cys	TAT → TGT	BHAP, DMP 266, HEPT, PYR, α-APA, NEV, PETT, TCA, TII	106, 107, 109, 111–113, 115, 119, 120, 123, 124, 127, 129–131, 134, 135, 146–151
181	Tyr → Ile	TAT → ATT	BHAP, HEPT, NEV	152
188	Tyr → Cys	TAT → TGT	HEPT, NEV	114, 135, 146
188	Tyr → His	TAT → CAT	α-APA, TIBO, BHAP	117, 127, 147
188	Tyr → Leu	TAT → CTT	α-APA	147
188	Tyr → Leu	TAT → TTA	DMP 266, TIBO	113, 145
189	Val → Ile	GTA → ATA	QUIN	104
190	Gly → Gln	GGA → CAA	QUIN	104
190	Gly → Thr	GGA → ?	QUIN	137
190	Gly → Glu	GGA → GAA	BHAP, QUIN, TCA‡‡, DMP 266	13, 120, 126, 153–156
190	Gly → Ala	GGA → GCA	α-APA, NEV, DMP 266	13, 107, 157
190	Gly → Ser	GGA → ?	DMP 266	13
225	Pro → His	CCT → CAT	QUIN††, DMP 226	13, 158, 159
227	Phe → Leu	TTC → TTA	TCA	160
233	Glu → Val	GAA → GTA	BHAP	127
236	Pro → Leu	CCT → CTT	BHAP, HEPT	146, 161
238	Lys → Thr	AAA → ACA	BHAP	127

HEPT derivatives include the hydroxyethoxymethyl phenylthiothymine derivatives HEPT, E-EBU, E-EBU-dM, E-EPSeU, E-EPU, and MKC-442 (I-EBU).
QUIN derivatives include the quinoxalines S-2720 and HBY-097.
TCA derivatives include the (thio)carboxanilides UC-10, UC-16, UC-32, UC-38, UC-42, UC-57, UC-68, UC-69, UC-70, UC-80, UC-81, UC-82, UC-84, and UC-781.
PYR derivatives include the pyridinones L-697,661 and L-697,593.
α-*APA derivatives* include the anilinophenylacetamides R89,439 (Loviride) and R18,893.
BHAP derivatives include the bisheteroarylpiperazines U-87201E (atevirdine), U-88204E, AAP-BHAP, and U-90152 (delavirdine).
TII derivative represents thiazolo-iso-indolinone BM +51.0836.
TIBO derivatives include the tetrahydroimidazobenzodiazepinones TIBO R82150, 9-chloro-TIBO (R82913), and 8-chloro-TIBO (R86183) (tivirapine).
PETT derivatives include the phenylethylthiazolylthioureas LY-300046 (trovirdine) and PETT-4 (MSH-204 prodrug).
NEV derivative represents BI-RG-587 (nevirapine).
TSAO derivatives represent the *tert*-butyldimethylsilylspiroaminooxathiole thymines TSAO-T, TSAO-m^{3}T, TSAO-e^{3}T, and TSAO-1,2,3-triazole.
*Compensatory mutation found in Gly 190 Glu background.
†Combined with Gly 190 Glu.
‡Combined with Gly 141 Glu.
§Combined with Tyr 181 Cys.
‖Combined with Leu 100 → Ile.
¶Mutation observed in TSAO-naïve patients.
**Combined with Lys 101 Ile.
††Combined with other mutations (i.e. V106A).
‡‡Combined with Lys 101 Glu.

TABLE 6. Sensitization of wild-type virus to NRTIs or NNRTIs upon addition of specific drug resistance mutations

Drug	Resistance mutation	Sensitization to drug	Degree of sensitization (-fold)	References
Foscarnet	Gln 161 Leu	AZT	11	100, 101
		Nevirapine	6	
		TIBO R82150	6	
Foscarnet	Gln 161 Leu + His 208 Tyr	AZT	45	100, 101
		Nevirapine	20	
		TIBO R82150	18	
NNRTIs	Tyr 181 Cys	(-)-7,8-Dihydro-calanolide B	10	32
3TC	Met 184 Val	Adefovir (PMEA)	3–4	165
		Tenofovir (PMPA)	~2	165, 166
Quinoxaline S2720	Pro 225 His	BHAP U-90152	8	158, 159
DMP-266	Pro 225 His	BHAP U-90152	~10	13, 167
BHAP U-90152	Pro 236 Leu	NNRTIs (i.e. nevirapine, pyridinone L-697,661, 9-chloro-TIBO)	10	161

single mutation is more common for the NNRTIs than for the NRTIs. The cross-resistance for the NNRTIs is highly determined by the nature of the amino acid mutation and the type of NNRTI used. It is important to realize that—in contrast with the first-generation NNRTIs—the currently most active second-generation NNRTIs (i.e. DMP-266, UC-781, HBY-097, and PETT-4) can usually deal very well with single mutations in the RT, and need several (two or more) NNRTI-specific mutations in the RT to achieve a high level of resistance against HIV-1 (i.e. Leu 100 Ile + Lys 103 Asn; Lys 101 Asn + Lys 103 Asn; Lys 103 Asn + Tyr 181 Cys; Lys 103 Asn + Val 108 Ile; and Lys 103 Asn + Pro 225 His) ([13, 162–164] and unpublished data).

It is amazing to note that, whereas the Val 108 Ile and Pro 225 His mutations did not confer measurable resistance of HIV-1 to DMP-266 as single mutations, addition of the Lys 103 Asn amino acid change to these mutations increased the DMP-266 resistance 17- to > 100-fold. Even more, the Leu 100 Ile mutation, while conferring 21-fold resistance (*in vitro*) to DMP-266, resulted in a > 4000-fold resistance (*in vivo*) to DMP-266 when combined with Lys 103 Asn [13].

The important difference in the speed and extent of resistance development between the first-generation (i.e. nevirapine, delavirdine, loviride, 9-chloro-TIBO) and second-generation [i.e. quinoxaline HBY-097 and GW420867X, efavirenz (DMP-266), UC-781, PETT-4] NNRTIs may have conceptual implications for how combination therapy should be performed. All available data suggest that it may be advisable to include a second-generation instead of a first-generation NNRTI in a drug combination cocktail. A first-generation NNRTI may too easily select for a NNRTI-specific mutation that easily results in pronounced resistance to the first-generation NNRTIs. The presence of such a mutation will partially compromise the effectiveness of second-line treatment with a second-generation NNRTI, since now addition of only one mutation instead of two might be sufficient to afford pronounced resistance to the second-generation NNRTIs. *In vitro* data showing a more pronounced long-term virus suppression in HIV-1-infected cell cultures by the second-generation NNRTIs compared with the first-generation NNRTIs (lower required virus knockout concentrations for the second-generation NNRTIs than for the first-generation NNRTIs in cell culture) are consistent with and corroborate the above-mentioned findings [9, 10].

DOES THE SENSITIZING EFFECT OF CERTAIN DRUG-SPECIFIC RESISTANCE MUTATIONS IN THE HIV-1 RT TO OTHER DRUGS PROVIDE PERSPECTIVES FOR RATIONAL COMBINATION THERAPY?

A number of amino acid mutations in the HIV RT have been reported to confer increased sensitivity of HIV to NRTIs or NNRTIs in the absence of additional mutations or to confer increased phenotypic sensitivity to NRTIs or NNRTIs when added to the genetic background of other NRTI- or NNRTI-specific resistance mutations. There exist several examples of amino acid mutations that afford a hypersensitization of the HIV to NRTIs and NNRTIs.

Mutations That Afford Sensitization to NRTIs and/or NNRTIs When Incorporated into a Wild-Type Background

A number of amino acid changes in the HIV-1 RT are identified to be able to sensitize HIV-1 to a number of well-defined NRTIs, ANPs, and NNRTIs (Table 6). Pro 225 His mutated RT, which appeared upon selection with quinoxaline S2720 [158, 159] and efavirenz (DMP-266) [13, 167], has been shown to acquire markedly greater sensitivity to BHAP U-90152 (delavirdine) but not to any of the other NNRTIs [158, 159, 167]. The hypersensitivity of the mutant RT enzyme and the corresponding mutant virus to delavirdine has been confirmed by site-directed mutagenesis and generation of recombinant viruses, and could be rationally understood by the molecular structural

TABLE 7. Suppression of phenotypic AZT resistance by other drug-resistance mutations

AZT resistance mutation background	Added resistance mutation	Drug that may select for the additional mutation	Increased sensitivity (-fold) to AZT	References
Thr 215 Tyr + Lys 219 Gln	Leu 74 Val	ddI	10	66
Clinical AZT-resistant isolates*	Lys 65 Arg	PMEA	>10	58
Met 41 Leu + Thr 215 Tyr	Met 184 Val	3TC, ABC	Complete	72, 168
Asp 67 Asn + Lys 70 Arg + Thr 215 Phe + Lys 219 Gln	Met 184 Val		Partial	72
Asp 67 Asn + Lys 70 Arg + Thr 215 Phe + Lys 219 Gln	Met 184 Ile		Partial	72
Asp 67 Asn + Lys 70 Arg + Thr 215 Tyr + Lys 219 Gln	Tyr 181 Cys	NNRTIs	30	169
Met 41 Leu + Thr 215 Tyr	Tyr 181 Cys		35	169
Asp 67 Asn + Lys 70 Arg + Thr 215 Tyr + Lys 219 Gln	Met 184 Val + Tyr 181 Cys	3TC + NNRTIs	Complete	
Asp 67 Asn + Lys 70 Arg + Thr 215 Tyr + Lys 219 Gln	Leu 100 Ile	NNRTIs	4000	170
Met 41 Leu + Thr 215 Tyr	Leu 100 Ile		1000	170
Lys 70 Arg	Leu 100 Ile		≥4	170
		Foscarnet		
Met 41 Leu + Thr 215 Tyr	Trp 88 Gly	Foscarnet	Complete	102
Met 41 Leu + Thr 215 Tyr	Trp 88 Ser		Partial	102
Met 41 Leu + Asp 67 Asn + Lys 70 Arg + Thr 215 Tyr	Glu 89 Lys		≥100	102
Met 41 Leu + Asp 67 Asn + Lys 70 Arg + Thr 215 Tyr	Leu 92 Ile		>100	102
Met 41 Leu + Asp 67 Asn + Lys 70 Arg + Thr 215 Tyr	Ser 156 Ala		~40	102

*Resistance mutations not determined.

determinants of the RT–BHAP complex [25, 158, 159]. The Pro 225 His mutation has been found recently to occur in drug-treated patients [167]. Interestingly, the Pro 236 Leu mutant virus that appears upon treatment with the BHAP derivatives atevirdine and delavirdine *in vitro* [161] was also shown to have a markedly higher sensitivity to the inhibitory effect of a variety of other NNRTIs including nevirapine, pyridinone, and 9-chloro-TIBO. However, the Pro 236 Leu mutation has not been reported thus far to occur in delavirdine-treated HIV-infected individuals. Also, the Tyr 181 Cys mutation that is selected under pressure of a variety of NNRTIs and causes varying degrees of cross-resistance to many NNRTIs specifically sensitizes the mutant virus to (-)-7,8-dihydro-calanolide B (another NNRTI) by 10-fold [32]. Virus strains harboring the 3TC-characteristic Met 184 Val mutation show a slight increase of sensitivity to the acyclic nucleoside phosphonates PMEA (adefovir) and PMPA (apropovir) [165, 166]. The Leu 92 Ile, Glu 89 Lys, and Ser 156 Ala mutations, appearing under PFA treatment, invariably increase AZT sensitivity 2.6 to 2.8 times, AzddU 4.1 to 36 times, and 9-chloro-TIBO and nevirapine 1.4 to 10.4 times, irrespective of the genetic background of the virus strains in which the PFA-characteristic mutations appeared [100, 101]. However, the Gln 161 Leu mutation that may also appear under PFA exposure afforded an 11-fold increased sensitivity to AZT (6-fold to nevirapine and 9-chloro-TIBO), whereas the double mutant Gln 161 Leu + His 208 Tyr results in a 45-fold hypersensitivity to AZT (20-fold to nevirapine and 18-fold to 9-chloro-TIBO). The structural basis of these observations on sensitization of HIV to NRTIs, ANPs, or NNRTIs upon appearance of NRTI- or NNRTI-characteristic mutations in most cases is not very well understood, but may open interesting perspectives for rationally combining well-defined drugs.

Mutations That Reverse the Phenotypic Resistance of HIV-1 RT to NRTIs and NNRTIs When Added to Drug-Resistance Genetic Backgrounds

The most prominent and most studied mutation-based resensitisation of the activity of RT inhibitors against HIV is that of AZT (Table 7). The very first mutation that was reported to restore phenotypic sensitivity of AZT-resistant virus strains (containing Thr 215 Tyr + Lys 219 Gln) to AZT was the Leu 74 Val mutation that appeared under ddI drug pressure [66]. However, as will be discussed later, the genetic background in which the Leu 74 Val mutation occurs plays an important role in the eventual degree of sensitization to AZT. Also, the Lys 65 Arg mutation that emerges under PMEA treatment *in vitro* was found to suppress resistance to AZT when added to the genotypic AZT resistance background. The 3TC-characteristic Met 184 Val and Met 184 Ile mutations were also able to restore AZT sensitivity when added to the genotypic AZT resistance background [168]. Typical NNRTI-characteristic mutations that confer increased phenotypic sensitivity of virus strains to AZT when introduced in an AZT resistance background are the Tyr 181 Cys and Leu 100 Ile mutations [169, 170]. Finally, PFA has been shown to select for a variety of resistance mutations, several of which markedly increased the sensitivity to AZT when present in an AZT resistance background [102]. Based on these observations, it would seem attractive to propose specific combination

therapies based on reversal of resistance against AZT or other RT inhibitors.

Thus, the above-mentioned characteristics may argue for a combined drug regimen containing AZT on the one hand, and ddI, PMEA, 3TC, ABC, PFA, and NNRTIs on the other hand. These drug combinations theoretically can be performed in two different administration modalities: (i) concomitant drug combination therapy expected to delay or suppress drug resistance development, or (ii) sequential drug therapy expected to back-mutate the existing resistance mutations due to the removal of the pressure of the first drug on the virus, or to resensitize the virus to the previous drug treatment when the sensitizing amino acid mutations characteristic for the second drug are added to the existing resistance mutations characteristic for the first drug. What happens in cell culture when a rational combination of drugs is exposed to HIV-1, or, more importantly, what happens in the HIV-infected patient who is subject to such treatment modalities? *In vitro* studies suggest that the virus can rather easily select for alternative mutations when exposed to two drugs with a complementary resistance spectrum. For example, it has been shown that the combination of two NNRTIs (i.e. TSAO-m^3T selecting for Glu 138 Lys RT and BHAP U88104 selecting for Leu 100 Ile RT in cell culture under monotherapy) selects for another NNRTI-specific mutation (i.e. Tyr 181 Cys) without significant delay of resistance development when compared with monotherapy [108]. The Tyr 181 Cys mutation results in cross-resistance to both NNRTIs. When AZT-resistant virus strains were passaged in the presence of nevirapine, they rapidly developed resistance to nevirapine due to the appearance of the Val 106 Ala mutation but not the Tyr 181 Cys mutation, resulting in virus strains that are cross-resistant to both AZT and nevirapine [170]. However, it has also been demonstrated that certain combinations of AZT resistance mutations (i.e. Met 41 Leu + Thr 215 Tyr but not Asp 67 Asn + Lys 70 Arg + Thr 215 Phe) with Leu 74 Val cause cross-resistance to AZT and ddI. Thus, if Met 41 Leu persists in the AZT resistance background, the ddI-characteristic mutation no longer efficiently suppresses AZT resistance [171]. Clearly, the virus can utilize different combinations of mutations to become multiply resistant to each double set of drugs that have been tried thus far in cell cultures.

What is our experience now in patients treated with pairs of drugs? The sensitizing effect of the PFA-specific Gln 161 Leu and Gln 161 Leu + His 208 Tyr mutations in the RT to AZT (and several NNRTIs) may help to explain why the PFA-specific Trp 88 Ser mutation is more commonly detected in clinical isolates derived from patients that were also taking AZT concomitantly [100]. Indeed, in this double treatment combination (AZT + PFA) therapy, the PFA-specific Trp 88 Ser mutation may have been preferred since it has no effect on AZT susceptibility, whereas the Gln 161 Leu mutation would be counterselected (suppressed) because of AZT hypersensitivity when AZT is part of the treatment cocktail. Thus, the virus has chosen to follow other resistance patterns when put under combined drug pressure than when exposed to drug monotherapy.

A similar observation has been made for resistance development against nevirapine (and also pyridinone) in the presence or absence of concomitant AZT therapy [32, 131]. For example, monotherapy with nevirapine rapidly selects for Tyr 181 Cys mutations (79% of patients). But when AZT is given in combination with nevirapine, the Tyr 181 Cys mutation does appear at a much lower frequency (10% of patients). Instead, a variety of other NNRTI-characteristic resistance mutations occur such as Lys 103 Asn (57% of patients), Tyr 188 His (50% of patients), Gly 190 Ala (50% of patients), and Val 106 Ala (14% of patients) [32]. Thus, mutations such as Tyr 181 Cys (for nevirapine) or Gln 161 Leu (for PFA) are less likely to emerge under concomitant AZT selective pressure combined with NNRTIs or PFA, respectively. A third example is the concomitant 3TC + AZT drug treatment. As mentioned above, the 3TC-characteristic Met 184 Val mutation resensitizes virus that would contain the characteristic AZT mutations to AZT [93, 94]. However, double (AZT + 3TC) combination therapy results in the appearance of an entirely new and unexpected mutation at position 333 of the HIV-1 RT (Gly 333 Asp/Glu), leading to cross-resistance of such virus to both 3TC and AZT. However, it should be mentioned here that the Gly 333 Glu/Asp change has only been shown to occur in a minority of patients. With the majority of individuals who fail 3TC/AZT therapy, other factors that are not always well-defined seem to play a role. Thus, it has been proven that the appearance of certain mutations could be prevented or at least markedly suppressed in rationally designed drug combinations in the clinical setting, whereas the virus also seems to be able to relatively easily select for other combinations of mutations that allow it to escape the concomitant (i.e. AZT + nevirapine, AZT + PFA, or AZT + 3TC) drug pressure.

Whereas it becomes increasingly clear that HIV can eventually escape the combined drug pressure by selecting for other known or even novel mutations, it should be kept in mind that for certain drug combinations (i.e. AZT + 3TC) a more pronounced suppression of virus and delay of cross-resistance development has been observed than is obtained with single drug treatment, still arguing for the application of such rational drug combinations in the patient. Moreover, triple combination therapy in which NRTIs are combined with either an NNRTI or a protease inhibitor has been shown to be by far superior over double drug combination therapy, in suppressing both virus replication and (cross-)resistance development. Thus, although these current rational combinations of drugs do not seem to be able to eradicate or completely suppress virus replication, they are worth administering to an HIV-infected individual because of their superior suppression and delay of resistance development compared with monotherapy. Moreover, since it has been demonstrated that drug-resistant HIV-1 strains (harboring AZT-, 3TC-, protease inhibitor- or even

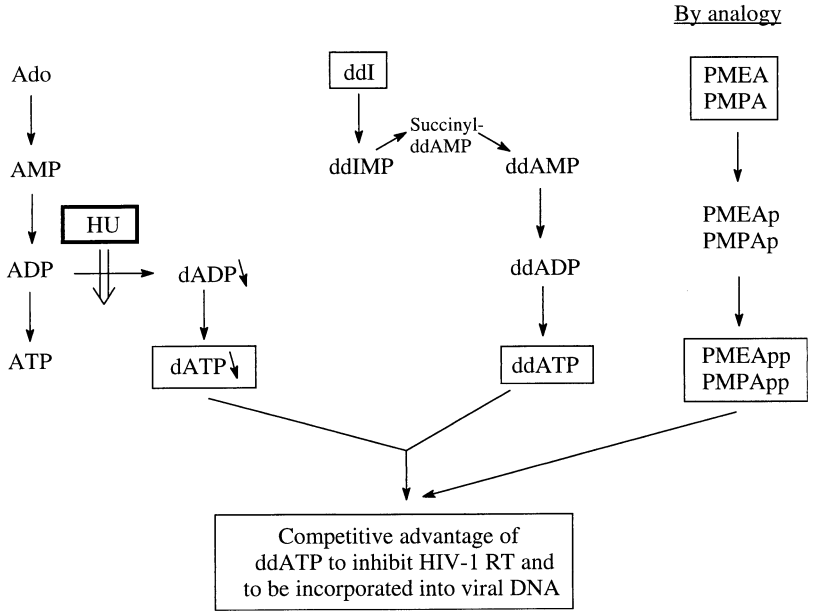

FIG. 6. Mechanism of HU potentiation of the antiviral activity of ddI.

multidrug-resistance mutations) can be transmitted to drug-naive individuals [172], careful attention should be given to the genetic and phenotypic analysis of the HIV strains present in such patients before choosing a specific drug therapy. The above-mentioned properties and interaction of resistance mutations should be taken into account before a specific patient-adapted treatment is initiated. Also, the transmission of drug-resistant virus strains to drug-naive individuals would urge for *in vitro* drug-resistance studies starting with virus strains that already contain AZT-characteristic or other NRTI-, ANP-, or NNRTI-characteristic mutations in their RT [173, 174] in an attempt to estimate or to predict the effect of additional mutations on the phenotypic resistance properties of such virus strains.

EFFECT OF ANTIMETABOLITES OF PURINE AND PYRIMIDINE NUCLEOTIDE METABOLISM ON THE ANTIVIRAL ACTIVITY OF NNRTIs AND NRTIs AND RELATED RESISTANCE DEVELOPMENT

It is now clear that HIV displays an enormous capacity to escape drug pressure by mutating its RT, even when combinations of several RT inhibitors with complementary resistance spectra are exposed to the virus. Therefore, additional strategies may be required to hit the virus even more efficiently and as much as possible in its replication capacities. One such strategy might be to target the host cells on which the virus is highly dependent for its replication, and on which a number of RT inhibitors, in particular NRTIs, depend for their conversion to the antivirally active metabolite. Although targeting of the host cells may lead to more severe (temporary?) side-effects, it may eventually prove necessary to explore more aggressive strategies by focusing on cellular targets, in addition to virus-specific targets, to efficiently beat the virus.

Since HIV does not encode for specific enzymes that activate or convert the NRTIs to the active (triphosphate) metabolites, the eventual conversion to the 2′,3′-dideoxynucleotide 5′-triphosphate (ddNTP) stage depends entirely on cellular enzymes and the metabolic machinery of the target cells. This property opens several perspectives for possible potentiation of the antiviral activity of NRTIs. Indeed, transient or sustained depletion of the endogenous purine or pyrimidine dNTP pools may favor the competitive effect of the ddNTPs with their natural counterparts for inhibition of RT and subsequent incorporation in the growing viral DNA chain. For example, AZT-TP or d4T-TP will be better incorporated into the growing viral DNA chain when the endogenous dTTP pools are lowered, whereas ddC-TP and 3TC-TP will more favorably inhibit the RT-catalyzed polymerisation reaction in the presence of lower dCTP pools. This principle explains the markedly higher activity of the purine dideoxynucleoside analogue ddA in HIV-1-infected monocytes/macrophages than in lymphocytes, since much lower (competing) levels of endogenous dATP pools exist in monocytes/macrophages relative to lymphocytes [175].

In Vitro *Studies with HU*

HU is probably the best known example among the antimetabolites that may afford lowered dNTP pools due to its ability to inhibit host cell ribonucleotide reductase (Fig. 6). HU has been combined with a variety of NRTIs, including ddI, ddC, and AZT [176–179]. The most marked antiviral potentiation was found for the combination of HU/ddI. Although at least two investigators have shown that HU has an anti-HIV effect in its own right in PBMC [180, 181], although only at relatively high (millimolar)

concentrations, the potentiating effect of HU against the NRTIs occurred at markedly lower concentrations (50–100 μM). These data illustrate that the observed effect of HU on ddI activity was not due to an intrinsic antiviral property of HU, but most likely to its indirect effect, namely, increasing the ddATP/dATP ratios to afford a better competitive effect of ddATP with dATP for inhibition of HIV RT. Indeed, it was shown that the dATP pool, although not the smallest of the four dNTP pools, was the most susceptible to depletion by HU [182]. For example, Palmer *et al.* [183] found > 10-fold and ~2.5-fold lower dATP and dGTP, but 25–50% increased dCTP and dTTP pools upon a 1 mM HU exposure of PBMCs than in the absence of HU. Due to the less pronounced decrease (or even increase) of the endogenous dTTP and dCTP pools, a much lesser potentiation by HU was found for AZT and ddC [176–178, 184–186]. The observed lack of drop of the pyrimidine nucleotide pools may be explained by the fact that the pyrimidine-2′-deoxynucleotide pools can be replenished relatively easily by their active salvage enzymes dThd kinase and dCyd kinase. The fact that there also exists an active HGPRT that can salvage guanine to GMP, which can then eventually be converted to GDP and to some extent to dGDP and dGTP through the action of the (inhibited) ribonucleotide reductase, can explain why the dGTP pools are decreased to a much lesser extent than the dATP pools. In the 1980s, Karlsson and co-workers [187] showed that HU (50–200 μM) increased phosphorylation of AZT and 3′-fluoro-2′,3′-dideoxythymidine (FLT) in CEM cells (K), and Palmer and Cox [188] found increased AZT-TP and 3TC-TP concentrations when AZT + 3TC were combined with HU. This can be explained by the fact that HU was shown to have a stimulatory effect on TK and dCK (the activating enzymes for AZT and d4T, and ddC and 3TC, respectively), since these enzymes are expressed at higher levels due to prolonged S-phase retention of the HU-exposed cells. It should be mentioned, however, that the HU effect on dTTP and dCTP levels may differ markedly from cell type to cell type and, thus, the degree of potentiation of the antiviral drugs may also differ from one cell type to another.

In Vivo *Studies with HU*

Clinical studies on the ddI/HU combination revealed a sustained reduction of the viral load in the majority of patients, but a lesser consistent improvement of CD_4^+-lymphocyte counts [189–191]. A number of patients developed a reversible leukopenia, likely due to the side-effects of HU at the 1 g/day dose level. Surprisingly, despite an observed decrease in the rate of virus replication, mutant virus containing the ddI-characteristic Leu 74 Val mutation developed more frequently in ddI/HU-treated patients than in patients receiving ddI alone [192, 193]. It may be speculated whether the imbalanced dNTP pools (due to decreased dATP levels) are responsible for an increased frequency of nucleotide substitution errors [182] and, thus, for the increased occurrence of the Leu 74 Val mutation. However, the cell culture studies of Palmer *et al.* [183] have shown that inhibition of Leu 74 Val mutated virus strains is also more pronounced in the presence of ddI + HU than in the absence of HU, thus counteracting, to some extent, the resistance of mutant virus against ddI as a single drug. As reported by Lori and colleagues at the recent Second Resistance Meeting at Lake Maggiori, Italy [194], HU/ddI-treated patients had a significant drop of plasma RNA after 40 weeks (1847 RNA copies/mL), and a further sustained decrease in plasma RNA levels after 122 weeks (254 RNA copies/mL). Remarkably, 6 of the 12 patients seemed to have a recovered CD4 proliferative response to p24, and this phenomenon, although we do not understand how it could have been caused by the prolonged HU/ddI treatment, may explain the lack of observed rebound of virus. Thus, the HU/ddI-treated patients consistently showed a progressive decrease of plasma viral load as a function of time, despite the presence of ddI-resistant mutants, and viraemia became even undetectable (< 500 copies/mL) in the majority of patients after prolonged treatment. Further studies to evaluate the long-term antiviral activity and efficacy of the ddI/HU combination are warranted in expanded clinical trials before firm conclusions on the potential beneficial efficacy of the combination of HU with ddI can be made. Johns and Gao pointed out in their overview on selective depletion of DNA precursors as a strategy for potentiation of NRTIs against HIV that the ddI/HU combination would not appear likely to replace the currently used triple combination therapy, due to the much higher response rate of the latter treatment modality [195]. However, in light of the most recent results, the addition of HU to the existing double and triple combination therapies may be warranted to further evaluate and determine its potential added value in the clinical setting. In particular, appearance of potential toxicity of long-term HU treatment should not be neglected, as shown by Milles *et al.* [196], who reported rather severe hematologic toxicity in d4T + 3TC + HU (500 mg BID)-treated patients with advanced disease. It remains also to be seen whether an HU-based combination therapy may preferentially be applied in special cases such as certain geographic areas where it is economically more feasible to be used (HU is a cheaper drug and easily made available at high quantities), or in cases where drug resistance or drug intolerability has appeared.

Strategies Other Than HU

Other strategies to increase the antiviral efficacy of NRTIs consist of a selective accumulation of endogenous ribo- or deoxyribonucleotide levels required as co-factors for the metabolic conversion (phosphorylation) of the NRTIs to their eventual active metabolite and/or of a stimulation of enzymes that are responsible for conversion of the NRTIs to their active ddNTP derivatives. These effects can be afforded by the addition of natural nucleosides (such as

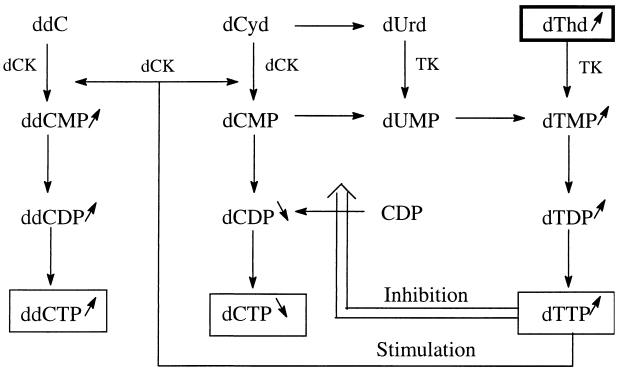

FIG. 7. Mechanism of dThd potentiation of the antiviral activity of ddC.

dThd) or antimetabolites of nucleotide metabolism (such as IMP-D inhibitors).

It was shown that high concentrations of thymidine enhanced the protective effect of ddC against HIV-1-infected ATH8 cells due to the combined action of a stimulation of ddC phosphorylation to ddCTP and decreased dCTP levels [197]. Indeed, dTTP, the active metabolite of dThd, feedback-inhibits ribonucleotide (CDP) reductase, resulting in lower endogenous dCTP levels. In turn, lowering the dCTP levels results in a stimulation of dCK, the activating enzyme of ddC. In addition, it has been shown that dTTP has a direct stimulatory effect on dCK as well (Fig. 7). One may assume that a similar phenomenon is expected to occur when 3TC is combined with dThd.

The metabolism (phosphorylation) and anti-HIV activity of d4T have been studied in combination with a variety of agents that lower the intracellular dTTP pools. Since TK (the putative enzyme that converts d4T to its monophosphate metabolite) is under feedback regulatory control by dTTP, lowering the intracellular dTTP pools may increase d4T phosphorylation on the one hand, and increase the competition of d4T-TP with the lowered dTTP pools for incorporation into the DNA during the RT-catalyzed polymerisation reaction on the other hand. Thymidylate synthase inhibitors [such as 5-fluoro-2′-deoxyuridine and methotrexate (through its inhibitory action against dihydrofolate reductase)] were shown to potentiate d4T phosphorylation and, consequently, also its anti-HIV-1 activity in PBMCs [198].

Ribavirin, an IMP-D inhibitor, enhances the anti-HIV activity of a variety of purine NRTIs including ddA, ddDAP, ddI, ddG, and FddA [199–204]. Ribavirin (and other IMP-D inhibitors such as EICAR, thiazofurin, selenazofurin, and mycophenolic acid) causes an increase in the levels of IMP, the preferred phosphate donor for the conversion of ddI to ddIMP by 5′-nucleotidase (Fig. 8) [205]. Consequently, ribavirin stimulates the conversion of ddI to its antivirally active metabolite ddATP. It should be recognized that the accumulation of the IMP pools does not result mainly from the direct inhibition of the IMP flow to XMP and GMP by ribavirin, but predominantly from the ribavirin-related decrease of GTP pools [202]. Indeed, GTP acts as an energy donor in the conversion of IMP to succinyl-AMP, and thus, lower GTP pools necessarily retard or stop the IMP → ATP flow, resulting in IMP accumulation. Unfortunately, this phenomenon also represents the biochemical basis for the limitations of this type of combination strategy. Any ddIMP formed by the increased IMP levels also needs to go through the same metabolic pathway as IMP, and thus (i) competition exists between higher IMP and higher ddIMP levels caused by ribavirin exposure, and (ii) due to GTP depletion, the eventual conversion of both IMP and ddIMP to ATP and ddATP, respectively, is affected. Consequently, this approach is self-limiting and will never result in a dramatic increase of the ddATP pools. Clinical trials have been performed with ribavirin and ddI, but the potential for an increased efficacy of such drug combination has never proven significant enough to justify follow-up studies.

One should also be cautious about combining ribavirin or other IMP-D inhibitors in the presence of pyrimidine NRTIs such as AZT or d4T. A reproducible antagonism between AZT and ribavirin was found to occur under a variety of experimental conditions [199, 206]. The underlying mechanism for this antagonism appeared to be inhibition of AZT phosphorylation by ribavirin, due to increased dTTP levels (caused by ribavirin) and subsequent feedback inhibition of TK, the activating enzyme for AZT

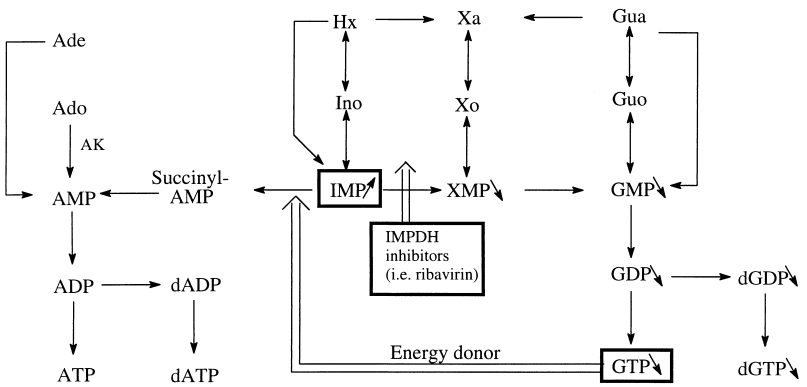

FIG. 8. Mechanism of ribavirin potentiation of the antiviral activity of ddI.

(and d4T). These observations make it clear that one should carefully investigate and rationally design combinations between antimetabolites and NNRTIs, because in some cases (i.e. AZT + ribavirin or d4T + ribavirin) adverse effects may be expected to occur.

Effect of dNTP Pool Imbalances on Resistance Development

Manipulating dNTP pools may not only have a beneficial effect on the potentiation of certain NRTIs, but it also has been shown recently to have a potential effect on shifting the resistance spectrum of NNRTIs and by analogy also of NRTIs and protease inhibitors [207]. It was shown that the continuous presence of dCyd + the deaminase inhibitors THU + dTHU in HIV-1-infected CEM cell cultures treated with the NNRTI TSAO shifted the TSAO-characteristic RT mutation Glu 138 Lys to the Glu 138 Gly mutation. Other antimetabolites such as 2′-deoxycoformycin, HU, and high dose dThd were not able to afford a similar effect. It was hypothesized that the increased ratios of intracellular dCTP/dTTP pools afforded by the treatment of TSAO-exposed HIV-1-infected cell cultures with dCyd + THU + dTHU forced the virus to shift its Glu (GAG) → Lys (AAG) mutation to a Glu (GAG) → Gly (GGG) mutation. This could be explained by an A → G transition mutation, combined with suppression of the G → A hypermutation as a result of the altered pyrimidine deoxynucleotide pool ratios. By analogy, it may be possible to shift the 3TC-characteristic resistance mutations from Met (ATG) → Val (GTG)/Ile (ATA) to Met (ATG) → Thr (ACG) in the presence of increased dGTP/dATP ratios. As discussed before, HU is able to afford such a shift in dNTP pools in PBMCs, and, thus, may be considered as a candidate compound to be combined with 3TC in the clinical setting after this concept has been proven in cell culture experiments. It is well-known that Met 184 Thr RT-mutated HIV-1 strains are highly attenuated and have a poor replication capacity compared with wild-type virus (~5%) [76]. Thus, it may be advantageous if a Met 184 Thr mutation could be selected under 3TC drug pressure instead of a Met 184 Val/Ile mutation. Although it has been observed that compensating mutations seem to occur relatively easily in mutated virus strains that contain resistance mutations against protease inhibitors, NRTIs, and NNRTIs, compromising the fitness of the mutant viruses, these drug resistance mutations proved to never directly affect the substrate-binding active site of the enzyme, but rather the template/primer positioning in the enzyme (as found for the majority of the NRTI mutations). In contrast, for the Met 184 Thr mutation, it has to be seen whether, and if so, how fast, compensatory mutations may occur to restore the replication competence of the mutant virus. It is rather unlikely that such a compensatory phenomenon will occur since it has never been noted thus far in 3TC-treated patients, in whom the appearance of Met 184 Ile/Val mutations in the HIV-1 RT results in a virus with lower replicative capacity and fitness than wild-type. The concept of influencing resistance development by creating endogenous dNTP imbalances is novel and should be further explored for its potential usefulness in the clinic.

In conclusion, a variety of combinations of NRTIs with antimetabolites has been proven to increase the antiviral efficacy of NRTIs. However, thus far, the effects have never been dramatic enough to prefer such combinations over the triple combination therapies currently used in humans and leading to a pronounced long-term suppression of virus replication. The additional benefit that inclusion of an antimetabolite in an already existing cocktail of drugs may afford is expected in most cases to be inferior to replacing the antimetabolite by an antiviral drug that directly interferes with the replication cycle of the virus. In addition, antimetabolites usually afford their perturbing effects on cellular nucleotide metabolism at concentrations that will be relatively close to the toxicity threshold, and thus the therapeutic window in which these type of compounds may be used will always be expected to be rather narrow. However, the recently described beneficial effects of HU/ddI in HIV-infected individuals and the findings that the resistance spectra of drugs may be altered in the presence of antimetabolites of nucleotide metabolism urge careful consideration and therapeutic exploration of the potential of these tools to find a place in antiretroviral combination therapy.

DOES A MUTATIONAL INCREASE OF THE FIDELITY OF HIV RT REPRESENT A USEFUL STRATEGY TO LOWER THE MUTATION RATE OF THE ENZYME (VIRUS) AND SUBSEQUENT RESISTANCE DEVELOPMENT OF THE VIRUS?

It has been hypothesized that increased RT fidelity may account for the lower emergence of virus variants in patients treated with 3TC, and for any delay of further resistance development against other NRTIs, NNRTIs, and protease inhibitors [i.e. generation of double-mutant (resistant) viruses] [208]. If such a hypothesis is viable, one would then assume that it may be advantageous to select for Met 184 Val mutant virus strains (being endowed with a mutant RT enzyme with higher fidelity than wild-type virus) in a patient, prior to starting a treatment regimen with other drugs. However, this hypothesis has been tested by Preston [209] in a relatively simple mathematical model of virus population dynamics, showing that the delayed variation of 3TC-resistant HIV-1 is likely the result of a decreased relative fitness of the virus rather than a decreased mutation rate due to an enhanced fidelity. Furthermore, standardized mutation assays using M13 templates have shown that the increased fidelity of the Met 184 Val enzyme does not result in a significant reduction of the overall error rate of this mutant enzyme [210]. Given the enormous virus replication dynamics, virus plasma load, and virus turnover *in vivo* [211–213], it is expected that mutations that may arise during such an intensive virus replication may easily

counteract any potential decreased mutation rate that results from the higher fidelity of RT. Moreover, several investigators have been able to show that 3TC-resistant virus strains harboring the Met 184 Val mutation easily mutate and select for double-resistant virus strains when put under NRTI or NNRTI pressure [10, 214, 215]. The speed of emergence of these double mutant viruses proved not to be significantly different from that of wild-type viruses exposed to the same NRTIs and NNRTIs. In these experiments it was also shown that the 3TC-resistant virus kept the Met 184 Val mutation when exposed to NNRTIs even in the absence of 3TC. These observations also proved that the concomitant presence of the Met 184 Val mutation and a variety of NNRTI-specific mutations in the HIV-1 RT are compatible and do not markedly affect the fitness and replication competence of the double mutant viruses. Also, the nature of the mutations that were added to the Met 184 Val genetic background under NNRTI treatment did not differ from those that were expected to appear in wild-type virus cultures under the selective pressure of the same NNRTIs.

Thus, the available data caution against strategies aimed at the accumulation of drug resistance mutations in the HIV-1 RT genome through the administration of single compounds in a sequential therapeutic treatment schedule even if the mutations increase fidelity of the RT. Such sequentially acquired mutations would make the virus not only easily resistant, but also highly (cross-)resistant to multiple drugs. Instead, the available observations strongly argue for the use of a combination of different HIV inhibitors such as 3TC and NNRTIs to suppress virus replication and to delay the emergence of drug-resistant virus in HIV-1-infected individuals due to the appearance of mutations in the HIV RT. The increased fidelity of certain mutant virus strains will not prevent continuing resistance development in the presence of any drug pressure.

CONCLUSION

Emergence of HIV drug resistance and the need for long-term treatment modalities are currently the main causes for the failure of antiretroviral therapy. Compared with the fast appearance of drug resistance mutations under monotherapy, the virus can be markedly suppressed for a relatively long period of time when exposed to multiple drug combination therapy (designated highly active antiretroviral therapy or HAART). However, the virus still has been shown to keep the capacity to replicate slowly in certain body compartments, thus keeping its ability to mutate and eventually escape the drug pressure. A few years ago, it was suggested that the different viral pools in the body (i.e. $CD4^+$ lymphocytes, tissue macrophages, follicular dendritic cells in lymphoid tissues) could be eliminated if effective treatment would be continued for 2–4 years, thereby raising the possibility of eradication of the virus [216, 217]. Nowadays, this view has been proven to be overly optimistic and unrealistic, since recent studies have shown that infectious HIV-1 persists latently in resting, memory CD4 lymphocytes in a post-integrated form despite 1–2 years of combination therapy [218, 219]. This latent reservoir of HIV-1 may represent a major hurdle to virus eradications due to the estimated long decay (~10 years) of these resting memory $CD4^+$ lymphocytes. Thus, even in people whose viral load is "undetectable," the virus continues to hide in cells throughout the body. Moreover, other obstacles such as viral sanctuaries may exist as well in the body out of reach of antiviral drugs, and have to be taken into account as an additional factor that may eventually lower the efficiency of the currently available drug cocktails. Consequently, it is important to realize that the existing armamentarium of drugs and treatment modalities is clearly not sufficient to keep long-term control of the virus replication. Whereas it is clear and evident that HIV-infected individuals need to be treated by concomitant multiple drugs at the highest possible dose to maintain a long-term control of the infection, the drug cocktails need to be carefully designed as to afford the most optimal treatment modality, dealing with and anticipating the nature of the resistance mutations that may be expected to occur, and the genetic (resistance) background that may already exist in an increasing number of patients. Given also the side-effects that seem to occur with several drugs (i.e. HIV protease inhibitors) upon long-term treatment of HIV-infected individuals, efforts must be continued to search for new drugs, not only directed against the RT and the virus-specified protease, but also against other targets of the virus replication and of the cellular machinery, to broaden our possibilities to suppress the virus more efficiently and to allow effective treatment shifts to other drug cocktails when the first-line combination treatments eventually fail.

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