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COMMENTARY

HIV RESISTANCE TO REVERSE TRANSCRIPTASE INHIBITORS

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Key words: human immunodeficiency virus, reverse transcriptase inhibitors, virus-drug resistance, dideoxynucleosides, non-nucleoside reverse transcriptase inhibitors, combination chemotherapy

Human immunodeficiency virus (HIV)† inhibitors targeted at the virus-associated reverse transcriptase (RT) can be divided into two groups, depending on whether they interact with the substrate or non-substrate binding site [1]. To the first group belong the 2',3'-dideoxynucleosides (ddNs), i.e. AZT, DDC, DDI, D4T, 3TC and FTC (Fig. 1), and also the acyclic nucleoside phosphonates (ANPs) [2], i.e. 9-(2-phosphonylmethoxyethyl)adenine (PMEA) [3], 9-(3-fluoro-phosphonylmethoxypropyl)adenine (FPMPA) [4] and 9-(2-phosphonylmethoxypropyl)-adenine (PMPA) [5]. What all these compounds have in common is that they need to be phosphorylated intracellularly to their triphosphate forms (for which the ddNs and ANPs take three or two phosphorylation steps, respectively) before they can act, at the RT level, as competitive inhibitors or alternate substrates (chain terminators) with respect to the natural substrates (dNTPs). That HIV can develop resistance to the various 2',3'-dideoxynucleoside analogues (AZT, DDC, DDI, D4T, 3TC and FTC) has been well documented (see *infra*). Yet, little, if any, evidence has been forthcoming on HIV resistance to the acyclic nucleoside phosphonates.

The discovery of the 1-(2-hydroxyethoxymethyl)-6-(phenylthio)thymine (HEPT) [6, 7] and tetrahydroimidazo[4,5,1-*jk*][1,4]-benzodiazepin-2(1*H*)-one and -thione (TIBO) [8, 9] derivatives as highly HIV-1-specific inhibitors heralded a new era of antiviral agents, viz. that of the non-nucleoside reverse transcriptase inhibitors (NNRTIs). These HIV-1-specific NNRTIs are highly potent inhibitors of HIV-1 but not of HIV-2 or any other (retro)viruses. They do not require any intracellular conversion but are able to interact directly with their target enzyme, HIV-1 RT, at an allosteric (non-substrate binding) site. Following HEPT and TIBO, various other HIV-1-specific RT inhibitors were described (Fig.

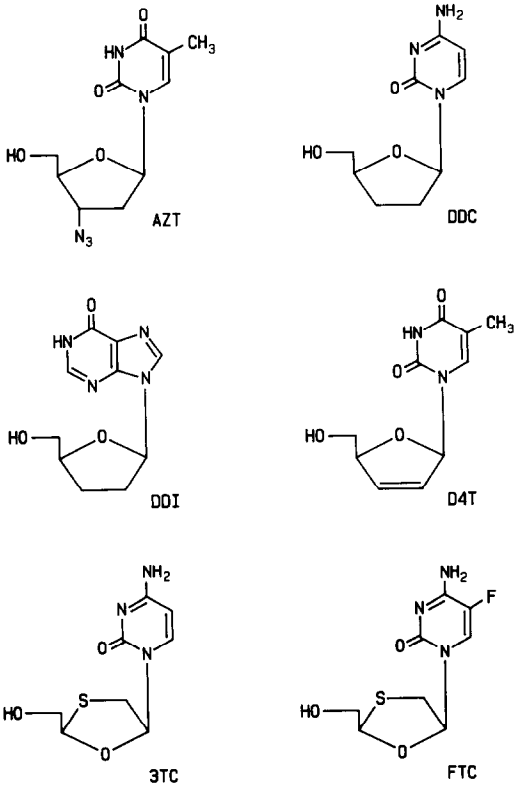

Fig. 1. 2',3'-Dideoxynucleoside (ddN) analogues: 3'-azido-2',3'-dideoxythymidine (AZT), 2',3'-dideoxycytidine (DDC), 2',3'-dideoxyinosine (DDI), 2',3'-didehydro-2',3'-dideoxythymidine (D4T), (−)-2',3'-dideoxy-3'-thiacytidine (3TC) and (−)-2',3'-dideoxy-5-fluoro-3'-thiacytidine (FTC).

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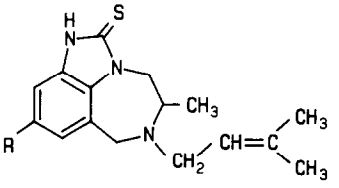
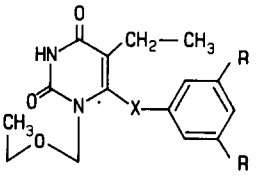
† Abbreviations: HIV, human immunodeficiency virus; RT, reverse transcriptase; ddNs, 2',3'-dideoxynucleosides; ANPs, acyclic nucleoside phosphonates; NNRTIs, non-nucleoside reverse transcriptase inhibitors; AZT, 3'-azido-2',3'-dideoxythymidine; DDC, 2',3'-dideoxycytidine; DDI, 2',3'-dideoxyinosine; D4T, 2',3'-didehydro-2',3'-dideoxythymidine; 3TC, (−)-2',3'-dideoxy-3'-thiacytidine; FTC, (−)-2',3'-dideoxy-5-fluoro-3'-thiacytidine; TIBO, tetrahydroimidazobenzodiazepinone; HEPT, hydroxyethoxymethylphenylthiothymine; Nev, nevirapine; Pyr, pyridinone; BHAP, bis(heteroaryl)piperazine; TSAO, *tert*-butyldimethylsilylspiroaminooxathioledioxide; α-APA, α-anilinophenylacetamide; and PETT, phenylethylthioureathiazole.



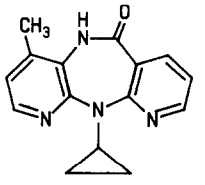
TIBO

R=H : R82150
R=CL : R82913

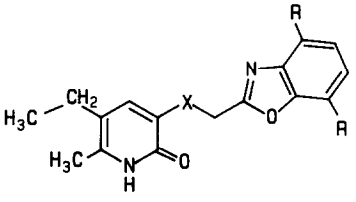


HEPT

X=S R=H : E-EPU
X=CH_2 R=H : E-EBU
X=CH_2 R=CH_3 : E-EBU-dM

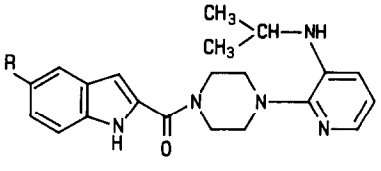


Nevirapine (BI-RG-587)



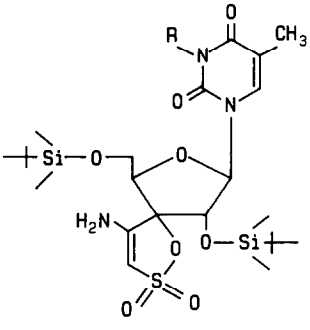
Pyridinone

X=CH_2 R=H : L-696, 229
X=NH R=Cl : L-697, 661



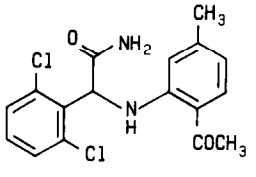
BHAP

R=H : U-88204
R=$NHSO_2CH_3$: U-90152

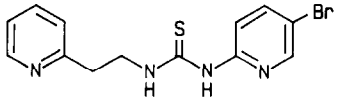


TSAO

R=H : TSAO-T
R=CH_3 : TSAO-m^3T

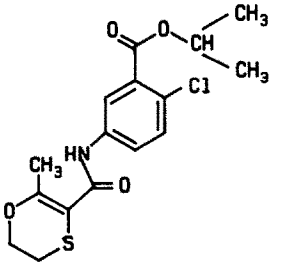


α-APA R89439

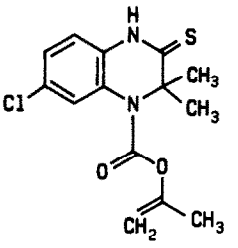


PETT : LY300046

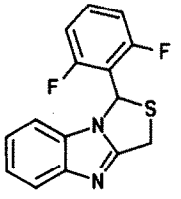
Fig. 2. HIV-1-specific non-nucleoside reverse transcriptase inhibitors (NNRTIs): tetrahydroimidazobenzodiazepinone (TIBO), hydroxyethoxymethylphenylthiothymine (HEPT), dipyridodiazepinone (i.e. nevirapine), pyridinone (i.e. L-696,229), bis(heteroaryl)-piperazine (BHAP), *tert*-butyldimethylsilylspiroaminooxathioledioxide (TSAO), α-anilinophenylacetamide (α-APA), phenylethylthioureathiazole (PETT).



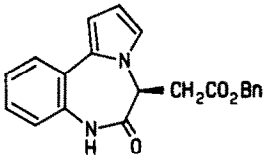
UNIROYAL
(oxathiin carboxanilide)



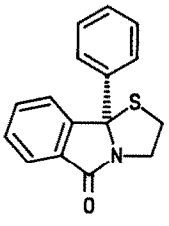
6-chloro-3, 3-dimethyl-4- (isopropenyl-oxycarbonyl) -3, 4-dihydroquinoxalin-2 (1H) -thione (S-2720)



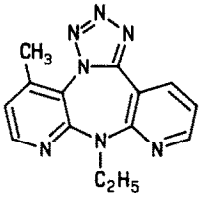
Thiazolobenzimidazole
(NSC 625487)



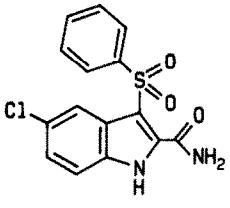
Pyrrolobenzodiazepinone



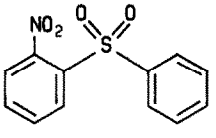
Phenylthiazoloisoindolone



Imidazodipyridodiazepine



5-chloro-3- (phenylsulfonyl) - indole-2-carboxamide
(L-737, 126)



Nitrophenylphenylsulfone
(NPPS)

Fig. 2. (continued). HIV-1-specific non-nucleoside reverse transcriptase inhibitors (NNRTIs): oxathiin carboxanilide (UNIROYAL), 6-chloro-3,3-dimethyl-4-(isopropenyloxycarbonyl)-3,4-dihydroquinoxalin-2(1*H*)-thione (S-2720), thiazolobenzimidazole (NSC625487), pyrrolobenzodiazepinone, 9b-phenyl-2,3-dihydrothiazolo[2,3-*a*]-isoindol-5(9b*H*)-one (phenylthiazoloisoindolone), imidazodipyridodiazepine, 5-chloro-3-(phenylsulfonyl)indole-2-carboxamide (L-737,126) and nitrophenylphenylsulfone (NPPS).

2), i.e. nevirapine (BI-RG-587) [10], pyridinone (L-696,229 and L-697,661) [11, 12], bis(heteroaryl)-piperazine (BHAP) (U-88204 and U-90152) [13, 14], 2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-3′-spiro-5′-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (TSAO) derivatives (TSAO-T, TSAO-m^{3}T) [15, 16], α-anilinophenylacetamide (α-APA) [17], phenylethylthioureathiazole (PETT) [18], 2-nitrophenylphenylsulfone (NPPS) [19], thiazolobenzimidazole [20], oxathiin carboxanilide (UNIROYAL) [21], 5-chloro-3-(phenylsulfonyl)indole-2-carboxamide [22], pyrrolo[1,2-d]-(1,4)-benzodiazepin-6-ones [23], imidazo[2′,3′:6,5]dipyrido-[3,2-b:2′,3′-e]-1,4-diazepines [24], 2,3-dihydrothiazolo[2,3-*a*]-isoindol-5(9b*H*)-ones [25] and quinoxaline derivatives [26]. Starting from HEPT, various new derivatives (i.e. E-EPU, E-EBU, E-EBU-dM) were prepared which excelled the parent compound in both potency and selectivity [27–31]. As a rule, all these compounds behave as allosteric inhibitors of HIV-1 reverse transcriptase [32–35]. They interact with a non-substrate binding site, which, for nevirapine, has been characterized as the pocket flanked by the tyrosine residues at positions 181 and 188 [36–39].

If, as a rule, the more specific an antiviral compound is in its antiviral action, the faster it should result in the development of virus-drug resistance, then the highly specific HIV-1 RT inhibitors may be expected to lead to the rapid emergence of drug-resistance HIV strains [40]. In fact, emergence of HIV mutant strains resistant to the HIV-1-specific NNRTIs should not come as a surprise, since, by definition, NNRTIs are active only against HIV-1 and not HIV-2, and thus HIV-2 may be considered as an *ab initio* NNRTI-resistant mutant.

It should be pointed out here that "resistance" is defined as reduced sensitivity to the antiviral action of the compound, as reflected by a significant (≥10-fold) increase in the EC_{50} (50% antivirally effective concentration). The implications of such an increase in EC_{50} are different from one compound to another, as the EC_{50} for the wild-type HIV-1 can range from nanomolar concentrations (e.g. AZT, TIBO, HEPT) to micromolar concentrations (e.g. DDI). Thus, a ≥10-fold increase in EC_{50}, while invariably interpreted as "resistance," has different consequences for the more potent compounds than for the less potent compounds. For the more potent compounds (e.g. AZT) it means that "resistant" virus can still be inhibited by drug concentrations (i.e. 1 μM) that are therapeutically attainable in human plasma, whereas for the less potent compounds (e.g. DDI) this is no longer the case.

HIV resistance to 2′,3′-dideoxynucleoside analogues

Resistance (i.e. reduced sensitivity) of HIV to the ddNs develops following prolonged therapy of HIV-infected individuals with these drugs, as first shown for AZT [41], and later with DDI [42] and DDC (for a review, see Ref. 43). Drug-resistant variants of HIV-1 have also been generated by *in vitro* passage of the virus in the presence of increasing concentrations of AZT, DDI, DDC or 3TC [44–46]. The mutations responsible for HIV-1 resistance to AZT have been identified at amino acid positions 41 (Met→Leu), 67 (Asp→Asn), 70 (Lys→Arg), 215 (Thr→Phe/Tyr) and 219 (Lys→Gln) of the HIV-1 reverse transcriptase [47, 48]. HIV-1 resistance to DDI is associated with Leu→Val substitution at position 74 [42], HIV-1 resistance to DDC is associated with Thr→Asp substitution at position 69 [49], and HIV-1 resistance to 3TC or FTC is associated with Met→Val substitution at position 184 [50]. The latter mutation confers high-level resistance (i.e. 1000-fold decreased sensitivity) to 3TC and FTC and low-level resistance (i.e. 4- to 8-fold decreased sensitivity) to DDI and DDC [46, 50, 51]. As mentioned above, the mutation Thr→Tyr at position 215 confers AZT resistance; further mutation of Tyr→Cys at this position would confer resistance to DDC [52]. Finally, the mutation Val→Thr at position 75 has been linked with resistance to D4T [53]. The RT mutations known to be involved in conferring resistance to the ddN analogues are listed in Table 1. In addition to these mutations, other mutations in the HIV-1 RT have been postulated to confer resistance, particularly to AZT [54, 55].

HIV-1 resistance to AZT would seem to develop in an orderly fashion [56–58] (Fig. 3). Mutation at codon 70 commonly occurs first during AZT

Table 1. Mutations in the HIV-1 RT gene conferring resistance to 2′,3′-dideoxynucleoside analogues

Amino acid number	Mutation		Compound(s)	References
	Codon	Amino acid		
41	ATG→TTG	Met→Leu	AZT	[48]
67	GAC→AAC	Asp→Asn	AZT	[47]
70	AAG→AGG	Lys→Arg	AZT	[47]
215	ACC→TTC	Thr→Phe	AZT	[47]
215	ACC→TAC	Thr→Tyr	AZT	[47]
219	AAG→CAG	Lys→Gln	AZT	[47]
74	TTA→GTA	Leu→Val	DDI	[42]
69	ACC→GAC	Thr→Asp	DDC	[49]
215	TAC→TGC	Tyr→Cys	DDC	[52]
75	GTA→ACA	Val→Thr	D4T	[53]
184	ATG→GTG	Met→Val	3T3, FTC	[50, 51]
			DDI, DDC	[46]

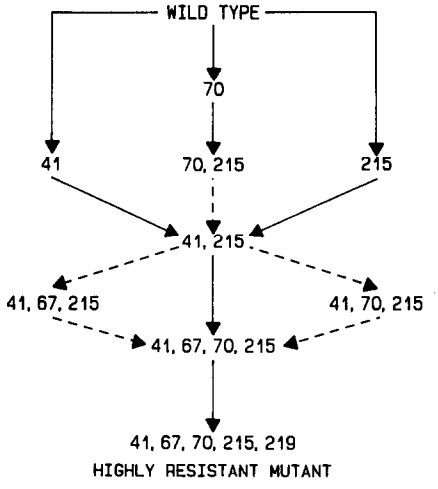


Fig. 3. Model for the sequential development of HIV-1 resistance to AZT, based on the mutations in the five codons: 41 (Met→Leu), 67 (Asp→Asn), 70 (Lys→Arg), 215 (Thr→Tyr) and 219 (Lys→Gln). According to C. A. B. Boucher ["Characterization of human immunodeficiency viruses during zidovudine treatment", thesis, University of Amsterdam, The Netherlands (1993)]. The model is based on a longitudinal study of HIV isolates obtained from 18 HIV-positive subjects [57].

treatment of HIV-1 positive symptom-free individuals, but is then replaced by a more stable mutation at codon 215. Upon prolonged treatment, other mutations (i.e. at codons 41, 67 and, again 70) join in so that the virus acquires increased resistance to AZT. This seems to occur only after progression to disease, as no highly resistant virus could be isolated from asymptomatic individuals [57].

HIV resistance to HIV-1-specific non-nucleoside reverse transcriptase inhibitors

Shortly after the first HIV-1-specific NNRTIs were described, it became evident that these compounds can promptly lead to the emergence of drug-resistant virus mutants upon passage of HIV-1 in the presence of the compounds. This was first shown for the pyridinones (Pyr) [58] and, subsequently, also revealed for nevirapine (Nev) [59, 60], TIBO [60–62], TSAO [62, 63], HEPT [64], BHAP [65], PETT [66] and quinoxaline [67]. In fact, given their specificity as HIV-1 RT inhibitors, all NNRTIs may be expected to be able to induce virus-drug resistance development. The amino acid substitutions within the HIV-1 RT that are responsible for this resistance are primarily the following: 100 Leu→Ile for TIBO; 103 Lys→Asn for Pyr; 106 Val→Ala for Nev; 138 Glu→Lys for TSAO; 181 Tyr→Cys for TIBO, HEPT, Nev, Pyr, α-APA; 188 Tyr→His for TIBO, HEPT, Pyr; 188 Tyr→Cys for Nev, Pyr; and 236 Pro→Leu for BHAP (Table 2).

Mutations at the HIV-1 RT positions 100, 101, 103, 118, 138, 179, 181, 188, 230 and 241 were observed during the development of HIV-1 resistance to PETT in cell culture [66]. Drug-resistant virus strains emerging upon passage of HIV-1 in the presence of the NNRTIs in cell culture may seem predictive of the mutations that could arise in the clinic, in patients treated with the NNRTIs. For example, following clinical trials with nevirapine, mutations of at least eight amino acid residues (No. 98, 100, 103, 106, 108, 181, 188, 190) of the HIV-1 RT were noted [70]. Most of these mutations had also been found *in vitro* following passage of the virus in the presence of nevirapine (Table 2).

The role of the amino acid residues at positions 100, 103, 106, 138, 181, 188 and 236 of the HIV-1 RT in the sensitivity/resistance of the virus to TIBO, HEPT, Nev, Pyr, TSAO and BHAP has been confirmed by site-directed mutagenesis, which can unequivocally link a single point mutation with the emergence of virus-drug resistance [38, 73–75]. These studies have revealed that TIBO, Nev and Pyr behave as a functional equivalent group with regard to their interaction with the amino acid residues 103, 181 and 188. Substitution of Asn for Lys at position 103 invariably leads to a decreased sensitivity of HIV-1 RT to all three classes of compounds, and the enzyme becomes totally refractory to the compounds if, in addition to the mutation at position 103, the tyrosine residues at

Table 2. Mutations in the HIV-1 RT gene conferring resistance to HIV-1-specific non-nucleoside reverse transcriptase inhibitors

Amino acid number	Mutation		Compound(s)	References
	Codon	Amino acid		
98	GCA→GGA	Ala→Gly	TIBO, Nev, Pyr	[78]
100	CTA→ATA	Leu→Ile	TIBO, Nev, Pyr, BHAP	[61, 62, 68, 69, 78]
103	AAG→AAC	Lys→Asn	TIBO, Nev, Pyr, BHAP	[22, 58, 62, 68, 78]
106	GTA→GCA	Val→Ala	HEPT, Nev	[64, 70, 71, 78]
108	GTC→ATC	Val→Ile	Nev, Pyr	[70, 71]
138	GAG→AAG	Glu→Lys	TSAO	[62, 63, 68, 69]
179	GTT→GAT	Val→Asp	TIBO, Pyr	[69, 72]
181	TAT→TGT	Tyr→Cys	TIBO, HEPT, Nev, Pyr, BHAP, α-APA	[17, 22, 58–60, 62, 64, 68, 73, 74]
188	TAT→CAT	Tyr→His	TIBO, HEPT, Pyr	[62, 64, 74]
	TAT→TGT	Tyr→Cys	TIBO, Nev, Pyr	[74, 78]
190	GGG→GAG	Gly→Glu	Quinoxaline	[26, 67]
236	CCC→CTC	Pro→Leu	BHAP	[65]

position 181 and/or 188 are mutated to cysteine [74].

Whereas substitution of Ile for Tyr 181, or Leu for Tyr 188 (i.e. the HIV-2 counterparts) annihilates the sensitivity of HIV-1 RT to TIBO, Nev and Pyr, substitution of Phe for Tyr at either position 181 or 188 had no influence on the drug sensitivity of HIV-1 RT. Substitution of Cys, Ser or His for Tyr at either position 181 or 188 results in a decreased sensitivity to all three compounds, except for the 188 Tyr→His substitution which does not appear to affect the sensitivity of HIV-1 RT to nevirapine [74].

Cross-resistance among different HIV-1-specific non-nucleoside reverse transcriptase inhibitors

The structure of the HIV-1 reverse transcriptase, complexed with either nevirapine [39] or a dsRNA template/primer and Fab fragment [76] has been solved at a 3.5 Å and 3 Å resolution, respectively. The locations of the ddN-resistance mutations (41/αA; 67, 69 and 70/β3-β4 connecting loop; 74/β4; 184/β9-β10 connecting loop; 215/β11a; and 219/β11b) as well as NNRTI-resistance mutations (100 and 103/β5-β6 connecting loop; 106 and 108/β6; 138/β7-β8 connecting loop; 181/β9; 188/β10; and 236/β13-β14 reverse turn) have been assigned. All these mutations are located in the p66 subunit of the p66/p51 RT heterodimer, except for the 138 Glu→Lys mutation conferring resistance to the TSAO compounds, which appears to be located in the p51 subunit [77]. However, this mutation, even if located in p51, is likely to participate in the non-nucleoside binding pocket at p66 [39, 40], due to its proximity to this pocket [77].

To the extent that the different mutations involved in resistance to the NNRTIs affect their binding to this non-nucleoside binding pocket, or at least affect the conformation of this pocket, cross-resistance may be expected among the different NNRTIs. Cross-resistance among most of HIV-1-specific RT inhibitors has indeed been observed if Tyr at position 181 is altered to Cys [17, 58–60, 62, 64, 68, 73, 74], but, for most of the other mutations, resistance is generally limited to one, two or three classes of the HIV-1-specific RT inhibitors (Table 2). If Glu 138 is mutated to Lys, only resistance to TSAO, and not to any other NNRTI, is seen [63]. This may be attributed to the fact that the TSAO compounds, unlike all other NNRTIs, specifically interact, probably via the 4″-amino group of the 3′-spiro substituent, with the carboxylic acid group of Glu 138 [75] of the p51 subunit [77] of HIV-1 RT.

Other amino acid substitutions, i.e. 100 Leu→Ile and 103 Lys→Asn, lead to resistance to TIBO but not HEPT [62, 64]. The 106 Val→Ala substitution confers resistance to nevirapine but not pyridinone [78]; it also confers resistance to TIBO but much less so than to nevirapine [78]. Also, α-APA is active against the TIBO-resistant 100 Leu→Ile mutant, while virtually inactive against the TIBO-resistant 181 Tyr→Cys mutant [17]. Quinoxaline is active against TIBO (R82150)-resistant virus [67], and PETT derivatives are active against TIBO-, TSAO-, Nev- and Pyr-resistant virus strains containing either the 100 Leu→Ile, 138 Glu→Arg, 181 Tyr→Cys or 188 Tyr→His mutation [79, 80]. The fact that PETT (LY300046) would be inhibitory to the 181 Tyr→Cys mutant [80] is of considerable interest, as this mutant is resistant to most other NNRTIs (Table 2).

Switching among different HIV-1-specific non-nucleoside reverse transcriptase inhibitors

The availability of so many different classes of HIV-1-specific RT inhibitors to which the virus may have retained (even if having acquired resistance to some of these inhibitors) is reassuring in terms of their chemotherapeutic potential for the treatment of HIV infections. Should resistance have arisen against one of the NNRTI classes, treatment could be readily switched to any of the other NNRTIs to which the virus has retained sensitivity.

Take, for example, 5-chloro-3-(phenylsulfonyl)-indole-2-carboxamide [22], which is still active against those HIV-1 strains that, because of the 103 Lys→Asn or 181 Tyr→Cys mutation, have acquired resistance to other HIV-1-specific RT inhibitors (i.e. TIBO, Nev, Pyr, BHAP). Admittedly, the (phenylsulfonyl)indole is less active against the mutant RTs than against the wild-type enzyme, but it is as potent against the mutant enzyme as AZT 5′-triphosphate [22].

Within a given class of HIV-1-specific RT inhibitors relatively minor modifications may improve, or even restore, activity against mutant HIV strains that have become resistant to the parent compound. Following this strategy, it is possible to identify new congeners within the different NNRTI classes (i.e. TIBO, HEPT, Nev, Pyr, BHAP), which are significantly more active against the mutant strains than the parent compounds: i.e. the new pyridinone congener L-702,019 (which differs from its parent L-696,229 only by the substitutions of two chlorine atoms for hydrogen in the benzene ring, and sulfur for oxygen in the pyridine ring) is markedly inhibitory to the HIV-1 mutants containing the 103 Lys→Asn or 181 Tyr→Cys mutation [81].

In attempts to circumvent the problem of virus-drug resistance, one can switch from one NNRTI to another, but also from one ddN to another, and, likewise, from ddNs to NNRTIs and back to ddNs. In several cases it has been observed that when the virus becomes resistant to one of the RT inhibitors, it not only remains sensitive to others, but may even acquire increased sensitivity to some of these other RT inhibitors. For example, the 236 Pro→Leu mutation causing resistance to BHAP increases by 10-fold the RT sensitivity to TIBO, nevirapine and pyridinone [65]. Similarly, AZT-resistant HIV strains (based on the mutations at positions 215 and 219 of the RT), when acquiring resistance to DDI (based on the 74 mutation), may regain sensitivity to AZT [42]. Yet, it should be noted that the suppressive effect of the 74 Leu→Val mutation on AZT resistance of HIV does not occur in all genetic contexts [82].

The 181 Tyr→Cys mutation that confers resistance to TIBO and most other NNRTIs (Table 2) suppresses resistance to AZT, so that AZT-resistant HIV strains (based on the 215 mutation) may regain sensitivity to AZT upon acquiring the 181 Tyr→Cys mutation [83]. This phenomenon may also hold *in*

vivo: in a patient, the appearance of the 181 Tyr→Cys mutation (following treatment with α-APA) was associated with reversal of AZT resistance [84]. These data thus point to the feasibility of switching from one RT inhibitor (whether NNRTI or ddN) to another, as a strategy to circumvent the virus-drug resistance problem.

Combination chemotherapy as an approach to circumvent virus-drug resistance

The most common strategy that has been envisaged to prevent virus-drug resistance from arising is based on the combined use of several anti-HIV drugs. The choice of the compounds to be used in combination should depend on the location of their resistance mutations. Hence, those compounds should be combined that give rise to different mutations, which, when combined, would suppress emergence of resistance to one another (Fig. 4).

Thus, the combination of AZT with DDI has been advocated, not only to prevent emergence of virus-drug resistance but also to exploit the (possible) synergism in the anti-HIV action and to reduce toxicity of the individual compounds. In patients, the combination of AZT with DDI would select against DDI resistance (74 Leu→Val mutation) [85], but does not prevent the emergence of HIV isolates with AZT resistance [86]. Yet, *in vitro* combination of AZT with DDI would prevent appearance of AZT-resistant HIV strains [87].

Recently, T. C. Merigan reported (at the Annual Meeting of the Laboratory of Tumor Cell Biology, National Cancer Institute, Bethesda, MD, 22–28 August 1993) that the combination of AZT with DDI seems to lead over a prolonged time period in patients to the emergence of five new mutations, namely at positions 151 (Gln→Met), 62 (Ala→Val), 75 (Val→Ile), 77 (Phe→Leu), and 116 (Phe→Tyr). The role of these mutations in the development of resistance to AZT, DDI and/or other drugs remains to be ascertained.

Another combination worth pursuing from a clinical viewpoint is that of DDI with ribavirin [88]. Ribavirin has been shown to potentiate the anti-HIV activity of DDI [89], essentially via IMP dehydrogenase inhibition, which leads, on the one hand, to an accumulation of IMP and increased phosphorylation of DDI, and, on the other hand, to a shut-off in the supply of dATP, the competitive substrate in the inhibitory action of ddATP (the active metabolite of DDI) at the HIV RT level [90]. As has been shown recently [91], ribavirin, when combined with DDI, restores its activity against DDI-resistant HIV strains.

According to some preliminary reports [70, 92], combination of AZT with nevirapine (whether simultaneous or alternating) would not prevent development of HIV resistance to nevirapine. However, other studies indicate that emergence of resistance to pyridinone (L-697,661) is prevented or delayed in patients receiving concomitant AZT [93, 94]. Also, D. D. Richman reported at the 1993 Annual Meeting of the Laboratory of Tumor Cell Biology (see above) that AZT, when combined with nevirapine, shuts off the emergence in patients of the 181 Tyr→Cys mutation, which is the mutation that arises most frequently if nevirapine is used only. From these data (concerning the impact of AZT on pyridinone or nevirapine resistance), as well as those mentioned above (concerning the impact of the 181 Tyr→Cys mutation on AZT resistance) [83, 84], it would appear that the AZT resistance mutation(s) (i.e. at position 215) and the pyridinone (or nevirapine) resistance mutation at position 181 (Tyr→Cys) are mutually suppressive.

Also, the 184 Met→Val mutation conferring resistance to 3TC, FTC, DDI and DDC (Table 1) has a suppressive effect on AZT resistance; and, if the 184 Met→Val mutation is accompanied by the 181 Tyr→Cys mutation, these mutations completely revert AZT resistance (based on mutations at codons 67, 70, 215 and 219) to AZT sensitivity [50]. The latter observations point to the potential usefulness of the combination of AZT with any of the NNRTIs (i.e. TIBO, pyridinone, α-APA) that lead to the 181 Tyr→Cys mutation and/or compounds such as 3TC (or FTC) which lead to the 184 Met→Val mutation.

Other drug combinations that may be worth pursuing would be those based on BHAP and any of the NNRTIs proned at Tyr 181, since the BHAP resistance mutation 236 Pro→Leu increases the

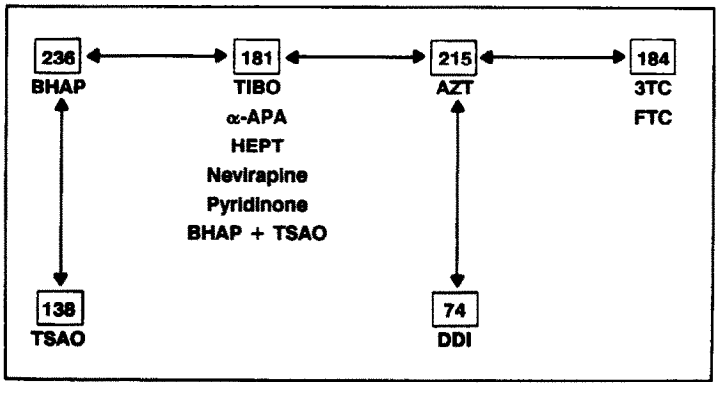


Fig. 4. Mutually suppressive drug-resistance mutations that may justify combinations of the corresponding drugs in attempts to prevent emergence of virus-drug resistance.

sensitivity of HIV-1 RT to TIBO, Nev and Pyr, even if the HIV-1 RT has been mutated at position 181 (Tyr→Cys) [65].

According to preliminary reports [95, 96], HIV-1 would not develop resistance to BHAP within a time frame of 6–12 weeks, if BHAP [atevirdine (ATV)] is given to patients in combination with AZT. This lack of resistance may be an intrinsic property of ATV or may be secondary to the combined use of AZT and ATV, in which case this combination should be explored further as a therapeutic modality to prevent virus-drug resistance.

A combination that should apparently be avoided is that of TSAO and BHAP [68]. When used individually, they lead to mutations at positions 138 (Glu→Lys) and 236 (Pro→Leu), respectively. When combined, they suppress each other's mutation, but elicit a third one, namely at position 181 (Tyr→Cys), which does not emerge when the compounds are used separately [68]. Thus, the combination of TSAO with BHAP does not seem advisable, unless AZT is added as a third component to this combination, since as mentioned above, AZT counteracts the 181 (Tyr→Cys) mutation-based drug resistance [93, 94].

Recently, the term "convergent combination therapy" has been coined for combinations of several drugs that lead to different mutations in the same target enzyme (reverse transcriptase), which, if combined, would attenuate the enzyme to such an extent that it would no longer function, and the virus would become nonviable [97]. Thus, the triple combination of AZT, DDI and pyridinone (or nevirapine) was found to shut off HIV-1 replication, and this was attributed to "evolutionary limitations that would restrict multidrug resistance (MDR) development" [97]. There is, however, a much simpler explanation for the fact that the combination of AZT, DDI and pyridinone (or nevirapine) prevented HIV-1 breakthrough, that is that the drug concentrations used (i.e. 0.3 μM AZT, 10 μM DDI and 0.09 μM pyridinone), when combined, sufficed to suppress completely ("knock out" [69]) virus replication from the beginning so that virus, whether drug-resistant or not, could not break through.

Also, the premise that a triple drug-resistant virus would *per se* be unable to replicate has proved to be faulty, as it has been demonstrated that, based on the specific mutations in the reverse transcriptase, HIV can acquire co-resistance to AZT, DDI and nevirapine [98]. In fact, the HIV-1 variant with the RT mutation 74 (Leu→Val), 103 (Lys→Asn), 215 (Thr→Tyr) and 219 (Lys→Gln) that was reported by Chow *et al.* [97] to be nonviable, appears to exhibit growth kinetics similar to the wild-type virus [99]. Chow *et al.* [100] have attributed this discrepancy to additional unintended mutations in the HIV-1 reverse transcriptase. Yet, they did not reveal the nature of these unintended mutations. Nor did they explain their role in rendering the virus nonviable. From the data reported by Emini *et al.* [99], it is evident that the HIV-1 variant with the four RT mutations (at positions 74, 103, 215 and 219) still retained susceptibility to AZT and pyridinone L-697,661 at concentrations (<1 μM) that are therapeutically attainable in human plasma.

In addition to the simultaneous drug treatment regimen, alternating drug regimens may be envisaged as an approach to overcome the development of virus-drug resistance. Thus, treatment could be switched from compound A to B, when resistance develops to A, and back to A, when resistance develops to B (and sensitivity to A is restored). The latter obviously depends on the reversibility of virus-drug resistance (see below). If sensitivity to compound A is not restored, and the virus has acquired resistance to B, treatment could be switched to C, and further onto D, when resistance develops to C, etc. As an example of an alternating drug regimen, AZT alternating with DDC has been pursued. This alternating drug regimen was actually installed in attempts to reduce the side-effects of the individual drugs. However, it did not seem to prevent the emergence of AZT-resistant HIV variants [101].

Alternating ddN/NNRTI drug regimens have not been thoroughly pursued in patients. As appropriate candidate drugs for such trials, one may think of using those compounds that lead to mutually exclusive resistance mutations, i.e. at positions 215 (AZT) and 181 (i.e. TIBO, α-APA). Also, different NNRTIs, giving rise to non-cooperative resistance mutations, i.e. at positions 236 (BHAP) and 181 (i.e. TIBO, Nev, Pyr), may be worth pursuing as alternating drug modalities. Finally, combinations of different drugs could be administered in an alternating fashion (i.e. compounds A and B, alternating with compounds C and D), which would result in an almost countless number of possibilities.

How reversible, transmissible and pathogenic are drug-resistant HIV variants?

Anecdotal reports suggest that drug-resistant HIV-1 strains, particularly AZT-resistant HIV-1 strains, can be transmitted from one person to another [102, 103]. Also, AZT-, DDI-, DDC- and Nev-resistant HIV-1 isolates have been obtained from patients who had never been treated with AZT, DDI, DDC or nevirapine [104, 105]. The latter observations do not necessarily indicate that the drug-resistant virus was transmitted as such. Given that HIV-1 exists *in vivo* as a population of diverse yet related viruses, resistance may develop spontaneously, perhaps due to the error-prone reverse transcription step [104]. The drug resistance that the virus may show *ab initio* should be taken into account when starting the appropriate drug (combination) treatment. Yet, it remains at present difficult to assess the transmissibility of the resistant phenotype as compared with that of the wild type.

The AZT-resistant phenotype appears to be quite stable [106], and, upon cessation of AZT treatment, it reverts only slowly to the wild type [107]. A period of 1 year without AZT may be required for reversion of the mutant (or mixed) virus population to the wild-type virus population [108, 109]. As development of full resistance to AZT requires 4 (or 5) mutations [47, 48], it is perhaps not surprising that it takes such a long time for the resistant virus to revert back to the wild type. It has not been determined how long it takes for the NNRTI-resistant HIV-1 mutants to revert to the wild type.

Resistance to at least some of the NNRTIs seems to emerge rapidly, i.e for pyridinone L-697,661 within 12 weeks of treatment [110]. Since, as a rule, HIV-1 resistance to the NNRTIs depends on only one mutation, its reversion to the wild phenotype, upon withdrawal of the drug, may not be as long as for the AZT-resistant phenotype. Furthermore, this reversion may be accelerated in the presence of a drug (i.e. AZT) that has a suppressive effect on the NNRTI resistance mutation (i.e. 181 Tyr→Cys).

Although the emergence of drug-resistant virus strains is generally assumed to delay, if not preclude, clinical improvement (and has, in some instances, led to termination of therapy [110]), the clinical relevance of HIV resistance development to any of the ddN or NNRTI analogues has remained unsettled. The possibility that drug-resistant variants may be "evolutionarily" handicapped in their replicative capabilities as compared with the wild type should be the subject of further studies. It is remarkable that the mutation (190 Gly→Glu) conferring resistance to quinoxaline also leads to a markedly reduced RT activity [26]. It would thus seem mandatory to examine whether such a mutant is also handicapped in its reproductive ability and pathogenicity. As far as the clinical data stand, resistance to AZT or DDI appears to be of unknown clinical significance [111–113]. Patients with AZT-resistant virus tend to have low CD4 cell counts [114], and, vice versa, high CD4 cell counts tend to be associated with lower rates of AZT resistance development [115]. Furthermore, disease progression in patients treated with AZT seems to be more closely associated with the syncytium-inducing (SI) phenotype than with AZT resistance [116].

Knocking out HIV-1, whether drug-resistant or not

Given the possibility that drug-resistant HIV strains can be transmitted, are relatively stable, and could be pathogenic, and thus contribute to disease progression, do we have any means to prevent or circumvent development of virus-drug resistance? One approach to counteracting the emergence of drug-resistance HIV strains is based on the combined (simultaneous or alternating) use of several anti-HIV drugs. As discussed above, a number of drug combinations have indeed been found to suppress development of resistance to the drug(s) present in the combination. However, due to the abundance of acceptable drug candidates currently available from both the ddN and NNRTI series, the number of drug combinations that could be envisaged, as simultaneous, alternating (or mixed) drug regimens, is virtually infinite, and thus by far exceeds the number that could be reasonably pursued in the patient. As this discrepancy in what is theoretically possible and practically feasible will increase rather than decrease in the future, the "ideal" drug combination to combat HIV infections will most likely never be materialized.

What would seem conceptually straightforward, potentially efficacious and practically feasible as an approach to preventing drug-resistant HIV strains from arising is that based on the use of "knocking-out" concentrations of the HIV-1-specific RT inhibitors [69]. If these inhibitors, i.e. BHAP (U-88204 or U-90125), are used from the start at a sufficiently high concentration (i.e. 1 or 3 μM, respectively), they completely suppress virus replication [117, 118], so that the virus is "knocked out" and does not have the opportunity to become resistant. If U-90152 is combined with AZT, the concentrations can be lowered, so that at a concentration of 0.5 μM each, the combination of U-90152 with AZT also achieves total virus suppression [118].

Not only BHAP, but also other HIV-1-specific RT inhibitors such as TIBO, nevirapine and pyridinone have been shown to "knock out" HIV-1 in cell culture when used at concentrations, i.e. 2.5 μg/mL (TIBO, pyridinone) or 10 μg/mL (nevirapine, BHAP), that are non-toxic to the cells (Fig. 5) [69]. That the virus is really knocked out, and the cell culture thus sterilized or cleared from the virus infection, has been ascertained by polymerase chain reaction (PCR) analysis of the infected cell cultures (Fig. 6): even with two successive 35-cycle PCR rounds, no trace of proviral DNA could be detected in HIV-1-infected cell cultures that had been treated from the start with a sufficiently high concentration (2.5 to 10 μg/mL) of the drugs (TIBO, pyridinone, BHAP, or nevirapine) for up to 40 days (10–15 subcultivations) [69].

In sharp contrast with the NNRTIs, the ddN analogues (i.e. AZT) proved unable to achieve this sterilizing effect; when used at a relatively high

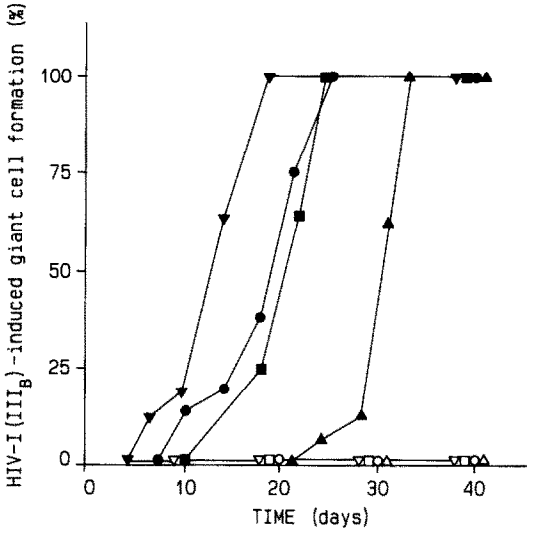


Fig. 5. HIV-1-induced giant cell formation in CEM cell cultures treated with nevirapine at 0.1 μg/mL (●) or 10 μg/mL (○); TIBO R82913 at 0.5 μg/mL (■) or 2.5 μg/mL (□); BHAP at 1 μg/mL (▲) or 10 μg/mL (△); and pyridinone L-697,661 at 0.1 μg/mL (▼) or 2.5 μg/mL (▽). CEM cell cultures (3×10^5 cells/mL) were infected with 200 times the 50% cell culture infective concentration ($CCIC_{50}$) of HIV-1(III_B). Passages were performed every 3–4 days by adding 0.5 to 1 mL of the virus-infected cells to 4.5 to 5.0 mL of fresh cell culture medium containing 3×10^5 uninfected CEM cells/mL and the inhibitors at the indicated concentrations. Data taken from Balzarini *et al.* [69].

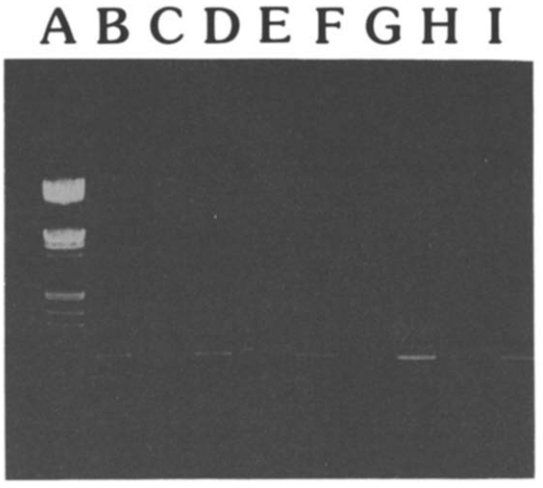


Fig. 6. Detection of proviral DNA in HIV-1-infected CEM cell cultures treated with nevirapine at 0.1 μg/mL (lane B) or 10 μg/mL (lane C); TIBO R82913 at 0.5 μg/mL (lane D) or 2.5 μg/mL (Lane E); BHAP at 1 μg/mL (lane F) or 5 μg/mL (lane G); and pyridinone L-697,661 at 0.1 μg/mL (lane H) or 2.5 μg/mL (lane I). PCR Amplification was performed on cells obtained after the 10–15th subcultivation of the HIV-1-infected CEM cells in the continuous presence of the test compounds at the indicated concentrations. Lane A represents several molecular weight markers. Data taken from Balzarini *et al.* [69].

concentration, i.e. 3 μM [118] or 1.3 μg/mL [69], AZT only achieved a transient suppression of virus growth and did not prevent virus breakthrough after a few days. In fact, earlier observations had indicated that even at concentrations up to 25 μM, AZT only delayed virus replication, but could not prevent resumption of virus production, so that drug-treated HIV-1-infected cell cultures eventually produced as much virus as did untreated infected cells, despite the continued presence of the drug [119].

The apparent suppression of virus replication that has been seen with nevirapine (or pyridinone) in combination with AZT and DDI [97] can also be ascribed to the "knocking-out" phenomenon, since the concentrations at which the compounds were used in the combination (see above) may suffice to completely block virus replication from the start. In addition to BHAP, TIBO, nevirapine, and pyridinone, other HIV-1-specific RT inhibitors may be expected to achieve a long-lasting suppressive effect on HIV replication when added to the virus-infected cells from the start at sufficiently high concentrations, and these concentrations could be reduced if any of the ddN analogues is added to the NNRTI.

Conclusion

While chemotherapy of HIV infections by reverse transcriptase (RT) inhibitors of both the nucleoside and non-nucleoside type seems to be compromised by the emergence of drug-resistant virus strains, several critical issues, i.e. as to the pathogenicity of these resistant mutants and their role in disease progression, remain unclear. It cannot be denied that the virus has a whole repertoire of mutational capabilities at the RT level, which enables its escape from the RT inhibitors whether belonging to the ddN or NNRTI type. However, some of the mutations that lead to drug resistance, e.g. those located at RT positions 215 (AZT) and 181 (several NNRTIs), are mutually suppressive. This offers the opportunity for combining the appropriate drugs in efforts to prevent or circumvent resistance. Furthermore, virus-drug resistance emerges subsequently to, and probably because of, the continued pressure of relatively low concentrations of the RT inhibitors. If one starts with sufficiently high, but still non-toxic, concentrations of the HIV-1-specific NNRTIs (i.e. TIBO, HEPT, nevirapine, pyridinone, BHAP), virus replication can be suppressed completely ("knocked out"). These "knocking out" concentrations completely prevent breakthrough of the virus, whether resistant or not.

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