Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs) for the Treatment of Human Immunodeficiency Virus Type 1 (HIV-1) Infections: Strategies to Overcome Drug Resistance Development

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I. INTRODUCTION

The first compounds ever shown to specifically inhibit HIV-1 (human immunodeficiency virus type 1), but not HIV-2 (human immunodeficiency virus type 2), replication were the 1-(2-hydroxyethoxymethyl)-6-(phenylthio)thymine (HEPT)[1,2] and tetrahydroimidazo[4,5,1-jk][1,4]benzodiazepin-2(1H)-one and -thione (TIBO) derivatives.[3,4] The unprecedented specificity of the TIBO derivatives, which were found to inhibit HIV-1 replication at concentrations that were 10,000- to 100,000-fold lower than the concentrations required to affect normal cell viability, was attributed to a specific interaction with the HIV-1 reverse transcriptase (RT).[3,4] For the HEPT derivatives it became evident that they also interact specifically with HIV-1 RT after a number of derivatives, such as E-EPU, E-EBU, and E-EBU-dM, had been synthesized that were more active than HEPT itself in inhibiting HIV-1 replication.[5,6]

Whereas the HEPT and TIBO derivatives were discovered as the result of a systematic evaluation for anti-HIV activity in cell culture, and later proved to achieve their anti-HIV activity through a specific interaction with HIV-1 RT, the following compounds emerged from a screening programme for detecting HIV-1 RT inhibitors, before they were shown to inhibit HIV-1 replication in cell culture as well: nevirapine (BI-RG-587),[7,8] pyridinone derivatives (L-696,229 annd L-697,661),[9,10] and bis(heteroaryl)piperazine (BHAP) derivatives (U-88204 and U-90152).[11,12]

Following TIBO, HEPT, nevirapine, pyridinone, and BHAP, the 2′,5′-bis-O-(*tert*-butyldimethylsilyl)-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide)pyrimidine (TSAO) nu-

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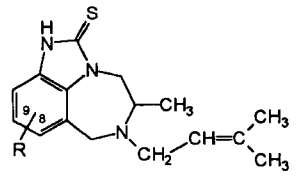
cleoside analogues (TSAO-T and TSAO-m^{3}T),[13–15] α-anilinophenylacetamides (α-APA R89439),[16] and various other compounds (Fig. 1) were found to inhibit HIV-1 replication through a specific interaction with HIV-1 RT. These compounds have been collectively referred to as NNRTIs or "non-nucleoside reverse transcriptase inhibitors."[17,18]

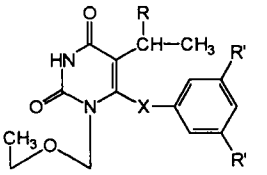
NNRTIs share a number of characteristics which distinguish them from, and makes them more specific than, the "conventional" dideoxynucleoside (ddN)-type RT inhibitors AZT (3'-azido-2',3'-dideoxythymidine, zidovudine), DDI (2',3'-dideoxyinosine, didanosine), DDC (2',3'-dideoxycytidine, zalcitabine), D4T (2',3'-didehydro-2',3'-dideoxythymidine, stavudine), and 3TC ((-)-3'-thia-2'-deoxycytidine, lamivudine).[17] NNRTIs achieve a highly selective suppression of HIV-1 replication in cell culture, with little, if any, cytotoxicity and without affecting the replication of HIV-2 or other retroviruses. They do not require intracellular metabolism, and interact as such with HIV-1 RT in a non-competitive manner with regard to the natural substrate (dNTP) at an allosteric ("non-nucleoside") binding site. Not only are they independent of the phosphorylating enzyme machinery of the cells [which may be unsatisfactory (i.e., in resting cells such as monocytes/macrophages)], their action at the RT level cannot be outweighed by competing dNTP pool levels. Howver, NNRTIs rapidly lead to the emergence of drug-resistant HIV-1 mutant strains,[19,20] the mutations being clustered around the putative binding site of the NNRTIs.[21,22]

As the emergence of virus–drug resistance is the major, if not the only, problem compromising the therapeutic usefulness of the NNRTIs, their future may be determined to a major extent by strategic approaches to prevent, circumvent or overcome drug resistance development. Among the different approaches that could be envisaged are the following:[18,19] (i) switch from one class of NNRTI inhibitors to another (or, even within each class, from one compound to another), since different NNRTI inhibitors do not necessarily lead to cross-resistance; (ii) combine different compounds that show differences in their resistance mutation profile, particularly when these mutations appear to counteract each other (as has been demonstrated among both NNRTIs and ddN analogues); (iii) start with sufficiently high drug concentrations so as to "knock out" the virus and prevent breakthrough of any virus whether resistant or not; and (iv), in combining approaches, (ii) and (iii), start with drug combinations that could achieve a total knock out (i.e., clearance of the cells from the virus) at lower drug concentrations than if the compounds were to be used individually.

Erik De Clercq was born in Dendermonde, Belgium, on March 28, 1941. He received his M.D. degree in 1966 and his Ph.D. in 1972 both from the Katholieke Universiteit Leuven (K. U. Leuven). After having spent two years at Stanford University Medical School as a postdoctoral fellow, he returned to the K.U. Leuven Medical School, where he became docent in 1973, professor in 1975, and full professor in 1977. He has been in charge of the courses of Microbiology (Virology) and Biochemistry in the second and third undergraduate years of the Medical School of the K.U. Leuven Campus Kortrijk. Prof. E. De Clercq served as Chairman of the Department of Microbiology from 1986 until 1991. In 1986 he became Chairman of the Directory Board of the Rega Institute, and since 1995 he is titular of the Professor P. De Somer Chair for Microbiology of the K.U. Leuven. Prof. E. De Clercq's scientific interests are in the antiviral chemotherapy field, and, in particular, the development of new antiviral agents for various viral infections, including AIDS. The present review focuses on the non-nucleoside reverse transcriptase inhibitors (NNRTIs) which are specific for HIV-1. The work that led to the discovery of this class of compounds started in the author's Laboratory in 1987 with support from the Janssen Research Foundation.

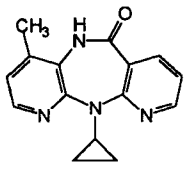


Tetrahydroimidazo[4, 5, 1-jk] [1, 4]-benzodiazepin-2(1H)-one (TIBO)
R=H : R82150
R=9-Cl : R82913
R=8-Cl : R86183
R86183.HCl : R91767 (8-Chloro-TIBO)

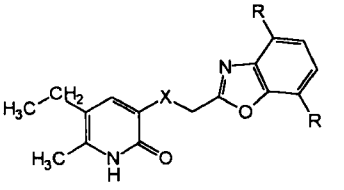


1-(2-Hydroxyethoxymethyl)-6-(phenylthio)thymine (HEPT)

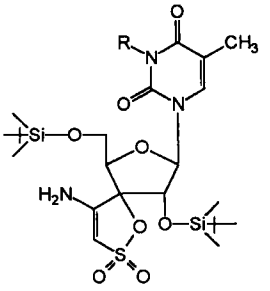
X=S R,R'=H : E-EPU
X=CH_2 R=H R'=CH_3 : E-EBU-dM
X=CH_2 R=CH_3 R'=H : I-EBU (MKC-442)



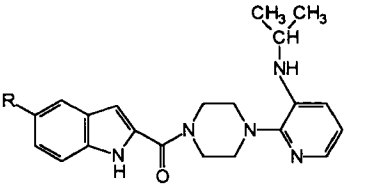
Nevirapine (BI-RG-587)



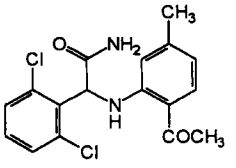
Pyridinone
X=CH_2, R=H : L-696, 229
X=NH, R=Cl : L-697, 661



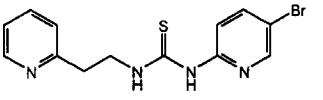
2',5'-Bis-O-(tert-butyldimethylsilyl)-3'-spiro-5''-(4''-amino-1'',2''-oxathiole-2'',2''-dioxide (TSAO) derivative
R=H : TSAO-T
R=CH_3 : TSAO-m^{3}T



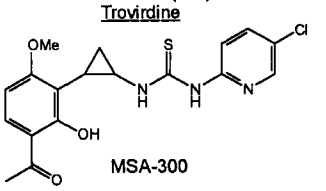
Bis(heteroaryl)piperazine (BHAP)
R=H : U-88204
R = OCH_3 : U-87201 (Atevirdine)
R=$NHSO_2CH_3$: U-90152 (Delavirdine)



α-Anilinophenylacetamide (α-APA)
R89439 (Loviride)



LY300046.(HCl)
Trovirdine

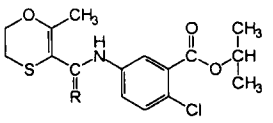


MSA-300

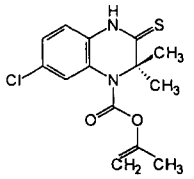
N-(2-Phenylethyl)-N'-(2-thiazolyl)thiourea (PETT)

(a)

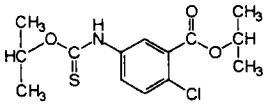
Figure 1.(a, b, c) Formulae of non-nucleoside reverse transcriptase inhibitors (NNRTIs).



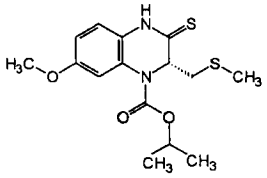
R = O Oxathiin carboxanilide UC 84 (NSC 615985)
R = S Oxathiin thiocarboxanilide UC 23



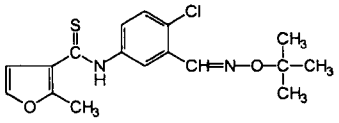
6-Chloro-3,3-dimethyl-4-(isopropenyl)-oxycarbonyl)-3,4-dihydroquinoxalin-2 (1H)-thione (S-2720)



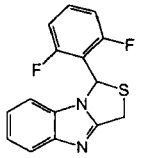
Thiocarboxanilide UC38 (NSC 629243)



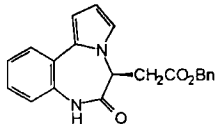
Quinoxaline HBY097



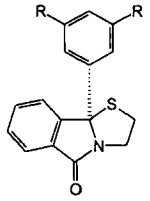
Thiocarboxanilide UC10



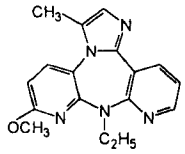
1-(2',6'-difluorophenyl)-1H,3H-thiazolo [3,4-a]benzimidazole (NSC 625487)



Pyrrolo-[1,2-d]-(1,4)-benzodiazepin-6-one

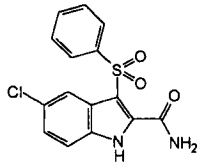


9b-Phenyl-2,3-dihydrothiazolo [2,3-a]isoindol-5(9bH) one (R=H) (BM 21.1298), and its dimethyl derivative (R=CH_3) (BM + 51.0836)

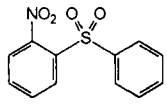


Imidazo[2',3':6,5]dipyrido [3,2-b:2',3'-e]-1,4-diazepine (UK-129, 485)

(b)

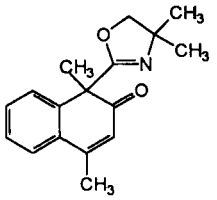


5-Chloro-3-(phenylsulfonyl)-indole-2-carboxamide (L-737, 126)

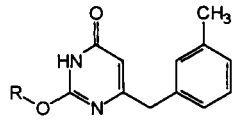


2-Nitrophenyl phenyl sulfone (NPPS)

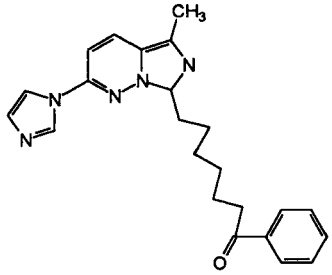
Figure 1. Continued



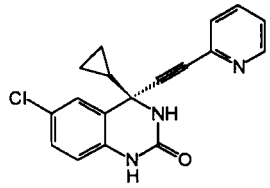
1,4-Dimethyl-1-(5,5-dimethyl-2-oxazolinyl)-naphthalene-2-one (TGG-II-23A)



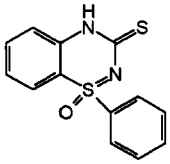
3,4-Dihydro-2-alkoxy-6-(3'-methylbenzyl)-4-oxopyrimidine (DABO) R=cyclohexyl



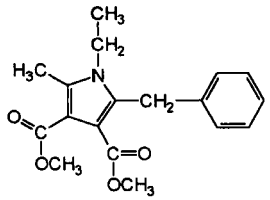
7-[2-(1H-Imidazol-1-yl)-5-methyl-imidazo[1,5-b]pyridazin-7-yl] 1-phenyl-1-heptanone



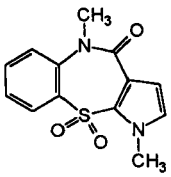
(-)-4-[2-(2-Pyridyl)ethyn-1-yl]-6-chloro-4-cyclopropyl-3,4-dihydro-quinazolin-2(1H)-one



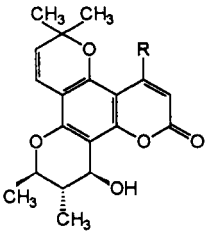
Benzothiadiazine-1-oxide (NSC 287474)



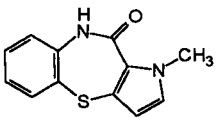
Dimethyl 1-ethyl-2-methyl-5-benzyl 1H pyrrole 3,4-dicarboxylate



1,5-Dimethyl-4-oxo(1H,5H)-pyrrolo[2,3-b] [1,5]benzothiazepine 10,10-dioxide (Compd 1)



R=propyl : Calanolide A
R=phenyl : Inophyllum B



(c) 1-Methyl-10-oxo(1H,9H)-pyrrolo [3,2-b] [1,5]benzothiazepine (Compd 2)

Figure 1. Continued

II. INHIBITION OF HIV-1 REPLICATION BY NNRTIs

Which requirements do the compounds have to fulfill to qualify as NNRTIs? They should specifically interact with HIV-1 RT, and inhibit HIV-1, but not HIV-2, replication in cell culture at a concentration that is significantly lower than the concentration required to affect normal cell viability. The latter should be evident from the selectivity index (SI), or ratio of the CC_{50} (50% cytotoxic concentration) to the EC_{50} (50% effective concentration) (Table I). Based on these premises, several classes of compounds (Fig. 1) could be considered as NNRTIs: TIBO derivatives,[3,4,23,24] HEPT dereivatives,[5,6,25–27] nevirapine[7,8,28] (as member of the dipyridodiazepinones), pyridinones,[9,10] bis(heteroaryl)piperazines,[11,12,29] TSAO derivatives,[13–15] α-anilinophenylacetamides,[16] PETT derivatives,[30] quinoxaline S-2720,[32] thiazolobenzimidazole (NSC 625487),[33] pyrrolobenzodiazepinone,[34] phenyldihydrothiazoloisoindolones (BM 21.1298 and BM + 51.0836),[35–37] imidazodipyridodiazepine (UK-129,485),[38] chlorophenylsulfonylindole carboxamide (L-737,126),[39] 2-nitrophenyl phenyl sulfone (NPPS),[40] naphthalenone TGG-II-23A,[41] DABO derivatives,[42,43] imidazopyridazinyl phenyl heptanone,[44] pyridylethynyldihydroquinazolinone,[45] benzothiadiazine-1-oxide (NSC 287474),[46] highly substituted pyrrole derivatives,[47] pyrrolobenzothiazepines,[48] and a number of natural products, i.e., calanolides[49] and inophyllums,[50] extracted from the tropical rain forest tree *Calophyllum lanigerum* and *Calophyllum inophyllum*, respectively.

As is evident from Table I the most potent and most selective NNRTIs were found to inhibit HIV-1 cytopathicity at nanomolar concentrations with selectivity indices up to 100,000. All the compounds listed in Table I were found to inhibit HIV-1 RT activity, albeit at widely varying IC_{50} values, the lowest value (0.65 nM) being recorded for 7-[2-(1*H*-imidazol-1-yl)-5-methylimidazo-[1,5-b]pyridazin-7-yl]-1-phenyl-1-heptanone.[44] The only compound listed in Table I for which no inhibitory effect on HIV-1 RT could be witnessed in the original experiments was oxathiin carboxanilide (UC84).[31] However, in follow-up experiments, oxathiin carboxanilide (UC84) proved to be as good an inhibitor of HIV-1 RT as the other NNRTIs, i.e., TIBO R82913, nevirapine, pyridinone L-697,661, BHAP U88204, and TSAO-m^3T;[51,52] in CEM cells, UC84 inhibited HIV-1 replication (cytopathicity) at an EC_{50} of 0.015 μg/ml, while being toxic to the host cells at a CC_{50} of 8.8 μg/ml, thus achieving a selectivity index of 587. The anti-HIV-1 potency of UC84 was markedly enhanced upon substituting sulfur for oxygen in the carboxamide moiety of the molecule: the resulting compound UC23 proved inhibitory to HIV-1 replication at an EC_{50} of 0.007 μg/ml, and, as its CC_{50} was 16 μg/ml, compound UC23 achieved a selectivity index of 2286. The thiocarboxanilide UC38 showed a similar potency (EC_{50}: 0.009 μg/ml) and selectivity index (1333), and various other thiocarboxanilide derivatives (i.e., UC10, UC16, UC42, UC68 and UC81 and UC84) were identified as potent inhibitors of HIV-1 RT, with IC_{50} values ranging from 0.05 to 0.20 μM.[52]

III. STRUCTURE-FUNCTION RELATIONSHIP CONSIDERATIONS

Extensive structure-activity relationship (SAR) studies have been conducted with a number of the NNRTIs, and these studies have revealed that, despite the heterogeneity of the different classes of NNRTIs, within each class stringent requirements exist with regard to the structural determinants for optimal anti-HIV activity in cell culture.

Within the 4,5,6,7-tetrahydro-5-methylimidazo-[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-one (TIBO) series, the 3,3-dimethylallyl group at the 6-position is an optimal substituent.[53] Variations in the five-membered urea ring are not tolerated, except for the substitution of the urea by thiourea, which, in fact, causes a significant increase in activity.[54] The anti-

TABLE I
Inhibitory Effects of NNRTIs on HIV-1 RT Activity and HIV-1 Cytopathicity

Compound	HIV-1 RT Activity	HIV-1 Cytopathicity			References
	IC_{50} (μM)[a]	EC_{50} (μM)[b]	CC_{50} (μM)[c]	SI[d]	
TIBO R82150	0.139	0.044	552	12545	3,4,23
R82913	0.015	0.033	34	1030	3,23,24
R86183	0.050	0.0046	138	30000	23
HEPT E-EPU	0.27	0.022	146	6636	5,25
E-EBU-dM	0.036	0.0022	249	113000	6,25
I-EBU (MKC-442)	0.012	0.014	>100	>7000	26,27
Nevirapine (BI-RG-587)	0.084	0.048	>50	>1000	7,8,28
Pyridinone L-696,229	0.022	≤0.05	>200	>4000	10
L-697,661	0.019	≤0.012	>60	>4800	9
BHAP U-88204	0.25	0.04	>10	>250	11
U-90152 (Delavirdine)	0.26	0.01	>100	>10000	12,29
U-87201 (Atevirdine)	1.3	0.04	>10	>250	11
TSAO-T	17	0.036	>50	>1400	13–15
TSAO-m^3T	4.7	0.034	139	4088	13,14
α-APA R89439 (Loviride)	0.2	0.013	710	54615	16
PETT LY300046.(HCl) (Trovirdine)		0.016	87	5438	30
Oxathiin carboxanilide UC84 (NSC 615985)		0.22	106	482	31
Quinoxaline S-2720	0.1	0.003	50	17000	32
Thiazolobenzimidazole (NSC 625487)	0.5	0.5	50	100	33
Pyrrolobenzodiazepinone	0.04 (μg/ml)	0.3 (μg/ml)[e]	10 (μg/ml)	33	34
Phenyldihydrothiazolo-isoindolone (BM 21.1298)	0.28	0.392	>50	>125	35,36
and its dimethyl derivative (BM + 51.0836)	0.016	0.01	>50	>5000	36,37
Imidazodipyridodiazepine UK-129,485	0.156	<0.002	>10	>5000	38
Chlorophenylsulfonylindole carboxamide L-737,126	0.003	<0.003			39
2-Nitrophenyl phenyl sulfone (NPPS)	0.61	5	125	25	40
Naphthalenone TGG-II-23A	136	26	550	21	41
DABO (R = cyclohexyl)	1.8	0.8	>335	>418	42,43
Imidazopyridazinyl phenylheptanone	0.0006	0.01	26	2600	44
Pyridylethynyl dihydroquinazolinone	0.012	<0.025			45
Benzothiadiazine (NSC 287474)	1.2	4.04	>130	>32	46
Substituted pyrrole dicarboxylate	0.64	2.3	175	76	47
Pyrrolobenzothiazepines					
(compd 1)	1.5	14	>362	>25	48
(compd 2)	28	16	>434	>27	48
Calanolide A	0.07	0.1	20	200	49
Inophyllum B	0.038	1.4	55	39	50

(*continued*)

TABLE I (*Continued*)

Compound	HIV-1 RT Activity	HIV-1 Cytopathicity			
	IC_{50} (μM)[a]	EC_{50} (μM)[b]	CC_{50} (μM)[c]	SI[d]	References
Oxathiin carboxanilide UC84	1.5	0.015(μg/ml)	8.8 (μg/ml)	587	51,52
Oxathiin thiocarboxanilide UC23		0.007 (μg/ml)	16 (μg/ml)	2286	51
Thiocarboxanilide UC38		0.09 (μg/ml)	12 (μg/ml)	1333	51

[a]Fifty percent inhibitory concentration, or concentration required to inhibit HIV-1 RT activity by 50%.

[b]Fifty percent effective concentration, or concentration required to inhibit HIV-1-induced cytopathicity by 50% (in principle, based on cytopathicity of HIV-1 III_B strain for MT-4 cells).

[c]Fifty percent cytotoxic concentration, or concentration required to reduce cell (i.e., MT-4 cell) viability by 50%.

[d]Selectivity index, or ratio of CC_{50} to EC_{50}.

[e]EC_{90}.

HIV activity in enantiospecifically dependent on the methyl group at the 5-position.[53] The C-5 methyl-substituted analogues maintain the highest desired activity, with the C-7 mono-methyl-substituted analogues being the only others showing comparable activity.[55] Substituents on the 8-position furnished the most rewarding results: these included halogen, thiomethyl, methyl, cyano, methoxy, and acetylene.[56] The 8-chloro compound R86183 with EC_{50} = 0.0046 μM[23] was chosen for clinical development (as its hydrochloride salt R91767).

Within the 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT) series, the importance of the methyl function at the 5-position, and of the phenylthio group at the 6-position, relative to other C-5 and C-6 substituents was ensured.[57] Also, various 2-, 3-, and 4-substituted analogues of HEPT were synthesized.[58] Of these analogues, the 2-thio analogue proved about 5- to 10-fold more potent than HEPT in inhibiting HIV-1 replication.[59] Replacement of the methyl group at C-5 by an ethyl[5] or isopropyl[60] group led to 50- to 100-fold incrase in anti-HIV-1 activity. The hydroxyl group of the (2-hydroxyethoxy)methyl side chain at N-1 is not mandatory, or even not desirable,[61] since the anti-HIV-1 activity increases if the 2-hydroxyethoxy function is replaced by ethoxy or benzyloxy.[5,6] Introduction of two methyl groups in the phenylthio ring further increases the activity by 2- to 20-fold depending on the precursor.[6,60]

Thus, starting from HEPT, the anti-HIV-1 activity is systematically enhanced if (i) methyl is replaced by ethyl (or isopropyl) at C-5, (ii) phenylthio is replaced by 3,5-dimethylphenylthio at C-6, (iii) (2-hydroxyethoxy)methyl is replaced by ethoxymethyl (or benzyloxymethyl) at N-1, and (iv) as the 6-phenylthio moiety can be replaced by a 6-benzyl group without loss of activity,[62] this leads to a final gain in anti-HIV-1 activity of more than three orders of magnitude as in E-EBU-dM.[25] The related I-EBU has been selected for further development [MKC-442].[26,27]

A number of HEPT analogues have been described in which the 6-(phenylthio) group was replaced by 6-(phenylselenenyl),[63] or in which the substituent linked at N-1 was eliminated, the phenylthio moiety was shifted from the 6- to the 2-position of the pyrimidine ring and an alkoxy or cycloalkoxy chain was bound at the 6-position.[64] However, these modifications did not enhance, or even abolish, the anti-HIV-1 activity of HEPT.

Nevirapine (BI-RG-587) emerged as one of the most potent HIV-1 RT inhibitors from a

series of more than one hundred pyridobenzo- and dipyridodiazepinones.[28] SAR studies with this class of compounds pointed to the need of an alkyl moiety (i.e., ethyl, cyclopropyl) at the diazepinone N-11 and a lipophilic substituent (i.e., methyl) at the 7-position of the pyridobenzodiazepinones (or 4-position of the dipyridodiazepinones). Both requirements are fulfilled by the dipyridodiazepinone derivative 11-cyclopropyl-5,11-dihydro-4-methyl-6*H*-dipyrido[3,2-b:2′,3′-*e*][1,4]diazepin-6-one (nevirapine). Various pyridobenzoxazepinones and dibenzoxazepinones have also been described as specific HIV-1 RT inhibitors,[65] but, as a class, oxazepinones are inherently less potent inhibitors of HIV-1 RT than are diazepinones and, in addition, they are also less soluble, and this poor solubility appears to be a critical impediment to the development of the oxazepinones.

Within the 2-pyridinone family,[66–70] the benzoxazole derivatives were found to be more active against HIV-1 RT than the phthalimide derivatives, and introduction of methyl or chloro groups in the benzoxazole ring further enhanced the inhibitory effect on HIV-1 RT. Also, substitution of the aminomethylene linker by an ethylene linker resulted in increased activity, and this has eventually led to the identification of 3-[(benzoxazol-2-yl)ethyl]-5-ethyl-6-methylpyridin-2(1*H*)-one (L-696,229) and the structurally related L-697,661, as highly selective inhibitors of HIV-1 RT (IC_{50}: ~ 0.020 μM), which were selected for clinical evaluation as antiviral agents.[70]

Of the BHAP series, the original lead compound was N-ethyl-2[4-[(methoxy-3,5-dimethylphenyl)methyl]-1-piperazinyl]-3-pyridinamine (U-80493E);[11] the methylene group linking the phenyl ring to piperazine was replaced by a carbonyl (U-85199E) without loss of anti-HIV-1 activity, and, when the phenyl ring of the latter was replaced by indole (U-85961), it resulted in a greatly enhanced inhibitory activity. Of U-85961, several derivatives [i.e., U87201 E (Atevirdine) and U-88204] were prepared,[11] and further substitution of U-88204 led to the identification of 1-[(5-methanesulfonamido-1*H*-indol-2-yl)-carbonyl]-4-[3[(1-methylethyl)amino]-pyridinyl]piperazine monomethanesulfonate (U-90152S) as a second-generation clinical candidate (Delavirdine).[12]

Structure-activity relationship studies with a large variety of TSAO derivatives have established that the simultaneous presence of the 3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″, 2″-dioxide) group and both the 2′- and 5′-*tert*-butyldimethylsilyl groups are essential for anti-HIV-1 activity.[14,71,72] The 5′-silyl protecting group seems to be more critical for activity than the 2′-silyl protecting group: thus, replacement of the silyl moiety at the C-5′ position by other groups (i.e., esters, benzylethers or silylethers) that mimic either the lipophilic or steric properties of the *tert*-butyldimethylsilyl, results in antivirally inactive TSAO-T derivatives, whereas similar modifications at the C-2′ position only slightly reduce the anti-HIV-1 activity.[73] The *ribo* configuration of the sugar ring is a prerequisite for antiviral activity, as the *xylo* enantiomer of TSAO-T is completely inactive.[71,74] Also, the D-ribofuranosyl configuration is called for, as the L-isomer did not show appreciable inhibition of HIV-1 replication.[75]

In contrast to the stringent structural requirements imposed upon the sugar part of the TSAO derivatives, the nature of the base part is less critical for anti-HIV-1 activity. The thymine moiety of TSAO-T can be replaced by a number of pyrimidines, purines, and triazoles without marked decrease of activity.[76,77] In particular, 5-substituted amido-, methylamido-, and dimethylamido-1,2,3-triazole derivatives are endowed with potent anti-HIV-1 activity, comparable to that of TSAO-T.[77] Introduction of a lipophilic (i.e., methyl, ethyl or allyl) entity at the N-3 of the thymine ring or N-1 of the purine ring results in a significant decrease of cytotoxicity, without concomitant decrease of anti-HIV-1 activity, thus yielding derivatives (i.e., TSAO-m^3T) with increased selectivity (relative to TSAO-T).[13,14,72,74]

Within the α-APA series,[16] both O-nitro- and O-acetyl substituted anilino derivatives were found to inhibit HIV-1 replication at concentrations that were 1,000- to 100,000-fold lower than the cytotoxic concentration. In all cases the (−)enantiomers were considerably more active than the (+)enantiomers. As the most active congener emerged R90385, the (−)enantiomer of R89439 (Loviride), which has been selected for clinical evaluation: R89439 inhibits the cytopathicity of HIV-1 in cell culture at a concentration of 4–40 nM without being toxic for the host cells at concentrations up to 710 μM, thus achieving selectivity indexes of about 100,000.[16]

The PETT compounds originated from a dismantling of the rigid tricyclic nucleus of the TIBO derivatives.[30] This generated N-(2-phenylethyl)-N'-(2-thioazolyl)thiourea (LY73497) as the lead compound. The phenyl and 2-thiazolyl moieties were then replaced by a 2-pyridyl and 2-(5-bromopyridinyl), respectively, to yield LY300046, which, as its hydrochloride, has been selected for further evaluation (Trovirdine).

Within the oxathiin carboxanilide (i.e., UC84) skeleton[31] four parts could be discerned: the oxathiin moiety (part A) linked through a carboxamide (part B) to 2-chlorobenzoic acid (part C) which is esterified with an isopropyl group (part D). Structural modifications have been made in all four parts of the molecule, and this has allowed to delineate the structural requirements for anti-HIV-1 activity:[51,78] the oxathiin moiety (part A) can be replaced by an alkoxy (i.e., isopropoxy) group (as in UC38) without loss of antiviral activity; replacement of the oxygen of the carboxamide (part B) by a sulfur increases the activity; an intact 2-chlorophenyl moiety (part C) seems to be a prerequisite for potent antiviral activity; and, although the nature of the ester in part D may seem to be the least stringent, the isopropyl group cannot be shortened or lengthened without a decrease in activity.[78] Quite a number of thiocarboxanilide derivatives could be envisaged as potential candidate drugs for clinical studies:[52] for example UC10 (also because of its high stability and oral bioavailability).

IV. MECHANISM OF ACTION OF NNRTIs

The ddN 5'-triphosphates (ddNTPs) are active against a broad range of retroviral reverse transcriptases, whereas the NNRTIs are active only against HIV-1 RT. This by itself indicates that the mechanism of action of these two groups of HIV RT inhibitors must be different.

HIV-1 RT can be considered as a heterodimeric complex (p66/p51), the p66 subunit being composed of a p51 and p15 component. It has three catalytic functions: (i) transcription of the viral RNA genome to DNA, thus leading to the formation of the RNA.DNA hybrid; (ii) degradation of the RNA strand by ribonuclease H (H for hybrid); and (iii) duplication of the remaining DNA strand, thus resulting in the formation of the double-stranded proviral DNA. All these functions seem to be associated with the p66 subunit, the ribonuclease H function being specifically linked to the p15 component thereof. The NNRTIs, i.e., TIBO (R82150),[4] nevirapine,[7,79] and BHAP (U-87201E)[80] preferentially inhibit the RNA-dependent DNA polymerization rather than the subsequent ribonuclease H or DNA-dependent polymerization step.

The NNRTIs show marked differences in their template/primer preferences, poly(C).oligo(dG) being the most desired, that is yielding lower IC_{50} values than, say, poly(A).oligo(dT).[3–5,9,15,79] TSAO-T is inhibitory to HIV-1 RT only with poly(C).oligo(dG), but not with poly(A).oligo(dT), poly(U).oligo(dA), or poly(I).oligo(dC) as template/primer.[15] Primed ribosomal RNA (16S/23S) appears to be an excellent primer/template for evaluating the inhibitory effects of NNRTIs on HIV-1 RT: with ribosomal

RNA as template TIBO R82913 inhibits the enzyme at an IC_{50} of 0.006 μM, which is more than 1,000-fold lower than the IC_{50} (8 μM) obtained for TIBO R82913 with poly(A).oligo(dT) as template/primer.[24]

Inhibition of HIV-1 RT by the TIBO derivatives (i.e., R82150),[4,81] HEPT derivatives (i.e., E-EBU-dM),[5,6] dipyridodiazepinones (i.e., nevirapine),[7,82] pyridinones (i.e., L-697,639),[9] bis(heteroaryl)piperazines (i.e., U-85961),[11] TSAO derivatives (i.e., TSAO-T),[15] and α-APA derivatives (i.e., R89439)[16] is noncompetitive with respect to both the substrate (dGTP) and the template/primer [poly(C).oligo(dG)]. This contrasts with the behavior of the ddNTPs (i.e., ddGTP), which competitively inhibit the incorporation of the natural substrates (i.e., dGTP) into the DNA product.

That TIBO derivatives (i.e., R-82150) preferentially inhibit the RNA-dependent DNA polymerization [function (i) of the HIV-1 RT (see above)], and do so in a noncompetitive manner with respect to the natural substrates, was confirmed in studies on the kinetics of the endogenous HIV-1 RT reaction.[83] The TIBO derivatives can be considered as allosteric inhibitors of both the exogenous and endogenous HIV-1 RT reactions.[83,84] They disturb the cooperativity between the two RT subunits (p66 and p51).[84] They must bind to a site at the HIV-1 RT that is different from the catalytic, substrate-binding, site.

While the TIBO-binding site, or for that matter the NNRTI-binding site at the HIV-1 RT, could be interpreted as a "nonsubstrate" binding site, the following observations suggested that this site may be functionally and possibly also spatially related to the substrate binding site.[85] Whereas TIBO R82150 is a noncompetitive inhibitor with respect to the natural substrate dGTP (Ki = 0.65 μM), the much weaker HIV-1 RT inhibitor HEPT (Ki = 30 μM) behaves competitively with respect to dGTP.[85] Other HEPT derivatives (i.e., E-EPU and E-EBU-dM)[5,6] have also proven to competitively inhibit HIV-1 RT with respect to dTTP if directed by poly(A).oligo(dT) as template/primer. If the HIV-1 RT reaction is carried out with poly(U).oligo(dA) as template/primer, TIBO R82150 also acts as a competitive inhibitor with respect to dATP.[15] Also, the relatively weak inhibitory effect of TIBO R82150 on the RT of simian immunodeficiency virus (SIV_{agm}) follows the competitive type of inhibition, even if measured with poly(C).oligo(dG) as template/primer.[86]

These findings led us to assume that the HIV-1 RT binding site for the NNRTIs must be functionally and possibly also spatially associated with the dNTP binding site.[17,85] The recent data of Spence *et al.*[87] provide direct evidence for communication between the NNRTI-binding site and the catalytic site. Not only were the NNRTIs (i.e., TIBO and nevirapine) found not to interfere with the binding of the natural substrates to HIV-1 RT, they even made the substrates to bind more tightly; i.e., dATP bound to the enzyme/inhibitor complex with a Kd of about 0.13 μM, as compared to the ground-state Kd of 5 μM in the absence of the inhibitor. In previous studies, Dueweke *et al.*[88] had found that the BHAP derivative U-88204 and the dideoxynucleotide ddGTP can also bind simultaneously to the HIV-1 RT, but, in this case, the presence of one ligand decreased the affinity of RT for the other.

V. HIV-1 RT TARGET SITE FOR NNRTIs

TIBO R82913 has ben aligned with nevirapine,[89] and this alignment has revealed that (i) the methyl group at position 4 in nevirapine and the methyl group at position 5 in R82913 are proximal, thus accounting for the enantio-specificity of the methyl group at position 5 of the TIBO derivatives; (ii) the carbonyl group at position 6 of nevirapine and the thiocarbonyl group at position 2 of TIBO are in close proximity; (iii) the cyclopropyl

group of nevirapine partially overlaps with the methylbutenyl group of TIBO. The proximity of the methyl groups, the carbonyl/thiocarbonyl moieties, and the N-linked lipophilic side chains, suggest that they may be relevant pharmacophores involved in the binding of the molecules to their target site at the HIV-1 RT.

Through photoaffinity-labeling experiments, using the 9-azido-substituted BI-RG-587 (nevirapine) analogue BI-RJ-70, Wu *et al.*[90] ascertained that the dipyridodiazepinones (i.e., nevirapine) and TIBO compounds must interact with a nonsubstrate binding site, since they both protected HIV-1 RT from photoinactivation, whereas the natural substrate dGTP failed to do so. The enzyme could be covalently labeled by BI-RJ-70, and, when the resulting photo adduct was subjected to enzymatic digestion, a single, highly labeled peptide containing the amino acid residues 174–199 was found with Tyr-181 and Tyr-188 as the labeled residues.[91] It was suggested that these tyrosine residues play a critical role in the binding of nevirapine and its congeners to HIV-1 RT.

Unequivocal proof that Tyr-181 and Tyr-188 are critical determinants for sensitivity to nevirapine and TIBO derivatives came from chimeric RT constructs whereby the tyrosine residues at position 181 and 188 of HIV-1 RT were replaced by the corresponding HIV-2 RT amino acid residues (Ile and Leu, respectively). The Y181I and Y188L RT constructs proved resistant to both nevirapine and TIBO R82913, while retaining sensitivity to AZT 5'-triphosphate.[92] Conversely, HIV-2 RT substituted with amino acids 176–190 from HIV-1 RT acquired sensitivity to the nonnucleoside RT inhibitors.[92] Through site-directed mutagenesis we have confirmed that Y181 and Y188 are indeed required for sensitivity of HIV-1 RT to TIBO derivatives.[93] The Y181I substitution leads to a significant decrease, and the Y188L substitution even to a total loss, of the sensitivity of HIV-1 RT to TIBO and HEPT derivatives, while not affecting its sensitivity to ddNTPs.[85]

Using HIV-1 RT/HIV-2 RT chimeras, Condra *et al.*[94] and Shaharabany and Hizi[95] confirmed that the sensitivity of the HIV RT to NNRTIs is largely dependent upon the RT region defined by amino acid residues 176–190. However, other regions, i.e., that defined by residues 101–106 were found to influence the activity of the three inhibitors studied (i.e., TIBO, nevirapine, and pyridinone).[94] For calanolide A the situation is more complex: the inhibition patterns of the chimeric RTs by calanolide A have provided evidence that, in addition to a segment between residues 94 and 157, another segment located between amino acids 225 and 427 in HIV-1 RT may be important for specifying susceptibility to the drug.[96]

The crystal structure of the HIV-1 RT has been successively refined at 7 Å,[97] 3.5 Å,[98] 3.0 Å,[99] and 2.2 Å resolution,[100,101] and this has allowed to delineate the different subdomains (i.e., fingers, palm, thumb, connection, and ribonuclease H in the p66 subunit). The p66 palm domain contains the polymerase active site, and the four active site residues (Tyr-183, Met-184, Asp-185, and Asp-186) invariant in all known HIV RTs, lie in a turn connecting β7 and β8, while Asp-110 lies in strand β4 adjacent to Asp-185 and Asp186 [Fig. 2(a)]. The three aspartic acid residues (i.e., at positions 110, 185, and 186) together constitute the catalytic triad. Nevirapine binds in a pocket that lies in the β-sheet of the "palm" at some 10 Å distance from the polymerase active site,[98,102] thus adjacent but not overlapping with the pol active site, exactly as predicted from the enzyme kinetics studies.[17] This pocket does not exist in the p51 subunit and accordingly nevirapine does not bind to p51.[90]

According to Unge *et al.*,[100] the amino acid residues of the β-sheet [composed of β7 (179–183), β8 (186–191), and β4 (105–110), according to Ren *et al.*],[101] could be lined up in different rows, row 1 (Met-184 and Asp-185) and row 2 (Tyr-183, Asp-186, and Asp-110) being part of the pol active site, and row 3 (Tyr-181, Tyr-188, and Val-108) and row 4

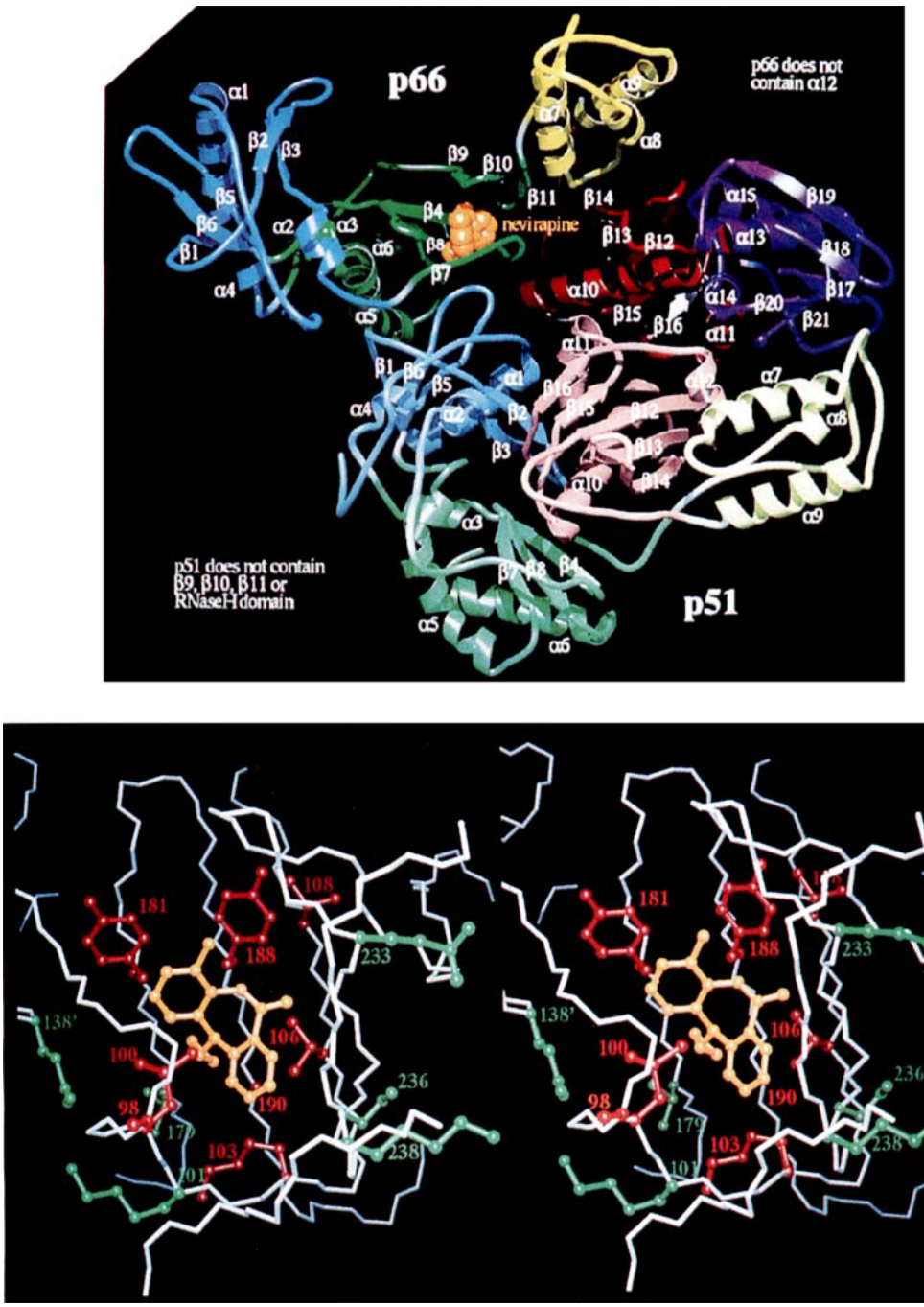


Figure 2. (a) A ribbon diagram showing the overall structure of the RT-nevirapine complex. The nevirapine molecule is shown as space-filling orange atoms; the protein is colored according to subunit and domain. Domains of the p66 subunit are drawn in saturated colors (in approximate spectral progression according to position in the polypeptide chain) and the domains of p51 are in pastel shades of corresponding hue. The domains are defined as: fingers (blue) 1–88 & 121–146; palm (green) 89–120 & 147–242; thumb (yellow) 243–311; connection (red) 312–425; and RNaseH (purple) 426–560. According to Ren *et al.*,[101] with permission. (b) Stereodiagram of known NNI resistance mutations mapped onto the RT-nevirapine structure. The nevirapine molecule is colored orange and the protein backbone white. Side chains where mutations have been observed to confer resistance to nevirapine are shown in red while those providing resistance to other NNRTIs are colored green. The diagram shows wild-type side chains, the mutations observed are: A98G, L100I, K101E, K103N/Q, V106A, V108I, V179D/E, Y188C/H/L, G190A/E, E233V, P236L, K238T, E138K(p51), and especially Y181C. According to Ren *et al.*[101] Reprinted from *Structural Biology* Vol. 2, Nature Publishing Company.

(Val-179, Gly-190, and Val-106) being part of the NNRTI-binding site.[100] The amino acid residues of rows 3 and 4 are all known to lead to mutations conferring resistance to the NNRTIs (see below) and they map at positions that allow direct contact with the inhibitor (i.e., nevirapine) [Fig. 2(b)].

The binding of NNRTI to its pocket may be assumed to indirectly affect the conformation of the nearby pol active site aspartic acid residues (Asp-185, Asp-186, and Asp-110). According to Esnouf *et al.*,[103] the binding of NNRTI would lead to a rigid-body movement of the three (β7, β8, and β4)-stranded β-sheet, and, consequently, repositioning of the catalytic aspartic acid residues. This means that NNRTIs may inhibit HIV-1 RT by locking the RT active site in an inactive conformation, reminiscent of the conformation observed in the p51 subunit.[103]

VI. NNRTI RESISTANCE MUTATIONS AT HIV-1 RT

Mutations at the amino acid residues surrounding the NNRTI-binding pocket [Fig. 2(b)] may give rise to drug resistance through a number of mechanisms:[104] (i) loss of favorable interaction between the inhibitor and the protein (i.e., at Tyr-181, the Y181C mutation may result in a loss of π stacking with the NNRTIs); (ii) creation of steric hindrances between the inhibitor and the protein (i.e., at Gly-190, mutations may lead to repulsive steric interaction with essential portions of the NNRTI, such as the cyclopropyl ring of nevirapine); and (iii) induction of main conformation changes in the binding pocket as the result of any of the mutations.

As first shown for the pyridinones,[105] HIV-1 readily develops resistance to the NNRTIs when passaged in cell culture in the presence of the NNRTIs. Resistance to the pyridinones appeared to be mediated by the amino acid mutations K103N and Y181C in the HIV-1 RT. Subsequently, emergence of drug-resistant HIV-1 mutants in cell culture have been demonstrated for nevirapine (due to the Y181C mutation),[106,107] TIBO derivatives (due to the Y181C, L100I or K103N mutation),[107–109] TSAO (due to the E138K mutation),[109,110] HEPT derivatives (due to the V106A, V108I, Y181C, Y188H or P236L mutation),[111–113] BHAP derivatives (due to the P236L mutation),[114] quinoxaline S-2720 (due to the G190E mutation),[115] oxathiin carboxanilide UC84 (due to the E138K mutation),[78] and the thiocarboxanilide derivatives (due to the L100I, K101E, K103N, and yet other mutations).[51,52]

Well-documented cases of mutations in the HIV-1 RT conferring resistance to the NNRTIs are listed in Table II. In principle, all these mutations are clustered around the pocket where the NNRTIs are assumed to bind (Fig. 2). This implies that all the NNRTIs must bind to the same site, with the exception of calanolide which may interact elsewhere at HIV-1 RT.[133] With the exception of the E138K mutation, which is located on the p51 subunit, all other NNRTI resistance mutations are located on the p66 subunit of the HIV-1 RT.[131,132] As for the E138K mutation, it must be located exactly where the p51 subunit makes contact with the pocket site within the p66 palm domain.

The importance of most of the mutations (listed in Table II) in engendering resistance to the NNRTIs has been confirmed by site-directed mutagenesis, which allows to unequivocally link a single point mutation with virus drug resistance.[92,93,124,127] Also, the drug-resistant virus strains emerging upon passage of HIV-1 in cell culture may seem predictive of the mutations that could be expected to arise in the clinic, in patients treated with the NNRTIs. For example, for nevirapine mutations were found at positions 98, 100, 103, 106, 108, 181, 188, 190 of the RT of patient isolates;[117] most of these

TABLE II
Mutations in the HIV-1 RT Conferring Resistance to NNRTIs

Amino Acid Number	Mutation		Compound(s)	References
	Codon	Amino Acid		
98	GCA → GCA	Ala → Gly	Nevirapine, pyridinone	116,117
100	TTA → ATA	Leu → Ile	TIBO, nevirapine, pyridinone, BHAP	108,109,116,118–121
101	AAA → GAA	Lys → Glu	Pyridinone, BHAP	116
103	AAA → AAC	Lys → Asn	TIBO, nevirapine, pyridinone, BHAP	105,109,116,119
106	GTA → GCA	Val → Ala	TIBO, HEPT, nevirapine, BHAP, TSAO	20,111,116,118,119, 122
108	GTA → ATA	Val → Ile	TIBO, HEPT, nevirapine, pyridinone	112,116,123
138[a]	GAG → AAG	Glu → Lys	TSAO	109,110,119,120, 124,125
179	GTT → GAT	Val → Asp	TIBO, pyridinone	116,126
181	TAT → TGT	Tyr → Cys	TIBO, HEPT, nevirapine, pyridinone, BHAP, TSAO, α-APA, quinoxaline	6,32,39,93,105–107, 109,111,112,116, 119,120,127,128
181	TGT → ATT	Cys → Ile	TIBO, nevirapine, pyridinone, BHAP, TSAO, quinoxaline	122,129
188	TAT → CAT	Tyr → His	TIBO, HEPT, pyridinone	109,111,127,130
188	TAT → TGT	Tyr → Cys	TIBO, nevirapine, pyridinone	116,127
190	GGA → GAA	Gly → Glu	Quinoxaline, TIBO, nevirapine, pyridinone, BHAP	32,115,122
236	CCT → CTT	Pro → Leu	BHAP, HEPT	113,114
138[a]	GAG → AAG	Glu → Lys	UC84	78
101 190	AAA → GAA GGA → GAA	Lys → Glu Gly → Glu	UC38	51,52
100	TTA → ATA	Leu → Ile	UC68	52
103	AAA → AAC	Lys → Asn	UC10, UC81, UC16	52
103	AAA → ACA	Lys → Thr	UC42	52
101 141	AAA → ATA GGG → GAG	Lys → Ile Gly → Glu	UC16	52

[a]Mutation located on the p51 subunit;[131,132] all other mutations located on the p66 subunit of HIV-1 RT.

mutations had also been found *in vitro* following passage of the virus in the presence of nevirapine (Table II). Also, drug-resistant virus strains isolated from patients treated with pyridinone L-697661[134] showed mutations at positions 103 and 181, which had been known to arise in cell culture as well.[105]

Whereas, as a rule, resistance to NNRTIs is engendered by single (point) mutations following exposure of the virus to a single drug, it has proven possible to increase resistance by accumulating several mutations, i.e., at positions 103, 181 and/or 188, into the same RT gene.[127] In cell culture, the Y181C mutation may further shift to the Y181I mutation under continuous pressure of the inhibitor (i.e., BHAP), thus engendering high-level resistance to most of the NNRTIs.[129] For quinoxaline S-2720, resistance development may temporarily pass through the V106A mutation before passing onto the apparently more durable G190E mutation.[122]

From the various studies that have been conducted with the NNRTIs (summarized in Table II), it has become clear that resistance to one NNRTI not necessarily leads to cross-

resistance to other NNRTIs. One of the most exclusive mutations is E138K which provokes resistance to TSAO[109,110] (and also to the oxathiin carboxanilide UC84[78]), but not to any other NNRTIs. This may be attributed to the fact that TSAO derivatives, unlike all other NNRTIs, directly interact, probably *via* the 4″-amino group of the 3′-spiro moiety with the carboxylic acid group of glutamic acid at position 138 of the HIV-1 RT p55 subunit.[124]

Other amino acid substitutions, i.e., L100I and K103N, lead to resistance to TIBO but not HEPT.[109,111] The V106A substitution confers resistance to nevirapine but not pyridinone.[116] Loviride (α-APA R89439) is active against the TIBO-resistant L100I mutant, while being virtually inactive against the TIBO-resistant Y181C mutant.[16] HIV-1 variants containing the P236L mutation that confers resistance to BHAP[114] and HEPT,[113] not only remain sensitive to other NNRTIs, but even exhibit enhanced sensitivity to these NNRTIs (i.e., TIBO, nevirapine, pyridinone, and thiazolobenzimidazole).

The mutation conferring the most generalized cross-resistance is Y181C (Table II). Although resistant to most NNRTIs, the Y181C mutant still proved relatively sensitive to the PETT compounds (i.e., LY300046.HCl),[30] 5-chloro-3-(phenylsulfonyl)indole-2-carboxamide,[39] calanolide A,[113] quinoxaline S-2720,[122] and a number of thiocarboxanilide derivatives (i.e., UC10, UC16, UC42, UC68, and UC81).[52] Even after the Y181C mutation had been shifted to the C181I mutation (which increased resistance to most NNRTIs by another 1–2 orders of magnitude), sensitivity to various HEPT derivatives (i.e., E-EBU-dM) was remarkably well retained.[129] These observations indicate that, should resistance arise against one class of inhibitors, therapy could be readily switched to other NNRTIs to which the virus has retained sensitivity.

VII. CLINICAL STUDIES

The NNRTIs have been the subject of only a limited number of clinical trials. The first compound to enter such trial was TIBO R82913. It was given to 22 patients with AIDS or AIDS-related complex by the intravenous route at doses of 10 to 300 mg daily for up to 50 weeks.[135] The compound was well tolerated, mean CD4 cell counts rose slightly during the second month of treatment, and median p24 antigen concentration fell by 41% during the first month of therapy.[135] A decrease of p24 antigenemia has also been noted in another study with intravenously administered TIBO R82913; however, oral bioavailability of the compound appeared to be low.[136] With the "second generation" TIBO derivative R91767 (8-chloro-TIBO or R86183.HCl), when given orally at 200 or 300 mg 3 times a day during 8 weeks, a clear reduction in plasma viral load was achieved, which, however, was only transient.[137] The rapid return of the viral load to baseline levels was accompanied by the occurrence of NNRTI-associated mutations, particularly the K103N mutation. From the patients treated with TIBO R82913 an HIV-1 strain with a 100-fold reduced sensitivity to R82913 was isolated after 3 weeks of treatment.[126] It was due to the Y188L mutation. On passaging this virus isolate in cord blood lymphocytes, the resistant virus was lost in favor of an HIV-1 strain containing the wild-type Y188 with normal sensitivity to R82913. This is a unique example of apparent reversibility of the mutant to the wild-type.

In clinical trials, nevirapine was given at doses of 2.5 to 400 mg. It showed a high level of oral bioavailability and long half-life: the terminal half-life and mean residence time exceeded 24 h in all but one subject; peak concentrations in plasma and areas under the plasma concentration-time curves increased proportionally with increasing the dose from 2.5 to 200 mg; and a daily dose of 12.5 mg was predicted to achieve trough

concentrations in plasma in the range required to totally inhibit replication of HIV-1 in cell culture.[138] Two phase I/II trials, one of nevirapine monotherapy and one of combination therapy with AZT, demonstrated reductions in the level of p24 antigen in serum and elevations in CD4 cell counts as early as 7 days, which returned to baseline values in a matter of weeks after the initiation of therapy.[117] This rapid loss of activity was associated with the emergence of nevirapin resistant virus containing mutations at RT amino acid residues 103 (K103N), 106 (V106A), 108 (V108I), 181 (Y181C, Y181S), 188 (Y188L, Y188H, Y188D), and 190 (G190A, G190S, G190L).[117] Interestingly, higher doses (i.e., 400 mg) of nevirapine afforded sustained reductions of p24 antigen and HIV RNA in serum for 6 months or longer.[139] At this dose, nevirapine must have been able to prevent the emergence of drug-resistant virus, or, at least, it must have achieved sufficient drug levels that exceeded the susceptibility of drug-resistant virus.

The pyridinone L-697,661 was given orally to HIV-1-infected patients at one of the following dosage schedules: 25 mg twice a day, 100 mg thrice a day or 500 mg twice a day. The compound was well tolerated and the patients showed a dose-related decrease in plasma p24 antigen levels.[134] Viral suppression induced by L-697,661 persisted through 8 weeks of treatment but rebounded thereafter.[140] This rebound paralleled the emergence of viral isolates showing resistance to L-697,661. Drug-resistance mutations appeared to be located at RT positions 181 (Y181C) and 103 (K103N, K103Q and K103R),[134] as could have been predicted from the cell culture data.[105]

For BHAP U-90152 (Delavirdine), resistance mutations of patient isolates collected after 8 weeks of treatment appeared to map at positions 103 (K103N), 181 (Y181C), and 236 (P236L),[141] which is again conform to the RT mutations arising in cell culture (Table II).

In a randomized, double-dummy, placebo-controlled trial in asymptomatic HIV-1 seropositive subjects, α-APA R89439 (Loviride) was found to induce a significant increase in CD4 cell counts that lasted for at least 24 weeks.[142] It was not clearly stated at which dosage the compound was administered (assumingly, at 3 × 100 mg per day orally) but it was mentioned that mutations at position 106 and 181 were not observed in plasma samples of patients treated with R89439 for 3 months.[142] In follow-up studies with Loviride,[143] the compound was found to lead to mutations at RT positions 98, 100, 101, 103, 181, 188, and 190 (irrespective of whether the compound has been used alone, or in combination with AZT, DDC or 3TC). The predominant mutation observed upon Loviride therapy was K103N.[143] This mutation has also been observed in an HIV-1 strain, harboring, in addition to the K103N mutation, multiple drug resistance mutations against ddN analogues.[144]

Thus, in all studies conducted to date with NNRTIs *in vivo* in patients, consistently three effects were observed that could be attributed to the compounds: (i) a marked, albeit transient, decrease in p24 antigenemia and/or plasma viremia; (ii) a significant, albeit modest, increase in CD4 cell counts; and (iii) the rapid emergence of drug-resistant virus strains. The emergence of virus drug resistance has been precipitately interpreted by some authors as "precluding" the clinical use of TIBO-like compounds,[145] or even as "clinical failures," "dashing the hopes for AIDS drugs."[146] According to Saunders,[146] the high selectivity of TIBO for HIV-1 may well have sounded the first alarm bells for what was later to become a serious issue acting to question the success of this class of compounds. Although the high specificity of TIBO may indeed be considered as a prelude for the swift development of virus drug resistance, this should by no means denigrate this uniquely meritorious class of compounds but rather encourage the design of strategies to overcome the resistance problem.

VIII. SECOND GENERATION NNRTIs

An important consideration alleviating the resistance problem is that, while having acquired resistance to one or more classes of NNRTIs, HIV-1 may still retain sufficient sensitivity to yet other NNRTIs. If thus resistance has developed to one or more NNRTIs, therapy could be switched to any of the other NNRTIs to which the virus has remained sensitive. Take, for example, Trovirdine (LY 300046.HCl),[30] which is still active against those HIV-1 strains, that, because of the Y181C or L100I mutation have become resistant to other NNRTIs (i.e., TIBO, nevirapine, pyridinone, etc.). Admittedly, Trovirdine is less inhibitory to the Y181C and L100I mutant than the wild-type, but it is still effective against these mutants at therapeutically attainable concentrations ($\leq$ 1 μM).[30] Another example is 5-chloro-3-(phenylsulfonyl)-indole-2-carboxamide,[39] which is inhibitory to the K103N and Y181C mutant RTs at an IC_{50} of about 0.1 μM, which is much lower than the IC_{50} values of TIBO R82913, pyridinone L-697,661, nevirapine or BHAP U-87201E.

Within a given class of NNRTIs, relatively minor modifications may improve, or even restore, activity against mutant HIV-1 strains that have become resistant to the parent compound. This has been most clearly demonstrated within the TIBO series, where a shift of the chlorine from the 9-position (R82913) to the 8-position (R86183), was found to partially restore activity against the Y181C mutant (EC_{50}: 0.13 μM).[23] Also, the new pyridinone derivative L-702,019 (which differs from its predecessor only by the substitution of two chlorine atoms for hydrogen in the benzene ring, and of sulfur for oxygen in the pyridine ring) remains markedly inhibitory to HIV-1 mutants that contain the K103N or Y181C mutation.[147]

Thus, in attempts to circumvent the problem of HIV-1 resistance to NNRTIs, one can switch from the "first generation" to the "second generation" NNRTIs. In some cases it has been observed that when the virus becomes resistant to one of the NNRTIs (i.e., HEPT or BHAP, due to the P236L mutation), it not only remains sensitive, but even becomes hypersensitive, to other NNRTIs such as TIBO, nevirapine, pyridinone,[114] and thiazolobenzimidazole.[113] Also the quinoxalines (i.e., S-2720) can be considered as "second generation" NNRTIs, as they retain marked activity against those HIV-1 mutants (i.e., V106A, E138K, Y181C) that have been totally refractory to other NNRTIs (i.e., nevirapine, TSAO-m^3T, and pyridinone, respectively).[122]

While oxathiin carboxanilide UC84 is virtually inactive against mutant HIV-1 strains containing the TIBO resistance mutation (L100I), or nevirapine resistance mutation (V106A), or TSAO resistance mutation (E138K), or pyridinone resistance mutation (Y181C) in their reverse transcriptase,[52] minor structural changes in the molecule such as replacing the oxygen of the carboxamide moiety by sulfur (as in compound UC23) restored the activity against these virus mutant strains: compound UC23 was only 2-fold more active than the parent compound UC84 against wild-type HIV-1, but 30- to 100-fold more active against HIV-1 mutant strains that contained the L100I, V106A, E138K or Y181C mutations in the reverse transcriptase.[78]

In contrast with the oxathiin carboxanilide UC84, the thiocarboxanilide derivatives UC10, UC16, UC42, UC68, and UC81, like compound UC23, also proved markedly active against the L100I, V106A, E138K, and Y181C mutants[52] (Table III). This is the most remarkable for the Y181C mutant, against which the thiocarboxanilide derivatives proved active at an EC_{50} of 0.1–0.2 μM, that is significantly lower than the EC_{50} of any of the "first-generation" NNRTIs (i.e., TSAO-m^3T, nevirapine, pyridinone L-697,661, BHAP U88204, and TIBO R82913).

TABLE III
Activity of Thiocarboxanilide and HEPT Derivatives Against Mutant HIV-1 Strains That Are Resistant to Other NNRTIs[52,148]

	EC_{50}[a](μM)					
Compound	HIV-1 (III_BU) Wild-Type	HIV-1 100 Leu→Ile	HIV-1 103 Lys→Asn	HIV-1 106 Val→Ala	HIV-1 138 Glu→Lys	HIV-1 181 Tyr→Cys
UC10	0.14	0.24	≥3	0.37	0.21	0.21
UC16	0.079	2.2	>3	0.19	0.20	0.20
UC42	0.014	0.47	1.7	0.11	0.10	0.12
UC68	0.023	0.54	1.3	0.21	0.17	0.20
UC81	0.011	0.45	3.2	0.15	0.09	0.19
E-EPU	0.006	0.033	1.54	0.65	0.13	0.65
E-EPU-dM	0.004	0.006	0.07	0.09	0.03	0.30
E-EPU-SdM	0.003	0.01	0.11	0.07	0.03	0.09
E-EBU-dM	0.001	0.008	0.15	0.06	0.009	0.04
I-EBU (MKC-442)	0.002	0.02	1.3	0.23	0.06	0.22
TSAO-m^3T	0.05	0.08	0.25	>75	>75	3.6
Nevirapine	0.024	0.34	5.1	7.8	0.10	7.8
Pyridinone L-697,661	0.02	0.51	1.4	0.69	0.49	10.5
BHAP U88204	0.11	2.1	4.7	1.1	0.19	1.1
TIBO R82913	0.05	5.4	13.4	1.6	0.93	6.2

[a]Fifty percent effective concentration, or compound concentration required to inhibit virus-induced cytopathicity in CEM cells.[52,148]

Also, various HEPT derivatives, i.e., E-EPU, E-EPU-dM, E-EPU-SdM, E-EBU-dM, and I-EBU (MKC-442) were found to inhibit the replication of the HIV-1 RT L100I, K103N, V106A, E138K, and Y181C mutants at concentrations that, albeit higher than for the wild-type HIV-1 (III_B) strain, were still sufficiently low to be therapeutically attainable[148] (Table III). Thus, if any resistance may arise to the "first generation" NNRTIs based on mutations at RT positions 100, 103, 106, 138 or 181, therapy should be switched to those "second generation" NNRTIs, i.e., thiocarboxanilides[52] and HEPT derivatives,[148] to which the HIV-1 mutants have retained sufficient sensitivity.

IX. DRUG COMBINATIONS

In the treatment of any infectious diseases, the combined use of different drugs has been advocated for mainly three reasons: (i) synergistic antimicrobial action; (ii) diminished toxicity, and (iii) reduced risk of drug resistance development. These principles also hold for anti-HIV drugs, including NNRTIs, which may be expected to achieve, when combined with other HIV inhibitors such as the ddN analogues (i.e., AZT), a synergistic inhibition of HIV replication, concomitantly with a counteracting effect on resistance development.

Thus, the combination of TIBO R86183 with either AZT or DDI was found to result in a synergistic inhibition of HIV-1 replication in cell culture,[23] and this observation was confirmed and extended to the combination of AZT with TIBO R82913.[149] Synergistic inhibition of HIV-1 replication in cell culture has also been reported for the combination of HEPT with

recombinant α-interferon,[150] for the combination of the HEPT derivative E-EPU with AZT,[151] for the combination of I-EBU (MKC-442) with AZT,[25,152] for the combination of I-EBU with the glycosylation inhibitor MDL-28,574 (6-O-butanoylcastanospermine),[152] for the combination of nevirapine with AZT,[153] for the combination of BHAP U-87201E (Atevirdine) with AZT,[154] and for the combination of BHAP U-90152 (Delavirdine) with AZT or DDC.[155]

In only few cases were drug combinations pursued at the enzymatic level, that is with the cell-free reverse transcriptase. The combination of BHAP U-85961 with AZT 5'-triphosphate (AZT-TP) achieved a synergistic inhibition of HIV-1 RT,[11] and so did the combination of I-EBU with AZT-TP.[26] However, combinations of AZT-TP with other NNRTIs such as nevirapine, pyridinone L-696,229, and the TIBO derivatives R82913 or R86183 did not show synergism at the enzymatic level.[26,149] It is at present unclear why I-EBU (MKC-442) [and BHAP (U-85961)] should behave differently from the other NNRTIs in this regard. As has been demonstrated specifically with nevirapine and the TIBO compounds, the binding of the NNRTI to the HIV-1 RT results in an even tighter binding of the dNTP to the enzyme.[87] This apparent cooperation between the NNRTI-binding site and dNTP-binding site may be interpreted as indicative of a synergistic action between the NNRTIs and ddNTPs. It led Spence *et al.*[87] to conclude that the interaction between the two sites may provide a means to increase the effectiveness of different drugs when combined, or of a single drug when combining the functionalities of a NNRTI and a nucleotide analogue.

The term *convergent combination therapy* has been coined for combinations of several drugs that lead to different mutations, which, when accumulated in the same enzyme (i.e., HIV-1 RT), would cripple the enzyme to such an extent that it would not longer function, and thus make the virus no longer viable.[156] The triple combination of nevirapine (or pyridinone) with AZT and DDI was indeed found to completely block virus replication, but, instead of attributing this shut off of virus replication to "evolutionary limitations that would restrict the development of multidrug resistance,"[156] a much simpler explanation is that the drug concentrations used (i.e., 0.3 μM AZT, 10 μM DDI, and 0.09 μM pyridinone), when combined, sufficed to completely suppress ("knock out") virus replication from the start (as will be further discussed in Sec. X),[52,120,122] so that virus, whether drug-resistant or not, did not have a chance to break through.

The original proposal[156] that the multiply mutated multidrug resistant virus would be unable to replicate has proved to be faulty. HIV can apparently acquire co-resistance to AZT, DDI, and nevirapine, and remain viable.[157] In fact, the HIV-1 variant with the RT mutations L74V, K103N, T215Y, and K219Q that was reported by Chow *et al.*[156] to be nonviable, turned out to exhibit growth kinetics similar to the wild-type virus.[158] The discrepancy was attributed to additional unintended mutations in the HIV-1 RT,[159] the nature of which was not revealed. It is noteworthy that the HIV-1 variant with the four RT mutations (L74V, K103N, T215Y, and K219Q) retained susceptibility to AZT and pyridinone L-697,661 at concentrations (< 1 μM)[158] that are therapeutically attainable in human plasma.

A rational approach toward drug combination may be based on the choice of those drugs that lead to mutually noncomplementary, or even better, mutually antagonistic drug-resistance mutations[19] (Fig. 3). For example, the AZT resistance mutation at position 215 (T → F/Y) is suppressed by the NNRTI resistance mutation Y181C, but not V106A.[160] Vice versa, the Y181C mutation is suppressed if AZT is co-administered with nevirapine to patients (although the appearance of mutations at positions 103, 106, 188, and 190 was increased).[117] AZT resistance reversal has also been observed in a symptomatic HIV-infected individual treated with α-APA R18893.[161] From these data it would seem that the AZT resistance mutation at position 215 and NNRTI resistance mutation at position 181 of the HIV-1 RT are mutually suppressive.

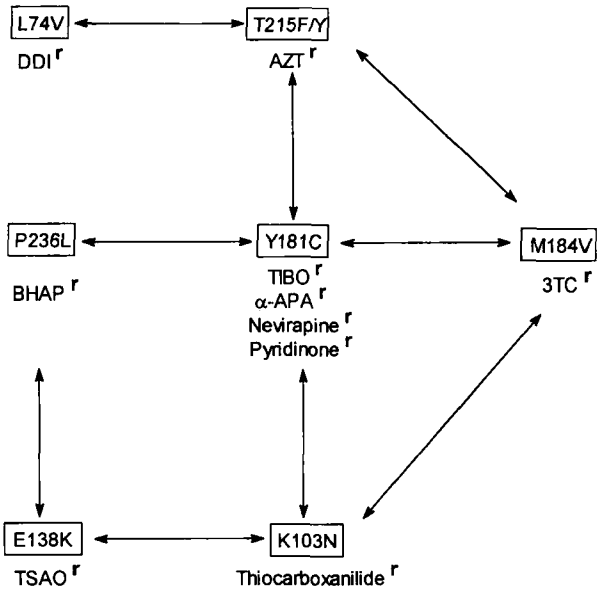


Figure 3. Mutually noncomplementary or antagonistic drug-resistant mutations in the HIV-1 reverse transcriptase.

Similarly, the AZT resistance mutation T 215 F/Y is suppressed by the 3TC resistance mutation M184V.[162] AZT-resistant mutants become phenotypically sensitive to AZT upon the Met → Val substitution at RT position 184, and this has been invoked as a potential mechanism for the sustained antiretroviral efficacy of the AZT-3TC combination therapy.[163] If the M184V mutation is accompanied by the Y181C mutation, AZT resistance (based on mutations at positions 67, 70, 215, and 219) is completely reverted to AZT sensitivity.[162] The latter observations point to the potential usefulness of the triple combination of AZT with 3TC and any of the NNRTIs (i.e., TIBO, α-APA, etc.) that lead to the Y181C mutation.

Other drug combinations that may be worth pursuing are those based on BHAP and any of the NNRTIs proned at Tyr-181, since the BHAP resistance mutation P236L increased the sensitivity of HIV-1 RT to TIBO, nevirapine and pyridinone, even if the HIV-1 RT had been mutated at position 181 (Tyr → Cys).[114] Also, the DDI resistance mutation L74V has been reported to suppress AZT resistance,[164] thus seemingly justifying the combination of AZT with DDI. However, the suppressive effect of the L74V mutation on AZT resistance does not occur in all genetic contexts.[165]

In addition to the 181 Tyr → Cys substitution, the 100 Leu → Ile substitution, another mutation engendering resistance to NNRTIs, has been found to suppress resistance to AZT when co-expressed with AZT-specific substitutions,[166] thus, again, pointing to the feasibility of combinations of AZT with NNRTIs leading to the resistance mutation L100I. Other combinations that may be worth envisaging are those based on the TSAO derivatives and thiocarboxanilides as they both lead to nonoverlapping resistance mutations [i.e., at positions 106, 138, and 181 for TSAO; and at positions 100, 101, and 103 for the thiocarboxanilides] (Table II).[52] The ddN analogue 3TC could be profitably added to this combination. When 3TC, TSAO-m^{3}T and the thiocarboxanilide UC10 were used individually, they rapidly led to the emergence of drug-resistant HIV-1 mutants containing the following mutations in their RT: E138K for TSAO-m^{3}T, M184V for 3TC, and K103 T/N for UC10.[167] If 3TC was combined with either TSAO-m^{3}T or UC10 or UC42, emergence of

drug-resistant virus was markedly delayed or even suppressed. The concomitant presence of the E138K and M184V mutations was noted in the RT of these mutant viruses that emerged under combination therapy of 3TC with either TSAO-m^{3}T or UC10, but the UC10 resistance mutation K103 T/N was no longer detected, which means that it must have been suppressed upon combining UC10 with 3TC.[167]

X. HIV-1 KNOCKING-OUT STRATEGIES

What would seem to be conceptually straightforward, potentially efficacious, and practically feasible as an approach to prevent drug-resistant HIV-1 strains from emerging is that based on the use of "knocking-out" concentrations of the NNRTIs.[120] When added to the HIV-1-infected cell cultures from the start at sufficiently high concentrations, the drugs may completely suppress virus replication and thus prevent the virus from becoming resistant. Thus, at a concentration of 2.5 μg/ml (TIBO R82913, pyridinone L-697,661) or 10 μg/ml (nevirapine, BHAP U-88204), these compounds were found to prevent virus (i.e., HIV-1 III_B) breakthrough in cell culture (i.e., CEM cells) for at least 40 days.[120] The HIV-1-infected cell cultures were apparently "cleared" from the virus infection, as attested by (i) the lack of cytopathicity, (ii) the lack of virus-specific envelope glycoprotein expression, (iii) the lack of viral p24 antigen production, and (iv) the apparent absence of proviral DNA in the cells: even with two successive 35-cycle PCR (polymerase chain reaction) rounds, no trace of proviral DNA could be detected.[120]

BHAP U-88204 (at a concentration of 1 μM) was found to completely block HIV-1 replication in MT-2 cells for at least 15 days, and, when the drug was removed on day 15, viral p24 antigen and proviral DNA remained undetectable for another 32 days.[118] Also, BHAP U-90152 (Delavirdine) has been shown to prevent HIV-1 spread *in vitro* for at least 85 days when added to the HIV-1-infected cells from the start at a concentration of 3 μM.[29] Again, when analyzed for proviral HIV-1 DNA, no evidence of the HIV-1 genome was observed, which means that the cells could be considered as completely cleared from the HIV-1 infection. In marked contrast with the NNRTIs, the ddN analogues (i.e., AZT) failed to achieve this sterilizing effect on HIV-1-infected cell cultures: when used at 3 μM[29] or 1.3 μg/ml,[120] AZT only achieved a transient suppression of virus growth and could not prevent virus breakthrough for more than a few days. In fact, earlier observations[168] had indicated that even at concentrations up to 25 μM, AZT could not prevent resumption of virus production after an initial delay so that drug-treated HIV-1-infected cell cultures eventually produced as much virus as did untreated infected cells despite the continued presence of AZT.[168]

As shown in Table IV, 3TC could not prevent virus breakthrough for more than 7–11 days when added to the HIV-1-infected cell cultures at a concentration of 0.5 or 2.5 μg/ml.[167] At a concentration of 2.5 μg/ml, nevirapine and TSAO-m^{3}T did not suppress virus breakthrough for more than 10 to 15 days. However, BHAP U-88204 at 2.5 μg/ml prevented virus breakthrough for at least 47 days; and the thiocarboxanilides UC10 and UC42 did so at a concentration of 0.5 μg/ml.[52] For quinoxaline S-2720, the concentration required to completely suppress virus breakthrough could be lowered to 0.1 μg/ml, which is considerably lower than the concentration required for the other NNRTIs (i.e., nevirapine, TSAO-m^{3}T, BHAP U-88204) to "knock out" the virus (Table IV). That at a concentration of 0.1 μg/ml, quinoxaline S-2720 was able to "knock out" the virus, was again ascertained by monitoring several parameters, including the presence of proviral DNA:[122] the HIV-1-infected CEM cells that had been treated with S-2720 at 0.1 μg/ml appeared to be completely free ("cured") of proviral DNA (Fig. 4).

TABLE IV
Suppression of Viral Breakthrough in CEM Cells Infected with HIV-1(III_B) and Treated with Different Compound Concentrations[a]

Compound	Concentration (μg/ml)	HIV-1-Induced Cytopathicity (Monitored by Syncytium) Formation, as % of Control, at Day . . . Postinfection											
		3	7	11	14	17	21	24	28	32	42	47	53
3TC	0.1	9	25	37	62	100	100	100	100	100	100	100	100
	0.5	0	0	6	25	100	100	100	100	100	100	100	100
	2.5	0	0	0	19	100	100	100	100	100	100	100	100
Nevirapine	0.1	37	87	100	100	100	100	100	100	100	100	100	100
	0.5	6	25	50	100	100	100	100	100	100	100	100	100
	2.5	0	0	6	25	62	100	100	100	100	100	100	100
TSAO-m^3T	1	0	0	12	75	100	100	100	100	100	100	100	100
	2.5	0	0	0	18	87	100	100	100	100	100	100	100
	10	0	0	0	12	62	100	100	100	100	100	100	100
BHAP U-88204	0.1	0	12	50	100	100	100	100	100	100	100	100	. . .
	0.5	0	0	6	6	25	75	87	100	100	100	100	. . .
	2.5	0	0	0	0	0	0	0	0	0	0	0	. . .
Thiocarboxanilide UC10	0.1	0	0	6	25	100	100	100	100	100	100	100	100
	0.5	0	0	0	0	0	0	0	0	0	0	0	0
	2.5	0	0	0	0	0	0	0	0	0	0	0	0
Thiocarboxanilide UC42	0.1	0	0	0	6	25	87	100	100	100	100	100	100
	0.5	0	0	0	0	0	0	0	0	0	0	0	0
	2.5	0	0	0	0	0	0	0	0	0	0	0	0
Quinoxaline S-2720	0.02	0	0	0	0	0	12	50	87	100	100	100	100
	0.1	0	0	0	0	0	0	0	0	0	0	0	0
	0.5	0	0	0	0	0	0	0	0	0	0	0	0

[a]Data taken from Refs. 52, 122, and 167.

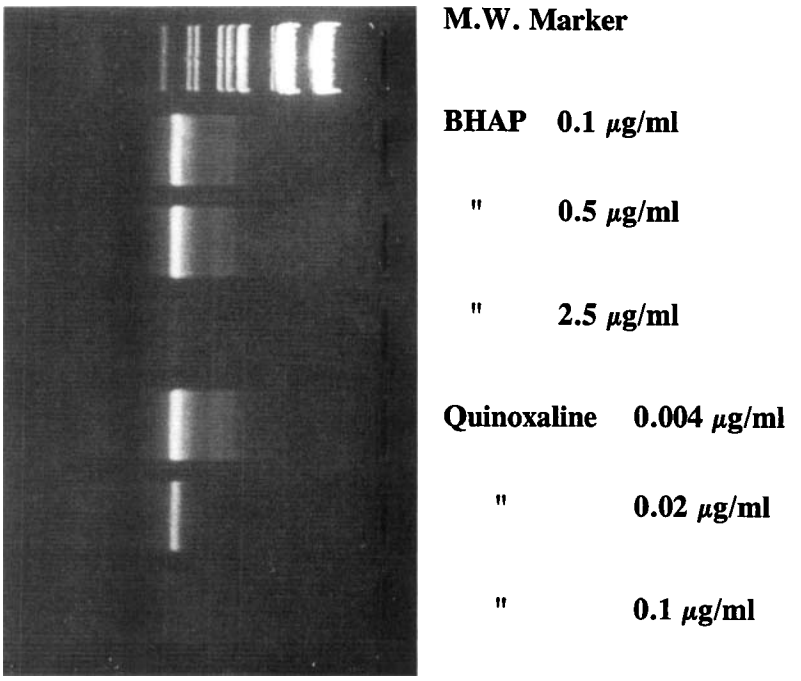


Figure 4. Detection of proviral DNA in HIV-1-infected CEM cell cultures treated with BHAP at 0.1, 0.5, and 2.5 μg/ml or quinoxaline S-2720 at 0.004, 0.02, and 0.1 μg/ml. The left lane represents several molecular weight (M.W.) markers. PCR amplification was performed on HIV-1-infected CEM cell samples that were obtained after the 10th subcultivation in the continuous presence of the test compounds at the indicated concentration. According to Balzarini *et al.*[122]

The "knocking-out" principle also holds for HIV-1 RT mutant strains: *viz.* in cell cultures infected with the TSAO-resistant E138K mutant, virus replication could be completely suppressed by micromolar concentrations of NNRTIs (i.e., TIBO, nevirapine, BHAP) that are not affected by the E138K mutation.[129] Furthermore, the "knocking-out" principle may extend to HIV protease inhibitors as well: with Ro 31-8959 (Saquinavir) long-term treatment of HIV-infected MT-4 cells at a drug concentration of 0.1 μM has been shown to result in a complete "cure" of the infection, so that after 3 months of treatment the drug could be removed safely and HIV was cleared demonstrably from the cell culture.[169]

When NNRTIs are combined with one another, or when they are combined with AZT or 3TC, the drug concentrations required to knock out the virus can be markedly reduced. For example, the concentration of BHAP U-90152 needed to prevent the spread of HIV-1 in cel culture is 3 μM, but, if combined with AZT (0.5 μM), the concentration of U-90152 can be lowered to 0.5 μM so as to totally prevent viral spread.[29]

While 3TC and TSAO-m^3T, when used individually at a concentration of 0.02, 0.05, 0.1 or 0.4 μg/ml, cannot prevent virus breakthrough in HIV-infected CEM cell cultures for longer than a few days,[167] these drugs, when combined, are able to totally prevent virus breakthrough; for more than 52 days as noted for the combination of 0.4 μg/ml of TSAO-m^3T with 0.05 or 0.1 μg/ml of 3TC (Table V). Similarly, at a concentration of 0.05 μg/ml, both 3TC and thiocarboxanilide UC10, when used as single agents, were unable to prevent virus breakthrough for more than 6 days,[167] but, at the same concentration,

TABLE V
Suppression of Viral Breakthrough in CEM Cells Infected with HIV-1 (III_B) and Treated With Dual Drug Combinations at Different Concentrations[a]

Compounds	Mean Day of Virus Breakthrough (50% Cytopathicity)			
3TC at / UC10 at	0 μg/ml	0.02 μg/ml	0.05 μg/ml	0.1 μg/ml
0 μg/ml	3	4	6	13
0.02 μg/ml	4	14	26	30
0.05 μg/ml	6	33	>52	>52[b]
0.2 μg/ml	21	42	>52[b]	>52[b]
3TC at / TSAO-m^{3}T at	0 μg/ml	0.02 μg/ml	0.05 μg/ml	0.1 μg/ml
0 μg/ml	3	4	6	13
0.05 μg/ml	4	6	26	30
0.1 μg/ml	6	19	34	>52
0.4 μg/ml	13	23	>52	>52[b]
UC42 at / TSAO-m^{3}T at	0 μg/ml	0.04 μg/ml	0.1 μg/ml	
0 μg/ml	5	16	20	
1 μg/ml	16	25	>77	
2.5 μg/ml	16	35	>77	

[a]Data taken from Refs. 52 and 167.
[b]After wash-out of the drugs at 52 days postinfection, virus breakthrough no longer occurred (i.e., the cell cultures remained virus-free for at least 12 subcultivations in the absence of the test compounds).

when combined, 3TC and UC10 suppressed virus breakthrough for more than 52 days (Table V).

With two NNRTIs (i.e., TSAO-m^{3}T and thiocarboxanilide UC42) combined (Table V), virus breakthrough could be suppressed for more than 77 days at drug concentrations (0.1 μg/ml for UC42 and 1 μg/ml for TSAO-m^{3}T) at which the individual compounds delayed virus breakthrough for 20 and 25 days, respectively.[52] Virus breakthrough could be suppressed for even longer, and at lower drug concentrations, if a third NNRTI (i.e., BHAP U-90152) was added to the combination of UC42 with TSAO-m^{3}T, which points to the feasibility of triple drug combinations in preventing virus breakthrough and resistance development.[52]

It thus appears that in cell culture, i.e., HIV-1(II_B)-infected CEM cells, a complete clearance ("cure") of the cells from the HIV infection can be accomplished with various NNRTIs,[52,120,122,167] but not with the ddN analogues AZT, DDI or 3TC.[120,167] However, the NNRTI concentrations required to knock out the virus could be lowered if they were combined with AZT[29] or 3TC.[167] Dual and triple NNRTI combinations were found to effect a complete viral clearance at drug concentrations that were lower than when the compounds were used individually.[52] These multiple NNRTI combinations could be advocated as potential treatment regimens in attempts to completely knock out HIV-1 infection.

XI. CONCLUSION

We now have at hand a number of NNRTIs (non-nucleoside reverse transcriptase inhibitors) such as the TIBO, HEPT, nevirapine, pyridinone, BHAP, TSAO, α-APA, PETT, quinoxaline, and oxathiin carboxanilide derivatives that, through a highly specific interaction with the HIV-1 RT, achieve a highly selective and potent inhibition of HIV-1 replication. These compounds therefore offer great potential for the treatment of HIV-1 infections. Yet, the virus may become rapidly resistant to NNRTIs due to mutations located at positions [i.e., 100 (Leu → Ile), 101 (Lys → Glu), 103 (Lys → Asn), 108 (Val → Ile), 138 (Glu → Lys), 179 (Val → Asp), 181 (Tyr → Cys → Ile), 188 (Tyr → Cys/His), 190 (Gly → Glu), and 236 (Pro → Leu)] that surround the pocket at HIV-1 RT where the NNRTIs are assumed to bind. The emergence of HIV-1 drug resistance is generally perceived as compromising the clinical usefulness of the NNRTIs.

However, several strategies could be envisaged to circumvent or prevent the development of resistance to the NNRTIs: (i) switching from one class of NNRTIs (to which the virus has developed resistance) to another class of NNRTIs (to which the virus has not developed resistance), or within a given class of NNRTIs, switching from one compound to another, as even small chemical modifications suffice to reinstall HIV-1 drug sensitivity; (ii) combination of different NNRTIs leading to mutations that do not confer cross-resistance and that in some instances may even engender hypersensitivity to one another; (iii) using the NNRTIs from the start at sufficiently high ("knock-out") concentrations so as to completely suppress virus replication and prevent resistance from emerging; and (iv) using from the start combinations of different NNRTIs (to which ddN analogues such as AZT and/or 3TC may be added) at "knock-out" concentrations so as to completely suppress virus replication and thus prevent the breakthrough of any virus, whether resistant or not, for a much longer time period and at much lower drug concentrations than could be achieved if the drugs were to be used individually.

Various NNRTIs, combinations thereof, as well as combinations of NNRTIs with ddN analogues have proved to be able to knock-out the virus in cell culture, but will the "knock-out" principle also work *in vivo* in the patient? According to the findings of Wei *et al.*[170] and Ho *et al.*,[171] AIDS is the consequence of continuous rounds of high-level replication of HIV-1, with dynamics (i.e., turnover of both virus and infected cells) that closely mimic the dynamics of HIV-1 replication *in vitro* in cell culture. If this view is correct, and if our findings on the *in vitro* "knock-out" of HIV-1 by the appropriate drug regimens can indeed by extrapolated to the *in vivo* situation, then it should *a priori* be possible to eradicate the virus from the site(s) where it actively replicates, provided, of course, that the compounds are able to penetrate to the sites of active virus replication. In designing the appropriate treatment protocols, drug combinations should be used from the start at sufficiently high concentrations so as to suppress the replication of all viral variants, including those quasispecies that may have pre-existing mutations conferring resistance to drugs to which the virus was not previously exposed.[172,173]

Conditio sine qua non for the "knock-out" principle to function is that the virus must replicate. If latent, and as long as it is not expressed, the proviral DNA is not going to be affected by the "knocking-out" approach. Enbrelson *et al.*[174] have shown that there are large numbers of latently infected CD4 lymphocytes and macrophages throughout the lymphoid system where HIV can persist. These latently infected cells far outnumber the actively replicating pool and constitute an intracellular reservoir large enough ultimately to contribute to much of the immune depletion in AIDS.[174] However, to drive the turnover of the CD4 lymphocytes, thus initiating the immune depletion, the virus must

first be switched on,[170,171] and, at this point, it should become vulnerable to chemotherapeutic intervention, and be susceptible to the envisaged "knocking-out" approach.

Clearly, the feasibility of the "knocking-out" strategy should now be assessed in the *in vivo* setting. Meanwhile, preliminary evidence is accumulating for both NNRTIs (i.e., nevirapine)[175] and HIV protease inhibitors [i.e., Ro 31-8959 (Saquinavir)[176] and ABT-538 (Ritonavir)[177]] that the higher the drug dosage levels (or the higher the plasma drug trough levels) the more durable the antiviral response (and the more delayed the emergence of drug-resistant virus). All three studies[175–177] concerned monotherapy. It would now seem mandatory to examine how long the duration of the antiviral response could be extended (or how long the appearance of drug-resistant mutants could be prevented) by using drug combinations at sufficiently high doses.

Progression to AIDS in HIV-infected individuals seems to be associated with a high viral burden, and, vice versa, long-term survival of persons who remain asymptomatic for many years despite HIV-1 infection seems to be associated with a low viral load.[178,179] Chemotherapeutic efforts should therefore aim at reducing the burden of HIV-1 to the levels seen in long-term survivors, or, if possible, to undetectable levels. There are cases, such as the perinatally infected infant reported by Bryson *et al.*,[180] where the HIV-1 infection was (apparently) spontaneously cleared so that the virus became undetectable. If, indeed, disease progression is linked to viral burden, strategies to eliminate the virus, as can be achieved *in vitro* by the "knocking-out" drug regimens, should be urgently pursued *in vivo*, so as to attempt to accomplish *in vivo* what *in vitro* may be interpreted as a complete recovery from the disease.

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