Polypharmacology in HIV inhibition: can a drug with simultaneous action against two relevant targets be an alternative to combination therapy?#

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#Dedicated to Professor Jan Balzarini on the occasion of his retirement and in recognition to many years of fruitful collaboration
Abstract

HIV infection still has a serious health and socio-economical impact and is one of the primary causes of morbidity and mortality all over the world. HIV infection and the AIDS pandemic are still matters of great concern, especially in less developed countries where the access to highly active antiretroviral therapy (HAART) is limited. Patient compliance is another serious drawback. Nowadays, HAART is the treatment of choice although it is not the panacea. Despite the fact that it suppresses viral replication at undetectable viral loads and prevents progression of HIV infection into AIDS HAART has several pitfalls, namely, long-term side-effects, drug resistance development, emergence of drug-resistant viruses, low compliance and the intolerance of some patients to these drugs. Moreover, another serious health concern is the event of co-infection with more than one pathogen at the same time (e.g. HIV and HCV, HBV, herpes viruses, etc). Currently, the multi-target drug approach has become an exciting strategy to address complex diseases and overcome drug resistance development. Such multifunctional molecules combine in their structure pharmacophores that may simultaneously interfere with multiple targets and their use may eventually be more safe and efficacious than that involving a mixture of separate molecules because of avoidance or delay of drug resistance, lower incidence of unwanted drug-drug interactions and improved compliance. In this review we focus on multifunctional molecules with dual activity against different targets of the HIV life cycle or able to block replication, not only of HIV but also of other viruses that are often co-pathogens of HIV. The different approaches are documented by selected examples.

Keywords

HIV, co-infection, multitarget drugs, polypharmacology, hybrids, dual drugs
**Introduction**

Infections by human immunodeficiency virus (HIV) and several other viruses (e.g. herpes viruses, hepatitis B and C, influenza, etc) are the main cause of morbidity and mortality worldwide. These viral infections represent a continuous serious concern and a global threat to human health in the twenty first century. In particular, HIV infection and the AIDS pandemic not only constitute a serious health problem but also have important social and economic consequences all over the world. HIV still causes approximately 3 million deaths annually [1]. Despite the outstanding advances in the treatment of HIV infection, AIDS still remains an incurable disease and represents one of the most important challenges for chemotherapy in this century and possibly one of the most common chronic infectious diseases in the near future. HIV is an unprecedented and fearful pathogen which rapidly mutates to escape the immune system. Moreover, HIV targets the CD4⁺ T lymphocyte cells and causes immunodeficiency, which often leads to a high susceptibility to opportunistic infections by several other pathogens including viruses, bacteria and protozoa.

Although there is no cure for HIV/AIDS, there are currently 24-27 approved drugs for the management of HIV infection that are used in combination in highly active antiretroviral therapy (HAART). It is generally accepted that antiretroviral monotherapy is not a good option to treat the HIV infection. The reason is that an incomplete inhibitory activity against HIV is associated with emergence of antiviral drug resistance, which has been observed with all antiretroviral agents discovered to date. HAART chemotherapy turned out to be useful to better suppress viral replication leading to undetectable viral loads, suppression of prominent drug resistance and to block progression of HIV infection into AIDS. However, long-term chemotherapy of AIDS and HIV infection suffers from several pitfalls, such as side-effects and eventual development of drug resistance (which compromises the long-term viral suppression), low compliance and the intolerance of many patients to drugs. Therefore, it can be assumed that drug resistance is probably the most important factor of the failure of treatment and eradication of HIV infection. There is indeed an increasing number of infected people who harbour HIV
strains resistant to multiple drugs. Moreover, the transmission of HIV variants resistant to at least one of the drug classes used in HAART has been reported in the literature [2-4]. Even more importantly, the presence of HIV mutations associated with drug resistance has been detected in treatment-naïve patients, that is, individuals who had never received any prior antiretroviral therapy.

Due to the difficulty of achieving viral eradication, combination antiretroviral therapy is nowadays a realistic modality for the prolonged reduction of the viral burden. Antiretroviral drugs acting on different viral targets and/or on the same target but with different mechanisms of action have proved successful in HAART. Indeed, the combination of currently available antiretroviral agents exploits drug interactions of compounds that are synergistic or have additive activity. When those drugs are combined, the virus needs to mutate simultaneously at multiple positions in the genome to circumvent the drug-directed blockade.

Another serious health concern is the event of coinfection of patients with more than one pathogen at the same time. Several cases of HIV patients coinfectected with other pathogens, such as hepatitis B virus (HBV), hepatitis C virus (HBC), herpes simplex virus (HSV), enteroviruses, tuberculosis, Leishmania, etc., have indeed been reported and also require combination therapy.

One alternative to combination therapy, which may help to increase the antiretroviral armamentarium and to address the devastating impact of HIV infection, is the use of hybrid (conjugated, multifunctional) molecules that combine pharmacophores in their structure that can simultaneously interfere with multiple targets [5]. These agents may eventually be more safe and efficacious than combination of single molecules, may delay the emergence of drug resistance or may prevent compromising drug-drug interactions. Multi-target drugs therefore represent an exciting strategy in the fight against complex infections and drug resistance.

This review will focus on multi-target molecules with dual activity against different targets of HIV or with dual concomitant activity against the replication of HIV and other co-pathogenic viruses. The different approaches will be illustrated with selected examples.
Multi-target drugs

A multitarget or hybrid drug can be defined as a chemical entity that combines the pharmacophores of two or more drugs with different mechanisms of action in a single molecule which is capable to interact simultaneously with two or more molecular targets [6]. Drugs of this kind are usually classified, according to the degree of framework merging, as “conjugated”, “fused” or “merged” hybrids [6]. Thus, in “conjugated” drugs both parent molecules are separated by a linker (cleavable or non-cleavable). “Fused” drugs are those in which both frameworks are directly coupled without a linker in between, and finally in “merged” drugs the frameworks integrate into a single scaffold that shares common structural features of the parent drugs to generate smaller and simpler molecular entities. On the other hand, hybrid drugs can also be classified according to the mode of action. Thus, the hybrid drug may act as a single molecule (non-cleavable hybrids) or be cleaved inside the cell releasing both parent drugs (co-drugs). Although most of the co-drugs use a linker to connect both parent drugs, there are examples in the literature of fused hybrids that release both intact compounds inside the cell. Finally, the classification can also be based on the targeted binding sites. For example, the sites can be (i) pockets of a single protein that are adjacent in space, (ii) pockets located on different proteins but recognizing similar endogenous ligands, or (iii) pockets of different proteins that recognize different ligands [7]. In order to simplify this scenario, this review follows the mode of action classification of the compounds.

1.- Conjugated non-cleavable hybrids

Non-cleavable hybrids are chemical entities with two different pharmacophores from two different drugs which are not cleaved inside the cell. Since the parent compounds are not released in the process, the biological target of each pharmacophore should be close enough to allow the interaction of the hybrid, but it does not necessarily have to be on the same enzyme or target. The design of this kind of hybrids is challenging and several factors should be taken into account: i) the stability of the linker under physiological conditions outside/inside the cell; ii) the length of the linker, which must be long and flexible enough to allow both ends of the hybrid to interact with their respective
targets; iii) the site of attachment of each parent drug, which should be carefully chosen to avoid the disruption of pharmacophores or to avoid changes in the conformation that may compromise the binding to the targets; and iv) potential negative interference on the efficient binding of the individual drugs to their target by the linker or by the other pharmacophore [8].

1.1.- Conjugates (hybrids) that bind at the same biological target

1.1.1.- HIV-1 reverse transcriptase as the target

An HIV regimen for the treatment of drug-naïve patients is generally based on the combination of two nucleoside reverse transcriptase (RT) inhibitors (NRTIs) with a third antiretroviral drug from one of the three following drug classes: allosteric nonnucleoside RT inhibitors (NNRTIs), protease (PR) inhibitors (PI) or integrase inhibitors (INIs) [9]. The close proximity between the NRTI- and NNRTI-binding sites in HIV-1 RT encouraged several researchers to design conjugated non-cleavable hybrids in order to potentially interact simultaneously at both binding sites in an attempt to have a synergistic effect to increase selectivity, and/or to eventually prevent or decrease the emergence of viral drug resistance [9].

Conjugates of an HIV-1 RT NRTI and an NNRTI were first reported by the Camarasa’s group [10]. The authors prepared different hybrids that combined in a single molecule an NRTI such as AZT with an NNRTI such as TSAO-T or HEPT. Both molecular entities were connected by non-cleavable polymethylene linkers of different lengths (n = 3-9) attached at the N-3 position of the thymine ring of each compound (1, 2, Figure 1). Hybrids of TSAO-T or HEPT linked to the natural substrate (thymidine, Thd) by a polymethylene linker (3, 4, Figure 1) were also prepared. The most active hybrid of this series was [TSAO]-([CH₂]₃-[AZT] 1 (n = 3) (EC₅₀ = 0.09 µM). Hybrids with 4- to 6-polymethylene spacers were endowed with pronounced antiviral activity, while those possessing longer polymethylene showed a substantial decrease in anti-RT activity. None of the hybrids were cytotoxic at 100 µM. The TSAO hybrids were devoid of anti HIV-2 activity which suggests that the inhibitory activity of the hybrids against HIV-1 was solely derived from the NNRTI part (TSAO-T). However, the HEPT hybrids were inactive and more cytotoxic than the parent HEPT. In retrospect, for these
hybrids the N-3 position was probably not a good choice for linker attachment. An explanation for the inactivity of AZT and HEPT hybrids could be rationalized in a subsequent work [11] where crystallographic studies demonstrated that in the RT-HEPT complex there is a stabilizing hydrogen bond between the N-3 of HEPT and the carbonyl oxygen of Lys101. When this interaction is blocked i.e. by binding the linker at the N-3, the compounds showed an important loss of inhibitory activity. The authors speculated [10] that the initial failure in increasing the inhibitory activity of the hybrids with respect to activity of the parent NRTI or NNRTIs could be due to several factors: (a) the lack of knowledge of the optimal length and position of the spacer between the NRTI and the NNRTI to place both ends of the molecule in contact for optimal interaction with their respective binding sites and/or (b) the possible lack of phosphorylation of the NRTI part (it is very unlikely that the hybrid molecules would be phosphorylated inside the cells), as it is known that only AZT-TP and not AZT interacts with RT.

![Chemical structures](image)

*Figure 1. Selected examples of NRTI and NNRTI conjugates*

Taking into consideration these findings, the authors designed a second series of hybrids in which both the nature and position of the spacer were explored. Also the NRTI part was modified and thus hybrids with an AZT-5’-phenylphosphoramidate, so as to obtain a stable masked monophosphate group resistant to phosphodiesterases, were prepared [12]. Several analogues of the prototype [TSAO]-(CH₂)₃-[AZT] (1, Figure 1) in which the NRTI AZT was replaced by d4T (5, Figure 2), TSAO-T was kept as the NNRTI, and the flexible
polymethylene linker was replaced by linkers of different nature and conformational freedom (6-9, Figure 2) were reported. Other attachment points different from the N-3 position for the linker in the nucleobase part of the NRTI were also explored (10-12, Figure 2) so as to preserve the potential base-pairing capacities of the hybrids with the template. Finally, to circumvent the obligatory dependence of the first phosphorylation step of the NRTI part of the hybrid by cellular kinases, hybrids with the NRTI bearing a 5’-phosphoramidate (a masked monophosphate) were also synthesized (13-15, Figure 2) and tested [13,14].

![Diagram](image)

*Figure 2. Structures of [TSAO-T]-(CH₂)₂-[AZT] and [TSAO-T]-(CH₂)₃-[d4T] hybrids*

Replacement of AZT by d4T in the prototype hybrid (1, n = 3, Figure 1) resulted in compounds (5) with superior anti-HIV activity. The best compound was the d4T hybrid [TSAO-T]-(CH₂)₃-[d4T] (5, n = 3) with a propyl spacer linked at the N-3 position of both NNRTI (TSAO-T) and NRTI. [TSAO-T]-(CH₂)₃-[d4T] (5, n = 3) was 10-fold more active (EC₅₀ = 0.04 µM) against HIV-1 in CEM cell cultures than 7 and 2-fold more potent than the unsubstituted parent TSAO-T [15,16]. The presence of a phosphoramidate in the NRTI hybrids also resulted in potent anti-HIV compounds [13-15]. However, no improvement in antiviral potency with respect to the nonphosphorylated analogues was observed (1 or 5, n = 3). Changing the nature and the position of the linkers on the NRTI resulted in
hybrids 6-9 (linker at position N-3) and 10-12 (linker at position C-5) that kept their anti-HIV activity. However, changes in the nature of the NRTI were important for the eventual anti-HIV-1 activity as hybrids containing d4T were more active than their respective AZT analogues [13].

Later Pontikis et al [17] also prepared NNRTI-NRTI hybrids using AZT or ddC as NRTI and HEPT analogues as the NNRTI (Figure 3). Linkers of different length and nature were used and they were attached either at the N-3 (16) or at the C-5 position of AZT (17,18), at the C-4 position of ddC (19) and at the N-1-acyclic chain of HEPT (20). Figure 3 shows some representative examples. Neither the attachment position (N-3 or C-5) of the linker to AZT nor its nature greatly affected the anti-HIV-1 activity of the hybrids (EC50: 2-5 μM). However, these compounds were inactive against the replication of either HIV-2 or the Y181C HIV-1 mutated strain. Overall, the activities of the hybrids were lower than those of the parent NRTI (AZT) and NNRTI (HEPT) components.

![Figure 3. Selected examples of AZT-HEPT hybrids](image)

Since, all AZT-HEPT hybrids lost the activity against the resistant Y181C strains the authors concluded that, as in the case of the above-mentioned TSAO-hybrids, the NNRTI (HEPT component) was the only part responsible for the activity of the AZT-HEPT hybrids. Regarding the interaction of the hybrids (AZT-HEPT) with HIV-1 RT, it was suggested [17] that it takes place by binding in the hydrophobic pocket in such a manner that both the linker and the AZT are left outside, interacting weakly either with the medium or with neighboring residues.
Finally, when AZT was replaced by ddC (19) the anti-HIV activity of the hybrids was increased by 10-fold. Interestingly, the ddC-HEPT hybrid 19 showed equipotent activity (EC$_{50}$ = 0.45 µM) against HIV-1, HIV-2 and Y181C HIV-1 strain in cell culture. Since NNRTIs are highly selective in their inhibition of HIV-1 RT, it was postulated that in this (ddC-HEPT) hybrid 19 the ddC alone contributes to the observed activity.

In a further piece of work, Hunter and co-workers [18,19] described hybrids of d4U (as NRTI) linked through a butynyl spacer to phenethylthiazolyl (PETT) derivative HI-236, a potent NNRTI against wild-type and NNRTI-resistant HIV strains [18-20]. The linker was attached at the C-5 position of d4U and at the C-2 phenolic oxygen. Several hybrids in which the length and nature of the linker were modified (21, 22, Figure 4), were also prepared [19], and a phosphoramidate prodrug of one of the d4U-Hi-236 hybrids (23) was described too [20]. Although the hybrids showed activity against HIV-1 in cell culture the simultaneous binding of both moieties (NRTI and NNRTI) in their respective binding sites on HIV-1 RT could not be demonstrated. As mentioned in the work carried out by other groups, this could probably be due to the lack of recognition by cellular kinases responsible for phosphorylating the NRTI part of the hybrid.

![Figure 4. Examples of d4U-PETT hybrids](image)

The group of Anderson [21] described NRTI-NNRTI hybrids using flexible polyethylene glycol (PEG) linkers to connect d4T (as NRTI) with TIBO (as NNRTI) (24-26, Figure 5). The PEG linker may favor drug solubility in the
cellular environment. The C-5 of d4T and the C-8 position of TIBO were used as attaching points of the linker. The d4T-PEG-TIBO conjugates 24-26 showed modest antiviral activity (mid-µM range), which decreased with increasing the length of the linker. The compound with 4-PEG fragments (24) showed the highest activity in cell culture (EC50 = 45µM). This rather poor activity could be attributed to the lack of phosphorylation of the nucleoside part or to the linker nature and/or location and/or length. To address these issues, the triphosphates of d4U-4PEG-TIBO and N3-d4U-6PEG-TIBO 27-30 were synthesized (d4U was used due to an easier chemical accessibility). However, the triphosphate derivatives did not improve activity. The observation that TIBO-6PEG showed a ~2000-fold decreased activity with respect to the parent drugs led to the suggestion that the effect of the linkers is underestimated and, rather than being inert, they can significantly alter the binding of the hybrids to RT and disrupt the proposed interaction altogether [21]. The use of 8-CI-TIBO as a pharmacophore was not an optimal choice since the addition of a 6PEG alcohol at the 8-position markedly diminished the antiviral activity of the hybrid molecule [22].

![Figure 5](Image)

**Figure 5. Structures of d4T-PEG-TIBO, d4TTP-PEG-TIBO, d4TTP-PEG-TMC and TMP-ALK-TMC hybrids**

In subsequent work, and in collaboration with Hunter and Jorgensen, hybrids bearing TMC compounds as the NNRTI and d4T as the NRTI [22] linked through a PEG spacer were described. Since phosphorylation of the nucleoside to the active triphosphate is a must, the authors described the synthesis of the
triphosphate of a d4T-TMC hybrid (28, Figure 5) and studied their interaction with HIV-1 RT. Compound 28 showed nanomolar inhibition (IC$_{50}$ = 3 nM). Although the nucleoside triphosphate moiety of the hybrid seems to bind at the active site of HIV-1 RT and to be incorporated in a base-specific manner, simultaneous binding at both sites could not be proved. Next, the study was continued by preparing thymidine (Thd) and TMC hybrids in which the length and nature of the PEG linker was changed [23]. Thus, the PEG was replaced by polymethylene alkyl linkers. Also a monophosphate and a triphosphate of the hybrids were prepared and studied as model compounds. TMP-ALK-TMC 29 was 3-fold more potent than the parent TMC-derivative against HIV RT and TTP-ALK-TMC (30) was incorporated in a correct base-pairing manner.

1.1.2.- HIV entry as the target

Several HIV entry inhibitors that generally bind either to the viral surface or to the human cell surface have been reported. These inhibitors block the virus before it can enter the cell. These inhibitors can be considered as promising microbicides to prevent viral infection. HIV-1 entry is a complex process in which the HIV-1 envelope glycoproteins gp120 and gp41 are involved. HIV entry can be inhibited by blocking one or more of the events that lead to viral infection. Binding of gp120 to the cellular surface receptor and (co)receptors CD4 and CCR5 or CXC4, respectively, initiates the fusion process [24]. Fusion inhibitors, such as peptides that bind to gp41, can block the 6-helix bundle formation [24]. T20 (enfuvirtide, Fuzeon®, Figure 6) was the first fusion inhibitor approved for the treatment of HIV-1-infected patients that do not respond to currently available drugs. The peptide is derived from the C terminus of gp41. T20 has several drawbacks that limit its use in the clinic, such as rapid proteolysis and emergence of T20-resistant virus strains. To overcome these drawbacks, next-generation fusion inhibitors with better pharmacokinetic profiles were discovered, such as T1144 [25].

In 2011, chimeric drugs that inhibit HIV-1 entry were described by Pan et al [26], who reported the preparation of a chimera protein-based HIV-1 fusion inhibitor targeting gp41 (one of the viral glycoproteins involved in the process of virus entry into the cell), named TLT35. This compound combines T20 (the first generation
fusion inhibitor), with **T1144** (belonging to the next generation of HIV-1 fusion inhibitors) linked by a flexible 35-mer (Figure 6). Both parent inhibitors (**T20** and **T1144**) target gp41 but they contain different and complementary functional domains. The authors explored different polypeptide linkers (10- to 40-mers). The 35-mer was found to contain the optimal length to bind both parent peptides. **TLT35** was more active than the parent compounds (**T20** and **T1144**) against different HIV-1 strains, including **T-20**-resistant virus strains. The compound was stable in human sera and was more resistant to proteolysis than the parent **T20** or **T1144**. This higher stability resulted in a longer half-life, and therefore the compound could eventually be used at a lower dosage and administered less frequently. The biological profile makes **TLT35** an interesting candidate of a new generation of HIV-1 fusion inhibitors able to prevent HIV-1 infection.

<table>
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<tr>
<th>Peptide</th>
<th>Sequence</th>
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<tr>
<td>T1144</td>
<td>TTWEAWDRAEYAARIEALLRALQEKKNEALREL</td>
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<tr>
<td><strong>T20</strong></td>
<td>YTSLHLIESEQNQEKKNEKELLELDKWASLWNWF</td>
</tr>
<tr>
<td>10-mer linker</td>
<td>GGGSSSgggg</td>
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<tr>
<td>15-mer linker</td>
<td>GGGSSSgggg</td>
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<tr>
<td>20-mer linker</td>
<td>GGGSSSgggg</td>
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<tr>
<td>25-mer linker</td>
<td>GGGSSSgggg</td>
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<tr>
<td>30-mer linker</td>
<td>GGGSSSgggg</td>
</tr>
<tr>
<td>35-mer linker</td>
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</tr>
<tr>
<td>40-mer linker</td>
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<tr>
<td><strong>TLT35</strong></td>
<td>TTWEAWDRAEYAARIEALLRALQEKKNEALRELGGGGSSSgggg</td>
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**Figure 6. Chimeras of T1144-T20 fusion inhibitors**

HIV infection can be also inhibited by binding to the co-receptors present at the human cell surface, in particular CCR5 [27-30], Chemokines (specially RANTES), the natural ligands for CCR5, were able to block the HIV infection [31].

Although there has been success in the different strategies for the inhibition of HIV entry, many of these inhibitors have drawbacks. For example, RANTES variants are extremely potent but since they work by binding to CCR5 they are only effective against R5-tropic viruses [27]. On the other hand, although peptides such as **T20** are effective against most of the HIV strains, the virus can mutate to reduce the ability of the peptide to bind gp41 [32-34].
In the HIV entry process there are some simultaneous events that can be inhibited. Thus, the simultaneous binding of fusion inhibitors and co-receptor inhibitors can be accomplished before the exposure of gp41 and after the interaction of gp120 with its co-receptor. Zhao et al [35] described the first chimeric inhibitors that target two different events of HIV entry. They combined, in a single molecule, one co-receptor inhibitor with one fusion inhibitor linked through an appropriated spacer (Figure 7). As the co-receptor inhibitor, the CCR5 ligand variants 5P12-RANTES or 5P14-RANTES (being among the most potent R5 entry inhibitors, mid-picomolar range) were used [27,28]. As the fusion inhibitor, the C-peptide C37 was used [35]. The C-terminus of the RANTES peptides were covalently linked to C37 via a flexible glycine-serine 10-amino acid peptide (GGGGSGGGGGS) (Figure 7). Both chimeric inhibitors 5P12-linker-C37 and 4P14-linker-C37 had a higher anti-HIV activity than that of the parent compounds (5P12-RANTES, 5P14-RANTES and C37) [35]. The chimeras showed an extremely high antiviral potency in the low picomolar range against R5-tropic viruses and a 6000-fold potency enhancement against X4-tropic viruses [35]. These molecules can be considered as excellent anti-HIV drug candidates.

<table>
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<th>Ligand</th>
<th>Sequence</th>
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<tr>
<td>C37 (C-peptide)</td>
<td>HTTWMEWDREINNYTSLIHSLIEESNOKEKNEQELL</td>
</tr>
<tr>
<td>5P12-RANTES (N-peptide)</td>
<td>GQPLMATOS</td>
</tr>
<tr>
<td>5P14-RANTES (N-peptide)</td>
<td>GQPLMSLQV</td>
</tr>
<tr>
<td>linker</td>
<td>GGGGSGGGGGS</td>
</tr>
<tr>
<td>5P12-RANTES-Linker-C37</td>
<td>GQPLMATOSGQGGGSGGSSHHTTWMEWDREINNYTSLIHSLIEESNOKEKNEQELL</td>
</tr>
<tr>
<td>5P14-RANTES-Linker-C37</td>
<td>GQPLMSLQVGGGGSGGSSHHTTWMEWDREINNYTSLIHSLIEESNOKEKNEQELL</td>
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5P12-RANTES and 5P14-RANTES represent the N-terminal sequence (0-9) of the chemokine analogues. This sequence is followed by the RANTES (10-68) sequence in the full proteins.

Figure 7. Chimera of a co-receptor inhibitor with a fusion inhibitor

2.- Conjugated cleavable hybrids: co-drugs

The co-drug approach exploits the prodrug strategy to improve the pharmacology of drugs. Two different drugs are linked via a labile covalent bond facilitating that each drug may act as a carrier for the other and vice versa (mutual prodrugs). Once inside the cell, chemical or enzymatic hydrolytic
processes release both drugs which then independently act either on the same viral enzyme or on two different enzymes of the virus life cycle.

2.1. **Co-drugs that target the same enzyme (HIV-1 RT)**

With the aim of exploring the anti-HIV-1 activity and eventually circumventing or delaying the emergence of drug-resistant virus mutants, Ijichi et al [36] prepared several nucleotide heterodimers (Figure 8) which combine a HIV-1 NRTI inhibitor, such as AZT or ddI, with (i) ddI (2’,3’-dideoxyinosine, also a NRTI) (AZT-P-ddl, 31), (ii) a NNRTI such as E-HEPU-dM (6[3,5-(dimethylphenyl)thio]5-ethyl-1-[(2-hydroxyethoxy)methyl]uracil) (AZT-P-E-HEPU-dM, 32,33) or (iii) ribavirin (AZT-P-Ribavirin, ddI-P-Ribavirin, 34, 35) linked through their 5’ positions by a phosphate bond.

![Figure 8. Selected examples of co-drugs of an NRTI and an NNRTI](image)

The heterodimers AZT-P-ddl 31, AZT-P-E-HEPU-dM 33, and AZT-P-Ribavirin 34 were potent and selective inhibitors of HIV-1 replication, while ddI-P-Ribavirin 35 was inactive. Compounds 33 and 34 showed anti-HIV activity comparable to that of AZT (EC\textsubscript{50} = 2 nM) and enhanced anti-HIV activity with respect to the second nucleoside (ddI, EC\textsubscript{50} = 13.3 µM; E-HEPU-dM, EC\textsubscript{50} = 0.007 µM). Moreover, these derivatives were also significantly active against AZT- and NNRTI-resistant virus strains. The authors carried out stability studies with these heterodimers and found that AZT-P-E-HEPU-dM 33 was completely degraded in culture medium after 72 h of incubation, while 57% of AZT-P-ddl (31), 36% of AZT-P-Ribavirin 34, and 44% of ddI-P-Ribavirin 35 were still detected after 72 h of incubation at 37°C. These findings suggest the
degradation of the heterodimers and the release of each nucleoside in cell cultures as a likely mechanism of inhibition by the heterodimers. The activity of the more potent component of the heterodimer may be responsible for the anti-HIV-1 activity of the co-drug.

The phosphate linker was also used by Japan Kokai Tokyo Koho Company [37], which reported trimer codrugs combining an AZT molecule with two molecules of ddI linked also through their 5’ positions by a phosphate bond (36, Figure 9). However, the incorporation of a second molecule of ddI not only did not enhance the activity of the trimer but decreased it (AZT-P-(ddl)2 EC₅₀ = 0.165 μM) with respect to AZT (EC₅₀ = 2 nM) and to the AZT-P-DDI 31 (EC₅₀ = 2 nM).

\[ \text{Figure 9. Structure of } \text{AZT-P-(ddl)₂ co-drug} \]

Co-drugs with other labile linkers, like carbonates and carbamates, have also been reported. Thus, Taourirte et al [38] prepared homo- and heterodimers (heterodinucleosides) of AZT and d4T bearing carbonate and carbamate linkers (Figure 10). The two parent drugs may be released inside the cell by intracellular hydrolysis and also some synergistic effects on their HIV-1 inhibition can be expected. Stability studies carried out with the carbonate linked codrug d4T-CO-d4T 37, as a model compound, at pH =7 and at pH = 11.7 indicated that the dimer is relatively stable under neutral pH and unstable at high pH. With respect to the anti-HIV-1 activity of the co-drugs, the biological results showed that while the homo and heterodimer carbamates showed weak anti-HIV activity (i.e, d4T-carbamate-AZT 40 EC₅₀ = 96.3 μM; AZT-carbamate-AZT 42 EC₅₀ = 26.0 μM), the carbonates were markedly active (i.e., d4T-CO-AZT 37 EC₅₀ = 0.006 μM; AZT-CO-AZT 39 EC₅₀ = 0.002 μM). The presence of AZT in the carbonate-linked co-drugs increased the activity (d4T-CO-d4T (37)
EC₅₀ = 0.24 µM; d4T-CO-AZT 38 EC₅₀ = 0.006 µM) being the AZT-CO-AZT homodimer 39 the most active compound (EC₅₀ = 0.002 µM). The authors concluded that, since the carbonate co-drugs could undergo a fast chemical and/or enzymatic hydrolytic cleavage, the activity observed might be attributed to the presence of the most potent nucleoside (AZT) among the two components of the co-drug.

Figure 10. Structures of co-drugs with labile linkers (carbamates and carbonates)

Velázquez et al [39] described the first example of hybrids which combined in a single molecule a TSAO-derivative as NNRTI and foscarnet (PFA) connected through a labile covalent ester bond (Figure 11). PFA is an effective antiviral agent approved for intravenous treatment of human cytomegalovirus (HCMV) retinitis in patients with AIDS, but also inhibits HIV RT by blocking the pyrophosphate binding site [40]. However, the ionic nature of PFA at physiological pH hampers its cellular uptake [41]. On the other hand, PFA was a potent inhibitor of TSAO-resistant HIV-1 strains (the activity against the TSAO-mutant was one order of magnitude higher than against the wild-type virus) and both compounds displayed additive antiviral activity [39]. These results inspired the authors to design the TSAO-PFA hybrids to explore whether the conjugation of the ionic foscarnet with the highly lipophilic TSAO derivatives might facilitate cell penetration and, if these compounds escape extracellular hydrolysis, the
parent compounds would be regenerated intracellularly. With the exception of 44, conjugates were active against wild-type HIV-1-RT ($IC_{50} = 3.7-11 \mu M$) and kept some residual activity against RT from TSAO-resistant HIV-1- strains ($IC_{50} = 293-430 \mu M$). Compound 48 was active against HIV-1-PFA-resistant HIV strains ($EC_{50} = \approx 0.16 \mu M$). The hybrids were inhibitors of HIV-1 replication ($EC_{50} = 0.08-4.5 \mu M$) although they were less active than the parent TSAO compound ($EC_{50} = 0.1 \mu M$). These results suggest a main role for the TSAO part but not for PFA in the eventual activity of the hybrids [39]. Stability studies of the hybrids showed that TSAO-PFA conjugates 43-48 were stable in PBS within 3 h of incubation (intracellular half-life $\approx 30$ min). However, some hybrids generated the parent inhibitors in CEM cell extracts.

The first phosphorylation step is often the bottleneck of the NRTI activation inside the cell. Petersen et al [42] prepared hybrids that combine D4T monophosphate, masked with one S-acyl-2-thioethyl (SATE) group, and one aromatic group to which analogues of MKC442 (an NNRTI) were linked through their N-3 position (Figure 12). These hybrids could be considered as double-prodrugs based on the mixed SATE prodrug approach [43]. The SATE group was first described by Imbach et al [44] as a carboxylesterase-labile protecting group for nucleotides and designed as mononucleotide prodrugs to bypass the first phosphorylation step. The mixed SATE pronucleotides described by Petersen [42] were designed to allow the cellular uptake of the hydrophilic nucleotide and, once inside the cell, deliver selectively both antivirals (d4T-MP and MKC442) to act at their corresponding binding sites. In particular, Petersen described hybrids that combine the masked SATE d4T monophosphate linked through the labile $p$-hydroxybenzoyl group in the N-1 position of MKC-442 (49).
or by a more stable phenolic linker that was part of the N-1 substituents of MKC-442 (50 and 51). The hybrids showed good activities against wild type HIV-1 (49 EC<sub>50</sub> = 0.03 µM and 50 EC<sub>50</sub> = 0.003 µM) and the MKC442-resistant Y181C virus strain (49 EC<sub>50</sub> = 2.7 µM and 50 EC<sub>50</sub> = 0.4 µM) and also displayed some activity against a HIV-2 strain resistant to NNRTis (49 EC<sub>50</sub> = 3 µM and 50 EC<sub>50</sub> = 2 µM). The hybrids released the d4T monophosphate and the MKC-442 derivatives inside the cells [42].

![Image](image.png)

**Figure 12. Co-drugs of d4TMSATE-MKC-442**

### 2.2.- Co-drugs that target different HIV enzymes (HIV RT, HIV PR or HIV IN)

The co-drug (double-drug) strategy has also been used to design hybrid molecules that combine two different classes of inhibitors acting on two different targets of the HIV life cycle with the aim of enhancing synergistically the anti-HIV efficacy. In the clinic, combination of HIV RT inhibitors with PIs is one of the successful treatments of HIV infection that maintains a good antiviral effect and eventually better prevents the emergence of drug-resistant virus strains [45-47]. Matsumoto et al [48,49] reported hybrids that combine in a single molecule a PI (KNI-684 or KNI-694) with an HIV RT inhibitor (AZT). In these hybrids, the carboxyl group of the PIs was directly esterified with the 5'-hydroxyl group of AZT (52, 53, Figure 13). Due to the known easy crossing of cell membranes by nucleosides [50], it was reasoned that the presence of the AZT might facilitate penetration of the hybrids across the cell membrane and, once inside the cell, release of the two parent inhibitors (AZT to interact with HIV RT after metabolisation to the 5'-triphosphate and KNIs to interact with HIV PR) can occur. The hybrids showed a more potent anti-HIV activity than that of the parent AZT and the PIs [48]. As an example, hybrid KIN-684 52 showed a
higher anti-HIV activity ($EC_{50} = 19$ nM) than that of the individual components (AZT = 126 nM; KNI-413 = 52 nM) [49].

Next, different kinds of linkers were explored, hybrids that combine KNI-727 with AZT using dicarboxylic acid linkers, such as succinic acid, succinylglycine, glutarylglycine, succinyl-$\beta$-alanine and glutaryl-$\beta$-alanine to covalently bind both hydroxyl groups of KNI-727 and AZT were prepared [51]. The best results were obtained with a hybrid that contained glutarylglycine as the linker (KNI-1039, 54, Figure 13). This compound showed potent anti-HIV activity ($EC_{50} = 0.1$ nM). This activity was 920 and 62 fold higher than that of either KNI-727 ($EC_{50} = 92$ nM) or AZT ($EC_{50} = 6.2$ nM), respectively. This synergistic effect suggests that the hybrid crosses the cell membrane and, once inside the infected cells both classes of inhibitors are released. However, it cannot be excluded that the higher antiviral activity is due to the interaction of the intact hybrid molecule with its target as such. Evaluation against KNI-727 and AZT-resistant virus strains might have helped to clarify this issue. Also, it would have been interesting to compare the antiviral activity of the hybrid molecule with those of mixtures of both parent molecules.

With the aim to enhance antiviral activity and to improve cell membrane permeability, Fossey et al [52] reported hybrids which combine two different classes of anti-HIV compounds, one NRTI with one integrase inhibitor (INI), bound by a spontaneously cleavable linker. In a first series of compounds, d4U,
d2U and d4T as NRTIs were linked to INI inhibitors belonging to the β-diketo acid (DKA) class [53] and glycine or β-alanine were used as cleavable linkers bound to the 5’ position of the nucleoside inhibitor [54] (Figure 14). An amino acid was chosen as a linker based on the following criteria: first, the hybrids should be stable outside the cell; second, after crossing the cell membrane, the hybrid must generate the parent inhibitors (Figure 14, B) that could then inhibit their different targets (HIV RT and IN). Only the d4T hybrids 55 showed significant anti-HIV activity (IC₅₀ = 3.0-5.6 µM) and among them, the hybrids containing a glycine linker (55, n = 1) showed better activity than hybrids with a β-alanine. However, all the active hybrids were less potent than the parent inhibitors from which they were derived.

Next, the authors explored the use of different spontaneously cleavable linkers, and reported hybrids of d4T as NRTIs with L-708,906 and L-731,988 to the DKA integrase inhibitors [52] (56-58, Figure 15). As self-immolative linkers in a physiological environment, ortho- or para-hydroxybenzylicarbonate (OABC or PABC) moieties were used. These linkers were expected to spontaneously decompose (by 1,6- or 1,4-elimination electronic cascade), inside the cell, after enzymatic cleavage (Figure 15, B). The linker was bonded to the 5’-OH of d4T by a carbonate group and to DKA by an amide bond. Although several hybrids
of this series, bearing PABC linkers 56, showed anti-HIV activity at submicromolar concentrations [55] (IC$_{50}$ = 0.43 µM, for 56, Figure 15), the hybrids bearing OABC linkers (structures not shown in Figure 15) showed a dramatically diminished activity.

![Chemical Structures](image)

**Figure 15. Selected examples of hybrids of HIV RT inhibitors and HIV PR inhibitors**

All the hybrids were less active an more cytotoxic, in CEM cells, when compared with DKA and d4T (the parent drugs). It was suggested that the linkers were not cleaved and that the hybrids could release the [INI]-p-aminobenzylalcohol or [INI]-o-aminobenzylalcohol and d4T, either inside or outside the cell, by hydrolysis of the carbonate bond [52]. d4T could then inhibit HIV RT after intracellular conversion to its 5'-triphosphate form. The increase in toxicity may be due to the partial release of [INI]-p-aminobenzylalcohol or [INI]-o-aminobenzylalcohol derivatives with an uncleavable spacer that do not release the parent DKA under physiological conditions. These results led to the
hypothesis that the amide bond that connects the INI and the linker is not spontaneously cleaved thus preventing the release of the INI. In later work [55], the authors reported a new series of [INI]-linker-[d4T] hybrids in which an enzymatically labile amino acid (L-alanine) linker connected to d4T by a self-cleavable PABC or OABC spacer was used. Although several of the novel hybrids showed anti-HIV activity comparable to that of the prototype 56, again they were less potent than the parent compounds and more cytotoxic [55].

3.- Dual drugs

A dual-action drug is a compound which combines two desired different pharmacological actions at a similarly efficacious dose. In the case of dual antivirals against HIV-1 replication, a single molecule may inhibit (a) two different activities of the same enzyme, (b) different targets of the same virus, or (c) may be active against two different viruses.

3.1.- Dual compounds targeting the same virus (HIV)

3.1.1. Dual compounds interacting at the same target (HIV RT)

HIV-1 RT) is a multifunctional enzyme with DNA polymerase activity (using RNA or DNA as a template) and RNase H activity. The latter is responsible for the selective degradation of the RNA strand of the RNA-DNA complex during DNA synthesis. Mutations in the polymerase domain affect the RNase H domain and vice versa since both HIV-1 RT catalytic sites are interdependent [56].

The first examples of anti-HIV-1 dual inhibitors were developed targeting HIV-1 RT. Himmel et al [57] solved the crystal structure of HIV-1 RT complexed with a dihydroxy benzoyl naphthyl hydrazine (DHBNH, Figure 16) derivative displaying sub-micromolar HIV-1 RT RNase H inhibition and very weak inhibition of HIV-1 RT polymerase activity, and found that this compound does not bind at the RNase H binding site. Instead it binds at a novel site approximately 50 Å away from the RNAse H subdomain and in between the NNRTI binding pocket and the polymerase active site, partially overlapping the NNRTIs binding pocket. From the information obtained from this crystal structure, it was predicted that DHBNH derivatives bearing bulky substituents at the para position of the benzoyl ring would partially interact with the NNRTI-binding pocket, and this
would result in increased inhibition of the RT polymerase activity while maintaining the inhibition of RNase H activity. On the basis of these premises the dual inhibitors 59 and 60 (Figure 16) were designed and synthesized. These compounds inhibit both the RNase H and DNA polymerase activities of HIV-1 RT at micromolar and at sub-micromolar potency, respectively. The structural data for the DHBNH family is consistent with the binding of the compounds close to the polymerase active site and this is believed to promote structural changes in the polymerase primer grip that may disrupt the trajectory of the template-primer between the polymerase and the RNase H domains, thus preventing the cleavage of an RNA-DNA duplex by RNase H.

![DHBNH](image)

**Figure 16. Dual inhibitors of HIV RT RNaseH and RT Pol activities**

Esposito *et al* [58] reported a series of alizarine derivatives as new dual inhibitors of HIV-1 RT-associated DNA polymerase and RNase H activities. This family of compounds was also effective in inhibiting the RNase H activity of NNRT-resistant HIV RTs. A series of anthraquinone derivatives, based on the alizarine structure (1,2-dihydroxyanthraquinone, Figure 17), substituted at positions 1 and 2 of the anthraquinone ring, were prepared. Compound 61 inhibited both enzyme-associated activities in the micromolar range (with IC$_{50}$ values of $\sim$ 12 µM). In contrast alizarine inhibited the HIV-1 RT-associated DNA polymerase function (IC$_{50}$ = 79 µM), but proved to be inactive against the HIV-1 RT-associated RNase H function (Figure 17).
Some derivatives inhibited the HIV-1 RT RNase H-associated function of K103N and Y181C HIV-1 RT resistant variants. Kinetic studies showed that this family of compounds bind neither to the NNRTI-binding pocket nor to the RNase H active site, and that they are non-competitive inhibitors of the enzyme activity. Docking studies and molecular dynamics simulations led to the hypothesis that this family of dual inhibitors bind in the pocket (as described by Himmel et al [57]) adjacent to the NNRTI-binding pocket and to the polymerase catalytic triad. Inhibition of the HIV-1 RNase H activity may be due to a modification in the accommodation of the RNA:DNA hybrid within RT. This will probably result in a variation of the nucleic acid trajectory towards the RNase H catalytic site, preventing the correct anchorage of the primer grip to the nucleic acid. Moreover, the deep occupancy of the NNRTI binding pocket and hydrophobic contacts with residues of this pocket by the compounds favor inhibition of the HIV-1 RT-associated RNA dependent DNA polymerase (RDDP) function by this family of compounds.

Next, the efforts of this group to discover and develop dual inhibitors of HIV-1 RT-associated functions, DNA polymerase and RNase H activities, continued with a program on ligand-based virtual screening (VS) [59]. Initially, they applied the shape-based screening method ROCS (Rapid Overlay of Chemical Structures) [60] on the National Cancer Institute (NCI) compound database and used as the query compound the hydrazone derivative DHBNH. Several compounds able to inhibit both activities of HIV-1 RT were found. A further parallel ligand-based VS (LBVS), using the most active compounds found in the

Figure 17. Selected examples of dual inhibitors of HIV RT RNaseH and Pol activities
first VS process, led to the identification of a new scaffold for dual inhibition of HIV-1 RT-associated functions [59]. Among them, 62 (Figure 17) was the best compound with IC\textsubscript{50} values in the low micromolar range for both activities (RNase H IC\textsubscript{50} = 3.2 µM, RDDP IC\textsubscript{50} = 0.9 µM). Compound 62 does not chelate Mg\textsuperscript{2+} ions [61] and its activity is slightly affected when tested on mutated HIV-1 RTs (K103N and Y181C), which suggests that 62 may behave as the previously reported hydrazone derivatives [57,62]. Indeed, biochemical and docking SIMULATIONS showed that 62 is able to bind to HIV-1 RT in the region described by Himmel et al [57] as the RNase H allosteric site, close to but not overlapping with the NNRTI-binding pocket that modulates both RT activities (DNA polymerase and RNase H). Next, modifications of 62 were carried out and a small library of compounds bearing the indolinone and thiazole moieties was prepared. Compound 63 (Figure 17) turned out to be a dual inhibitor that showed IC\textsubscript{50} values of 9.8 and 1.4 µM on HIV-1 RT-associated DNA polymerase and RNase H activities, respectively. The nature of the aromatic substituent in the position 4 of the thiazole ring plays a key role in modulating the activity of the two RT-associated functions [63,64].

Vernekar et al [65] reported, in 2015, novel dual inhibitors of HIV-1 RT-associated RNase H and DNA polymerase based on the 2-hydroxyisoquinoline-1,3-dione (HID) scaffold 64 (Figure 18). A series of biaryl-substituted HID 65 was designed and synthesized. All HID analogues bearing benzyl orbiarylmethyl substituents were potent dual inhibitors of RNase H and DNA polymerase-associated functions of HIV-1 RT (IC\textsubscript{50} RNase H = 0.4-5.4 µM, IC\textsubscript{50} RT\textsubscript{Pol} = 0.5-6.5 µM).

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Figure 18. Structures of dual inhibitors of HIV RT RNaseH and RT Pol activities (64-66)
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Selected compounds of this series were assayed against NNRTI-resistant HIV RT mutants (Y181C or L100I/K103N). The compounds inhibited also the mutated RT forms with IC\textsubscript{50} values similar to those of the wild-type. This suggests that the compounds do not bind to the NNRTI binding pocket. In the
presence of the chelating triad, these HID analogues may either compete for the DNA polymerase active site or bind at an unknown site of RT and disturb both the DNA polymerase and RNase H activities. Docking calculations suggested favourable binding of the compounds at the HIV RNase H active site. Moreover, some of the compounds moderately inhibited HIV IN at low micromolar concentrations, with IC$_{50}$ values ranging between 3.8 and 36 µM. Finally, a number of analogues also inhibited HIV replication at low micromolar concentrations. The best compound of this series was 66 (IC$_{50}$ RNase H = 0.8 µM, IC$_{50}$ RT Pol = 1.6 µM, EC$_{50}$ HIV-1 = 3.0 µM, CC$_{50}$ = 50 µM).

3.1.2. Dual compounds interacting at the same target (HIV entry)

Dual compounds that interfere with the HIV entry process have also been described. HIV entry into the cell is a complex process that involves the interaction of HIV gp120 and gp41 glycoproteins with human cell surface receptor CD4 and with a chemokine co-receptor (CCR5 or CXCR4, depending of the virus tropism). There are several reports which demonstrate that HIV gp120 triggers chemotactic and signaling events specifically through CXCR4 and CCR5 in a CD4-independent manner [66]. Princen et al [68] postulated that a CCR5/CXCR4 antagonist may effectively block these effects induced by the viral envelope. The N-pyridinylmethyl cyclam compound AMD3451 (Figure 19) was described as the first dual inhibitor (CCR5/CXCR4 antagonist) with activity against both X4 and R-5 tropic viruses [67]. AMD3451 efficiently blocked HIV infection at a very early stage and was active in the micromolar concentration range (1.2-26.5 µM) in various T-cell lines, and CCR5 or CXCR4-transfected cells. The specificity of the effect was demonstrated since AMD3451 did not induce an intracellular Ca$^{2+}$ response at a concentration of up to 400 µM (discarding an agonistic activity of the compound) through seven other chemokine receptors. However, AMD3451 enhanced the binding of several anti-CXCR4 monoclonal antibodies (in contrast to the specific CXCR4 antagonist AMD-3100). Focusing on CCR5-antagonist, unlike the CCR5-antagonist SCH-C, AMD3451 did not interfere with the binding of the anti-CCR5 Mab clones 45,523 and 45,531. These differences suggested to the authors that AMD3451 has a mode of binding to the CXCR4 and CCR5 coreceptors different from that of the previous entry-blocking drugs [67]. The dual interaction
of AMD3451 with both CCR5 and CXCR4, and hence, its potential ability to block the cellular infection of R5, X4 and R5/X4 viruses, showed the potential of AMD3451 to be further developed as a microbicide.

![Chemical structures](image)

**Figure 19. Dual inhibitors of HIV-entry involving HIV gp120, gp41 glycoproteins and CCR5 or CXCR4 cell surface receptors**

Following on the premise that development of dual CCR5/CXCR4 antagonists could lead to the next generation of chemokine-based entry inhibitors [68], a novel family of dual entry inhibitors which combined dual host-pathogen pharmacology was reported in 2015 [69]. The new class of antiviral agents (bearing a pyrazolo-piperidine core) interact with three distinct targets, the two GPCRs (CCR5 and CXCR4) and one viral protein (HIV RT). Following a ligand-based model approach for multitarget GPCR ligand design [70], the Aldrich Marketplace Select library (containing ≈ 5 million compounds) was screened for potential dual CCR5/CXCR4 antagonists. Biological testing of anti-HIV-1 activity against separate viral tropisms, using CD4/CCR5/CXCR4-expressing MAGI cells, led to the discovery of 67 (Figure 19), weak inhibitor of both CCR5 and CXCR4 (IC$_{50}$ = 25 and 17 µM, respectively). A further sequential and focused screening approach (based on the structure of 67) led to the prototype compound 68 (Figure 19), which also inhibited both CCR5 and CXCR4 (IC$_{50}$ = 0.8 and 3.8 µM, respectively) and blocked viral entry. Surprisingly, compound 68 inhibited isolated HIV-1 RT suggesting that this compound may also act as an NNRTI in addition to its chemokine-related activities [71]. This was confirmed when the compound was tested against an NNRTI-resistant viral strain that contained the K103N/Y181C mutations and it was shown that its antiviral activity decreased by 20-fold. It was concluded that, although the anti-HIV-RT activity seems predominant, the dual chemokine activity of this family of compounds may help to delay resistance compared to other NNRTIs. With the discovery of 68, an important tool to study viral infectivity and prototype
multitarget compounds that block HIV-1 entry through dual chemokine receptor binding became available [69].

It could be questioned whether such type of dual inhibitors may, on the one hand, delay resistance development by the multiple targeting but, on the other hand, trigger resistance due to potential sub-optimal application of the drug against at least one of the targets. Compound 68 may be a good example to help to get interesting insights into this important issue.

3.1.2. Dual compounds interacting at different targets

The development of dual drugs targeting two different steps of the HIV life cycle might also be an approach for a more favorable outcome to beat the virus. In this scenario the RNase H domain of HIV-RT and IN represent a good opportunity to develop such dual compounds, given the striking structural and functional similarities between the catalytic cores of the two enzymes [71]. Moreover, both enzymes share a similar mechanism of catalysis [72,73]. The homologies between IN and RNase H paved the way for screening several IN inhibitors against RNase H activity and vice versa [74-76]. These assays led to the discovery of new dual antiviral compounds, as will be described below.

Billamboz et al [75,76] considered the HID 69 (Figure 20) as a potential lead compound for the development of novel dual IN/RNase H compounds (IC$_{50}$ IN = 6.3 µM; IC$_{50}$ RNase H = 5.9 µM). This compound is the prototype of a series of N-hydroxyimides bearing three oxygen atoms at optimal distances for interaction with the two divalent metal ions (Mg$^{2+}$) present in the active site of both enzymes. The synthesis of other N-hydroxy imides differently substituted at position 7 or 4 were reported (Figure 20) [76]. The majority of 7-substituted compounds were inhibitors of HIV-1 RNase H and IN at micromolar concentrations, being 70 one of the most active compounds (IC$_{50}$ IN = 0.56 µM; IC$_{50}$ RNase H = 6.6 µM). A number of compounds showed a very poor inhibition of the DNA polymerase activity of HIV-1 RT even at 50 µM. The 4-substituted compounds were moderate inhibitors of HIV-1 RNase H (IC$_{50}$ RNase H = 25-50 µM) [76]. On the other hand, some of the compounds were good inhibitors of IN
at low µM concentrations. Unfortunately, the cytotoxicity in cell culture of this kind of compounds limited their application as antiviral agents [75,76].

Figure 20. Dual inhibitors of RT RNase H activity and IN

Following the same approach of testing IN inhibitors against HIV-1 RNase H, Costi et al in collaboration with Di Santo [77,78] designed in 2013 a small library of DKA derivatives analogues of 71 (Figure 21, an IN inhibitor that also inhibit the HIV-1 RNase H function) [79]. In this small library of pyrrolyl diketohexenoic derivatives, the fluorine atom of the 4-fluorobenzyl moiety of 71 was replaced by different substituents with various steric and electronic properties with the aim to obtain dual inhibitors of IN and RNase H. The compounds were prepared by a simple and efficient parallel solution-phase approach [77]. Most of the compounds inhibited both IN and RNase H activities. The best compounds showed low µM inhibition of RNase H and nanomolar IC50 values against IN in recombinant assays. The 2-Me derivative 72 (IC50 RNase H = 3 µM; IC50 IN = 32 µM), the 3,4- F derivative 73 (IC50 RNase H = 3 µM; IC50 IN = 0.60 µM), and the 4-F derivative 74 (IC50 RNase H = 2.5 µM; IC50 IN = 0.026 µM) emerged to be the most active compounds of the series. The antiviral activity was also studied in cell-based assays with a general good correlation with the inhibition data obtained against recombinant enzymes [77]. Compounds bearing an ester group showed lower activity against IN than their acid counterparts while an ester group was preferred for inhibition of RNase H function of RT. Thus, if a dual inhibition is pursued, the ester group is needed. The best dual inhibitor of this series was compound 74 (IC50 RNaseH = 2.5 µM; IC50, IN = 0.026 µM, and EC50 = 2 µM).
Next, the same group prepared new series of pyrrolyl DKA to gain further insight into the structure-activity relationships (SAR). The pyrrole ring and the DKA chain of 71 were maintained, while the other parts of the molecule were modified, in particular aromatic substituents at position 4 of the pyrrole ring were introduced; the diketo hexenoic chain was shifted from 2 to 3 position of the pyrrole or shortened into a diketo butanoic group; the fluorine moiety was replaced by alkyl or aryl groups; the carboxylic function was replaced by a triazole ring; alkyl groups were introduced at the DKA branch and finally the keto group of the DKA moiety was replaced by a NH2 [78]. Most of the compounds showed good potency against IN and moderate activity against RNase H function. In general, as expected, when comparing the activity data of the ester versus the acid derivatives, the acids were more potent as IN inhibitors than the corresponding esters, while the esters were generally more potent as HIV-1 RNase H inhibitors than the acids. Among all the new compounds, the 2-pyrrolyl diketohexenoic ester 77 (Figure 21) was the best dual inhibitor with similar IC50 values against both IN and HIV-1 RNase H function (IC50 IN = 1.2 µM; IC50 RNase H = 1.8 µM). However, 77 was 20 times less potent in cell-based assays. It should be noted that this compound bears the ester group that is necessary for dual inhibition [77].

Wang et al [80] reported dual inhibitors of HIV RT and IN by merging a NNRTI such as HEPT [81] (Figure 22) and DKA INIs. These bifunctional compounds
target the IN- and the NNRTI-binding sites. *Portmanteau inhibitors*, i.e. compounds that contain two scaffolds merged into one structure, which bind multiple sites that do not recognize similar endogenous ligands, were designed. Their design was based on the known fact that an aromatic ring immediately connected to a DKA group is an essential structural requirement for the DKA class of INIs such as 79 (Figure 22) [82,83]. A DKA moiety was incorporated into 80, a very potent second generation HIV-1 RT inhibitor derived from HEPT, to obtain the merged compound 81 [81]. X-ray crystallography of RT in complex with 80 revealed that the N-1 substituent phenyl group is positioned near the binding pocket opening [81,84], oriented at the protein/solvent interface. Docking studies of the merged compound 81 confirmed their design, showing the DKA group located at the protein/solvent interface without disturbing the binding of the NNRTI core structure [80]. A series of substituted merged inhibitors (Figure 22) were also prepared. All the merged inhibitors were evaluated against HIV RT and IN, and inhibited both enzymes with IC<sub>50</sub> values in the nanomolar (0.0092-0.23 μM) to low micromolar (1.8-7.7 μM) range, respectively. In a cell-based assay the compounds showed nanomolar activities (0.0097-0.052 μM) and very low toxicity, which resulted in high therapeutic indices.

Figure 22. Selected examples of “merged” dual inhibitors of HIV RT and IN

Compound 81 was identified as the best compound of this series (IC<sub>50 RT</sub> = 24 nM; IC<sub>50 IN</sub> = 4.4 μM; EC<sub>50 HIV-1</sub> = 10 nM) [80]. To improve the dual activity of these compounds, to determine the pharmacophore and to delineate the SAR of integrase inhibition of this family of compounds, the Wang group [85]
prepared a second series of analogues of compound 81 modified at different positions of the HEPT ring (N-1 linker length, N-3 linker, C-2 linker, additional benzyl in N-1 linker, etc.) (Figure 22).

These compounds contain an IN pharmacophore merged with the known RT pharmacophore through a shared C-6 benzyl group. The results of this study demonstrated that the C-6 benzyl group not only constitutes part of the RT pharmacophore but is also essential for the hydrophobic binding requirement for IN. The optimal activity against IN is achieved with the DKA substituent at either N-1 or N-3 position of the HEPT moiety. The optimal linker between the two minimal pharmacophores must be ~ 2-3 atoms [85]. Moreover, since the N-3 substitution annihilates the anti-HIV-1 RT activity, to have dual inhibition the DKA must be linked at the N-1 position. The best compound in this second series was 82 (IC$_{50}$ RT = 28 nM; IC$_{50}$ IN = 14 µM) (Figure 22).

Continuing with this work, the group reported in 2011 [86] a new dual IN/RT inhibitor scaffold based on pyrimidine 2,4-diones NNRTIs, containing a N-hydroxyimide functionality that resulted from a 3-N-hydroxylation of the pyrimidine ring of the HEPT molecule.

This minimal substitution resulted in HEPT compounds active against HIV-1 RT while gaining activity against IN (83, Figure 23) [86]. The dual RT/IN inhibitor 83 contained a chelating triad (C(2)O-N(3)OH-C(4)O) of two Mg$^{2+}$ together with a hydrophobic benzyl group at N-1 or C-6, thus fulfilling the minimal structural requirements for binding to IN [87]. On the other hand, the N-3-OH allows the HEPT molecule to hydrogen bond to K101 in the NNRTI binding site of HIV-1 RT [88]. With these premises in mind, the N-3-OH 83 was prepared (which was dually active against IN and RT at low sub-micromolar concentrations), and its
N-3 amino analogue 84 (being active against HIV-1 RT but not against IN). Next, a series of analogues of 83 bearing fluorine substituents at one or both of the aromatic rings [86] (Figure 23) were also synthesized. SAR studies indicated that the 3-OH is essential for integrase binding and that the benzyl group at the N-1 side chain is more important for binding than that at C-6. Fluorine substitution at the para position of the C-6 benzyl is detrimental for RT binding while the same substitution at the benzyl group of N-1 side-chain is well tolerated. Most of the compounds inhibited IN and RT at low micromolar and sub-micromolar concentrations, respectively (IC$_{50}$ IN = 3.5-8.2 µM; IC$_{50}$ HIV-1 RT = 0.17-9.4 µM) and in general showed excellent antiviral potency (EC$_{50}$ HIV-1 = 0.0080-1.1 µM) and low toxicity when tested in cell-based antiviral assays against HIV-1 replication. The best anti-HIV-1 compounds of this series were 85 and 86 with low nanomolar activity in cell culture (IC$_{50}$ HIV-1 RT = 0.0080 and 0.024 µM, respectively). Next, Wang and coworkers reported a new series of analogues of 83 substituted with a benzoyl, instead a benzyl group, at C-6 position of the pyrimidine ring [89]. Most of the compounds inhibit IN, RT and HIV-1 replication at low micromolar range. Compound 87 (Figure 23) was one of the best compounds that inhibited IN and RT at micromolar to low micromolar concentrations, respectively and HIV-1 at nanomolar concentration (IC$_{50}$ IN = 41 µM; IC$_{50}$ HIV-1 RT = 1.0 µM; EC$_{50}$ HIV-1 RT = 0.061 µM). Finally, this group reported in 2016 [89] novel dual inhibitors of IN and of the RNase H activity of HIV, based on the above mentioned 3-hydroxypyrimidine-2,4-dione scaffold that incorporated a 5-N-benzylcarboxamide moiety. The best compound of this family was 88 (Figure 23) that inhibits HIV-1 replication, IN and RNase H activity at low nanomolar concentrations (IC$_{50}$ IN = 0.021 µM, IC$_{50}$ RNase H = 0.029 µM, EC$_{50}$ HIV-1 = 0.015 µM).

3.2. **Dual drugs that target different viruses (HIV and others)**

Dual drugs acting against HIV and other viruses have also been reported. The HIV infection weakens the immune system allowing the emergence of opportunistic infections/diseases. The coinfection of HIV with other viruses is a real health risk that affects millions of people all over the world. In the treatment of coinfected patients the use of a dual compound that may block the replication
of both viruses (HIV and another) would be desirable, since this will eventually prevent, for instance the emergence of virus drug resistance, drug-drug interactions, etc. Some selected examples of dual drugs that inhibit the replication of HIV and other viruses will be presented below.

Opportunistic infections by human cytomegalovirus (HCMV) are common among HIV-infected patients reaching an incidence of 90%-100% [90]. In the search for a “second generation” of TSAO molecules (NNRTIs) more resilient to the development of drug resistance than the “fist generation”, De Castro et al [91] designed a series of TSAO derivatives substituted at the 4”-position with carbonyl groups to interact with the amino group of the Lys138 of the TSAO-resistant HIV-1 E138K strain. Compounds showed a good activity against HIV-1 wild-type virus but still were 12-15 fold less sensitive to the mutated virus although the levels of resistance were much less pronounced than those observed for the prototype compound TSAO-m³T (>1,000 fold). The compounds were also tested against the replication of other viruses in cell culture. Surprisingly, several derivatives 89-91 (Figure 24) gained activity HCMV and showed dual antiviral activity against the replication of HIV-1 and HCMV in the low micromolar range at subtoxic concentrations (EC₅₀ HIV-1 = 0.028-0.7 μM; EC₅₀ HCMV = 0.29-6.5 μM). The best dual HIV-1/HCMV compound of this series was 91 (EC₅₀ HIV-1 = 0.24 μM; EC₅₀ HCMV = 0.29 μM). These derivatives were considered as the first examples of an NNRTI with dual anti-HIV-1 and anti-HCMV activity [91].

Coinfection with HIV and hepatitis C virus (HCV) is a real health challenge that affects more than 10 million people [92,93]. Tsou et al described in 2010 [96] a dual inhibitor of HIV and HCV based on a calix[4]arene scaffold. Calix[4]arene derivatives block vascular endothelial growth factor (VEGF) which is elevated in HIV-infected T-cells and is one of the main factors associated to the development of Kaposi’s sarcoma [95]. Interestingly, some calixarene compounds show activities against viruses, bacteria, fungi and cancer cells [96]. These facts prompted the authors to study the antiviral activity of some calix[4]arene derivatives that were previously found to inhibit the binding of VEGF and platelet-derived (PDGF) to their receptors [99]. They found that compound 92 (Figure 24) was a good dual inhibitor of HIV and HCV replication.
with activities in the low micromolar concentration \((EC_{50}^{HIV-1} = 0.36 \, \mu M; \, EC_{50}^{HCV} = 1.8 \, \mu M)\). Computational studies calculations suggest that a substituted calix[4]arene at the lower rim with four hydroxyl or alkoxy groups stabilizes the scaffold into a cone conformation. Further SAR studies showed that aromatic spacers at the top of the cone are essential for anti-HIV activity while the diacid substituents at the aromatic rings are required for anti-HCV effects. In summary, the effects of alkylation in the lower-rim to maintain the cone conformation and the interaction of the head groups (substituted aromatic rings) in the upper-rim of the calix[4]arene play an essential role in the potent dual anti-HIV/HCV activities [97].

Figure 24. Selected examples of dual TSAO and calix[4]arene inhibitors of HIV and HCMV and HIV and HCV, respectively.

An important family of dual drugs against HIV and other viruses belongs to the acyclic nucleotide phosphonate (ANPs) class of compounds. ANPs were designed to bypass the first phosphorylation step leading to the nucleoside triphosphates and prevent the hydrolysis of the phosphorylated nucleosides by phosphomonoesterases. This family of compounds was nicely reviewed in 2007 by De Clercq [98] Adefovir (PMEA) (93, Figure 25) was one of the first ANPs described with dual activity against HIV and HBV \((EC_{50}^{HIV} = 2 \, \mu M; \, IC_{50}^{HBV} = 0.7 \, \mu M)\). Although it was initially used for the treatment of HIV-infected patients [99,100], its oral prodrug, adefovir dipivoxyl (Hepsera®) was licensed in 2002 for the treatment of chronically-infected HBV patients [98]. Several structural modifications were carried out on the PMEA molecule that had an impact on the activity profile. For example, the introduction of an amino group at the C-2 position of the heterocyclic base (94, Figure 25) improves the antiretroviral activity of the compound but 94 is more toxic than adefovir (93) [101]. On the other hand, the simultaneous introduction of a methyl group into the acyclic
chain and an amino group at the C-2 position (95) improved the anti-HIV activity while retaining the HBV potency. Another dual representative member of this family of compounds is PMPA (Tenofovir, 96, Figure 25) [101]. 96 was approved in 2001 as its oral prodrug tenofovir disoproxil fumarate (Viread®) for the treatment of HIV-infected patients, and later on for the treatment of HBV-infected individuals [102]. Furthermore, in 2011, Tenofovir (96) was considered for its use as a microbicide, under topical administration, effective against HIV and HSV-2 replication [103].

A novel subclass of multi-targeted ANPs, the 6-[2-(phosphonomethoxy)alkyloxy]2,4-diamino pyrimidines (PMEO-DAPyms) (Figure 25) was reported by the groups of Holý and Balzarini [104-106]. These compounds are active against HIV, HBV and herpes virus replication [107-109]. In these derivatives, the nucleobase is linked to an aliphatic alkyloxy phosphonate through an ether bond to the C-6 position of the pyrimidine base. Due to the presence of amino groups on the pyrimidine ring of these compounds, they retain the pharmacophore of a purine for base-pair recognition. Thus, the nucleobase of PMEO-DAPyms can be considered as purine mimics [110]. PMEO-DAPym (97, Figure 25) was 20-fold superior to tenofovir against HSV replication [111] (EC_{50} = 6.3 μg/mL; tenofovir EC_{50} = 105 μg/mL) and exhibit marked activity against both HIV and HBV (EC_{50 HIV} = 0.8 μg/mL; EC_{50 HBV} = 0.3 μM) [104, 105].

![Figure 25. Selected structures of dual ANPs against HIV and HBV replication](image)

In 2015 De Castro et al [112] designed and prepared a PMEO-diamino triazine (PMEO-DAT) 99 as an 5-aza-analog of PMEO-DAPym 97. Derivative 99 showed a biological profile similar to that of the parent PMEO-DAPym 97, as it inhibited HIV (EC_{50} = 2.4 μM, HIV-2 = 3.7 μM), HBV (EC_{50} = 3.1 μM), murine sarcoma virus (MSV) (EC_{50} = 0.35 μM) and VZV (EC_{50} = 3.1 μM) at low
micromolar concentrations. The compound was more selective than PMEO-DAPym since it did not induce secretion of β-chemokines in PBMC cultures.

There are also dual antiretroviral compounds isolated from natural products with activity against Chicungunya virus (CHICV), an emerging arthropod-borne virus that causes massive epidemics for which no specific antiviral therapy is currently available [113,114]. Looking for anti-CHICV drugs, Nothias-Scalia et al [115] isolated jatrophone diterpenes from a bioassay-guided whole plant extract of *Euphorbia amygdaloides* ssp. *semiperfoliata*. Six new and six known jatropane esters were isolated. The anti-CHICV activity of the isolated compounds, as well as their activity against Sindbis virus (SINV), Semliki Forest virus (SFV) (both *Alphaviruses* as CHICV), HIV-1 and HIV-2, were studied. The ester derivative 100 (Figure 26) exhibit anti-CHIKV activity and activity against HIV-1 and HIV-2 at submicromolar concentrations (EC$_{50}$ CHIK = 0.76 µM; IC$_{50}$ HIV-1 = 0.34 µM; IC$_{50}$ HIV-2 = 0.043 µM). The authors suggested that the mechanism of action of this compound could be related to a stimulation of PKCs in both viruses.

![Figure 26. Structures of dual antiretroviral compounds 100 (against HIV and CHICV) and 101 (against HIV and HSV)](image)

Another example of dual compounds isolated from natural products are the lantibiotics produced by bacteria. Lantibiotics are peptides bearing noncanonical amino acids in their structure. Labyrinthopeptin A1 (LabyA1, 101, Figure 26) is a carbocyclic lantibiotic isolated from the actinomycete *Actinomadura namibiensis* DMS6313 by Férir et al [116]. 101 displayed dual activity against HSV and HIV replication (EC$_{50}$ HSV = 0.29-2.8 µM; EC$_{50}$ HIV = 0.70-3.3 µM) in cell culture. LabyA1 showed activity against wild-type and TK-deficient HSV-1 and HSV-2 and against clinical strains comparable to that of acyclovir and cidofovir. Moreover, LabyA1 kept antiviral activity against acyclovir-resistant strains. Time of addition experiments indicated that 103 interfere with the HIV viral entry
process by interacting with the HIV envelope viral gp120 glycoprotein. Since 101 does not affect the growth of vaginal bacteria, the compound can be considered as a potential microbicide in the prevention of sexual transmission of HIV and HSV.

Discussion, conclusions and perspectives

It is generally accepted that HIV infection is a chronic disease for which a vaccine is still a difficult and challenging goal. Due to the lack of a vaccine and thanks to the efforts of the scientific and clinical communities, it is possible to treat HIV-infected patients with the currently available drugs that target different steps of the life cycle of the virus. Also, it is accepted and demonstrated that a single drug is not a good option to treat the infection, mainly due to the rapid emergence of viral drug resistance. Nowadays, treatment with a combination of different drugs acting by different mechanisms of action, under what is known as highly active antiretroviral therapy (HAART) is the therapy of choice although it is not free of pitfalls such as drug resistance development, unwanted long-term side effects, intolerance to the drugs, the transmission of HIV drug-resistant strains to at least one of the drug types used in HAART, etc. Another serious concern is the appearance of individuals co-infected with HIV and other pathogens at the same time, which require combination therapy for their treatment. One alternative to combination therapy would be the use of multi-target drugs that combine, in a single molecule, pharmacophores that may interact simultaneously with different targets. The use of multi-target drugs may be safer than that of single drugs administered in combination because of a lower probability of drug-resistance development and/or avoidance of some of the unwanted side effects resulting from drug-drug interactions. However, some possible disadvantages should also be taken into consideration since a multtarget drug (whatever the type, hybrid, dual or merged) that has different IC$_{50}$ values for its two molecular targets may eventually be active mainly against its target with the lowest IC$_{50}$ because it may not efficiently suppress its second target. As a consequence, this second target may be more prone to resistance development which will soon weaken the drug in its action and compromise other drug actions against the mutated target.
Moreover, in the case of dual inhibitors with similar EC_{50} values against two pathogens, but even more in case the EC_{50} for one target proved to be markedly higher than the EC_{50} for the other target, it would be imperative to ascertain that drug levels that can be achieved in the treated patients are high enough to efficiently suppress the pathogen that has the least sensitivity for the drug. Therefore, an extensive SAR should be performed to select the most suitable hybrid drug molecule to be used to optimally suppress both pathogens. Sufficiently high drug levels are also a prerequisite to ascertain that resistance development against the hybrid drug by the least sensitive pathogen would be suppressed as much as possible.

Finally, as is the case for any drug combination used in drug treatment, the effect of the combination of the drugs on clearance of the individual drugs is always a matter of concern and need to be carefully addressed before drug combinations (or eventually hybrid drugs) are applied. Increased clearance may usually be a disadvantage weakening the drug suppression of the pathogen, but decreased clearance will be advantageous, resulting in potent and sustained clinical activity as is clearly shown for combinations of ritonavir and the booster cobicistat not active as such in the HAART cocktails for HIV treatment.

In this review we gathered different strategies followed to develop multi-target agents: design and synthesis of (i) hybrid drugs that combine two different molecules linked through cleavable or non-cleavable linkers, that act either on the same molecular target of HIV, or (b) on different molecular targets of HIV; (ii) dual drugs that interact only with HIV or with different viruses; and (iii) merged drugs that combine two different activities in a single structure.

From our personal point of view the rational design and discovery of multitarget drugs with activities against two different pathogens can be useful to administer to individuals who are infected not only by both pathogens (on the strict condition that both pathogens are efficiently suppressed by the drug at the given dose), but also in the case of individuals infected by one of both pathogens. In such cases, the intrinsic activity against the other pathogen might prevent superinfection by this pathogen. Thus, such drug may serve as a
prophylactic agent against the second pathogen in addition to the suppressive activity against the first pathogen.

Currently, combination therapy is the treatment of choice for HIV infected individuals, while the use of multitarget drugs is not available yet, although perhaps it is worth to mention the case of tenofovir that has activity against both HIV and HBV, and can be used to treat patients co-infected by both viruses. Since tenofovir is more active against HBV than against HIV, the recommended dose for HIV treatment can be used to co-treat HBV without fear of resistance development of HBV against the drug.

The rational design and development of multitarget single drugs is an exciting and challenging field of research that hopefully may offer a real complement or an alternative to combination therapy of HIV infection in a near future.

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