HIV-1 RNase H: Recent Progress in an Exciting, yet Little Explored, Drug Target

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Abstract: HIV-1 reverse transcriptase-associated ribonuclease H (RNase H) is an attractive non traditional target for drug development which has been, so far, little explored. All drugs shown to inhibit HIV-1 RNase H are reported, including the recently described classes of compounds that interact with the metal ions in the active site.

Keywords: HIV-1, Reverse transcriptase, Ribonuclease H, Inhibition, Drug development, N-hydroxyimides, Diketo acids.

INTRODUCTION

The Human Immunodeficiency Virus (HIV) is the etiological agent of the acquired immune deficiency syndrome (AIDS), a global epidemic which has become a major public health problem worldwide. Recently, it has been estimated that nearly 40 million people live today with HIV and that, just in the year 2004, almost 5 million people have been newly infected by HIV while approximately 3 million persons died for AIDS (www.unaids.org). Among the infected people, more than 50% live in the sub-Saharan Africa, whereas 1 million of HIV-1 positive patients are present in US and 600,000 in western Europe. Given the great difficulties in developing an HIV effective vaccine, in the last 15 years major efforts have been addressed towards the identification of valuable therapeutic strategies. Their successful development has effectively turned a dreadful disease into a manageable chronic infection, at least for the patients who can somehow access the therapy. However, even for these patients, despite the current presence of 20 antiretroviral drugs approved for the treatment, factors such as the persistence of viral replication reservoirs, the selection (and spreading) of resistant mutants, and the occurrence of several drug-side-effects lead to the compulsory need of implementing the existing therapeutic armamentarium with new drugs which could possibly block steps of the virus life cycle which are different from the ones which have been already targeted [1].

Among the novel, 'non traditional', targets that have been investigated in order to identify new HIV inhibitors, one is the ribonuclease H (RNase H) function associated to the viral reverse transcriptase (RT) which hydrolyses the RNA of the RNA/DNA replication intermediate. In fact, several studies have demonstrated that the abolition of this enzyme function stops the virus replication and that, therefore, it is an attractive step for drug development [2]. However, RNase H is a drug target which has been so far little explored and, as a result, all RT inhibitors currently approved, and/or under investigation in clinical trials, inhibit the RT-associated polymerase activity, while none of them blocks the RT-associated RNase H activity. Given these considerations, in the present review I will focus on the RNase H function and its structural interaction with the other RT domains, the relationship between the RNase H activity/inhibition and the selection of RT resistant mutants, and all the compounds which are able to inhibit the RNase H activity.

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1. HIV-1 RNASE H FUNCTION

The HIV genome is replicated through several steps, shown in (Fig. **1**), that are carried out by the HIV-1 RT, a multifunctional enzyme which displays two associated activities: i) a DNA polymerase activity, that can use both RNA and DNA as a template; ii) an RNase H activity, that selectively degrades the RNA strand of the hybrid RNA/DNA which is formed during the synthesis of the minus (-) strand DNA that uses (+) RNA as template. The initial step for the (-) strand DNA synthesis is the hybridization of a host-derived tRNA to the primer binding site (PBS) near the 5′ end of the HIV genome [2]. The subsequent RNA-dependent DNA synthesis proceeds until the RT reaches the 5′ end of the RNA genome, leading to a (-) DNA strong stop. This process exposes the repeat (R) sequence at the 3' of the (-) strand DNA which can be thus hybridized to the R sequence at the 3'-end of the (+) strand RNA, allowing then a strand translocation that can take place either inter- or intramolecularly. Noteworthy, the RNase H activity is absolutely required for strand transfer and, in fact, mutations that selectively abolish its function stop strand transfer and accumulate (-) strand DNA strong stop [3]. After the first strand transfer, the polymerization of (-) strand DNA continues until conclusion, while the RNase H degrades the (+) strand RNA. However, a purine-rich run of 15 bases, known as the polypurine tract (PPT), is initially resistant to the RNase H degradation and is used to prime the (+) strand DNA synthesis which is than elongated through the U3, R and U5 sequences, and also through a portion of the tRNA. At this point, RNase H selectively removes the PPT and tRNA, thereby exposing the (+) strand DNA PBS sequence and allowing a second strand transfer to occur. In fact, the (+) strand DNA is translocated to the 3′ end of the (-) strand DNA strand through a PBS complementarity. Finally, it is assumed that the replication intermediate

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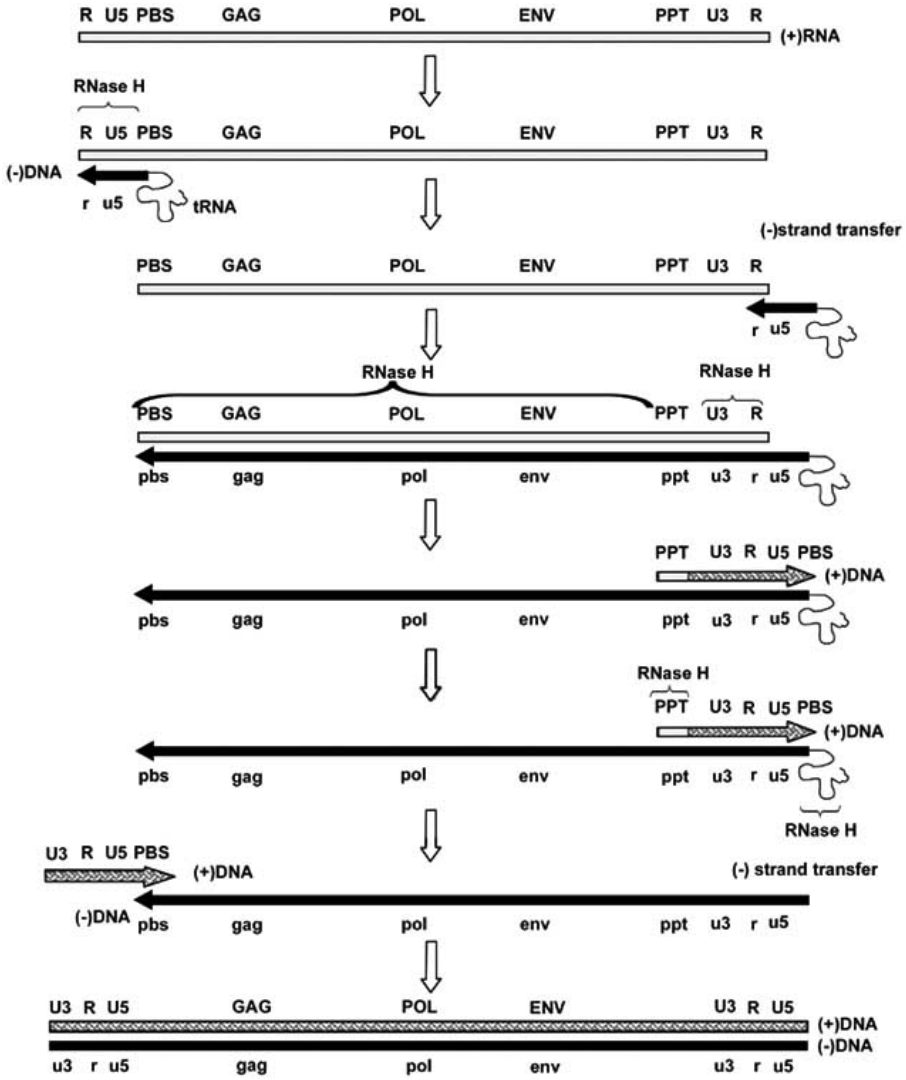

Fig. (1). HIV-1 RNA genome conversion into DNA by RT.

is circularized and that bidirectional DNA synthesis continues, during which a strand displacement function of RT is required.

Together with transposases, retroviral integrases and RuvC resolvase, the RNase Hs belong to the polynucleotidyl transferase family and catalyzes the phosphoryl transfer through nucleophilic substitution reactions on phosphate esters [4]. Several studies have shown that the RNA hydrolysis catalyzed by the HIV-1 RNase H may be performed through two different modes of cleavage. The first takes place in concert with the DNA synthesis and is generally referred to as polymerization-dependent RNase H mode of cleavage [5, 6]. The second takes place independently of the DNA synthesis and is referred to, therefore, as polymerization-independent RNase H mode of cleavage [7]. The latter mode of cleavage has been proposed to be particularly important for viral replication [8]. In fact, it has been demonstrated that during the HIV-1 reverse transcription process the nucleotide addition rate of the polymerization reaction is 7-10 fold faster than the RNase H degradation rate [9]. This observation suggested that the polymerizing RT may not remove all the RNA during the (-) strand DNA synthesis [7, 10, 11]. Further complementation studies with polymerase-deficient and RNase H-deficient HIV-1 RTs have demonstrated that the former RT (which has only a polymerase-independent mode of cleavage) is sufficient to allow virus replication [12]. Overall, these findings demonstrated that, during viral infection, RNA-dependent DNA polymerase (RDDP) and RNase H RT-associated activities are not necessarily coupled, and it has been consequently proposed that the polymerizing RT may leave behind non degraded RNA fragments in its *in vivo* function. Therefore, given that a single virion contains 50-100 RT molecules, many RTs may be actually available to bind to the RNA/DNA duplex and to degrade the residual RNA fragments through a polymerization-independent cleavage, so that the entire reverse transcription process would not need to be completed by a sole RT molecule.

2. HIV-1 RT STRUCTURE AND FUNCTIONAL CORRELATION OF ITS DOMAINS

The HIV-1 RT is an asymmetric heterodimer composed of a p66 kDa subunit (p66) and a p51 kDa subunit (p51), the latter deriving from the p66 polypeptide by a specific HIV-1 protease-mediated cleavage of its p15 C-terminal (which

contains the RNase H domain) [13, 14]. The p66 subunit shows a 3D-structure similar to the one of several polymerases such as the Klenow fragment of the *E. coli* DNA polymerase, the DNA polymerase β, the *Taq* polymerase and others, and consists of the fingers (residues 1-85 and 118-155), palm (residues 86-117 and 156-237), thumb (residues 238-318), connection domain (residues 319-426) and RNase H domain (residues 427-560) [14]. Differentently, even though it is composed by the same 440-amino-acids of the p66 N-terminal that comprise the polymerase site, the p51 subunit has a spatial arrangement diverse from the p66 subunit. It has no cleft for the template binding, the residues required for catalysis are buried into the subunit and, as a result, it does not contain a functional polymerase site. Hence, the two RT-associated enzymatic functions are carried out by two distinct catalytic sites which reside in the p66 subunit and are positioned at a distance of approximately 18 base pairs from each other [15]. The two catalytic sites are inter-dependent and, in fact, mutations in the polymerase domain affect the RNase H activity and, *vice versa*, mutations in the RNase H domain affect the polymerase function.

The HIV-1 RNase H domain have been characterized both as a part of the whole RT [14, 16, 17] and as an isolated domain [18] and shown to form a central five-stranded mixed -sheet, surrounded by 4 -helices and 8 connecting loops [2, 15]. The core domain of the RNase H active site contains a three-amino-acid DDE motif which is highly conserved: mutations in any of the D443, D498 and E478 residues abolish enzyme activity [19, 20]. The HIV-1 RNase H, similarly to other polynucleotidyl transferases, is metal-dependent and requires either Mg^{2+} or Mn^{2+} for enzyme function [21]. However, differently from the structure of the *E. coli* RNase H which needs a single divalent cation in the active site [22], the structure of the HIV-1 RNase H domain has been proposed to need two divalent cations which, consistently with the phosphoryl transfer geometry, seem to be coordinated by the active site carboxylates D443, E478, D498 and D549 [23]. Catalysis occurs by deprotonation of water to form a nucleophilic hydroxide group that attacks the scissile phosphate group on RNA, according to the general phosphodiester hydrolysis reaction scheme which requires the concerted action of a general base activating the nucleophile and a general acid protonating the leaving group [24].

X-ray crystal structure studies have shown that two important regions of the HIV-1 RT, named "primer grips", are involved in the proper positioning of the template primer since they guide the nucleic acid into the two active sites: a "polymerase primer grip" and an "RNase H primer grip" [25-27]. The RNase H primer grip makes extensive contacts with the DNA primer and mutations in this amino acid region have been examined. Important amino acids probably helping to control the trajectory of the RNA template into the RNase H catalytic center and contributing to the RNase H grip include the T473-N474-Q475-K476-quartet, Y501, and I505 [28]. Noteworthy, they have been shown to be conserved among retroviral and bacterial RNase Hs [27] and their mutation leads to a decrease in catalytic activity [29]. Interestingly, contacts among the RNA/DNA hybrid bases, the RNase H grip amino acids and the RNase H catalytic center occur primarily through the sugar-phosphate backbone, while only three direct proteins contacts with the bases have been observed. Two of them have been found in the RNase H active site where the scissile bond is cleaved.

Structural evidences suggest that the HIV-1 RT has an high intrinsic conformational flexibility which is essential in controlling the mechanism of both polymerase and RNase H catalysis and which plays an important role in RT function. In fact, upon binding with the template-primer duplex, highly concerted motions of different RT domains has been observed [30]. Several studies indicated an hinge-bending motion between the fingers and the palm domains of p66 and the rest of the molecule. The p66 thumb, in particular, seems to be extremely flexible and a strong cross-correlation of opposite sense has been found between the p66 thumb and the RNase H domains after template binding. Similarly, an anti-correlated motion has been observed between RNase H and the p66 finger domains while, on the contrary, a positive cross-correlation occurs between the RNase H and the p51 thumb domains [30]. Globally, the movements of the RNase H domain may complement those of the above p66 domains to control the closing/opening of the p66 cavity for DNA binding. This motion has been proposed to correspond to the processing motion in which the duplex RNA/DNA is moved along the surface of the protein and exhibits a coordinated release by hand and pull by RNase H domain or, alternatively, a push by the closed hand followed by its release and pull by the RNase H domain. A more detailed knowledge of this dynamic will be certainly important for new therapeutic intervention given that RT mutants in the fingers, palm and thumb domains can affect the specificity and/or efficiency of the RNase H cleavage [31-36].

3. RELATIONSHIP BETWEEN POLYMERASE RT INHIBITORS (RTI) AND RNASE H ACTIVITY

Currently, there are three classes of polymerase RTIs: nucleoside analogues (NRTI), nucleotide analogues and non nucleoside compounds (NNRTI) [1]. NRTI and nucleotide analogues are phosphorilated to triphosphate by the cellular machinery and are incorporated into the nascent viral DNA where they stop the chain elongation process. NNRTIs do not need cellular activation, they bind to an hydrophobic specific region of the p66 subunit (known as NNRTI binding pocket) which is adjacent to the polymerase active site, and induce an allosteric change in the protein conformation that inhibits the DNA polymerization.

Upon binding, NNRTIs have a drastic effect on the RT domains collective motions, particularly on the p66 thumb flexibility, which is almost suppressed, and on the RNase H domain mobility, which is enhanced [30]. These significant structural rearrangements have been recently proposed to be linked to the movements of a stretch of 20 amino acids at the interface between the p66 connection and the RNase H domain, near to the NNRTI binding pocket, which act as the target site controlling the global reorientation of the RNase H domain following NNRTI binding [37]. The correlation between NNRTI binding and RNase H activity has been also explored by mutation studies. It is well known that HIV-1 resistance to NNRTIs involves mutations in several amino acid residues that line the NNRTI binding pocket and whose

substitution reduces the drug binding to RT [1]. A number of studies have examined the effects of these mutations on the RT enzymatic functions showing that some of the most common mutations selected under the drug treatment, such as K103N, V106A, Y181C and P236L, alter the rate and specificity of the RNase H cleavage with no significant effects on RNA- or DNA-dependent DNA polymerization [38-40]. Noteworthy, these studies confirmed that greater reductions in RNase H activity are associated with greater reductions in replication fitness [38, 39]. Straightforward evidences of the NNRTIs influence on the RNase H function came also from studies that showed that the binding to RT of NNRTIs such as nevirapine and a thiobenzimidazolone (TIBO) derivative directly affected the RNase H function, increasing the polymerase-independent RNase H catalytic activity and altering its cleavage pattern [41, 42]. Recently, these results led to test the behavior of NNRTI and RNase H inhibitor combinations on the RT polymerization and cleavage functions. Such drug combinations have been actually found to be synergistic, possibly involving an effect on the strand transfer process [43].

A very essential functional interaction has been also proposed to take place between NRTI and RNase H inhibitors. In fact, it has been recently hypothesized that NRTI incorporation leads indeed to chain termination, impeding further DNA synthesis, but only until a nucleotide excision occurs allowing so the DNA synthesis to resume [44]. In this view, the degradation of the RNA template catalyzed by the RNase H function leads the RT, whose polymerization process is blocked, to dissociate from the DNA and is, therefore, an essential component of the NRTI inhibition mechanism. The observation that mutations decreasing the RNase H RNA hydrolysis rate also confer resistance to 3'-azido-3'-deoxythymidine and 2',3'-didehydro-2',3'-dideoxythymidine supports this hypothesis and implies that RNase H inhibitors should be better examined in their combination with NRTI since this drug association may be somehow antagonistic [44].

4. HIV-1 RNASE H INHIBITORS

Despite the fact that the RT-associated RNase H is an attractive target for HIV inhibition, so far only few studies have been conducted to identify inhibitors of this enzyme function as compared to the vast amount of studies dedicated to the RT-associated polymerase inhibition. As a result, most of the compounds which were shown to inhibit the HIV-1 RNase H activity in the commonly used enzyme assays are actually lacking in specificity and selectivity. In fact, several of them have been found to inhibit in enzymatic assays also the HIV-1 RT-associated polymerase activity and/or several viral and cellular RNase Hs; moreover, only very few of them were actually able to block the viral replication in cell-based assays. Obviously, the specificity/selectivity of viral inhibitors is a very important issue in view of their therapeutic use, particularly given the recent observation that the disruption of the RNase H1 allele in mice confers a lethal embryonic defect [45]. Nonetheless, this issue needs to be appropriately addressed yet and, to this aim, an adequate understanding of the results obtained until now is certainly required. Given these considerations, in order to present a complete picture of the studies that have been focused so far on the HIV-1 RNase H inhibition, I will review all the agents which have been reported to affect the HIV-1 RNase H activity despite of their specificity/selectivity, dividing them according to their origin and chemical structure.

4.1. Natural Product-Derived Compounds

Sulfated polyanions such as heparin, xylan polysulfate and dextran sulfate, extracted from algae or fungi and known for their anticoagulant and antilipemic action, were reported to inhibit the RNase H activity in enzyme assays with IC_{50} values of 0.1-8 nM while they inhibited the RDDP activity at concentrations 250-5000 fold higher [46]. However, their mode of inhibition of the viral replication probably involves inhibition of virus-cell binding and fusion and their efficacy upon parental administration has not been demonstrated yet [47].

Illimaquinone (**1**), (Fig. **2**), a natural marine sesquiterpenoid quinone, isolated as secondary metabolite from the Red Sea sponge *Smenospongia sp.* and displaying a variety of pharmacological activities such as inhibition of chemically induced inflammation and cell division [48], was reported to inhibit HIV-1 RNase H and RDDP activities with IC_{50} values of 15 μM and >140 μM, respectively [49]. Illimaquinone inhibited also the Murine Leukemia Virus (MLV) RT-associated RNase H and RDDP activities with IC_{50} values of 30 and 60 μM, respectively, and both HIV-2 and *E. coli* RNase Hs at 40 and 63 μM concentration, respectively [49, 50]. Substitution of the 6'-OH group of the quinone ring with a methyl or acetate group led to compounds which were completely inactive against the HIV-1 RNase H activity while were less active against the RDDP (IC_{50} values of 134 and 85 μM, respectively), indicating that the hydroxyl in *ortho*-position to the one of carbonyl groups is a requisite for its anti-RNase H activity. Differently, introduction of a methoxy group in the 4' position led to a compound, Avarone (**2**), Fig. (**2**), which showed a preferential inhibition of the RDDP activity vs. the RNase H function (IC_{50} values of 3 and 42 μM, respectively) [51]. Mode of action studies suggested that illimaquinone may interact either directly with the RT residue C280 or with amino acids in its proximity [50]. No data on its effect on viral replication have been reported.

Another marine natural product obtained from a different Red Sea sponge, *Verongia sp*., the 3,5,8-trihydroxy-4-quinolone was also found to inhibit the RDDP more potently (IC_{50} value of 2 μM) than the RNase H (IC_{50} 200 μM) [52]. Differently, a similar natural product belonging to the naphthoquinone series, compound (**3**) (Fig. **2**), inhibited in enzyme assays the RNase H more potently than the RDDP (9 μM versus 69 μM) and was inactive on the *E. coli* DNA polymerase I [53]. The introduction of a methyl group in position C-5 of the naphthoquinone ring reduced by 10-fold its potency against both retroviral functions, while the presence of an hydroxyl substituent in position C-2 led to a derivative inactive on the RNase H but more active on the RDDP (IC_{50} 10 μM) [53]. In addition, other naphthoquinone and naphthalene derivatives tertagalloylglucopyranose analog was found to inhibit in enzyme assays the RNase H and the RDDP activities with an IC_{50} values of 39 μM and 0.04 μM, respectively [54]. Noteworthy, none of

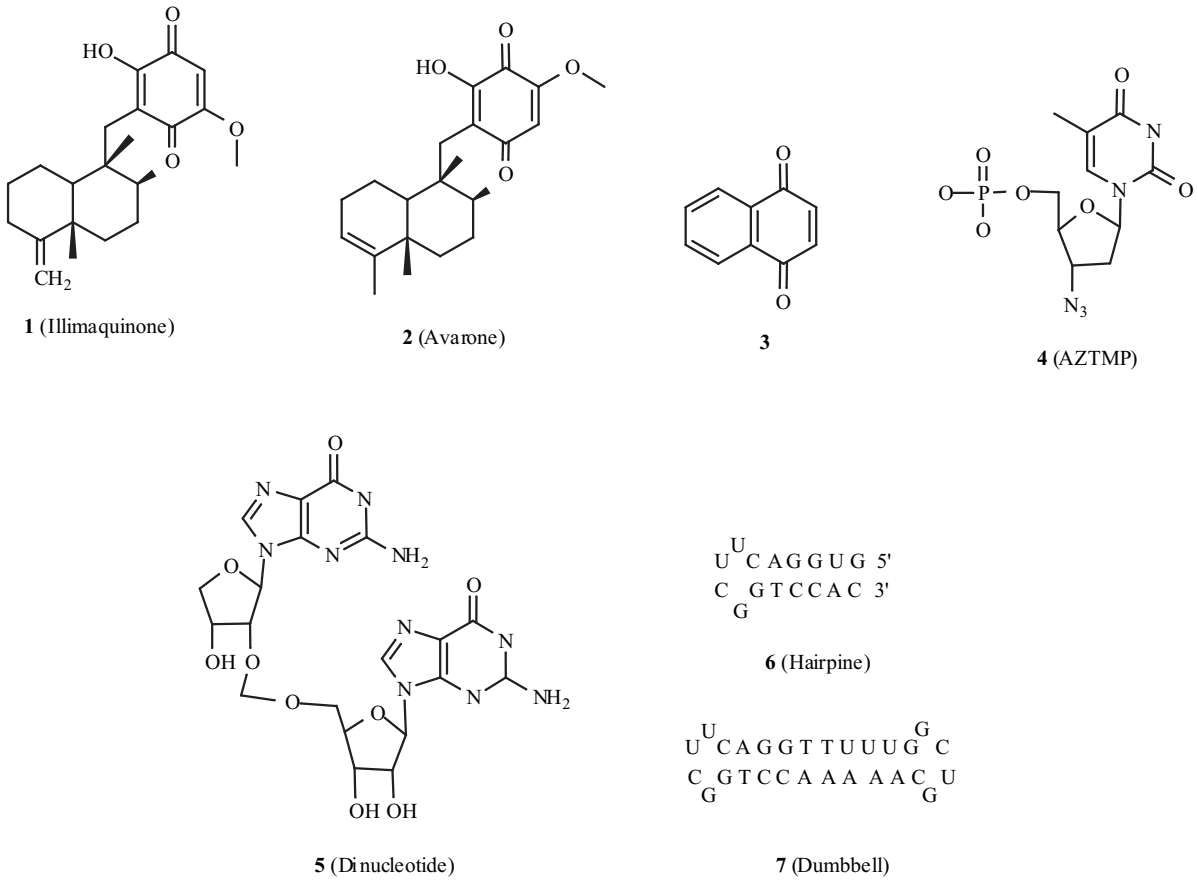

Fig. (2). Chemical structures of HIV-1 RNase H natural product derived and nucleotide inhibitors.

above natural compounds were reported to stop viral replication in cell-based assays.

Other natural product-derived agents which have been reported to have some activities on the HIV-1 RNase H function are the extracts from Argentine medicinal plants *Achyrocline flaccida* and *Phyllanthus sellowianus* whose infusions inhibited RNase H and RDDP activities at 2-4 μg/mL and 1-2 μg/mL, respectively, viral replication at 0.25-2 μg/mL, while they were not cytotoxic at 100 μg/mL [55]. Methanol extracts from the Korean plant *Agrimonia pilosa* slightly inhibited RNase H activity [56], and the kaempferol acethylrhamnosides from the Chinese herbal medicine *Dryopteris crassirhizoma* showed IC_{50} values of 25 and 4 μg/mL for, respectively, RNase H and RDDP activities [57]. No hypothesis on the mode of action of the above agents have been proposed.

4.2. Nucleotides and Oligonucleotides

The monophosphate form of the well known NRTI 3'-azido-3'-deoxythymidine (AZT), 3'-azido-3'-deoxythymidine 5'-phosphate (AZTMP), (**4**) (Fig. **2**), has been shown to inhibit the RNase H activity with an IC_{50} value of 0.05 mM, when Mn^{2+} was used as the divalent cation in the reaction mixture, and with an IC_{50} value of 5 mM when Mg^{2+} was present in the reaction [58]. Other nucleoside monophophates that inhibited the HIV-1 RNase H were 2',3'-dideoxyadenosine 5'-monophosphate (ddAMP) and 2',3'-dideoxyguanosine 5'-monophosphate (ddGMP), that showed IC_{50} values of 0.2 and 0.5 mM, respectively, in the presence of Mn^{2+}, while they were inactive in the presence of Mg^{2+} [59]. The potency of inhibition of these nucleoside monophosphate analogues was dependent on the hydrolysis substrate composition and, consistently, the AZTMP kinetic of inhibition differed according to the reaction substrate used resulting to be competitive with poly(rA)-poly(dT) and uncompetitive with poly(rG)-poly(dC). Overall, the precise mode of action of these nucleoside monophosphate has not been clarified yet.

The nucleotide dimer diguanosine, rGrG, was also shown to stop RNase H reaction with an IC_{50} value of 15 μM while it was inactive on cellular RNase Hs [60]. Substitution of its 3'-5'-phosphodiester linker with a 2'-5'-formacetal led to compound (**5**), (Fig. **2**), that showed a little increase in potency against the RT-associated RNase H (IC_{50} value of 5 μM), no effect on its RDDP and a modest inhibitory potency on bacterial *E. coli* RNase H (IC_{50} value of 50 μM) [60].

Phosphorotioate oligonucleotides were first reported to inhibit the RNase H activity, but they were not specific [61]. Later, the development of the systematic evolution of ligands by exponential enrichment (SELEX) approach allowed the isolation of oligonucleotides, named "aptamers", which have been proposed to specifically recognize with high affinity the HIV-1 RT RNase H domain [62]. Particularly, a SELEX G-rich 35-mer oligonucleotide, ODN93, was shown to inhibit the HIV-1 RNase H and RDDP activities with IC_{50} values of 0.5 and 1.5 μM,

respectively, while it was not able to inhibit the *E. coli* and human RNase Hs and the Avian Myeloblastosis virus (AMV) RDDP [62]. Therefore, ODN93 could be considered specific for HIV-1 RT but it was not able to discriminate between RNase H and RDDP activities. ODN93 was also reported to inhibit the viral replication in cell-based assays with an EC_{50} value of ~0.03 μM [62]. However, given that several G-rich oligonucleotides may form G-quartets and interfere with the virus adsorption and/or penetration into the infected cells, its actual mode of inhibition in cell-based assays needs still to be determined.

More recently, small hairpins and dumbbells were shown to inhibit selectively the HIV-1 RNase H activity without affecting neither the HIV-1 RDDP function nor the *E. coli* and human RNase Hs [63]. In particular, the most potent agents were the RNA hairpin (**6**) and the ligated RNA dumbbell (**7**),(Fig. **2**), which showed IC_{50} values for HIV-1 RNase H of 8 and 3 μM, respectively. Both RNAs were not able to interact with a mutated RT lacking the RNase H domain, indicating that they bind specifically to this enzyme domain with which they were proposed to interact noncompetitively [63]. Further studies will be needed to ascertain the molecular basis of their mode of action and their effect on virus replication.

4.3. Naphtalenesulfonic Acid Derivatives

A first series of naphtalenesulfonic acid derivatives was shown to slightly inhibit the HIV-1 RNase H activity, whereas they were more potent on its RDDP activity [64]. In particular, a *N*-palmitoylated derivative (**8**), (Fig. **3**), exhibited IC_{50} values of 27 μM and 2 μM, for RNase H and RDDP activities respectively, while a double palmitoylated analogue of the 4-amino-3-hydroxy-1-naphthalene-sulfonic acid (**9**), Fig. (**3**), showed IC_{50} values of 17 μM and 1 μM, respectively. Unfortunately, both derivatives were not active on virus replication [64]. Differently, a symmetric derivative with an aromatic spacer (**10**), Fig. (**3**), was reported to inhibit RNase H and RDDP activities (IC_{50} values of 14 and 3 μM, respectively) as well as viral replication (EC_{50} and CC_{50} values of 81 and >500 μM), even though it seems unlikely that it may enter inside the cells and, hence, it has been proposed that it may interfere with virus adsorption [64].

A second series of naphtalenesulfonic acid derivatives has been obtained by structure-based design and combinatorial medicinal chemistry approach [65]. The most potent of these compounds was compound (**11**), (Fig **3**), which, in enzyme assays, inhibited the HIV-1 RNase H and RDDP activities at 25-100 nM and 90 nM, respectively, it had a K_d value for DNA-RT binding of 40 nM, and in cell-based assays it showed an EC_{50} value of 2.5 μM and a CC_{50} value of 112 μM [65]. Further enzyme studies showed that (**11**) did not affect T7, T4 and Klenow DNA polymerases while it inhibited the MLV RDDP activity [65]. Probably, this compound prevents retroviral RT interaction with the substrate heteroduplex, even though the two orders of magnitude between the inhibitory potencies obtained in enzyme and cell-based studies do not allow to exclude that its *in vivo* mode of action could involve viral components other than RT.

4.4. Hydrazones

A possible approach for inhibiting metal-dependent enzymes is to identify agents that, selectively, may extract or bind to the metal cation in the active site, thereby inactivating the protein. According to this rationale, among several screened compounds the metal chelator *N*-(4-*tert*-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone,

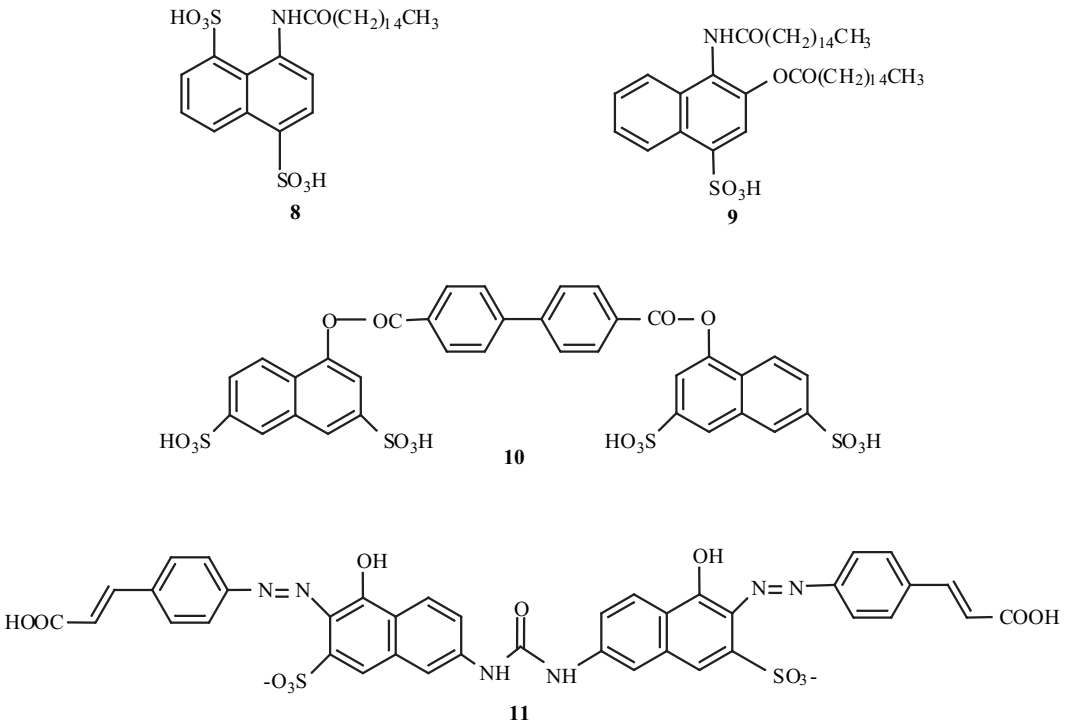

Fig. (3). Chemical structures of HIV-1 RNase H naphtalenesulfonic acid inhibitors.

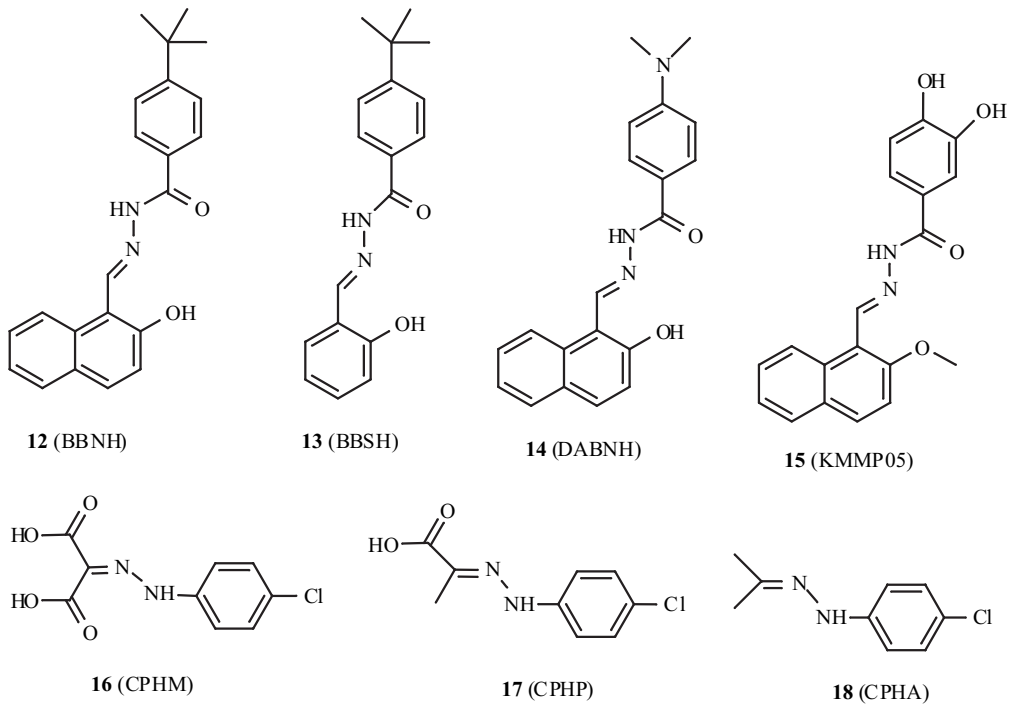

Fig. (4). Chemical structures HIV-1 RNase H hydrazone inhibitors.

BBNH (**12**) (Fig. **4**), was shown to inhibit, in enzyme assays, the HIV-1 RT-associated RNase H and RDDP activities with IC_{50} values of 3 μM and 0.8 μM, respectively [66]. BBNH also inhibited the RNase Hs from *E. coli* and Moloney Murine Leukemia Virus (Mo-MLV) with similar potency, whereas it was not active against the HIV-2 and AMV RNase Hs and other metal-dependent DNA polymerases. In cell-based assays, BBNH inhibited the viral replication with an EC_{50} value of 1.5-5 μM being cytostatic at 10 μM concentration and cytotoxic at 25 μM concentration [24, 66].

Mode of action studies revealed that the BBNH kinetic of RNase H inhibition was competitive with respect to the substrate heteroduplex. BBNH inhibited both RNase H and RDDP activities of HIV-1 RTs mutated in residues K103N, Y181I, Y188H, Y188L with potency similar to the one obtained for wild type RT. Differently, when assayed on the mutated RT Y181C BBNH was not active on the RNase H activity while it inhibited the RDDP function with an IC_{50} value of 1.6 μM. Therefore, also on the basis of photoprotection studies, it was proposed that BBNH could bind to two RT sites, a first site in the polymerase domain, close to the NNRTI bonding pocket, and a second site in the RNase H domain [66]. Further molecular modeling and mutagenesis studies demonstrated that the RT Y501 residue is integral in the BBNH-RNase H domain binding interaction [67]. Y501 residue has been proposed to be involved in assisting the appropriate positioning of the substrate in the active site and to be essential for enzyme activity. In fact, several mutated RTs bearing amino acid substitutions in the Y501 residue were RNase H-inactive. Noteworthy, the only mutated RTs which showed enzyme activity (Y501W and Y501R RTs) were not sensitive to the RNase H inhibition by BBNH [67]. Other RT residues that have been suggested to have contacts with BBNH are R448, N474, Q475, S499 and H539.

These studies have also identified two major binding determinants in the BBNH structure. The first binding determinant involves the coordination of one of the hydrazone linkage nitrogen atoms and of the hydrazone carbonyloxygen to the RNase H active site metal. In fact, docking studies have showed that BBNH is a flat molecule with an extended planar structure whose moieties could interact with the metal in the RNase H active site. The second binding determinant is an aromatic π-π stacking interaction between the phenyl ring of Y501 and the second BBNH naphthyl moiety. In order to confirm this hypothesis, the naphthyl double ring system of BBNH was replaced by a single aromatic ring to obtain the compound *N*-(4-*tert*-butylbenzoyl)-2-hydroxy-1-salicylhydrazone, BBSH (**13**) (Fig. **4**), which, in fact, was not active against the HIV-1 RNase H activity but still maintained an IC_{50} value of 3.6 μM against its RDDP function [67]. In order to further confirm that it was possible to differently modulate the antiviral activity of these derivatives, the (4,*N,N*-dimethylaminobenzoyl)-2-hydroxy-1-naphthyl hydrazone, DABNH (**14**) (Fig. **4**), was synthesized and shown to bind only to the RNase H domain. DABNH inhibited the RNase H activity with an IC_{50} value of 4 μM, while it was inactive against the RDDP function [68]. Interestingly, both BBNH and BBSH were found to reduce the RT dimer stability, whereas DABNH did not alter it. Overall these studies suggested that N-acylhydrazone derivatives interact with two different RT binding sites and could be developed to target either one of the two RT-associated activities. In fact, in enzymatic studies they were shown i) to affect the protein-protein RT interaction (even though they do not dissociate the enzyme, but probably induce conformational changes that affect the RDDP activity); ii) to interact, through the

hydrazone moiety, with the metal in the RNase H active site. However, since it has been shown that the treatment of HIV-1 chronically infected H9 cells with an appropriately formulated iron chelate of BBNH led to attenuation of nascent virus infectivity, but the p24 viral production and the HIV-1 protein processing in the nascent virions were unaffected by this formulation [69], the destabilization of the RT dimer interactions may be actually their primary mode of virus inhibition. More recently, further efforts to improve hydrazone selectivity led to the synthesis of KMMP05 (**15**), (Fig. **4**), a compound which inhibited the RNase H activity with an IC_{50} value of 0.5 μM, it was inactive on the RDDP function and showed EC_{50} and CC_{50} values of 4.5 μM and > 100 μM, respectively[1]. However, crystal structure studies demonstrated that KMMP05 binds to the polymerase domain close to the NNRTI binding pocket (including amino acid residues Y188 and W229) and to the polymerase active site (including residue D186), while no metal ion interactions were observed[2]. Therefore, more studies will be needed to fully understand the mode of action of these derivatives and their real significance as RNase H inhibitors.

Another hydrazone derivative which was found to have anti-RNase H activity is the 4-chlorophenylhydrazone of mesoxalic acid, CPHM (**16**) (Fig. **4**), that selectively inhibited the HIV-1 RNase H with an IC_{50} value of 3 μM, while it was not active against its RDDP function [70]. CPHM has been proposed to target the HIV-1 RNase H activity and, as a consequence, to inhibit the DNA strand transfer process [70, 71]. Specificity studies have showed that CPHM inhibited also the *E. coli* RNase H with the same potency (IC_{50} value of 2.6 μM), but it did not stop the AMV and Mo-MLV strand transfer process. Binding studies determined that the divalent cation Mg^{2+} is able to chelate CPHM with a K_d of 2.4 mM [71]. Structure-activity relationship (SAR) studies proved that the dicarboxylic moiety of CPHM is essential for its inhibitory action. In fact, CPHM analogues lacking one or both carboxylic groups, namely 4-chlorophenylhydrazone of pyruvic acid, CPHP (**17**) (Fig. **4**), and 4-chlorophenylhydrazone of acetone, CPHA (**18**) (Fig. **4**), displayed no enzyme inhibition and no ability to chelate Mg^{2+} [71]. However, later studies led by a different research team proposed that CPHM was able to inhibit the HIV-1 RDDP activity with the same potency (IC_{50} value of 2.3 μM) observed for its RNase H activity (IC_{50} value of 6.1 μM) [72]. While this incongruence has to be explained yet, further studies would be needed to identify the actual CPHM binding site. No data on its impact on virus replication have been reported.

4.5. Diketo Acid Derivatives

As outlined above, RNase H proteins belong to the polynucleotidyl transferase family that comprises also the retroviral integrase (IN) proteins among which is the HIV-1 IN that has been studied for long time as target for drug development and for which, recently, some new classes of inhibitors have been identified [73]. Given the structural similarities between the domains of the two viral enzymes, the DNA aptamer ODN93 originally shown to inhibit the RNase H activity [62] has also been tested against the HIV-1 IN and shown to be active on this enzyme too (74) and, *vice versa*, several diketo acid (DKA) analogues initially developed for IN inhibition were also screened against the HIV-1 RNase H activity. In particular, the 4-[5-(benzoylamino)thien-2-yl]-2,4-dioxobutanoic acid, BTDBA (**19**) (Fig. **5**), was reported to inhibit the HIV-1 RNase H activity with an IC_{50} value of 3.2 μM, while it was not active on the RDDP at 50 μM concentration [43, 72]. BTDBA also inhibited the isolated HIV-1 RNase H domain chimera carrying the *E. coli* basic helix loop motif (IC_{50} value of 4.7 μM) while it did not affect the *E. coli* RNase H [72]. However, BTDBA also inhibited the HIV-1 IN enzyme reaction with an IC_{50} value of 1.9 μM, showing therefore to be not able to discriminate between the two HIV-1 targets, while it did not block the viral replication in cell-based assays [72]. Interestingly, as previously delineated, BTDBA and NNRTI combinations were synergistic in an enzyme system that was able to measure at the same time the RDDP and RNase H activities, while they were additive against the HIV-1 RT-associated RDDP alone [43]. Similarly, combinations of BTDBA with high AZTTP concentrations were synergistic in the coupled assay [43]. Further characterizations with isothermal titration calorimetry demonstrated that i) BTDBA requires the presence of a divalent cation in order to bind to the RNase H domain; ii) its metal-dependent K_d value is comparable to its IC_{50} value; iii) the nucleic acid substrate is not needed for its binding to RNase H. Therefore, it has been proposed that, similarly to what has been described for DKA interaction with the HIV-1 IN [73], DKAs may sequestrate the active site divalent metals having a specific binding site on the HIV-1 RNase H domain [72]. More recently, BTDBA has been modeled into the HIV-1 RNase H active site assuming that the DKA triple-oxygen motif may interact with the protein active site metal ions [24]. According to this model, its aromatic moiety may extend towards the W266, L422 and W426 amino acid residues on the p51 subunit [24].

A second DKA derivative that has been reported to have anti-RNase H activity is the 6-[1-(4-fluorophenyl)methyl-1*H*-pyrrol-2-yl)]-2,4-dioxo-5-hexenoic acid ethyl ester, RDS 1643 (**20**) (Fig. **5**) [75]. In enzyme assays, RDS 1643 inhibited the HIV-1 RNase H activity with an IC_{50} value of 13 μM, therefore it was less potent than BTDBA, it did not affect neither the HIV-1 RDDP function nor the AMV and *E. coli* RNase H activity, while it slightly inhibited the HIV-1 IN reaction (IC_{50} value of 92-98 μM) [75]. Noteworthy, in cell-based assays it was able to block the replication of wild type HIV-1, showing an EC_{50} value of 13 μM and a CC_{50} value of 63 μM, and the replication of three HIV-1 NNRTI resistant viral mutants (RT mutations were Y181C; K103N/Y181C; K103R/V179D/P225H) showing EC_{50} values of 7-19 μM [75]. Mode of action studies demonstrated that the RDS 1643 maximum adsorbance shifted in the presence of the Mg^{2+} ions, suggesting a mechanism of action similar to BTDBA [75]. Consistently, further modeling studies proposed that RDS 1643 may bind to the HIV-1 RNase H domain similarly to BTDBA [24]. However, RDS 1643 would not reach towards

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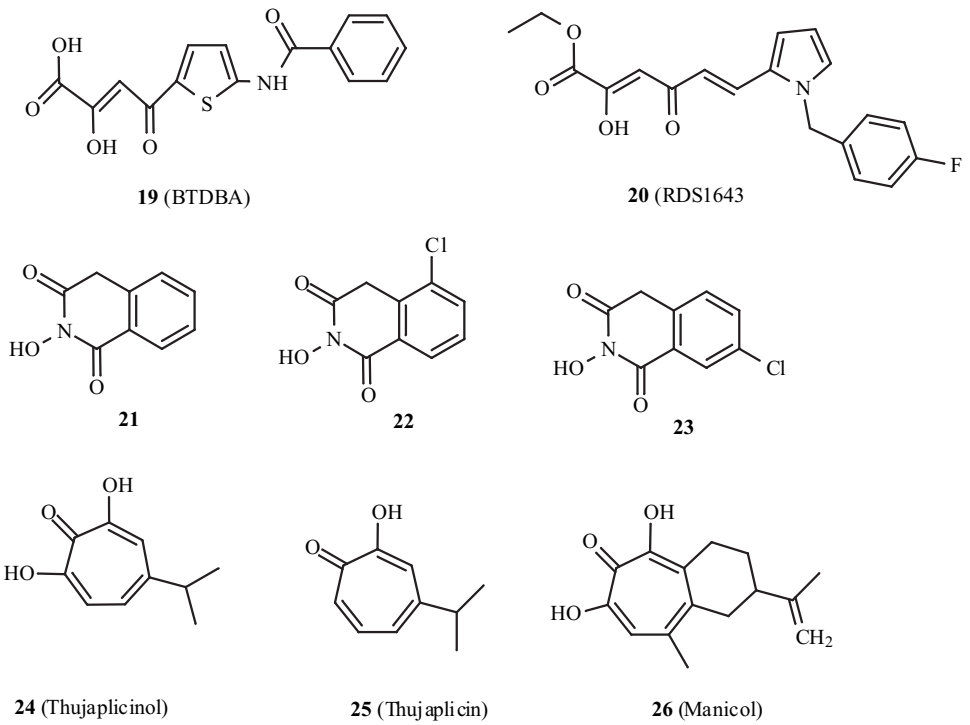


Fig. (5). Chemical structures of HIV-1 RNase H DKA, N-hydroxyimide and tropolone inhibitors.

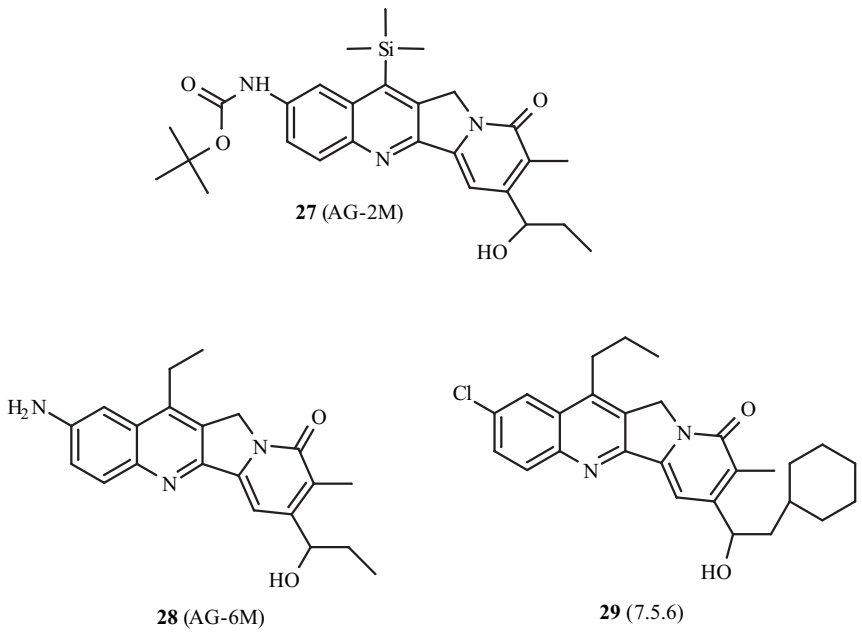
the p51 subunit as far as BTDBA, showing therefore a less favorable interaction with RT, consistently with the lower potency of RDS 1643 inhibition as compared to BTDBA inhibition [24]. Therefore, even though no straight demonstration that RDS 1643 inhibits the RNase H activity inside the cells has been published yet, these docking results reinforce the claim that RDS 1643 is the first compound that inhibits HIV-1 replication through the selective inhibition of the RT-associated RNase H function [75].

4.6. N-Hydroxyimides

Drug development studies have recently led to the design of novel series of influenza endonuclease inhibitors which bind to the endonuclease two-metal active site structure [76]. The major feature of their pharmacophore is the specific arrangement of three oxygen atoms to mimic the metal ion-mediated protein interaction with substrate, nucleophile and leaving group oxygens in the enzyme active site [76]. Based on these studies, a series of small N-hydroxyimides were tested for their activity against the HIV-1 RNase H function [77]. The prototype analogue N-hydroximide 2-hydroxy-4H-isoquinoline-1,3-dione, (**21**) (Fig. **5**), inhibited in enzyme assays the HIV-1 RNase H activity with an IC_{50} value of 0.6-1 μM, the HIV-1 RDDP function with an IC_{50} value of 40 μM, whereas it did not affect the one-metal structure of the *E. coli* RNase H [77]. Compound (**21**) was also shown to interact with an active isolated domain of the HIV-1 RNase H (IC_{50} value of 0.4 μM) and to inhibit the influenza endonuclease with a 15-fold lower potency [77, 78]. SAR studies showed that its N-hydroxyl group was essential for interaction with metal ions, since compounds where it was replaced by a methoxy or an amino group were inactive. Differently, substitutions on the phenyl moiety could somehow modulate its inhibition potency: compounds (**22**) and (**23**), (Fig. **5**), showed IC_{50} values for RNase H inhibition of 0.3 and 7 μM, respectively [76]. NMR studies confirmed that the interaction of these compounds with the RNase H domain occurs only in the presence of the divalent cation, whereas fluorescence quenching demonstrated that compound (**22**) has an apparent K_d value for the RNase H domain of 0.2 μM [77, 78]. Crystal structure determinations confirmed the metal-ion binding of these analogues to the RNase H active site [24].

4.7. Hydroxylated Tropolones

A recent high-throughput screening of the American National Cancer Institute library of pure natural compounds led to the identification of two tropolone (2-hydroxy-2,4,6-cycloheptatrien-1-one) derivatives with a 7-OH substitution [79]. The most potent analogue, β-thujaplicinol (**24**) (Fig. **5**), derived from the heartwood of several cupressaceous plants (e.g. *Thuja plicata*, *Thuja occidentalis* and *Chamaecyparis obtusal*), inhibited both HIV-1 and HIV-2 RNase H activities with IC_{50} values of 0.2 μM and 0.7 μM, respectively, while it did not affect their RDDP activities [79]. β-thujaplicinol also inhibited the human and *E. coli* RNase Hs but 30-fold and 250-fold less potently, respectively, than the HIV-1 enzyme. SAR studies showed that the hydroxyl function at position 7 of the heptatriene ring is required for RNase H inhibition since the β-thujaplicin analogue, (**25**) (Fig. **5**), is completely inactive; while the presence of other groups in the tropolone structure led to a compound, manicol (**26**) (Fig. **5**), which is more active on the human RNase H (IC_{50} value of 3.5 μM) than on the HIV-1 RNase H (IC_{50} value of 60 μM). Interestingly, combinations of β-thujaplicinol with the NNRTI calanolide A were reported to be synergistic, and the β-diketone moiety of thujaplicins has been proposed to chelate, and/or alter the



27 (AG-2M)

28 (AG-6M)

29 (7.5.6)

Fig. (6). Chemical structures HIV-1 RNase H mappicine inhibitors.

coordination geometry, of the divalent metal in the RNase H active site [79]. Unfortunately, all tropolone analogues tested were inactive on HIV replication in cell-based assays.

4.8. Mappicine Analogues

Finally it is worth to note that some mappicine analogues, (**27**-**29**) (Fig. **6**), have recently been reported to inhibit the HIV-1 RNase H activity, in enzyme assays, with IC_{50} values of 2-10 μM, and the viral replication, in cell-based assays, of wild type and NNRTI resistant mutant HIV-1 (RT mutations were K103N/Y181C and V106A/Y181C) [24, 80].

CONCLUSION

Despite the fact that the HIV-1 RT-associated RNase H function is an attractive target for drug development, only recently the search for agents targeted to alternative viral sites led to address an adequate attention to this enzyme activity. As a consequence of the little effort developed in the past years, the great majority of the compounds that in the last 15 years have been reported to inhibit the HIV-1 RNase H activity in enzyme assays are either not specific for this activity (inhibiting also the HIV-1 RDDP function or cellular RNase Hs), or are not able to stop the viral replication in cell-based assays. Recently, the use of the knowledge obtained in studies previously performed on the HIV-1 IN and the influenza endonuclease inhibition have allowed to identify small molecules that may selectively bind to the HIV-1 RNase H active site through the interaction with the metal divalent cations needed for the enzyme catalysis. This is certainly an important milestone in the search of anti-RNase H agents. In the near future, molecular modeling docking studies will surely further enrich the information needed to design new compounds which can be more potent and more selective than the actual derivatives. In this view, the chemical structure of the compounds reported in this review may represent a good starting point for further drug development. Finally, it is worth to note that in consideration of the possible use on the RNase H inhibitors in combination with both NRTIs and NNRTIs, further studies would be required to better understand the effects of the reciprocal interactions on the HIV replication.

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