Antiviral drug discovery: HIV-1 RNase H, the next hit target

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Antiviral drugs are a class of medication used for selectively treating viral infections. In the last three decades, the antiviral drug field has greatly developed through the interaction of several disciplines such as virology, biochemistry, chemistry, structural biology, and have reached enormous achievements. Paradigms of them are the treatments for HSV and HIV-1 infections. The latter is a striking example of the development of drugs which have turned a dreadful disease into a manageable chronic infection. However, still new anti-HIV drugs are needed, particularly drugs targeted to viral functions which are not inhibited yet by the current treatments. The HIV-1 reverse transcriptase-associated ribonuclease H (RNase H) activity is an attractive non traditional target for drug development which has been, so far, little explored. The present review is focused on the approach needed to identify valid RNase H inhibitors and lists the agents which have been reported, until now, to have an impact on the HIV-1 RNase H activity.

Keywords Antiviral; drug development; HIV-1; reverse transcriptase; ribonuclease H; RNase H

1. Antiviral drug development short history

The birth of drug research can be set around one hundred years ago, when chemistry reached a degree of maturity that allowed it to apply its principles and methods outside itself and when pharmacology began to be recognized as a scientific discipline (1).

During the 20th century, the isolation and purification of active ingredients from medicinal plants demonstrated their value for medicine. As active principles were available, the problem of providing standardized preparations of these drugs, often still impure, was addressed. On the ground of anti-infective drug discovery programs, the first screening programs were focused on the discovery of compounds with antimicrobial activities. The identification of the natural antibiotics, penicillin, from *Penicillium notatum* (Alexander Fleming) and cephalosporin from *Cephalosporium acremonium* (Giuseppe Brotzu), the semi-synthetic antibiotic, tetracycline, produced from natural chlortetracycline elaborated by *Streptomyces aureofaciens* (Benjamin Duggar), the anti-tubercular aminoglycoside, streptomycin, from *Streptomyces griseus* (Salman Waksman), were all landmark discoveries of the 1930s and 1940s.

Antivirals are a class of drugs used specifically for treating viral infections. In the 1940s, it seemed clear that viruses were not susceptible to antibiotics and for the two flowing decades virologists were taught that selective toxicity for these obligate intracellular parasites was unattainable (2). In 1957 came the first description by Isaacs and Lindenmann of interferon. Human interferons were subsequently developed for the treatment of particular virus infections, i.e. hepatitis B and, more recently, hepatitis C virus infections, as pegylated interferon, combined with ribavirin (3, 4).

The most important early milestone in the antiviral drug field was the description of 5-iodo-2′-deoxyuridine (idoxuridine, IDU) in the early 1960s (William Prusoff) and the realization of its antiviral properties (5). The first publications on this and similar nucleoside analogues appeared in cancer journals since the aim was the development of cytostatic or cytotoxic drugs for the treatment of neoplastic disease (6). However, an important by-product of this work was the discovery that IDU was a specific inhibitor of certain large DNA viruses, most notably herpes simplex virus (HSV). The compound is cytotoxic and was therefore only suitable for topical application, for which it remains in use to the present day.

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Influenza was also an early antiviral target, and in 1964 it was reported (C.E. Hoffmann) that amantadine was a specific inhibitor of the negative RNA strand virus, influenza A (7). Amantadine and its sister compound, rimantadine, were later shown to act by interaction with the viral M2 protein which forms an ion channel during the early stages of virus replication. Despite the data relating to their use in man were not so easily forthcoming, they provided a platform for the development of the next generation of anti-influenza drugs that resulted from a programme of rational drug design targeted to the virus neuraminidase which are now in clinical use (8).

In 1972 Ribavirin was reported to be a broad-spectrum antiviral compound acting against many different virus families, among which is - notably - the negative RNA strand virus, respiratory syncytial virus (9). Another discovery in the 1970s was the antiherpesvirus activity of the pyrophosphate analogue phosphonoformic acid (phosphonoformate, PFA) (10). Despite PFA suffers from toxicity problems, including nephrotoxicity, it continues to have a role in managing HSV infections in immunocompromised patients who are resistant to the current antiherpetic compounds.

The next milestone which had an enormous impact in the field was the discovery of the compound acyclovir, which selectively inhibits the HSV replication, made by the Nobel prize winner Gertrude Elion. Also acyclovir was a side result of a programme not primarily aimed at antivirals but aimed to identify DNA-based antimetabolites, which eventually led also to the discovery of modified purines with anticancer activity. Noteworthy, its particular mechanism of HSV inhibition and its pharmacology made it one of the safest drugs of all times with almost no adverse effects described during three decades of use (11). Acyclovir was the very first highly selective antiviral compound, demonstrating the possibility of inhibiting the viral replication safely. Since than, the field has changed dramatically and today, dozens of antiviral treatments are available, and scientists feel that they are only scratching at the surface of what can be done with these new drugs.

2. Drug discovery today

Over the last three decades, the methods for discovering new drugs have tremendously developed. Chemistry, pharmacology, microbiology, and biochemistry helped to shape the course of drug discovery and to bring it to a level where new drugs are no longer generated solely by the imagination of chemists but result from a direct dialogue between biologists and chemists. This dialogue, centred on the biochemical mechanisms of drug action, stems from the understanding of biological target structure and function and gives rise to the creation of novel chemical structures (12).

In fact, the studies generated in the field of molecular biology have greatly influenced the drug discovery process, allowing that the genetic information could be taken into account so that these information has become a very important player in the drug development. At first, the influence of molecular biology appeared to be restricted to cloning and expressing genes that encode therapeutically useful proteins (such as monoclonal antibodies). Later however, it appeared clear that the information that could be obtained by studying recombinant proteins could allow a much deeper understanding of the disease mechanisms at the molecular level. The deeper molecular understanding was essential to determine the new optimized molecular targets for drug intervention. Therefore, the interplay between molecular biology, biochemistry, genetics and chemistry have led important additions to the drug discovery rational and to the therapeutic armamentarium.

More recently, the advent of genomic sciences, rapid DNA sequencing, combinatorial chemistry, cell-based assays, and automated highthroughput screening (HTS) has led to a “new” concept of drug discovery. In this new concept, the critical interplay between chemists and biologists and the quality of scientific reasoning are implemented by the possibility of analyzing large numbers of data. Large numbers of compounds are now readily tested in computer-based *in silico* screening and than assayed in biological assays to confirm/disprove their biological activity, with great saving of time and money. Such “hits” - compounds that elicit a positive response in a particular biological assay - are then further developed to “leads”, i.e., compounds that continue to show the initial positive response in more complex models in a dose-dependent manner. Among the leads, the compounds which show good *in*

vitro pharmacological and toxicological profiles are chosen as preclinical candidate for further *in vivo* studies (12).

The identification of a good pre-clinical candidate is than followed by its development into a clinical candidate and its clinical development. The "perfect " drug is typically required to satisfy these three points: i) it must be safe; ii) it must be effective in treating the specific disease for which it was designed; iii) it can be manufactory in a clean and reproducible process. At the end of the four phases of the clinical development the drug is finally ready to be safely used by the patients.

3. Drug discovery applied to HIV-1

In the last two decades, the wide distribution of the human immunodeficiency virus type 1 (HIV-1), the etiological agent of the acquired immune deficiency syndrome (AIDS), a global epidemic which has become a major public health problem worldwide, has dramatically turn the attention of scientists to drug development programs aimed to the inhibition of this retrovirus. In fact, given the great difficulties in developing an HIV effective vaccine, in the last 20 years major efforts have been addressed towards the identification of valuable therapeutic strategies. Their development is a very successful story that has turned a dreadful disease into a manageable chronic infection, at least for the patients who can somehow access the therapy.

Noteworthy, the drugs currently employed for treating HIV infected patients are only a very minimal part of the thousand compounds which were found to inhibit *in vitro* the viral replication and which were not able to pass all the needed control phases to reach the clinics. The current HIV-1 therapeutic armamentarium is aimed to block three fundamental stages of the replication cycle of the virus: the virus entry, the genome replication (accomplished by the viral enzyme reverse transcriptase, RT), and the viral protein processing (accomplished by the viral protease, PR). The bases of the successful therapy is the use of cocktails containing different drugs.

The viral RNA reverse transcription, which comprises the synthesis of a DNA strand and the formation of a viral RNA-DNA hybrid, the removal of its RNA component and the synthesis of the second DNA strand using the firstly synthesised DNA strand as template, has been the first targeted steps. Zidovudine (azidothymidine, AZT), a compound firstly synthesised by Horwitz et al. in 1964 (13), was the first compound proven to have an HIV-inhibitory effect (14). AZT is a prodrug that is phosphorylated intracellularly by cellular kinases to an active triphosphate metabolite (14). This AZT-triphosphate has a specific, selective affinity for the HIV-1 RT, causing a chain termination and inhibition of this enzyme. This mechanism of inhibition is shared also by the other nucleoside RT inhibitors (NRTIs) such as Didanosine (15), Zalcitabine (16), Stavudine (17), Lamivudine (18), Abacavir (19), Tenofovir (20) and Emtricitabine (21).

In addition to the NRTI several classes of small molecules have been screened against the HIV-1 RT and found to inhibit its activity. The first compound described in the non-nucleoside RT inhibitor (NNRTI) class was Nevirapine (22, 23). Following it, hundreds of molecules have been studied but only Nevirapine, Delavirdine (24) and Efavirenz (25) have been approved for clinical treatment.

Also the viral enzyme PR, that cleaves the viral proteins activating them, has been deeply studied and several compounds have been found to stop its activity. Among them are Amprenavir, Atazanavir, Darunavir, Fosamprenavir, Indinavir, Lopinavir, Ritonavir, Nelfinavir, Saquinavir, Tipranavir (26). Furthermore, the viral entry is inhibited by Enfuvirtide which blocks the tie between the viral envelope and the cellular surface (27).

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 (28).

Among them are the virus-cell interaction, whose precise mechanisms (involving the interactions of virus particles with negatively charged cell-surface molecules such as galactosyl ceramides, mannose receptors, C-type lectins and others) however are not known yet; the coreceptor contribution (the

requirement, both genetic and physical, of seven transmembrane G-protein coupled chemokine receptors); the RNase H activity associated to the viral RT (which selectively hydrolyses the RNA strand of the RNA:DNA hydrid); the integration of the proviral DNA into the host cell chromosome, catalyzed by the virus-coded integrase (which has been extensively studied with very little success); the viral p7 nucleocapsid protein which has functions at multiple sites in the HIV replication cycle; the transcriptional regulatory viral proteins such as Tat and Rev; the virus maturation (29).

4. A study case: the HIV-1 RNase H as a drug target

The RNase H activity is considered an optimal target for the discovery of new inhibitors. It is an enzymatic function associated to the viral RT which replicates the HIV genome through several steps (Fig. 1) and 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 (30). 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 (31). 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 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 (32). 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 (33, 34). The second takes place independently of the DNA synthesis and is referred to, therefore, as polymerization-independent RNase H mode of cleavage (35). The latter mode of cleavage has been proposed to be particularly important for viral replication (36) and has been proposed to be sufficient to allow virus replication (37).

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 the action of the HIV-1 PR cleavage of its p15 C-terminal (which contains the RNase H domain). 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 or the DNA polymerase β 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). Differently, 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. The two catalytic sites are

interdependent 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.

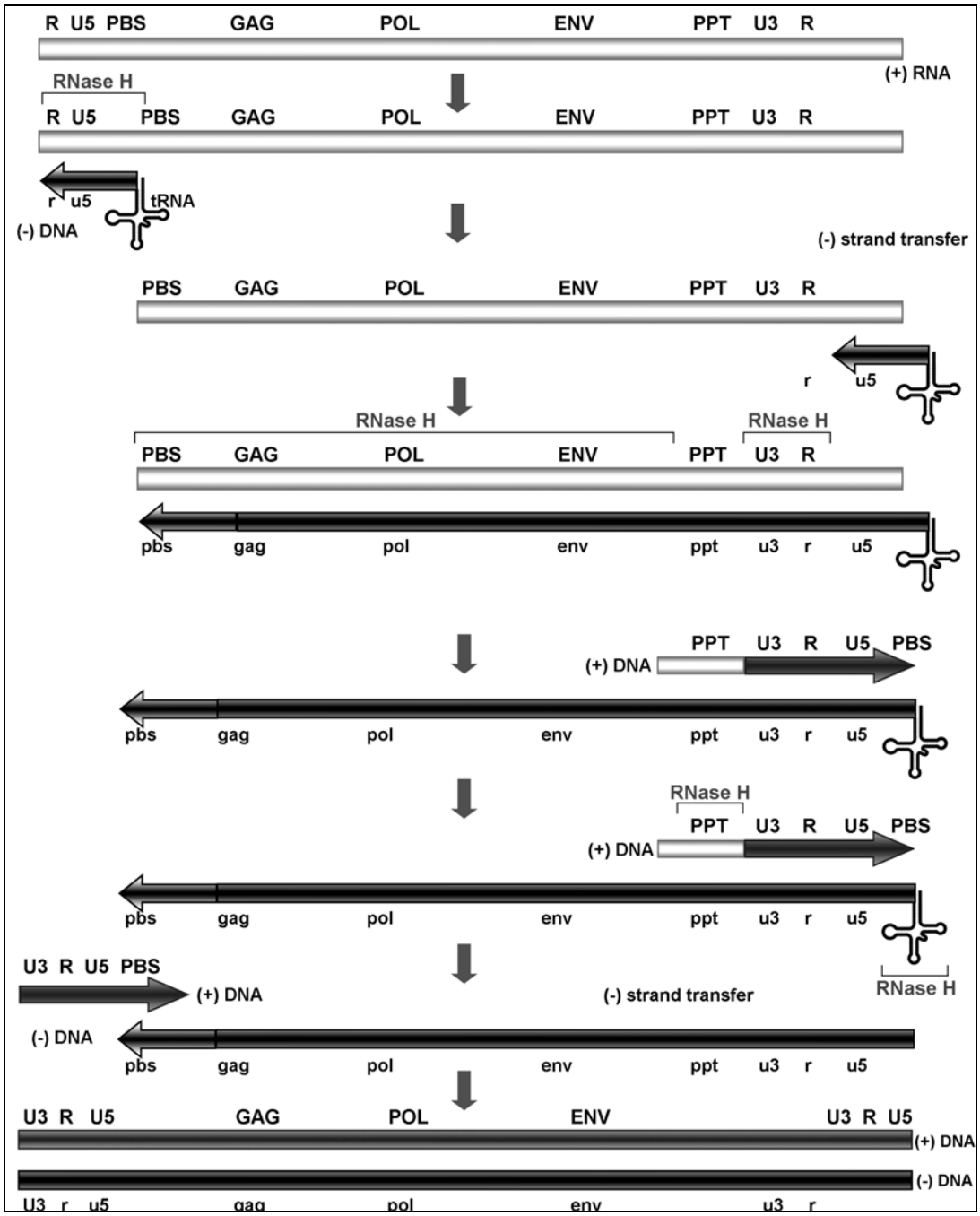

Fig. 1 Schematic representation of the HIV-1 RNA genome conversion into DNA by RT.

The HIV-1 RNase H domain have been characterized both as a part of the whole RT and as an isolated domain and shown to form a central five-stranded mixed β-sheet, surrounded by 4 α-helices and 8 connecting loops. 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 (38, 39). The HIV-1 RNase H is metal-dependent and requires Mg^{2+} for enzyme function. In fact, 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. 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.

In the last years, 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 (30). However, the RNase H function 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 (40).

5. RNase H polymerase-independent cleavage assay

As explained above, the current drug discovery rational needs first of all a biological assay in which the viral target, in this case the HIV-1 RT, is obtained as a recombinant protein and used to screen potential inhibitors. In our system, heterodimeric recombinant RT (rRT) is expressed in *E. coli* M15 strain containing the p6HRT-prot vector which is grown up to an OD_{600} of 0.8 and whose protein synthesis is induced by the addition of 1.7 mM IPTG 1.7mM (41). The rRT obtained has a C-terminus histidine tail (of 6 amino acids) which allow a fast and simple purification by affinity chromatography.

In fact, protein purification is carried out with a Ni^{2+}-Sepharose resin using a Biorad Biologic Duo Flow Chromatography System. The *E. coli* M15 cell pellet is resuspended in Lysis Buffer (20 mM Hepes pH 7.5, 0.5 M NaCl, 5 mM β-mercaptoethanol, 5 mM imidazole, 0.4 mg/mL lisozyme), incubated on ice for 20 minutes, sonicated and centrifuged at 30,000 x g for 1h. The supernatant is applied to the Ni^{2+}-Sepharose resin column and washed thoroughly with Wash Buffer (20 mM Hepes pH 7.5, 0.3 M NaCl, 5 mM β-mercaptoethanol, 60 mM imidazole, 10% glycerol) which eliminates all the non specific protein bindings. Subsequently, the rRT is gradient-eluted using for Elute Buffer the Wash Buffer plus 0.5 M imidazole. Fractions are collected. Protein purity is checked by SDS-PAGE (and usually found to be higher than 90%), protein concentration is determined and the enzyme activity is assessed. The enzyme containing fractions are pooled and dialyzed against the Storage Buffer (50 mM Tris-HCl pH 7.0, 25 mM NaCl, 1mM EDTA, 10% glycerol).

In our experimental system, the compounds to be tested in order to evaluate their ability to inhibit the RNase H polymerase-independent cleavage activity are screened in a biochemical assays which uses the above purified rRT. In this assay, the enzyme uses as reaction substrate a radiolabeled Poly(dC)-[^{3}H]Poly(rG) hybrid in a reaction mixture of 50 µL volume containing 50 mM Tris-HCl pH 7.8, 6 mM $MgCl_2$, 80 mM KCl, 1 mM DTT and 2 nM rRT. The reaction is incubated for 1 h at 37 °C. During this period, rRT cleaves the [^{3}H]Poly(rG) portion of the hybrid substrate into small pieces (Fig. 2) which are, therefore, the reaction products. At the end of the incubation period, 40 µL aliquots are spotted on glass fiber filters (Whatman GF/A) and processed for determination of trichloroacetic acid-insoluble radioactivity. The macromolecules in the reaction mixture, i.e. the radiolabeled Poly(dC)-[^{3}H]Poly(rG) hybrid substrate, are acid-insoluble and remain bound to the glass fiber filter, while the small molecules, i.e. the small radiolabeled [^{3}H]Poly(rG) oligonucleotides reaction products, are acid-soluble and are washed out from the glass fiber filter. The measurement of the acid-insoluble radioactivity, therefore (made with a Beckman Coulter scintillation counter) allows to evaluate the rate of the enzymatic reaction in the absence of any compound and its eventual inhibition determined in the presence of a small compound.

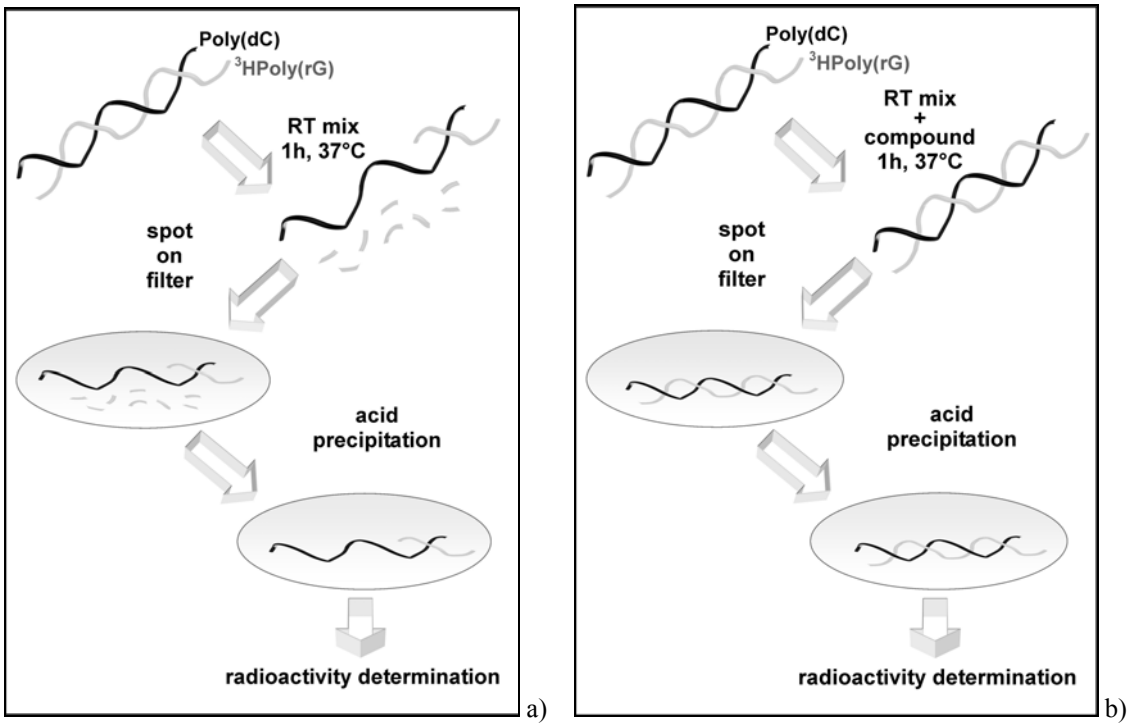

Fig. 2 Schematic representation of the RNase H polymerase-independent cleavage assay for the identification of RNase H inhibitors. Panel a). In the control reaction, the radiolabeled Poly(dC)-[^{3}H]Poly(rG) hybrid substrate is incubated in the RT reaction mixture for 1 hr at 37 °C. The rRT selectively cleaves the RNA portion of the RNA:DNA substrate, giving raise to small radiolabeled [^{3}H]Poly(rG) oligonucleotides as reaction products. The reaction mixture is than spotted on glass fiber filters and the small radiolabeled product oligonucleotides are separated by the longer radiolabeled substrate by acid precipitation. The radioactivity remained bound to the filter is determined as mean of measure of the enzyme activity. Panel b). When the test RT reaction mixture includes an RNase H inhibitor, the rRT does not produce the small radiolabeled [^{3}H]Poly(rG) oligonucleotides. Therefore, all the radiolabeled nucleotides are retained on the filter after the acid precipitation. The difference between the radioactivity measured in the control and test reactions shows the presence of an inhibitor.

6. HIV-1 RNase H inhibitors

The use of the above described biochemical assay, or others functionally analogue, have allowed the identification of compounds that inhibits the HIV-1 RNase H function *in vitro*. However, due to the limited number of studies dedicated to this enzyme so far, most of the compounds with a reported anti-HIV-1 RNase H activity lack 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 (RDDP) and/or several viral and cellular RNase Hs; moreover, only very few of them are 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 and, until now, only two compounds show biological properties promising for further development. Regardless of their specificity/selectivity, the agents which have been reported to affect the HIV-1 RNase H activity can be divided into 8 main classes based on their origin and chemical structure.

Natural product-derived compounds. Sulfated polyanions such as heparin, xylan polysulfate and dextran sulfate, extracted from algae or fungi, were reported to inhibit the RNase H activity in enzyme assays with IC_{50} values (i.e. the compound concentration needed to inhibit 50% of the enzyme activity) of 0.1-8 nM (42). Illimaquinone, 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 was reported to inhibit HIV-1 RNase H and RDDP activities with IC_{50} values of 15 μM and >140 μM, respectively (43). 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) (44). 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*, the methanol extracts from the Korean plant *Agrimonia pilosa* and the kaempferol acethylrhamnosides from the Chinese herbal medicine *Dryopteris crassirhizoma* (40).

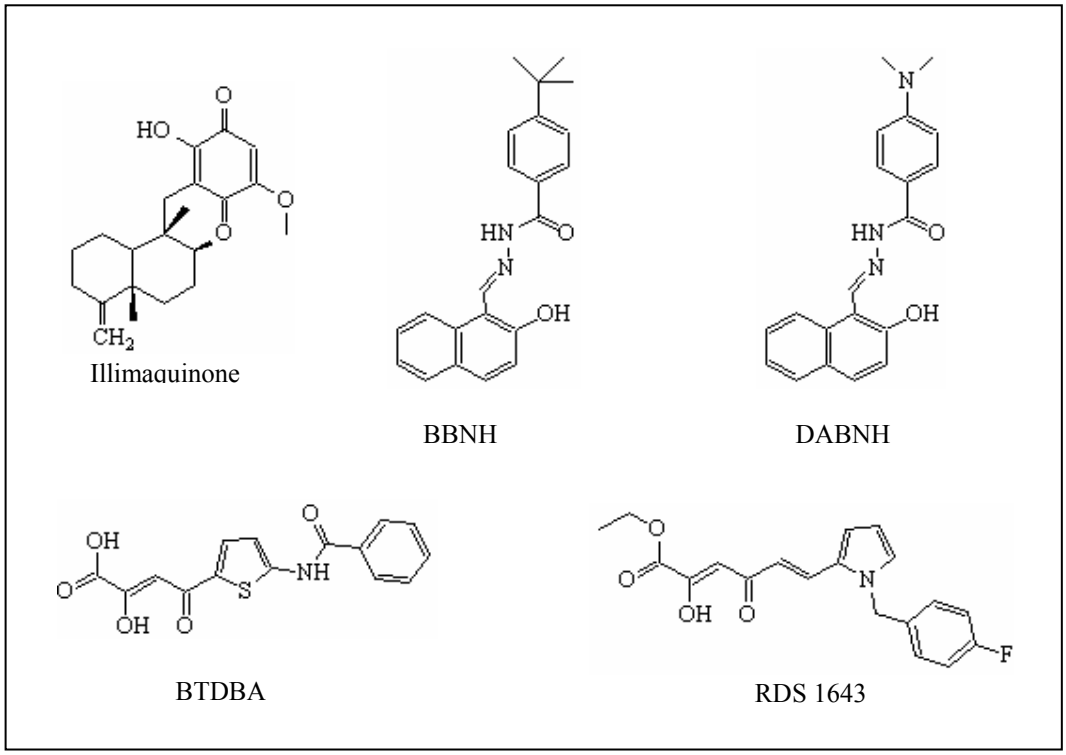

Fig. 3 Chemical structures of HIV-1 RNase H inhibitors.

Nucleotides and oligonucleotides. The monophosphate form of the well known NRTI 3'-azido-3'-deoxythymidine (AZT), 3'-azido-3'-deoxythymidine 5'-phosphate (AZTMP) and other nucleoside monophophates such as 2',3'-dideoxyadenosine 5'-monophosphate (ddAMP) and 2',3'-dideoxyguanosine 5'-monophosphate (ddGMP) slightly inhibited the RNase H activity. The potency of inhibition of these nucleoside monophosphate analogues was dependent on the hydrolysis substrate composition, however their precise mode of action has not been understood 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 (45).

Naphtalenesulfonic acid derivatives. A first series of naphtalenesulfonic acid derivatives was shown to slightly inhibit the HIV-1 RNase H activity, while a second series of naphtalenesulfonic acid derivatives, obtained by structure-based design and combinatorial medicinal chemistry approach, inhibited the HIV-1 RNase H and RDDP activities at 25-100 nM and 90 nM, respectively (40). However, probably these compounds does not interact with the RNase H site but prevent retroviral RT interaction with the substrate heteroduplex, and their effect on virus replication is 100 fold lower than the one observed on the enzyme activity.

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, BBNH, 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 (46). BBNH also inhibited the RNase Hs from *E. coli* and Moloney Murine Leukemia Virus with similar potency. 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 (46, 47). Mode of action studies allowed to identify the presence of two major binding determinants and led to the synthesis new derivatives among which is the (4,*N,N*-dimethylaminobenzoyl)-2-hydroxy-1-naphthyl hydrazone, DABNH, that inhibited the RNase H activity with an IC_{50} value of 4 μM, while it was inactive against the RDDP function (48). More recently, the novel analogue (*E*)-3,4-dihydroxy-*N'*-((2-methoxynaphthalen-1-yl)methylene)benzohydrazide (DHBNH) has been crystallized in complex with the HIV-1 RT and shown to bind to a novel site of the RT protein, > 60 Å away from the RNase H active site, and near both the polymerase active site and the non-nucleoside RT inhibitor binding pocket (49). This report has opened a new window of opportunity in the RNase H field.

Diketo acid derivatives. The diketo acid (DKA) derivative 4-[5-(benzoylamino)thien-2-yl]-2,4-dioxobutanoic acid, BTDBA, was reported to inhibit the HIV-1 RNase H activity with an IC_{50} value of 3.2 μM (50). However, it also inhibited the HIV-1 integrase 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 (51). A second DKA derivative that has been reported to have anti-RNase H activity by our group is the 6-[1-(4-fluorophenyl)methyl-1*H*-pyrrol-2-yl)]-2,4-dioxo-5-hexenoic acid ethyl ester, RDS 1643 (52). 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. However, noteworthy, in cell-based assays it was able to block the replication of wild type HIV-1 at the concentration of 13 μM and it was cytotoxic at a concentration of 63 μM. Furthermore, it inhibited the replication of three HIV-1 NNRTI resistant viral mutants (RT mutations were Y181C; K103N/Y181C; K103R/V179D/P225H) at concentrations of 7-19 μM (52). Mode of action studies demonstrated that the RDS 1643 maximum adsorbance shifted in the presence of the Mg^{2+} ions, suggesting that, similarly to BTDBA, it may sequestrate the active site divalent metals having a specific binding site on the HIV-1 RNase H domain. Consistently, further modeling studies proposed that RDS 1643 may bind to the HIV-1 RNase H domain similarly to BTDBA (47).

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 (53). 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 (53). Based on these studies, a series of small N-hydroxyimides were tested for their activity against the HIV-1 RNase H function (54). The prototype analogue N-hydroximide 2-hydroxy-4H-isoquinoline-1,3-dione inhibited in enzyme assays the HIV-1 RNase H activity with an IC_{50} value of 0.6-1 μM (40).

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 (55). The most potent analogue, β-thujaplicinol, 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. Unfortunately, these compounds were inactive on HIV replication in cell-based assays.

Mappicine analogues. Finally it is worth to note that some mappicine analogues 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) (47, 56).

7. Conclusions

In spite of its relatively short history, the field of antiviral drug discovery has already obtained several magnificent successes. The discovery of the inhibitors of the HSV and the HIV-1 infections are clearly bright examples that now allow to treat millions of infected patients. Given that the HIV-1 pandemic diffusion still needs to be fight also through the continuous development of new antiviral drugs, currently unexplored HIV-1 targets are under evaluation to this aim. Among these targets, one of the most promising is the HIV-1 RT-associated RNase H activity that only recently has attracted adequate attention. The biochemical methods available to assay this enzyme activity allow to quickly screen hundreds of compounds in search of new inhibitors. These methods, coupled with the structural biologist and chemist expertise, will bring in the next few years the development of promising drug candidates for clinical trial testing. At the moment, the two most promising known inhibitors that can be further developed are the DKA RDS 1643, the first selective RNase H inhibitor (certainly a milestone in the field), and the hydrazone derivative DHBNH. Based on these compounds, it is possible to foresee that the HIV-1 RNase H will be the next target hit in the antiviral drug discovery field.

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