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New insights into the interaction between pyrrolyl diketoacids and HIV-1 integrase active site and comparison with RNase H

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ABSTRACT

HIV-1 integrase (IN) inhibitors are one of the most recent innovations in the treatment of HIV infection. The selection of drug resistance viral strains is however a still open issue requiring constant efforts to identify new anti-HIV-1 drugs. Pyrrolyl diketo acid (DKA) derivatives inhibit HIV-1 replication by interacting with the Mg^{2+} cofactors within the HIV-1 IN active site or within the HIV-1 reverse-transcriptase associated ribonuclease H (RNase H) active site. While the interaction mode of pyrrolyl DKAs with the RNase H active site has been recently reported and substantiated by mutagenesis experiments, their interaction within the IN active site still lacks a detailed understanding. In this study, we investigated the binding mode of four pyrrolyl DKAs to the HIV-1 IN active site by molecular modeling coupled with site-directed mutagenesis studies showing that the DKA pyrrolyl scaffold primarily interacts with the IN amino residues P145, Q146 and Q148. Importantly, the tested DKAs demonstrated good effectiveness against HIV-1 Raltegravir resistant Y143A and N155H INs, thus showing an interaction pattern with relevant differences if compared with the first generation IN inhibitors. These data provide precious insights for the design of new HIV inhibitors active on clinically selected Raltegravir resistant variants. Furthermore, this study provides new structural information to modulate IN and RNase H inhibitory activities for development of dual-acting anti-HIV agents.

1. Introduction

Despite the multiple combinations of the approved 26 drugs for the human immunodeficiency virus (HIV) infection, the life-long treatment often leads to the selection of multi-drug resistant viral strains, causing high numbers of antiviral therapy failures (Frentz et al., 2014; Schneider et al., 2016). These data, together with recent reports of selection of highly aggressive HIV-1 recombinant variants, causing a very rapid progression to AIDS (Kouri et al., 2015), require the effort of developing new inhibitors effective on existing drug resistant variants. The latest class of drugs approved for HIV-1 treatment is composed by active site inhibitors of Integrase (IN) (Esposito and Tramontano, 2013), a key enzyme in the HIV-1 replication cycle responsible for the chromosomal integration of the newly synthesized double-stranded viral DNA copy into the host genomic DNA. IN operates within a large nucleoprotein assembly, named pre-integration complex (PIC) (Di Santo, 2014) exerting 3'-processing and strand-transfer (ST) reactions. Both these activities require enzyme multimerization and coordination of the nucleic acid substrate by two Mg^{2+} ions within the IN catalytic core (Faure et al., 2005; Guiot et al., 2006).

Diketo acid (DKA) derivatives are among the first compounds reported to interact with the Mg^{2+} cofactors within the IN active site (Hazuda et al., 2000; Marchand et al., 2002) and to inhibit the

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IN ST reaction. Similarly, DKA derivatives were also reported to inhibit HIV replication targeting another viral coded enzyme belonging to the polynucleotidyl transferase family, the reverse transcriptase (RT)-associated ribonuclease H (RNase H) function (Distinto et al., 2013; Tramontano et al., 2005). IN and RNase H share remarkable structural similarities: in both cases, in fact, the catalytic core domain (CCD) is formed by five-strand β sheets and four-to-six α-helices, respectively (Dyda et al., 1994; Esposito and Tramontano, 2013; Lapkouski et al., 2013). In the HIV-1 IN structure, there are two additional helices that are not present in RNase H. Both catalytic sites coordinate two Mg^{2+}ions acting as enzymatic cofactors, however they have different electrostatic properties. In particular, the IN active site is positively charged since only three acidic residues (D64, D116, E152) counterbalance the four positive charges of the two catalytic Mg^{2+}, whereas in the RNase H catalytic core four catalytic acid residues (D443, E478, D498 and D549) neutralize the charge of the two cations (Goldgur et al., 1998).

We previously reported a number of pyrrolyl DKA derivatives as IN inhibitors, RNase H inhibitors and dual IN and RNase H inhibitors (Di Santo, 2011; Corona et al., 2014a; Costi et al., 2014; Cuzzucoli Crucitti et al., 2015). Our structure activity relationship (SAR) studies showed that pyrrolyl diketoesters are generally more active on the RNase H function, while the corresponding acid derivatives are more potent against IN. Recently, by a combination of molecular modeling, site directed mutagenesis and cell based assays, we investigated the mode of RNase H inhibition of a series of pyrrolyl DKAs (Corona et al., 2014a), analogues of the 6-[1-(4-fluorophenyl) methyl-1H-pyrrol-2-yl)]-2,4-dioxo-5-hexenoic acid ethyl ester (RDS1643) (Tramontano et al., 2005), that was predicted by NMR analysis to inhibit HIV-1 RNase H activity interacting with highly conserved residues within the RNase H active site (Corona et al., 2014c). While most of the reported pyrrolyl DKA derivatives inhibit the HIV-1 IN, their binding to the IN active site still lacks a detailed analysis, impeding their further optimization as IN or dual IN/RNase H inhibitors. In the present work we selected four compounds with different structural properties, determining their effects on HIV-1 replication in cell-based assays and their interaction with the IN active site, identifying the aminoacid residues involved in their binding. In addition, comparative analysis of IN and RNase H active sites allowed to better delineate the DKA structural features required to modulate the potency towards either of the two enzymes.

2. Material and methods

2.1. Compounds synthesis

Compounds RDS1611, RDS1644, RDS1712 and RDS1823 have been synthesized and purified as previously reported (Costi et al., 2013; Cuzzucoli Crucitti et al., 2015). Compound purities were always >95% as determined by high-pressure liquid chromatography (HPLC).

2.2. Site-directed mutagenesis

HIV-1 IN mutants were obtained by site-directed mutagenesis using the Quick change Lightning site-directed mutagenesis kit (Agilent). This mutagenesis was performed on the pET15b encoding the full His-tagged HIV-1 IN, as previously described (Malet et al., 2015).

2.3. Expression and purification of recombinant HIV-1 IN

HIV-1 IN was expressed essentially as reported in (Zamborlini et al., 2011). Briefly, His-tagged IN was produced in *E. Coli* BL21-CodonPlus (DE3)-RIPL (Agilent, Santa Clara, USA) and purified under non-denaturing conditions. Protein production was induced at OD_{600} of 0.6 to 0.8, by adding Isopropyl β-D-1-thiogalactopyranoside (final concentration of 0.5 mM). Cultures were incubated for 3 h at 37 °C and then centrifuged 20 min at 1100 g, 4 °C. Cells were resuspended in buffer A (50 mM Tris-HCl (pH 8.0), 1 M NaCl, 4 mM β-mercaptoethanol) and lysed using French press. The lysate was centrifuged (30 min at 12,000 g at 4 °C), and the supernatant was filtered (pore size 0.45 μM) and incubated with nickel-nitrilotriacetic acid agarose beads (Qiagen, Venlo, The Netherlands) for at least 2 h at 4 °C. The beads were washed with buffer A and then with buffer A supplemented with 80 mM imidazole. IN was then eluted from the beads in buffer A supplemented with 1 M imidazole and 50 μM zinc sulfate and then dialyzed overnight against 20 mM Tris-HCl (pH 8.0), 1 M NaCl, 4 mM β-mercaptoethanol and 10% glycerol. Aliquots of the purification products were rapidly frozen and stored at −80 °C. The purity of enzyme was analyzed by SDS PAGE and calculated as higher than 90%.

2.4. HIV-1 IN activity

IN ST reaction was performed as described previously (Delelis et al., 2007). Oligonucleotides (Eurogentec), HIV-1B (5′-TGTGGAAAATCTCTAGCA-3′) and HIV-1A (5′-ACTGCTAGAGATTTTCCACA-3′) were used for the strand transfer-reaction. HIV-1B was radiolabelled with T4 polynucleotide kinase (Biolabs) and γ-[^{32}P]ATP (3000 Ci/mmol) (Amersham), hybridized and with HIV-1A and purified on a Sephadex G-10 column (GE Healthcare). ST reaction was carried out at 37 °C, in a buffer containing 10 mM HEPES (pH 7.2), 1 mM DTT, 7.5 mM $MgCl_2$ in the presence of 12.5 nM DNA substrate. Products were loaded on denaturing 18% acrylamide/urea gels. Gels were analyzed with a Molecular Dynamics STORM phosphoimager and quantified with Image Quant™ 4.1 software.

2.5. Cells and viruses

MT4 and 293T cells were cultured in RPMI1640 medium and in DMEM medium, respectively, supplemented with 10% fetal calf serum. HIV-1 stocks were prepared by transfecting 293T with the HIV-1 molecular clone derived from the pNL4-3 (Δenv viruses) (Thierry et al., 2015). Δenv viruses NLENG1-ES-IRESwt encode the wt IN. Pseudotyping of Δenv viruses was performed by co-transfection of 293T cells with VSV-g plasmid (Addgene) expressing the G glycoprotein of the vesicular stomatitis virus using the calcium phosphate method. Viral supernatants were filtered (0.45 μm) and frozen at −80 °C· HIV-1 $p24^{gag}$ antigen contents in viral inocula were determined by enzyme-linked immunosorbent assay (Perkin-Elmer Life Sciences). 120 ng of $p24^{gag}$ antigen per 10^6 cells, corresponding to a multiplicity of infection of 0.3, was used for infection. Flow cytometry analysis was performed on a FACSCalibur flow cytometer (BD Bioscience) and results were analyzed using the ImageQuant software.

2.6. Quantification of viral DNA genomes

Quantitative PCR (qPCR) was performed as described previously (Abram et al., 2015; Munir et al., 2013). Briefly, MT4 cells were infected and divided in two group, the first group was treated with compounds at time of infection, the second group was treated 16 h post infection. DKA inhibitors were used at concentration of 10 μM, Raltegravir (RAL) and Dolutegravir (DTG) (500 nM) (Selleckchem) or Efavirenz (EFV) (100 nM) (Sigma Aldrich) were used as controls. Samples were collected at 24 h post infection for total viral DNA quantification and at 48 h post infection for quantification of 2-LTRc