Chromenone derivatives as a versatile scaffold with dual mode of inhibition of HIV-1 Reverse Transcriptase-associated Ribonuclease H function and Integrase activity

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ABSTRACT: A number of compounds targeting different processes of the Human Immunodeficiency Virus type 1 (HIV-1) life cycle have been developed in the continuing fight against AIDS. Coumarin-based molecules proved to act as HIV-1 Protease (PR) or Integrase (IN) inhibitors and also to target HIV-1 reverse transcriptase (RT), blocking the DNA-dependent DNA-polymerase activity or the RNA-dependent DNA-polymerase activity working as common NNRTIs. In the present study, with the aim to exploit a coumarin-based scaffold to achieve the inhibition of multiple viral coded enzymatic functions, novel 4-hydroxy-2*H*, 5H-pyrano (3, 2-c) chromene–2, 5–dione derivatives were synthesized. The modeling studies calculated the theoretical binding affinity of the synthetized compounds on both HIV-1 IN and RT-associated Ribonuclease H (RNase H) active sites, confirmed by biological assays. Our results provide a basis for the identification of dual HIV-1 IN and RT RNase H inhibitors compounds.

KEYWORDS: Coumarin derivatives, HIV-1, RNase H, Integrase, dual inhibitors, LEDGF/p75 cellular cofactor.

INTRODUCTION

During the viral infection, the Human Immunodeficiency Virus type 1 (HIV-1) genome is retrotranscribed by the viral enzyme reverse transcriptase (RT), a multifunctional protein endowed of three main enzymatic functions: an RNA-dependent DNA-polymerase (RDDP) activity, that synthesizes the RNA:DNA intermediate, the Ribonuclease H (RNase H) activity, responsible of the hydrolytic cleavage of the RNA strand of the RNA:DNA hybrid and the DNA-dependent DNA-polymerase (DDDP) activity, that completes the synthesis of the integration-competent double strand DNA [1]. After the retrotranscription process, the viral DNA is integrated into the host genome through two enzymatic reactions accomplished by the viral enzyme integrase (IN) that operates within a preintegration complex (PIC), composed of viral DNA, viral and cellular proteins [2]. Among the cellular factors involved in the integration process there is the nuclear protein Lens-

Epitelium-derived (LEDGF/p75) that establishes specific interactions with chromatin and the catalytic core domain (CCD) to the IN dimer through its IN binding domain (IBD) [3].

The current HIV treatment is based on the highly active antiretroviral therapy (HAART), that allowed a major decrease in morbidity and mortality for AIDS patients [4]. However, in a prolonged therapy, the lack of compliance and compartmentalization phenomena still consent the selection of drug resistant strains, that can also affect multiple class of drugs [5] with increased transmission of drug-resistant viral strains detected in antiretroviral-treatment–naïve patients [6]. Therefore, the search of novel inhibitors continues, aiming to achieve drugs with novel mode of action and also lower long-term toxicity of the treatments. In this respect, the inhibition of two viral functions by a single molecule could provide a higher barrier to drug resistance selection, reducing the number of drugs administered and the consequent long-term toxicity [7].

Among the different categories of anti-HIV drugs, the RT inhibitors are the most prescribed drugs to treat HIV infection [8], in fact the RT inhibitors play a crucial role in the treatment of HIV-infected patients and IN inhibitors (INIs) are the last innovation, representing a good second line therapy together with protease inhibitors. The approved RT inhibitors structurally belong to two families: the nucleoside/nucleotide RT inhibitors (NRTIs /NtRTIs) and the non-nucleoside RT inhibitors (NNRTIs) [9]. The NRTIs were the first approved anti-HIV agents, derived from former known anticancer agents and characterized by heavy side effects. With respect to the NRTIs, the NNRTIs are characterized by a higher potency, lower toxicity, higher selectivity and specificity [10]. Though the approved NNRTIs have different chemical structures, all of them bind into the same site of RT, selectively inhibiting its polymerase function. No approved inhibitor of HIV-1 RT-associated RNase H activity is currently available [11], even if this function, essential for viral replication [12], is a well validated target for drug development [11,13-20], with some promising *hits* being currently investigated [21,22]. Among the INIs, all the approved drugs are active-site inhibitors [1], while allosteric inhibitors are under development [23-27]. The IN inhibitors are unable to bind IN alone but they bind to the pre-integration complex formed by IN and viral DNA and subsequently inhibit the strand-transfer reaction [28]. Interestingly,

some molecules able to target both RT and IN have been reported recently,[27,29-32] enlightening the possibility of a dual inhibition of the two viral functions [33].

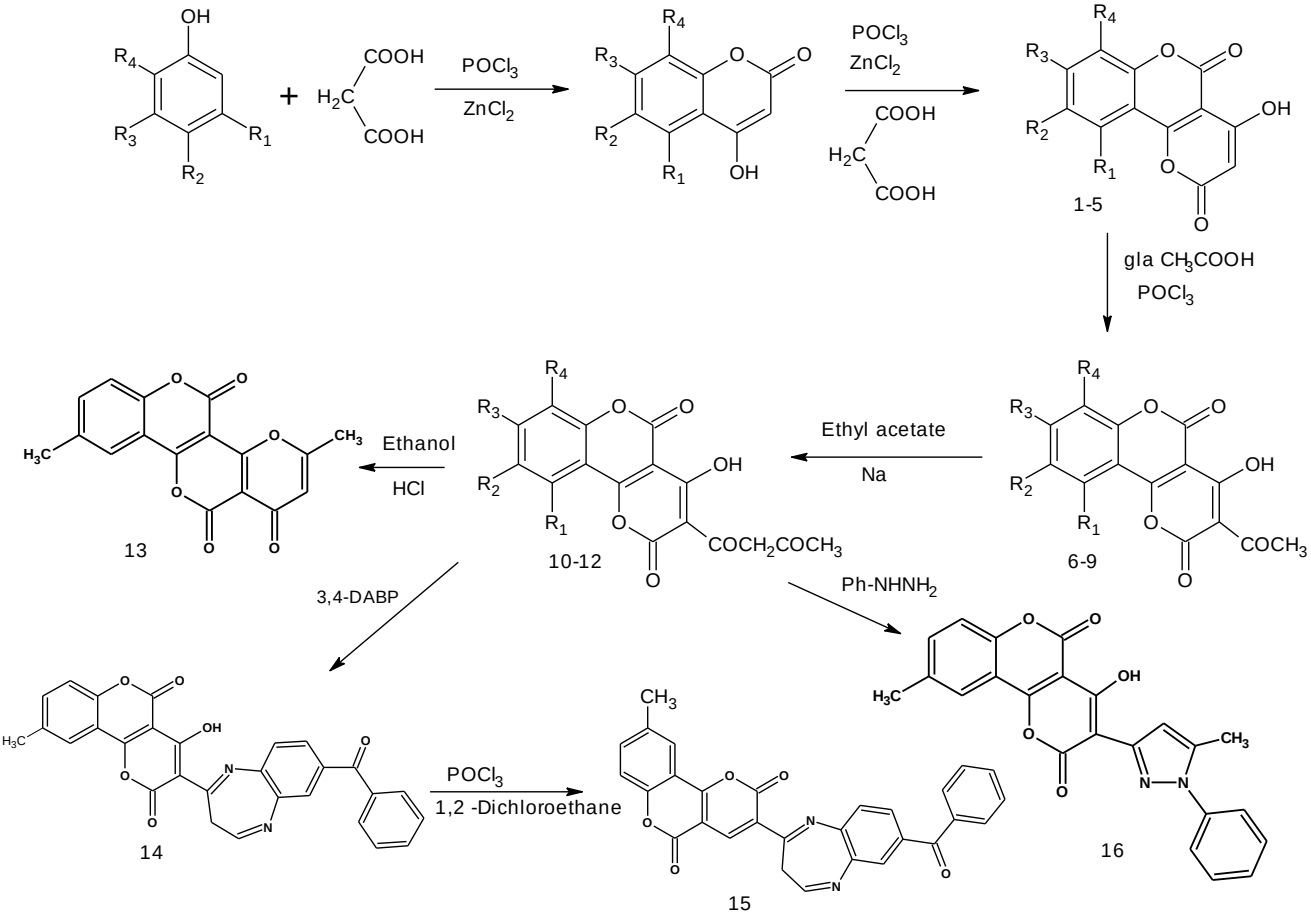
With the aim to develop novel drugs that could target more enzymatic functions, in this study we exploited the coumarin scaffold. It has been reported that coumarin (2H-chromen-2-one; 1-benzopyran-2-one) derivatives possess a wide range of activities. While antibiotic and anticoagulant activities are the most common actions [34], several coumarin derivatives, such as 4-hydroxy-3-(5- methyl-1-phenyl-1H-pyrazol-3-yl)pyrano[3,2-c]chromene-2,5- dione derivatives [35], (as khellactone derivatives [36], dihydroseselins [37] and 2,3-dimethyl-4-chromanol derivatives [38], were associated to anti-HIV activity, and this activity has been referred in many cases at the inhibition of PR [39] and IN activities [40-43], while RT inhibition has not reported for many of these compounds, such as khellactone derivatives [34]. Distinct modes of action include interaction with the HIV-1 encoded proteins Tat [44] and Vpr [45]. (+)-Calanolide was found to be an NNRTI and interestingly, 3',4'-di-O-(-)-camphanoyl-(+)-*cis*-khellactone derivatives were found to target RT, inhibiting selectively its DDDP activity and not its RDDP activity, differently form most of the known NNRTIs [36,43]. Starting from structural and physicochemical characteristics of known active compounds against HIV-1 RT [35,41] and IN enzymes and given the structural similarities between the viral RT-associated RNase H domain and the IN domain [30], 16 coumarin (4-hydroxypyranobenzopyran) derivatives were synthesized and their theoretical binding affinity versus *both* the enzymes were evaluated through computational studies. Finally, the biological activity against HIV-1 RNase H and IN functions were tested, thus identifying novel chemical structures with dual HIV inhibition.

RESULTS AND DISCUSSION

Chemistry

Sixteen novel coumarin derivatives (Figure 1) were synthesized according to Scheme 1. In the first stage 4-hydroxy coumarine derivatives were prepared by reaction of phenol and malonic acid in the presense of phosphorous oxychloride and zinc chloride. These hydroxy coumarine

derivatives were furhter treated with malonic acid, phosphorous oxychloride and zinc chloride resulting in derivatives of 4-hydroxy-2-methylenepyrano[3,2-c] chromene-2,5-dione (**1-5**). The acetylation of substituted 4-hydroxy-2-methylenepyrano[3,2-c] chromene-2,5-diones gave various acetyl substituted coumarine compounds (**6-9**). Reaction of these acetyl derivatives with ethyl acetate and sodium metal funirshed substituted derivatives of 4-hydroxy-3-(3-oxobuta-noyl)pyrano[3,2-c]chromene-2,5-dione (**10-12**). Acidic hydrolysis resulted in compound **13**, while cyclization of acetoacetyl derivatives of coumarine (**10-12**) with 3,4-diaminobenzophenone and phenyl hydrazine resulted in 3-(7-benzoyl-3H-benzo[b][1,4]diazepin-2-yl)-4-hydroxypyrano [3,2-c]chromene-2,5-dione (**14,15**) and 4-hydroxy-3-(5-methyl-1-phenyl-1H-pyrazol-3-yl)pyrano [3,2-c]chromene-2,5-dione (**16**) respectively. The corresponding reactions proceeded smoothly and resulted in considerably good yields (56%-82%). Structures of synthesized compounds were confirmed satisfactory by IR, ^{1}H-NMR, EI-MS and elemental analysis. For the NMR detailed for each compound see the Supplementary Material.



COMPOUNDS	R_1	R_2	R_3	R_4
1	H	CH_3	H	H
2	H	H	CH_3	H
3	H	H	H	CH_3
4	Ph		H	H
5	H	H	Ph	
6	H	H	H	H
7	H	CH_3	H	H
8	H	H	CH_3	H
9	H	H	H	CH_3
10	H	H	H	H
11	Ph		H	H
12	H	H	Ph	

Scheme 1: Synthesis of compounds **1-16**.

Physicochemical properties of selected compounds

Theoretical calculation of the physicochemical properties of selected compounds (Table S2) was performed in order to estimate the possibility of compounds of this series to become orally active

drugs and to evaluate the probable association of biological activity with the physicochemical properties of the compounds. Average logP of the compounds was between 0.49 and 3.50. Consequently, all compounds fulfill the related criterion of Lipinski and Ghose rules concerning membrane penetration. Compounds have intermediate aqueous solubility with ALOGS between -1.69 and -4.98. Topological polar surface area (TPSA) was between 90.6 and 131.9 that is lower than 140 $Å^2$ that is the upper limit of Ghose fifth criterion. Polarizability was between 27.196 and 52.73 cm^{-3}, and molar refractivity ranged from 68.60 to 133.01 cm^{-3}. Compound **14**, with predicted molar refractivity equal to 133.01 cm^{-3}, is the only one that slightly surpasses the upper limit of 130 cm^{-3} of Ghose second criterion. All compounds fulfill the criterion of molecular weight of both Lipinski and Ghose rules with MW of compounds being between 290.3 and 494.5 while the calculated molecular volume of the compounds ranges from 233.3 to 412.0 $Å^3$. In general, according to theoretical calculations, all compounds fulfill the Lipinski's rule of cell membrane penetration by passive diffusion and all of them with a minor aberration at the molar refractivity of compound **14**, also fulfill all Ghose's criteria.

Molecular modeling results

The docking analysis was performed by using two metal sites as constrain [46] in order to better understand the theoretical affinity and the binding modes of the studied compounds *versus* both HIV-1 RT-associated RNase H and IN activities. RDS1643 [47] and Raltegravir were used as reference compounds for the molecular recognition analysis respectively of the HIV-1 RT-associated RNase H function and IN enzymes. The HIV-1 RNase H active site contains a highly conserved and essential DDE motif comprising the carboxylate residues Asp443, Glu478 and Asp498, which coordinates two divalent Mg^{2+} cations [46]. Our docking results suggested that most of the synthesized coumarin derivatives were well accommodated into the RNase H active site and were able to chelate the metal cofactor ions thanks to the chelating core present in their scaffold. The Docking score values (D-score) for each studied compound are reported in Table 1.

Table 1: Docking score values of all the studied coumarin derivatives *versus* the HIV-1 RNase H active site and the PFV IN catalytic binding site in the presence of LEDGF cofactor, respectively. D-score values are expressed in kcal/mol.

COMPOUNDS	RT RNASE H ACTIVE SITE	PFV IN CATALYTIC BINDING SITE IN PRESENCE OF LEDGF COFACTOR
	Docking score value	Docking score value
7	-6,22	-9,96
6	-6,29	-9,93
8	-3,04	-9,90
9	-6,37	-9,91
12	-4,71	-8.83
4	-5,52	-8,69
3	-6,38	-8,46
10	-4,92	-8,46
2	-4,06	-8,36
1	-6,31	-8,35
5	-6,74	-8,29
11	-5,00	-8,21
14	-5,08	-7,83
RAL	-	-7,82
13R	-4,25	-6,75
13S	-4,30	-6,43
15	-4,94	-6,12
16	-4,94	-5,65
RDS1643	-5,81	-

Ligands with the lowest docking scores are predicted to have a better theoretical binding affinity towards the protein. Specifically, compounds **7**, **5**, **6**, **3** and **1** were the best predicted within the active site of RNase H due to the interactions between their chelating core and the two Mg^{2+} ions, which are coordinated to the active site acid residues Asp443, Glu478, Asp498 and Asp549, crucial for the RT-RNase H activity [48].

Additionally, compound **7** was involved in hydrophobic interactions with His539, Gly444 and Asn474. Compound **9** was well stabilized into the enzyme binding pocket through the coordination with the two metal cofactors, hydrophobic interactions with Gln500 and Asp498 and a pivotal π-π interaction with His539. The predicted binding mode of best pose for compounds **3**, **1**, **6** and **7** within the RT RNase H active site are shown in Figure 1, while the binding mode of the best poses of the other compounds are reported in the Figure S1 of the Supplementary Material. Compounds **10** and **12** showed a similar binding mode characterized by the chelation of the two metal cofactors

and the interaction with the catalytic triad, meanwhile their benzene rings were exposed outside from the active pocket. Due to their highly similar structure, also compound **14** and **15** are associated to the same binding mode, whereas **16** showed a slightly different binding mode. Compounds **2** and **8** have a disadvantaged theoretical binding affinity (D-Score values) for the RT RNase H active site, due to the loss of the interaction between the chelating core of the compounds and the two Mg^{2+} ions.

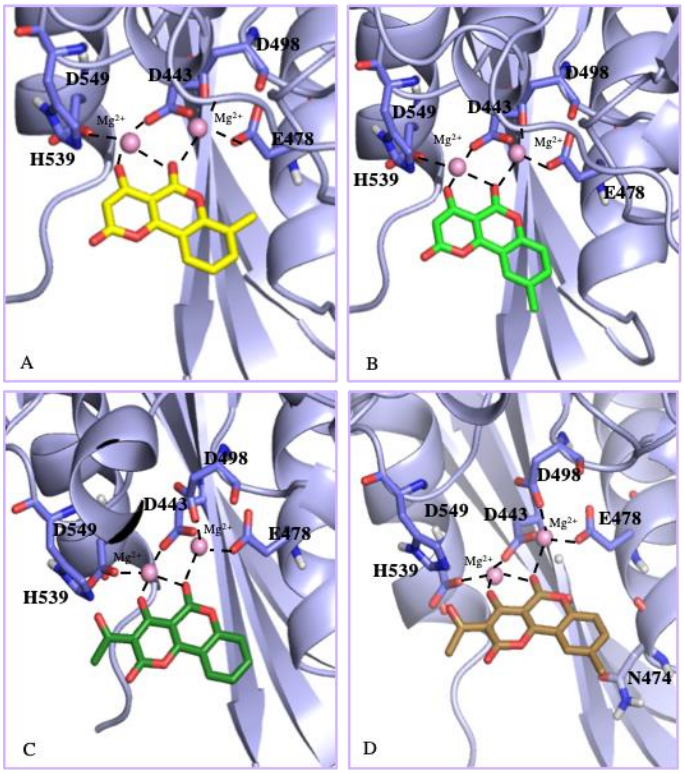


Figure 1: 3D representation of the best docking poses of compounds A) **3**; B) **1**; C) **6** and D) **7** into the HIV-1 RT RNase H active site. The ligands are shown, respectively, as yellow, green, dark-green and gold carbon sticks, while the receptor and the Mg^{2+} ions are reported as light blue cartoon and pink spheres, respectively. Amino acids involved in ligand binding are highlighted as slate carbon sticks.

HIV-1 IN and RT-RNase H share similar active site configurations, thus inhibitors of these enzymes functions involve overlapping pharmacophore features [46]. Therefore, at the same time we performed molecular recognition studies of the same compounds *versus* the PFV IN, in the presence of the LEDGF cofactor, in order to identify dual-acting inhibitors as new approach in HIV-1 drug development. After Molecular Dynamics simulations (MDs), the best receptor structure, selected according to the to the Boltzmann population and Prime energy parameters [57], was

adopted for further docking studies of the synthetized compounds. Our docking results highlighted that most of the analyzed compounds were able to bind into the PFV IN catalytic site in the presence of the LEDGF cofactor establishing interactions with the three acidic residues such as Asp128, Asp185 and Glu211 (corresponding to Asp64, Asp116 and Glu152 in HIV-1 IN) of the IN active site, by coordinating the essential Mg^{2+} metal cations [49]. In the Table 1 the theoretical binding affinity of the compounds *versus* the PFV IN active pocket, in the presence of the LEDGF cofactor, are reported.

Compounds **7**, **6**, **8**, **9** and **12** were associated to the best theoretical binding affinity *versus* the active site of PFV IN thanks to the interactions established between their chelating core and the two Mg^{2+} ions, coordinated to the crucial active site acid residues such as Asp128, Asp185, Glu221 (corresponding to Asp64, Asp116 and Glu152 in HIV-1 IN), and additional hydrophobic contacts with Pro214 and Tyr212 (corresponding to Pro145 and Tyr143, respectively in HIV-1 IN). Compounds **3**, **10**, **11**, **13R** and **14** were found to chelate the metal cofactors and showed their aromatic rings oriented toward the viral DNA, thus establishing a staking interaction with the nucleobase C16 of the viral DNA. Compounds **1**, **2**, **4** and **5** shared the same arrangement into the PFV IN catalytic site, while **1** and **4** were better stabilized by extra hydrophobic interactions with Tyr212 and Gln186 (corresponding to Tyr143 and Gln117 in HIV-1 IN). The predicted binding mode of best pose of compounds **7**, **6**, **3** and **1** within the PFV IN catalytic site are shown in Figure 2, while the binding mode of the best poses of the other compounds are reported in Figure S2 of the Supplementary Material. For compounds **15** and **16** our simulations highlighted that both compounds were involved in hydrophobic contacts with the tyrosine at position 212, but missing the coordinating interaction with the two Mg^{2+} ions.

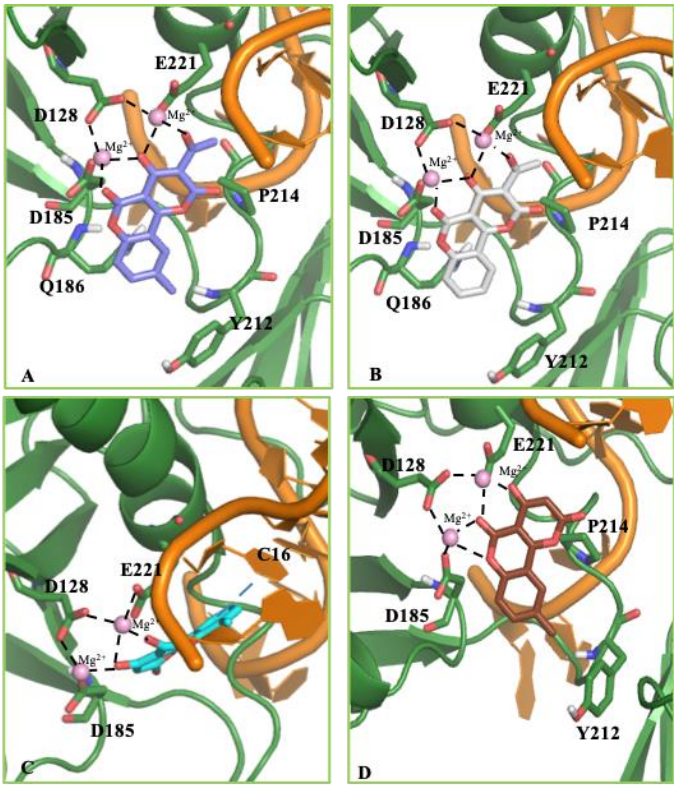


Figure 2: 3D representation of the best docking poses of compounds A) **7**; B) **6**; C) **3** and D) **1** into the PFV IN catalytic site. The ligands are shown, respectively, as slate, gray, cyan and chocolate carbon sticks, while the receptor and the vDNA are showed as green and orange cartoon, respectively. Amino acids involved in ligand binding are highlighted as green carbon sticks. The Mg^{2+} ions are showed as pink spheres.

Effect of coumarin derivatives on HIV-1 RT-associated RNase H activity

It has been reported that coumarin derivatives are able to target HIV-1 RT polymerase activity, and a moderate affection of *E. coli* RNase H function was once reported [50], even as the activity of this class of molecules on the HIV-1 RT-associated RNase H function was never further investigated. In addition, some compounds reported to allosterically inhibit the RDDP function, were also able to interfere with the RNase H activity [51]. Since the RT-associated RNase H function is a good target for the development of promising drugs, to confirm the docking prediction, the results of which show that coumarin derivatives interact with the catalytic site of HIV-1 RNase H, we tested a series of 16 coumarin derivatives for their ability to inhibit HIV-1 RNase H function in biochemical assay, using the diketoacid RDS1643 as a positive control [47]. As shown in Table 3, the different position of methyl group in the phenyl ring of compounds **1**, **2** and **3** consistently changed the

potency of inhibition, in fact differently from compound **2**, compounds **1** and **3** were able to inhibit HIV-1 RNase H activity with IC_{50} values around 16 and 30 uM, respectively. The introduction of an additional phenyl ring on compounds **4** and **5** did not improve their potency of inhibition (IC_{50} values of 61 and 25 µM, respectively). Then, we asked if the introduction of acetyl substituent on compounds **1**, **2** and **3** might improve the potency of these derivatives. Results showed that when the phenyl ring was unsubstituted (compound **6**) the potency of inhibition was not improved (IC_{50} value of 26 µM). Also compound **9** showed an IC_{50} value similar to the compound **3**, and compound **8** was not active, similarly to **2**. Differently, the insertion of acetyl group in compound **1**, led to compound **7** derivative that potently inhibited the HIV-1 RNase H function (IC_{50} around 7 µM). We then further improved the length of the chain with insertion of an acetylacetone (compounds **10**, **11** and **12**) and obtained an increase in potency only for compound **11** that inhibits the HIV-1 RNase H activity with an IC_{50} value of 13.8 µM. While the potency of **10** and **12** was negatively affected if compared with the acetyl substituted counterparts. Instead, the insertion of an additional pyranonic ring on compound **6** led to compound **13** with a slightly improved HIV-1 RNase H inhibitory activity (IC_{50} value of 16.8 µM). Finally, the introduction of additional heterocyclic groups (compounds **14**, **15** and **16**) led to derivatives that slightly inhibited the HIV-1 RNase H activity, with an IC_{50} value ranging between 38 and 52 µM.

Effect of coumarin derivatives on HIV-1 IN activity in presence of LEDGF/p75 cellular cofactor

It has been reported that coumarin-based compounds present HIV-1 IN catalytic activity inhibition [52]. Our docking results predict that our coumarin derivatives were able to bind the HIV-1 IN taking interactions in the active site. In order to support these results, we tested them for their ability to inhibit HIV-1 IN in presence of LEDGF/p75 cofactor, using the strand transfer inhibitor Raltegravir as a positive control (Table 2). Results show that the different substitutions on coumarin derivatives led to compounds that inhibit HIV-1 IN in presence of LEDGF/p75 with a different potency of inhibition.

Table 2: Coumarine derivatives effects on the HIV-1 RT-associated RNase H and HIV-1 Integrase activities.

Compounds	[a]IC_{50} HIV-1 RNase H (μM)	[c]IC_{50} IN LEDGF-dependent activity (μM)	[d]IC_{50} IN-IN subunit exchange (μM)	[e]MI_{50} IN-multimerization (μM)	[f]IC_{50} IN-LEDGF binding (μM)
1	16.5 ± 0.76	9.5 ± 1.5	69 ± 3	>100	>100 (55%)
2	>100 (89%)[b]	41.5 ± 8.5	ND	ND	ND
3	29.8 ± 2.12	24.0 ± 4.0	ND	ND	ND
4	61.3 ± 3.17	21.5 ± 2.5	ND	ND	ND
5	24.7 ± 2.02	7.5 ± 0.5	57.0 ± 10	>100	>100 (53%)
6	26.31 ± 0.92	8.5 ± 1.5	ND	ND	ND
7	6.75 ± 0.51	6.45 ± 0.45	56.5 ± 6.5	>100	>100 (52%)
8	>100 (63%)	22,35 ± 3,65	ND	ND	ND
9	25.5 ± 2.29	18.5 ± 5.5	ND	ND	ND
10	85.8 ± 3.77	42.0 ± 2.0	ND	ND	ND
11	13.8 ± 0.91	47.5 ± 8.5	ND	ND	ND
12	51.2 ± 0.57	11.0 ± 2.0	85 ± 7	>100	>100 (73%)
13	16.8 ± 2.08	14.0 ± 3.0	>100 (78%)	>100	>100 (56%)
14	38.4 ± 6.02	20.0 ± 3.0	>100 (85%)	>100	>100 (81%)
15	42.9 ± 1.78	>100 (78%)	ND	ND	ND
16	51.8 ± 6.68	>100 (61%)	ND	ND	ND
LEDGIN-6	-	9.0 ± 2.0	>100 (100%)	10 ± 1	13 ± 3
RAL	-	0.058 ± 0.01	>100 (100%)	>100 (100%)	>100 (100%)
RDS1643	11.2 ± 2.4	-	-	-	-

[a] Compound concentration required to inhibit the HIV-1 reverse transcriptase RNase H activity by 50% - [b]Percentage of control activity measured in the presence of 100 μM concentration. [c]Compound concentration required to inhibit the HIV-1 IN catalytic activities by 50% in the presence of LEDGF. -. -[d]Compound concentration required to inhibit the HIV-1 IN-IN subunit exchange by 50%. -[e]Compound concentration required to inhibit the multimerization increase by 50%. -[f]Compound concentration required to inhibit the HIV-1 IN-LEDGF interaction by 50%. -[f] –

Similarly to what found for the RNase H activity, compounds that presented a methyl group in position **2** on the phenyl ring were more active if compared with their isomers, with compound **1** showing IC_{50} value of 9.5 μM, being more potent than **2** and **3** (with 41.5 μM and 24 μM, respectively). A similar behavior was found also in the presence of the acetyl substituent, with compound **7** showing an IC_{50} value of 6.4 μM, being more potent than compounds **6**, **8** and **9** (IC_{50} values of 8.5, 22.4 and 18.5 μM, respectively). Differently from the HIV-1 RNase H activity, the introduction of an additional phenyl ring led to compounds **4** and **5** that retained a good HIV-1 IN inhibition (IC_{50} values of 21.5 μM and 7.5 μM, respectively). The elongation of the lateral chain of these two derivatives with insertion of an acetylacetone group had a detrimental effect on the potency of inhibition, obtaining compounds **10**, **11** and **12**, that inhibit the HIV-1 IN activity with an IC_{50} values of 42, 47.5 and 11 μM, respectively. The insertion of a closed chain of compound **6**,

led to compound **13** which decreased the HIV-1 IN inhibitory activity by two fold. Finally, the introduction of an additional heterocyclic ring phenyl led to compound **14** that slightly inhibited the HIV-1 IN activity, and compounds **15** and **16** that were not active.

Characterization of the mode of IN inhibition by coumarin derivatives

It has been previously reported that compounds binding to the LEDGINs binding pocket can allosterically modulate the dynamic interplay between IN subunits, inhibiting the IN subunit exchange, promoting and stabilizing the multimerization form of IN [53]. To further support the computational studies, we verify if also coumarin derivatives were able to bind into the LEDGINs binding pocket, testing them in a HTRF IN subunit exchange assay, using the allosteric inhibitor LEDGIN-6 as control [53] (Table 3). In this assay, when a compound inhibits the IN subunit exchange the HTRF signal decreases, while when a compound promotes the IN multimerization, the HTRF signal increases. Our findings showed that compounds **1**, **5**, **7** and **12** were able to weakly inhibit the IN-IN subunit exchange (IC_{50} value range between 56 and 85 μM), but differently from the control LEDGIN-6, none was able to affect IN multimerization, showing that IN inhibition through interactions with the catalytic site reveals a profile of inhibition different from the one of the already reported allosteric inhibitors. To evaluate if also coumarin derivatives possesses the same LEDGINs inhibitory mode of action, we tested them for their ability to inhibit IN/LEDGF/p75 binding, using LEDGIN-6 as a positive control (Table 3). Results showed that compounds **1**, **5**, **7** and **13** weakly inhibited the IN/LEDGF/p75 binding (IC_{50} values around 110 uM), to support the fact that by binding to the catalytic site probably disturbing the correct binding between IN and LEDGF/p75 cofactor. All the other derivatives were not able to inhibit the LEDGF/p75-IN binding. Otherwise from LEDGIN-6, coumarin derivatives can allosterically modulate the dynamic interplay between the IN subunits without stabilize the IN multimeric form, suggesting that these compounds do not interact with IN at the LEDGF binding site.

Unfortunately, when tested in cell-based assays these coumarin derivatives were not active (data not shown).

CONCLUSION

In the optimization of the coumarin scaffold to achieve compounds able to inhibit multiple HIV-1 enzymatic functions, we performed docking analysis of coumarin derivatives on HIV-1 IN and RNase H active sites. Most of the synthesized coumarin derivatives were well accommodated into both the investigated active sites. Mode-of-action and docking studies revealed compounds **1**, **3**, **6** and **7** as promising HIV-1 IN and RT RNase H dual inhibitors. Overall, compound **7** resulted the most interesting derivative since it inhibits both HIV-1 IN and RNase H activities in the low micromolar range, thus paving the way for a future rational optimization process for dual HIV inhibitors.

EXPERIMENTAL SECTION

Chemistry

The synthesis of coumarin derivatives and their characterization are reported in Supplementary Material. All the melting points were determined in open glass capillaries in a liquid- paraffin-bath and are uncorrected. Purity of the compounds was checked by TLC using silica gel-G as adsorbent and visualization was accomplished by UV light or iodine. IR spectra were recorded on FT-IR spectrophotometer and PMR spectra in DMSO-d_6 on a BRUKER AC (300 MHz) FT NMR spectrometer using TMS as internal standard (chemical shifts in δ ppm). Elemental analysis was carried out in the Saurashtra University, Rajkot on Perkin Elmer Elemental Analyzer. The theoretical calculation of physicochemical properties of the coumarin derivatives are reported in Table S2 of the Supplementary Material.

Molecular modeling studies

The molecular modeling analysis was performed by means of Schrodinger package [54]. The ligands were prepared by means of LigPrep tools at pH 7.4 and after submitted to 10000 steps of energy minimization, using OPLS_2005 as force field [55] (software: MacroModel, Schrodinger, LLC, New York, NY, 2018). For the modeling studies on the RT RNase H active site, we used the crystallographic structure of an N-hydroxythienopyrimidine-2,4-dione RNase H active site inhibitor with multiple binding modes to HIV-1 RT retrieved from the Protein Data Bank (PDB) with the code 6AOC [48]. This model was chosen for its recently release (2017) and its better X-ray resolution (1.8 Å), with respect to the other RT PDB models. In order to evaluate the reliability of our docking calculations, we used the Standard Protocol (SP) Glide algorithm, that was able to reproduce the experimentally determined binding modes, since we obtained a Root Mean Square Deviation (RMSD) value between the best docking pose and the ligand co-crystallized into the RNase H active site equal to 0.992Å. The receptor was prepared by means of the Protein Preparation Wizard implemented in Maestro, using OLPS_2005 as force field [56]. Residual crystallographic buffer components were removed, missing side chains were built using the Prime module [57], hydrogen atoms were added, zero-order bonds to metals were created followed by the generation of metal binding states and side chains protonation states at pH 7.4 were assigned. The structure was submitted to 10000 minimization steps using OPLS_2005 as force field [55] (software: MacroModel, Schrodinger, LLC, New York, NY, 2018). For the grid generation, a box of 26 Å by 26 Å by 26 Å, centered on the active site Mg2_ ions, was created, the docking studies were carried out by means of Glide SP v. 6.7 [54] generating 100 poses for ligands.

For the IN studies, we generated structure theoretical model in order to elucidate the binding mode and the interactions between the compounds and the IN catalytic core domain in the presence of the LEDGF/p75 cofactor.

The Prototype Foamy Virus (PFV) IN-LEDGF complex was constructed by assembling two crystallographic structures, the crystal structure of the PFV intasome in complex with magnesium and Raltegravir at 2.65 Å resolution (PDB code 3OYA) [58], and the experimental structure of the LEDGF/p75 cofactor (PDB code 2B4J) [59]. We used the PFV IN model due to the high level of

conservation between retroviral INs, especially in their active site [49,60]. The obtained receptor model was submitted to 10000 steps of energy minimization, carried out using OLPS_2005 as force field, (software: MacroModel, Schrodinger, LLC, New York, NY, 2018) and further 100ns of Molecular Dynamics simulations (MDs) were carried out. MDs were run using Desmond package v. 3.8 at 300 K temperature and ensemble NPT class; the system was put in an orthorhombic box of TIP3P water molecules, extending at least 10 Å from the protein, and counter ions were added to neutralize the system charge [61]. The resulting trajectory was clustered with respect to the RMSD, calculated on all atoms of the enzyme, thus obtaining ten representative structures. In detail, for the further molecular recognition studies, we selected the most populated structure, according to the Boltzmann population value, which also corresponded to the Prime lowest-energy structure [57]. For the grid generation, a box of 40 Å by 40 Å by 40 Å, centered on the active site Mg^{2+} ions, was created. The docking studies were carried out by means of Glide software v. 6.7 [54] by using SP algorithm and generating 100 poses for ligands.

Expression and purification of recombinant HIV-1 RT, INs and LEDGFs

His-tagged p66/p51 HIV-1 RTs were expressed in *E. coli* strain M15 and purified as described. [21,62] Full-length IN and LEDGF proteins were expressed in *E. coli* BL21 (DE3) and purified as described [3,63,64].

HTRF LEDGF-dependent assay

The IN LEDGF/p75-dependent assay measure the inhibition of 3'-processing and strand-transfer IN reactions in the presence of recombinant LEDGF/p75 protein [30,65,66]. 50 nM IN was preincubated with increasing concentration of compounds for 1 hour at room temperature in reaction buffer containing 20 mM HEPES pH 7.5, 1 mM DTT, 1% Glycerol, 20 mM $MgCl_2$, 0.05% Brij-35 and 0.1 mg/ml BSA. To this mixture, 9 nM DNA donor substrate (5'-ACAGGCCTAGCACGCGTCG-Biotin-3' annealed with 5'-CGACGCGTGGTAGGCCTGT-Biotin3') and 50 nM DNA acceptor substrate (5'-Cy5-ATGTGGAAAATCTCTAGCAGT-3' annealed with 5'-Cy5- TGAGCTCGAGATTTTCCACAT-3') and 50 nM LEDGF/p75 protein (or without LEDGF/p75 protein) were added and incubated at 37 °C for 90 minutes. After the

incubation, 4 nM of Europium-Streptavidine were added at the reaction mixture and the HTRF signal was recorded using a Perkin Elmer Victor 3 plate reader using a 314 nm for excitation wavelength and 668 and 620 nm for the wavelength of the acceptor and the donor substrates emission, respectively.

HTRF-based Integrase-LEDGF interaction assay

His-IN was pre-incubated with different concentrations of compound in a buffer containing 150 mM NaCl, 2 mM $MgCl_2$, 0.1% Nonidet P-40, 1 mg/ml BSA, 25 mM Tris (pH 7.4) for 30 minutes at room temperature.[3] Then, FLAG-LEDGF was added to the reaction and a mixture of anti-His6-XL665 and anti-FLAG-EuCryptate antibodies were then added to the reaction. After 4 hours at 4 °C, the HTRF signal was recorded using a Perkin Elmer Victor 3 plate reader using 314 nm for excitation wavelength and 668 and 620 nm for the wavelength of the acceptor and donor emission, respectively. The HTRF signal is defined as the emission ratio 665 nm/620 nm multiplied by 10,000.

HTRF-based IN Subunit Exchange Assay

His and FLAG-tagged INs were mixed in 25 mM Tris (pH 7.4) buffer containing 150 mM NaCl, 2 mM $MgCl_2$, 0.1% Nonidet P-40, 1 mg/ml BSA [67]. Test compounds were then added to the mixture and incubated for 2.5 hours at room temperature. A mixture of anti-His6-XL665 and anti-FLAG-EuCryptate antibodies were then added to the reaction and incubated at room temperature for 3 hours. The HTRF signal was recorded as above.

RT-associated RNase H polymerase-independent cleavage assay

The HIV-1 RT-associated RNase H activity was measured as described [68-71] in 100 µL reaction volume containing 50 mM Tris HCl pH 7.8, 6 mM $MgCl_2$, 1 mM DTT, 80 mM KCl, hybrid RNA/DNA (5'-GTTTTCTTTTCCCCCCTGAC-3'-Fluorescein, 5'-CAAAAGAAAAGGGGGGACUG-3'-Dabcyl) and 3.8 nM RT. The reaction mixture was incubated for 1 hr at 37 °C, the reaction was stopped by addition of EDTA and products were measured with a Victor 3 (Perkin) equipped with excitation/emission filters of 490/528 nm.

ASSOCIATED CONTENT

Supplementary Material

Supplementary Material related to this article can be found at…

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. Francesca Esposito and Francesca Alessandra Ambrosio contributed equally to this work.

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Declaration of Interest statement

The authors report no conflict of interest

ABBREVIATIONS

Human Immunodeficiency Virus type 1 (HIV-1), Protease (PR), Integrase (IN), Reverse Transcriptase (RT), Ribonuclease H (RNase H), RNA-dependent DNA-polymerase (RDDP), DNA-dependent DNA-polymerase (DDDP), Preintegration complex (PIC), Lens-Epitelium-derived (LEDGF/p75), IN binding domain (IBD), Highly active antiretroviral therapy (HAART), IN inhibitors (INIs), nucleoside/nucleotide RT inhibitors (NRTIs /NtRTIs), non-nucleoside RT inhibitors (NNRTIs), coumarin (2H-chromen-2-one; 1-benzopyran-2-one), coumarin (4-hydroxypyranobenzopyran), Molecular Dynamics simulations (MDs), Protein Data Bank (PDB), Root Mean Square Deviation (RMSD), Prototype Foamy Virus (PFV).

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