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Original article

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ABSTRACT

Data regarding the activity of metal complexes against HIV virus in cell are surprisingly scarce. In this study, we present the antiviral activity against HIV-infected cells of different types of chelating ligands and of their metal complexes. In particular, the carboxamide chelating scaffold and the corresponding coordination compounds demonstrated an interesting antiviral profile in the nanomolar range. These molecules inhibit not only HIV integrase catalytic activity, but they also interfere with the function of the RNase H component of the HIV reverse transcriptase. Here we also discuss the thermodynamic characterization in solution of the metal complexes of the most active ligands, affording to the best of our knowledge for the first time this type of data for complexes with anti-HIV activity.

1. Introduction

Metal chelating agents represent an important class of enzyme-inhibitors [1–4] and the metal binding strategy is of particular interest for the design of effective antivirals [2,5]. In the last decade, HIV-1 integrase (IN) has been validated as an important pharmacological target for the development of new drugs to be inserted in the Highly Active Antiretroviral Therapy (HAART). IN has a crucial role in the viral life-cycle, since it catalyzes the integration of proviral cDNA into the host cell genome through two different steps, 3′-processing (3-P) and strand-transfer (ST) [6–8]. This enzyme contains a catalytic core domain with an amino acidic triad (the so-called "D,D(35)E" motif), which is involved in the coordination of two divalent Mg^{2+} cofactors [6–8] that are essential to the catalytic process, according to the 'two-metal-ion' mechanism [9]. Chelation of the magnesium cofactors of IN has proven to be a successful strategy in the design of IN inhibitors, and it resulted in the approval, in late 2007, of the chelating inhibitor raltegravir (Isentress®, Fig. 1) as the first drug targeting IN [10,11]. In 2012, elvitegravir [12] (Fig. 1) and in August 2013 dolutegravir [13,14] jointed the therapeutic pool and other chelating IN inhibitors entered in clinical trials [15].

A great number of compounds have been studied as IN inhibitors [16–19]; an important class is represented in particular by the diketoacids (DKAs, Fig. 1). All these compounds respond to the necessity to achieve effective chelation of the metal cofactors and in fact they comprise a chelating region with a two-metal binding pharmacophore [2,5,20,21].

In previous studies, we demonstrated that the DKAs and some ligands synthesized as model of well-known potent IN inhibitors, such as raltegravir, elvitegravir and 5-CITEP (1-(5-chloroindol-3-yl)-3-hydroxy-3-(2H-tetrazol-5-yl)-propenone), effectively chelate divalent metal ions in solution, forming metal complexes with different stoichiometric ratios [22–26]. We isolated some metal complexes with these ligands and different metals. We chose the metal ions used in the enzymatic assays (Mg^{2+}, Mn^{2+}), but also other biologically essential (Cu^{2+}, Zn^{2+}) or less common (Ni^{2+}, Co^{2+}) transition metals, and tested them for their ability to inhibit

Abbreviations: HIV, human immunodeficiency virus; IN, integrase; HAART, highly active antiretroviral therapy; 3-P, 3′-processing; ST, strand-transfer; DKA, diketoacid; RT, reverse transcriptase; RNase, ribonuclease; ITC, isothermal titration calorimetry; ATR, attenuated total reflectance; $CCID_{50}$, 50% cell culture infectious dose; CC_{50}, 50% cytotoxic concentration; EC_{50}, 50% effective concentration.

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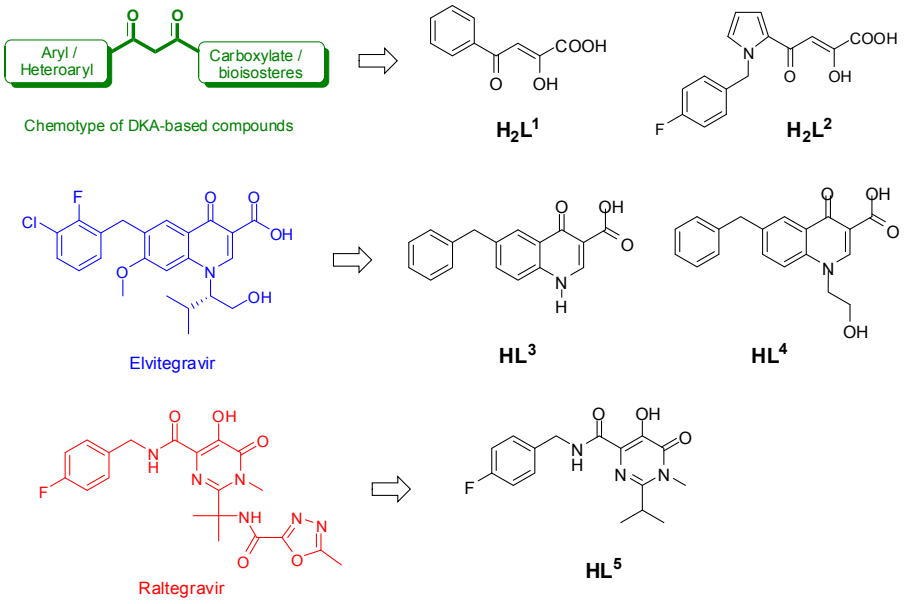


Fig. 1. Chemical structures of the model ligands H_2L^1, H_2L^2, HL^3–HL^5, and of their parent compounds.

IN in enzymatic assays, in order to verify if a metal-dependence can be highlighted. We found that some preformed complexes are active at a high nanomolar to low micromolar range [22–27].

Since data regarding the biological activity of metal complexes in HIV-infected cells are surprisingly scarce [27–31], now we were interested in evaluating their antiviral properties in cell-based assays, in order to establish if the activity observed in enzymatic studies can be maintained.

Firstly, we present here the results of the anti-HIV activity and cytotoxicity of the ligands H_2L^1, H_2L^2, HL^3–HL^5 (Fig. 1) and of the corresponding metal complexes (Fig. 2) in HIV-infected MT4 cells. HIV-1 replicates through the process of reverse transcription, that is accomplished through the enzyme reverse transcriptase (RT). RT has a ribonuclease (RNase) H domain that shows structural homologies with IN [2,32]. It is also well known that some DKAs are able to inhibit both enzymes [33,34]. For these reasons, we evaluated the activity of HL^5 and its metal complexes, which showed the best antiviral profile as well as the highest potency toward HIV-1 IN, toward HIV-1 RNase H enzymatic activity. Finally, to corroborate our previous studies on the speciation model of these metal complexes [22–25], we also performed calorimetric experiments to define the thermodynamics for the formation in solution of the most active coordination compounds.

2. Results and discussion

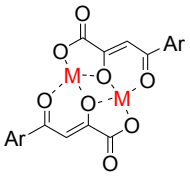
2.1. Chemistry

Our previous studies have highlighted the capability of the DKAs ligands H_2L^1 and H_2L^2 (Fig. 1) to give rise to dimeric species M_2L_2 (M = Mg, Mn, Ni, Co, Cu, Zn) both in solid state and in solution (complexes **1a**–**f**, **2a**–**f**, Fig. 2) [22,23]. These ligands can coordinate in the hydroxy-carboxylate form or in the acetyl-acetonate form; they can also use both the coordinating mode to give a dimer [22]. In all the cases the coordination sphere of the metals is completed by water molecules. The potentiometric, IR and mass measurements are in line with the hypothesis of a dimeric structure, where the acetyl, the hydroxyl, and the carboxylate groups coordinate to the metal [22].

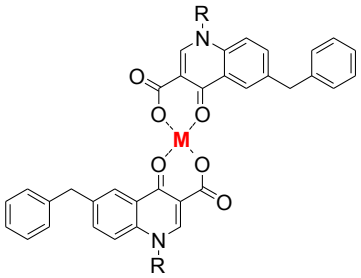
The quinolonic ligand HL^3 was introduced as a model of the selective ST inhibitor elvitegravir [24], while HL^4 was synthesized to increase the solubility and the lipophilicity of HL^3 (Fig. 1). We previously studied the coordinating behavior of the quinolonic scaffold towards magnesium and manganese divalent ions, highlighting the formation of 1:2 metal to ligand coordination compounds [24]. X-ray diffraction analysis on the manganese complex **3b** confirmed the proposed structure: the ligand coordinates to the metal through the pyridone and one of carboxylate oxygens and the coordination sphere is completed by two water molecules [24]. A similar coordinating behavior was found for HL^4, which was reacted with Mg(II), Mn(II), Zn(II) and Co(II) acetates in presence of a base, and the corresponding metal complexes **4a**–**d** were isolated and characterized (Scheme 1).

The use of a base is necessary to ensure deprotonation of the carboxylic moiety and subsequent coordination to the metal. In the IR spectra, the absorption of the ν(C=O) at 1610 cm^{-1} in the free quinolone was replaced in the complexes by two strong characteristic bands at about 1570 and 1490 cm^{-1}, attributable to the asymmetric and symmetric ν(O–C–O) vibrations, respectively. The $\Delta[\nu(CO_2)_{asym} - \nu(CO_2)_{sym}]$ is indicative of a monodentate coordination mode of the carboxylate [35]. The pyridone stretching vibration is shifted upon coordination from 1697 to about 1625 cm^{-1}. The changes in the IR spectra suggest that the ligand coordinates to the metal through the pyridone and one of carboxylate oxygens. The poor solubility of the magnesium and zinc complexes prevent the registration of NMR data, but elemental analysis confirmed the proposed ML^4_2 stoichiometry. Moreover, in order to verify the possible formation of complexes with a different stoichiometry, the conditions of the reactions were varied by changing pH, metal/ligand *ratio*, and anion. However, only the 1:2 metal to ligand species **4a**–**d** were isolated, as confirmed by elemental analysis.

HL^5 forms similar 1:2 metal to ligand species (**5a**–**d**) as well [24]. As we already reported [24], ^1H and ^{13}C NMR data indicate the involvement of the hydroxyl and of the secondary amide group in coordination, with the formation of bis-chelated coordination compounds (Fig. 2). The structure was confirmed both by potentiometric measurements and by X-ray diffraction analysis on **5a** and **5d**: the six-coordinated metal centers bind two bis-chelated



1a-f; 2a-f

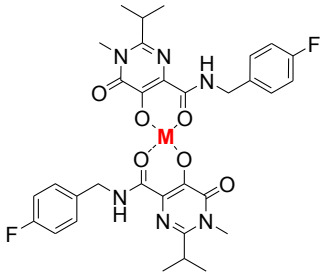


3a,3b; 4a-d

1 Ar = Phenyl, H_2L^1
2 Ar = (4-fluorobenzyl)-1H-pyrrol-2-yl, H_2L^2

3 R = H, HL^3
4 R = CH_2CH_2OH, HL^4

M = divalent metal: **a**= Mg(II), **b** = Mn(II), **c** = Zn(II), **d** = Co(II), **e** = Cu(II), **f** = Ni(II)



5a-d

Fig. 2. Chemical structures of the metal complexes.

deprotonated ligands in the equatorial plane and two water molecules at the apexes of a distorted octahedron [24].

Therefore, quinolonic and carboxamidic pharmachophores have a coordinating behavior that is different from that of the DKAs: in the case of **HL^4** and **HL^5** the monometallic, bis-chelate coordination compounds ML_2 can be isolated in the solid state and, as it is clear from the potentiometric measurements, they are also the predominant species at physiological pH in solution [24]. The DKAs **H_2L^1** and **H_2L^2**, on the other hand, give rise to dimeric M_2L_2 complexes [22,23].

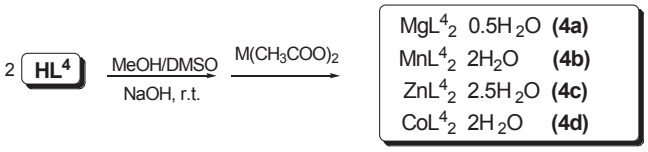
2.2. Isothermal titration calorimetry

HL^5, the most active ligand for HIV-1 IN inhibition in this study, was chosen to complete the thermodynamic characterization of the chelating behavior. We have reported and discussed elsewhere [24] the speciation in solution of methanol/water = 9/1 of **HL^5** with Mg(II), Mn(II), Co(II) and Zn(II) and the values of the formation constants (methanol/water = 9/1; ionic strength 0.1 M KCl). The species found in solution were $[ML^5]^+$ and ML^5_2, with formation constants that increased in the order $Zn^{2+} < Mg^{2+} < Mn^{2+} < Co^{2+}$ for $[ML^5]^+$ and in the order $Mg^{2+} \sim Zn^{2+} < Mn^{2+} < Co^{2+}$ for ML^5_2, according to the Irving–Williams sequence [24]. The stability constants allow to evaluate the affinity between the metal and the ligand in terms of ΔG°

$$\Delta G^\circ = -RT \ln \beta_q$$

where β_q is the formation constant of the complex ML_q, but, if measured at only one temperature, do not provide information about the entropy and enthalpy components ($\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$). To distinguish between enthalpic and entropic contributions, i.e. to find out the "thermodynamic signature", could provide valuable information for gaining a deeper insight in the mechanism of action of these compounds, or in decision making in lead discovery and optimization [36]. Isothermal titration calorimetry (ITC) is the method of choice for obtaining these parameters in a single experiment. Our ITC experiments were carried out in methanol/water = 9/1, the same mixture of solvents used in potentiometric measurements. In order to obtain reliable results, ITC measurements in this mixed solvent require a very accurate setting up [37]. In Table 1, the thermodynamic values derived from calorimetric measurements by using the HYPΔH software [38] are reported.

This program enables the calculation of either formation enthalpies, by using previously determined stability constants, or both formation enthalpies and stability constants, in systems where one or more complexes are in equilibrium in solution with a set of reagents. We have chosen to fix the speciation and the stability constants previously obtained by potentiometry [24] and to



[a] Reagents and conditions: NaOH, CH_3OH/DMSO, r.t. 4 h.

Scheme 1. Synthesis of the complexes **4a**–**4d**.

Table 1
Thermodynamic parameters for the formation of the complexes $M[L^5]_q$ with Mg(II), Mn(II), Co(II) and Zn(II) in methanol/water = 9/1 solution at 25 °C and I = 0.1 M KCl.

q	Mg(II) $-\Delta G°$ kJ/mol $\Delta H°$ kJ/mol $\Delta S°$ J mol^{-1} K^{-1}	Mn(II) $-\Delta G°$ kJ/mol $\Delta H°$ kJ/mol $\Delta S°$ J mol^{-1} K^{-1}	Co(II) $-\Delta G°$ kJ/mol $\Delta H°$ kJ/mol $\Delta S°$ J mol^{-1} K^{-1}	Zn(II) $-\Delta G°$ kJ/mol $\Delta H°$ kJ/mol $\Delta S°$ J mol^{-1} K^{-1}
1	29.12 (0.23) 5.9 (0.7) 118 (5)	35.79 (0.07) −2.0 (0.6) 107 (3)	39.38 (0.03) −1.4 (0.5) 128 (2)	27.75 (0.20) −17.04 (0.08) 37 (4)
2	51.74 (0.22) 1.7 (0.8) 180 (5)	58.01 (0.07) −6.2 (0.8) 175 (3)	66.50 (0.06) −5.5 (0.7) 205 (3)	52.94 (0.11) −28.56 (0.04) 83 (3)

evaluate from the microcalorimetric titrations only the heats of formation of the complexes. Results are compared in a pictorial way in Fig. 3. The formation of the two types of complexes, ML^5 and ML^5_2, is essentially entropy driven, with the exception of the complexes of Zn(II); the most stable ones are the complexes with Co(II) ($\Delta G° = -39.38$ kJ/mol for CoL^5 and −66.50 kJ/mol for CoL^5_2). In particular, due to the role that the magnesium ions plays in the activity of IN and RT, it is interesting to note that MgL^5 and MgL^5_2 are the only species with a positive formation enthalpy (complexation is endothermic). The few available thermodynamic data for the formation of complexes active against HIV virus do not allow any plain correlation with their biological activity. Anyway, it seems that for a ligand it not necessary to have high selectivity for Mg(II) or Mn(II) to have antiviral activity.

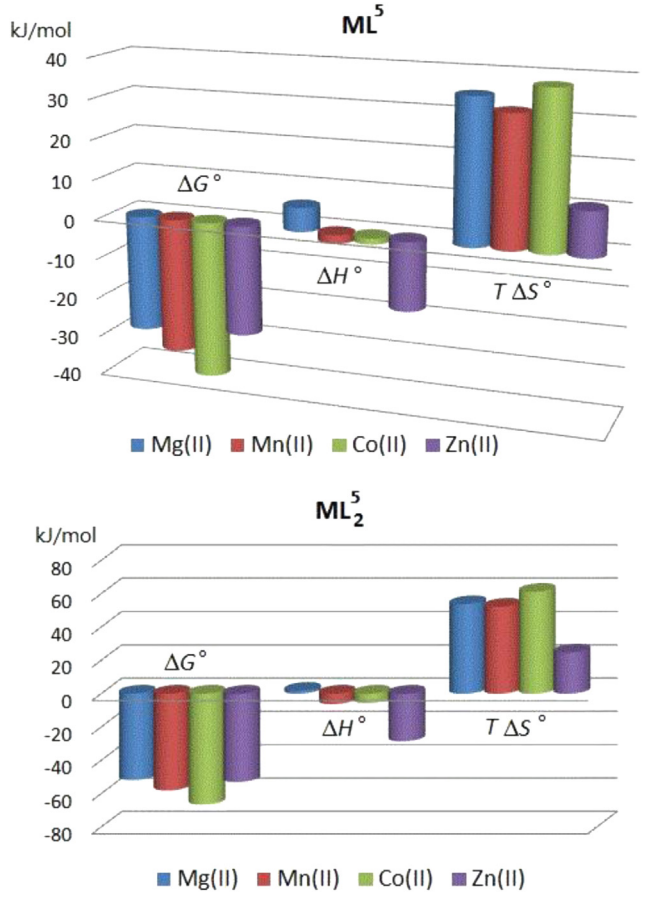


Fig. 3. Pictorial comparison between the thermodynamic parameters for the formation of the complexes ML^5 and ML^5_2 with Mg(II), Mn(II), Co(II) and Zn(II) in methanol/water = 9/1 at 25 °C and I = 0.1 M KCl.

2.3. Biological activity

The ligands $\mathbf{H_2L^1}$, $\mathbf{H_2L^2}$, $\mathbf{HL^3}$–$\mathbf{HL^5}$ were previously tested [22–26] in enzymatic assays with IN. In particular, $\mathbf{HL^5}$ showed a good inhibitory potency, with significant selectivity toward ST ($IC_{50} = 0.14 \pm 0.03$ μM), and some of its metal complexes similarly inhibit the IN enzymatic function (IC_{50}s from 0.09 ± 0.02 μM to 0.41 ± 0.2 μM, Table 3).

In order to verify the antiviral effect in cell-based assays, complexes **1b**–**f**, **2a**–**f**, **3a**, **3b**, **4a**–**d**, **5a**–**d,** and the corresponding free ligands were tested for their antiviral activity and cytotoxicity in MT4 cells (Table 2). Overall, with few exceptions, the metal complexes retained or improved the antiviral profile, with respect of that of the related free ligand.

As far as the DKA derivatives is concerned, the antiviral activity is greatly affected by the portion linked to the two-metals chelating region (Ar, Fig. 2), as already seen in the enzymatic assays [22,23]. In fact, $\mathbf{H_2L^2}$ (Ar = (4-fluorobenzyl)-1H-pyrrol-2-yl, Fig. 2) and its metal complexes **2a**–**f** share low micromolar activity (EC_{50}s from 2.4 to 9.4 μM, with the exception of **2d**) in cells infected with HIV-1 (strain IIIB) or HIV-2 (strain ROD), perhaps with a slight sensitivity against the HIV-1 strain (Table 2). Conversely, the complexes **1b**–**f** (Ar = phenyl, Fig. 2) are ineffective in preventing the HIV cytopathic effect in infected cells (Table 2). These data could be related to the balance of two parameters: for example, the aromatic portion proved to have a crucial role in determining the interactions with the target enzyme, as evidenced by several studies [39–41], and on the other hand it determines variations in the pK_as of the ligands and on the speciation of the corresponding metal complexes as a

Table 2
Antiviral activity and cytotoxicity for tested ligands and complexes.

Compound	Antiviral EC_{50} (μM)[a]		Cytotoxicity CC_{50} (μM)[b]
	HIV-1 (strain IIIB)	HIV-2 (strain ROD)	
1b	>62	>62	62
1c	>55	>55	55
1d	>39	>39	39
1e	>27	>27	27
1f	>34	>34	34
$\mathbf{H_2L^2}$	5.8	9.4	≥59
2a	2.7	5.1	71
2b	4.8	9.2	62
2c	3.0	5.7	60
2d	7.2	≥23	37
2e	2.4	3.1	14
2f	3.2	8.9	43
$\mathbf{HL^3}$	>19	>19	19
3a	>17	>17	17
3b	>11	>11	11
$\mathbf{HL^4}$	3.1	4.0	49
4a	4.5	>36	≥36
4b	>28	>28	28
4c	≥3	≥4.09	26
4d	>6.1	>6.1	6.1
$\mathbf{HL^5}$	0.063	0.086	61
5a	0.025	0.026	41
5b	0.025	0.030	35
5c	0.028	0.039	53
5d	0.047	0.049	34
Nevirapine	0.18	>15	>15
Zidovudine	0.0056	0.0060	>94
Zalcitabine	1.4	1.4	>95
Didanosine	12	19	>212

[a] Effective concentration required to reduce HIV-1-induced cytopathic effect by 50% in MT-4 cells.
[b] Cytotoxic concentration to reduce MT-4 cell viability by 50%.

Table 3
Inhibition of HIV-1 IN and RT-associated RNase H catalytic activities for **HL^5** and its metal complexes **5a**, **5b** and **5d**.

Compound	HIV-1 IN enzymatic activity, IC_{50} (μM)[a]		HIV-1 RNase H enzymatic activity, IC_{50} (μM)[b]
	3′-P	ST	
HL^5	10 ± 8	0.14 ± 0.03	11.9 ± 2.3
5a	8 ± 5	0.41 ± 0.2	9.77 ± 0.00
5b	7 ± 4	0.18 ± 0.1	17.45 ± 1.47
5d	1.9 ± 1.1	0.09 ± 0.02	14.16 ± 0.36

[a] Data from Ref. [24]. Compound concentration required to inhibit HIV-1 IN for 3′-P and ST catalytic activities by 50%.
[b] Compound concentration required to inhibit HIV-1 RT-associated RNase H activity by 50%.

function of pH [22]. These issues are also strictly related to the lipophilicity, that can directly affect the optimal physicochemical properties needed for an effective cell uptake.

Among the compounds belonging to the quinolonic series, **HL^4** and its magnesium complex **4a** gave better results as far as the antiviral profile (Table 2). The substituent at the pyridone nitrogen in **HL^4** could be involved in additional interactions with protein side chains *via* hydrogen bonding, as well as modulate the lipophilicity of the system. Such consideration could explain the antiviral activity found for **HL^4**-based compounds with respect to **HL^3** series.

HL^5, which bears a carboxamidic chelating scaffold, and the corresponding metal complexes **5a**–**d** proved to be the most active compounds, with an antiviral activity in the nanomolar range (EC_{50}s from 0.025 to 0.086 μM) and a very good cytoprotective effect (selectivity indexes are in general >500), with similar activity toward HIV-1 and HIV-2 strains; the metal complexes **5a**–**d** showed a lightly better antiviral profile *vs* the free ligand (Table 2). Note that **HL^5** and **5a**–**d** have activities that are interesting also in comparison with those of the compounds used as references (the commercial RT inhibitors nevirapine, zidovudine, zalcitabine and didanosine, Table 2). Therefore, influence of the metal on activity can be observed, as already found in the enzymatic assays [22–25]. The fact that metal complexes are active not only in the enzymatic assays, but also in the infected cells (with an antiviral profile better than the free ligand in the **HL^5** series) enforces our previous observation that they are quite stable in solution (as stated by potentiometric measurements [22–24]) and that the complexes, not necessarily only the free ligand, might be involved in the inhibition mechanism. The concentration of Mg^{2+} *in vitro* and *in vivo* makes in fact plausible the hypothesis that the free ligands could also act as complexes in their active form, as they could coordinate ions in solution before interaction at their putative binding site. When the metal complex is dissolved, equilibria between the different metal species in solution arise, according to the different formation constants and concentrations, thus resulting in slightly different antiviral activities.

Since, as anticipated in the introduction, DKAs were developed as IN inhibitors, but they were also able to inhibit the HIV-1 RNase H activity of RT, **HL^5** and its metal complexes have been tested to evaluate their inhibitory efficacy also toward such target. The effect of **HL^5** and of the corresponding metal complexes on the RT-associated RNase H function is reported in Table 3: both the free ligand and the metal complexes are active in the low micromolar range (IC_{50}s from 9.77 ± 0.00 to 17.45 ± 1.47 μM).

These data could suggest a multivalent mode of action: these chelating agents can exert their inhibition not only against IN, but also toward other metal-dependent enzymes of HIV, like the RNase H component of the RT, which shares with IN an active site with two magnesium ions and a two-metals catalysis [2,32]. Although the selectivity index (the ratio of the IC_{50}s RNase H/IC_{50}s IN) ranged from ~26 to ~150, thus highlighting a prevalent inhibition of IN, the interesting inhibition ability demonstrated for the RT-associated RNase H function should be kept into account in developing more potent dual and/or RNase H specific inhibitors.

Therefore, the good antiviral activity of the metal-chelating inhibitors could probably be ascribed to their capability to target different metal-dependent enzymes in the viral lifecycle, thus enforcing the idea of metal chelation as a successful strategy in drug design.

3. Conclusions

Some preformed metal-complexes have shown the capability to inhibit IN in *in vitro* enzymatic assays [22–26]. In line with these studies, here we presented the thermodynamic characterization and the antiretroviral activity against HIV-infected cells of some chelating ligands and of their metal complexes, highlighting that the activity of the complexes is retained or it is even higher in cellular assays. In a scenario of few available data on the activity of isolated metal complexes in HIV infection [27–31], we found that the ligand **HL^5** with the carboxamidic scaffold and some of its metal complexes have antiviral activity in the nanomolar range. These compounds inhibit IN and, even if with minor efficacy, also interfere with the function of the RNase H component of the RT. Therefore, chelating ligands could probably exert a multivalent mode of action during the virus lifecycle, having as a target different metal-dependent viral enzymes with analogous metal-based catalysis. This enforces the concept of metal chelation as a suitable strategy for the development of novel and effective antivirals.

4. Experimental section

4.1. Materials and methods

4.1.1. Chemistry

All reagents of commercial quality were used without further purification. The ATR-IR spectra were recorded by means of a Nicolet-Nexus (Thermo Fisher) spectrophotometer by using a diamond crystal plate in the range 4000–400 cm^{-1}. Purity of compounds was determined by elemental analysis; elemental analyses were performed by using a FlashEA 1112 series CHNS/O analyzer (Thermo Fisher) with gas-chromatographic separation.

The synthesis and characterization of **H_2L^1**, **H_2L^2**, **HL^3**–**HL^5** and of the metal complexes **1a**–**f**, **2a**–**f**, **3a**, **3b**, **5a**–**d** have been published elsewhere [22–24,26]. Complexes **4a**–**d** (Fig. 2) have been synthesized by using a previously established procedure [24]. **HL^4** was dissolved in methanol with few milliliters of dimethyl sulfoxide, and then NaOH was added. The solution was stirred at room temperature for 30 min, and then an aqueous solution of the metal salt was added: immediately, a precipitate was formed. The solution was stirred for additional 4 h, then it was concentrated on vacuum and the solid filtered off and washed with water.

HL^4. White powder. IR (cm^{-1}): ν_{OH} = 3312; $\nu_{C=O}$ = 1697, 1610. ^{1}H NMR (DMSO): δ 15.32 (s, br, 1H, OH); 8.86 (s, 1H, =CH); 8.23 (s, 1H, ArH); 8.02 (d, 1H, ArH); 7.83 (d, 1H, ArH); 7.30–7.21 (m, 5H, ArH); 5.02 (t, br, 1H, OH); 4.60 (t, 2H, CH_2); 4.17 (s, 2H, CH_2); 3.74 (*q*, 2H, CH_2). ^{13}C NMR (DMSO-d6): δ 178.2, 166.6, 144.8, 140.6, 139.9, 138.1, 135.1, 128.9, 128.7, 126.3, 124.5, 124.1, 120.0, 107.5, 40.6. Anal. Calcd. For $C_{19}H_{17}NO_4$: C 70.58, H 5.30, N 4.33. Found: C 70.39, H 5.27, N 4.20. ESI/MS (+, *m*/*z*): 324.3 [MH^+].

MgL^4_2 0.5H_2O (**4a**). White powder. Yield: 77%. IR (cm^{-1}): ν_{OH} = 3475, $\nu_{C=O}$ = 1627, 1585, 1492. Anal. Calcd for

$C_{38}H_{32}MgN_2O_8 \cdot 0.5H_2O$: C 67.26, H 4.86, N 4.13. Found: C 67.19, H 4.72, N 4.00.

MnL^4_2 2H_2O (**4b**). Yellow powder. Yield: 83%. IR (cm^{-1}): ν_{OH} = 3495, $\nu_{C=O}$ = 1625–1613, 1570, 1490. Anal. Calcd for $C_{38}H_{32}MnN_2O_8 \cdot 2H_2O$: C 61.99, H 4.89, N 3.81. Found: C 62.27, H 4.82, N 3.54.

ZnL^4_2 2.5H_2O (**4c**). White powder. Yield: 63%. IR (cm^{-1}): ν_{OH} = 3480, $\nu_{C=O}$ = 1625–1614, 1570, 1491. Anal. Calcd for $C_{38}H_{32}N_2O_8Zn \cdot 2.5H_2O$: C 60.39, H 4.90, N 3.71. Found: C 60.32, H 4.51, N 3.40.

CoL^4_2 2H_2O (**4d**). Pink powder. Yield: 68%. IR (cm^{-1}): ν_{OH} = 3473, $\nu_{C=O}$ = 1625–1614, 1564, 1489. Anal. Calcd for $C_{38}H_{32}CoN_2O_8 \cdot 2H_2O$: C 61.65, H 4.86, N 3.78. Found: C 61.43, H 4.73, N 3.41.

4.1.2. Isothermal titration calorimetry (ITC)

ITC measurements were carried out on a CSC model 5300 N-ITC III42 isothermal titration calorimeter (Calorimetry Sciences Corporations, USA) at 25 °C. Fifty portions of 5 μL of KOH solution in methanol/water = 9/1 (0.065–0.145 M) were stepwise injected into a 960 μL reaction cell filled up with a methanol/water = 9/1 solution containing a metal/ligand ratio equal to ½ or ¼ at 0.1 M KCl ionic strength, with an interval of 300 s between two successive injections. Blank experiments were also done, in which the KOH solution in methanol/water = 9/1was injected into the same solvent at 0.1 M KCl ionic strength. A continuous stirring at 150 rpm was maintained throughout the experiments. Binding isotherms were obtained by the integration of each injection peak followed by subtraction with blank experiment, using the BindWorks 3.1 software provided by CSC. The software HYPΔH [38] was used to evaluate the protonation and complexation enthalpies from the binding isotherms.

4.1.3. Cell-based assays

The detailed procedures to determine the anti-HIV activity and cytotoxicity of the compounds in human lymphocyte MT-4 cells can be found elsewhere [42]. Briefly, serial dilutions of the compounds were added to 96-well plates containing the MT-4 cells. To the virus-infected wells, 100–300 $CCID_{50}$ (50% cell culture infectious dose) of HIV-1 (strain IIIB) or HIV-2 (strain ROD) was added. The mock-infected wells received the compounds without virus. After five days incubation, the spectrophotometric MTT assay was performed to assess the effect of the compounds on the viability of the mock- and HIV-infected cells. The 50% cytotoxic concentration (CC_{50}) was defined as the compound concentration that reduced the viability of the mock-infected MT-4 cells by 50%. The concentration achieving 50% protection from the cytopathic effect of the virus in infected cells was defined as the 50% effective concentration (EC_{50}).

4.1.4. HIV-1 RT-associated RNase H assays

HIV-1 heterodimeric RT was expressed and purified as previously described [43]. The HIV-1 RT-associated RNase H activity was measured as described [44]. Briefly, in 100 μL reaction volume containing 50 mM Tris HCl (pH 8.1), 6 mM $MgCl_2$, 1 mM ditiotreitol (DTT), 80 mM KCl, hybrid RNA/DNA (5′-GTTTTCTTTTCCCCCTGAC-3′-Fluorescein, 5′-CAAAAGAAAAGGGGGACUG-3′-Dabcyl) and 2 nM RT. The reaction mixture was incubated for 1 h at 37 °C, then stopped by addition of EDTA and products were measured with a Victor 3 (Perkin) at 490/528 nm.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.06.055.

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