Novel tris-hydroxypyridinone complexes with gallium and lanthanides for potential medicinal applications. Biological evaluation of hydroxypyridinone derivatives.

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Abstract

A considerable research effort has been made in the discovery and development of new chelating agents for medical applications, including chelation therapy and diagnostic imaging techniques. Herein, the chelating capacity of a new compound, $NTP/PrHP)_3$, resulting from the attachment of three 3-hydroxy-4-pyridinone units in a nitrilopropionic acid skeleton, was evaluated. Complexation studies in the presence of metal ions, namely gallium and lanthanides, were performed by potentiometric and spectrophotometric techniques and the stability constants and coordination modes of the complexes determined. $NTP(PrHP)_3$ shows high affinity for the metals studied, forming 1:1 complexes (metal:ligand) and also 1:2 complexes for the lanthanide ions La, Pr and Gd. The coordination to the metal ion is done through the hydroxypyridinone units and does not involve the nitrogen atom of the skeleton.

The *in vivo* studies of the radiocomplex $^{67}Ga/NTP(PrHP)_3$ showed a good excretion of the compound after 24h of injection, with almost all the radioactivity being cleared off from tissues and organs. The studies performed on the gallium and lanthanide complexes of $NTP(PrHP)_3$ point towards their potential use as contrast agents in diagnostic techniques (PET, CG and MRI).

A series of N-functionalized bidentate 3-hydroxy-4-pyrydinone chelators were also biologically evaluated in terms of toxicity, DNA interaction and capacity of altering cells morphology or membrane fluidity. It was found that none of the compounds studied was toxic, and by the contrary, some of them increased the cell viability, depending on the chemical and lipophilic properties associated to the side chain.

Keywords: 3-hydroxy-4-pyrydinones, chelating agents, diagnostic agents, gallium, lanthanides, biological studies.

Introduction

Inorganic chemistry is giving a great contribution to medicine, namely associated to the research and development of new drugs based in organic compounds that bind metals inside the organism or that are already bounded to them before entering the body. In the first case, the organic compound can be used as a chelating agent in the treatment of serious pathological disorders associated with metal overload in the body, like iron overload in β Thalassemia; in the second case, the metal ion is the form of a metallic complex that can be used as a diagnostic imaging agent.

The first chelating agent used for the treatment of iron and aluminium overload was desferrioxamine[1], DFO, a natural siderophore produced by bacteria. Although, due to its oral inactivity and toxic side effects[2], efforts were made in the research of new and efficient chelating agents[3]. Both Fe and Al are considered hard metals and therefore they have affinity for hard atoms such as oxygen. Among the O,O donor ligands, the hydroxypyridinones, especially the 3-hydroxy-4-pyridinones (3,4-HP), have demonstrated high affinity for hard metal ions in physiologic conditions, because of their hydroxyl group in *ortho* position related to the cetone group, forming a bidentade ligand and a 5-membered coordinating ring around the metal ion. The importance of this class of compounds emerged after the use of 1,2-dimethyl-3-hydroxy-4-pyridinone (DFP) as a therapeutic alternative to DFO[4]. In fact, 3,4-HP are quite stable under biological conditions and resistant to oxidation or hydrolysis. Moreover, hydroxypyridinones can be N-functionalized in order to adjust some important physico-chemical properties, improving their biological activity and promoting a lower toxicity of the compound.

Hydroxypyridinone complexes as diagnostic agents

The use of metal complexes in diagnostic imaging techniques, including gamma scintigraphy (GC), Positron Emission Tomography[5] (PET) and Magnetic Resonance Imaging[6] (MRI) is quite usual.

In particular, gallium radioisotopes are commonly used in GC and PET techniques, due to their emitting radiation (gamma or positron emission) and appropriate half-lives. In fact, this Group III metal has two radioisotopes: ^{67}Ga (γ emission, $t_{1/2}$=78h) and ^{68}Ga (β^+ emission, $t_{1/2}$=68m). Since Ga presents similarities with Fe and Al, and is also considered a hard metal, it can be coordinated by hydroxypyridinones[7,8,9] forming stable hexacoordinated complexes in physiologic conditions[10]. In the case of MRI technique, lanthanide ions are frequently used because they induce the relaxation time decreasing of the water protons around them[6], including both inner and outer sphere molecules[11]. Among the lanthanide ions, Gd is the most considered one. In fact, there are Gd nine-coordinated complexes clinically approved as contrast agent[12]. In this study, the chelating capacity of a tri-functionalized structure (Fig. 1) will be evaluated. This compound was obtained from the coupling of three aminopropil-HP units to a nitrilopropionic acid (NTP) skeleton, forming a strong hexadentate ligand

(NTP(PrHP)$_3$) that functions like an anchor for the metal ion[13,14,15].

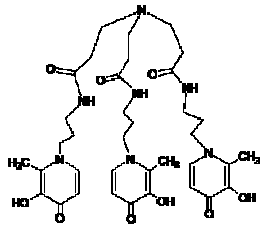

Figure 1: Molecular structure of NTP(PrHP)$_3$.

Potentiometric and spectrophotometric (UV) studies of NTP(PrHP)$_3$ in the presence of Ga(III) or a series of lanthanide ions (La, Pr, Gd, Er and Lu) were performed to evaluate the physico-chemical properties of the complexes. The lanthanide "contraction effect" was investigated in order to correlate the electronic density of the ion with the stability of the complexes formed *In vivo* assays were also made in order to study the behaviour of the radiocomplex ^{67}Ga-NTP(PrHP)$_3$, aimed for potential application in GC.

Hydroxypyridinones as anti-degenerative agents

All organisms and cells require essential elements, such as transition metal ions (Fe, Zn and Cu) for their normal growth and development. Iron is one of the most important elements, participating in quite a variety of cellular events. Although important, it's toxic when present in excessive concentration. In fact, the accumulation of iron in cells and several organs, like liver, pancreas and heart, results in dysfunction and death. Associated to the "free" iron in blood and tissues is the formation of reactive oxygen species like hydroxyl radicals ($OH^{\bullet}$), through the *Fenton reaction*[16]. Once formed, these radicals can initiate a metal catalysed protein and lipid oxidation and also both sugar and DNA interaction, resulting into tissue damage. On the other hand, Fenton reaction will promote Fe^{3+} formation, which will reduce back to Fe^{2+} through reductive agents inside the cell, leaving an excess of oxidative species that will promote oxidative damage and cellular death.

The diseases associated to iron overload are mostly genetic (β-thalassemia or hemochromatosis), but there are also other diseases related to the accumulation of iron in specific cells like brain cells, leading to neurodegenerative disorders such as Parkinson and Alzheimer diseases[17]. Since human body doesn't have any protective mechanism to overcome iron overload, chelation therapy can give a determinant contribution to the prevention of the free radical damage, responsible for several pathological disorders. While searching for new chelating drugs, several 3,4-HP derivates were developed[13], in which the ring nitrogen atoms were modified to improve the biological response and diminish the toxicity of the compound. Herein, a series of HP derivates (Fig. 2) were biologically evaluated in order to analyse their toxicity in human neuroblastoma cells, their ability for changing the morphology of the cell or the membrane fluidity, as well as their capability to interact with DNA.

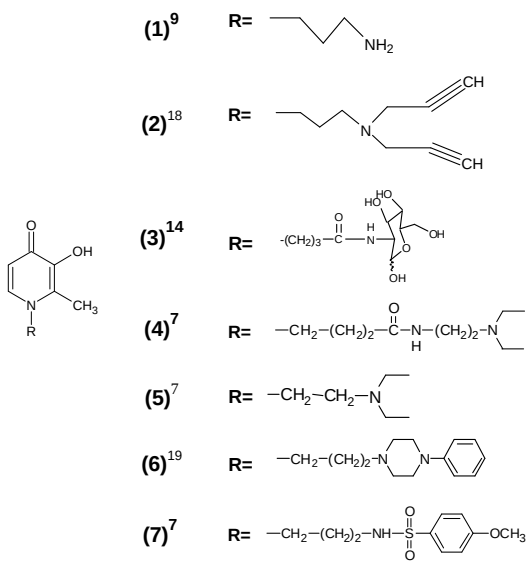

Figure 2: Structures of the 3,4-HP derivatives, biologically evaluated in this study.

Results and Discussion

Acid Base Properties of the ligand

The log Ki values determined potentiometrically for NTP(PrHP)$_3$ are summarized in table 1 and seem to be according to the expected. In fact, the protonation constants for the pyridyl nitrogen atoms (log K_i, i=5-7) are very similar to those found in the literature. For example, the commercial drug DFP and the compound **(1)** represented in figure 2 have corresponding protonation constants of 3.7[20] and 3.2[9], respectively, very close to the herein obtained values. The high acidity of the pyridinium proton is due to the delocalization by aromatic resonance of the nitrogen pair of electrons. In the case of the values obtained for the hydroxyl protonation constants (log K_i, i=1-3), they are also similar to those to DFP and **(1)**, since their protonation constant values are 10.07 and 9.86, respectively. This behaviour is common for all the HP derivatives, once their protonation constants are independent from the side chain groups[3]. Finally, the log K_4 value corresponds to the protonation constant of the apical nitrogen of the NTP skeleton. Since this protonation constant is 9.30[21] for NTP, probably there is the formation of hydrogen bonds of CONH---N type, between this tertiary nitrogen and the amide groups, resulting in a higher acidity of the NTP(PrHP)$_3$ protonation constant.

Table 1: Protonation constants (log K_i) of NTP(PrHP)$_3$, global stability constants of the correspondent gallium complexes (log β_i) and pGa values.

H_iL	log K_i	$M_mH_hL_l$	log β_i	pGa*
HL	9.946(9)			
H_2L	9.84(1)	1,5,1	46.68(1)	
H_3L	9.091(8)	1,3,1	42.98(3)	27.2
H_4L	6.77(1)	1,1,1	37.88(2)	
H_5L	3.81(1)	1,0,1	33.00(2)	
H_6L	3.14(1)			
H_7L	2.76(2)			

* pGa=-log[Ga^{3+}] (pH=7.4, C_L= 10^{-6}M, C_L/C_M=10)

Analysis of the distribution species diagram (Fig. 3) of the ligand as a function of pH shows that, for the range of pH

between 4 and 6, the most abundant specie is H_4L (90% formation) and for pH between 7.5 and 8.5 is H_3L. H_4L is the monoprotonated ligand and corresponds to all three hydroxyl groups protonated and also the apical nitrogen. In the H_3L specie occurs the deprotonation of the nitrogen. At physiological conditions, the solution is a mixture of 50% H_4L and 50% H_3L. The molar absorptivity values specified in figure 3 and determined by spectrophotometric titration of the ligand, indicate that at 280 nm, both H_4L and H_3L species absorb and at 310 nm, the absorbent species corresponds to the deprotonated ligand (L).

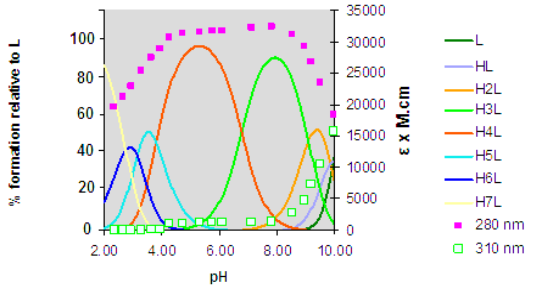

Figure 3: Distribution species diagram of $NTP(PrHP)_3$, together with the molar absortividades at 280 and 310 nm (C_L=5.35 ×10^{-5} M).

Gallium complexation

The global stability constants of the Ga*(*III*)* complexes of $NTP(PrHP)_3$ were determined from spectrophotometric titration of the 1:1 Ga(III)/L system, for pH≤2 and pH>2. The analysis of the speciation diagram of the gallium complexes as a function of pH *(*Fig. 4*)* shows that the complex formation starts at pH<2 thus suggesting that these ligand has strong affinity for Ga*(*III*)*. This diagram also indicates that in the range of pH 2–3, the main species are GaHiL (i=5, 3) and that for pH between 3 and 4.5, GaHL specie presents 90% formation. For pH 5, both GaHL and GaL coexist in solution and between pH 5 to 6 the neutral GaL specie is predominant. After pH 6 there was a precipitate formation which probably corresponds to the neutral GaL species.

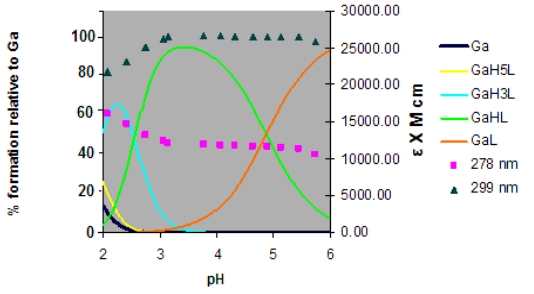

Figure 4: Distribution species diagram for the Ga^{3+}/$NTP(PrHP)_3$ system (C_L/C_M = 1, C_L = 5.3 ×10^{-5} M).

In relation to the absorptivity values at 278 and 299 nm also represented in figure 4, it can be seen that both hexacoordenated complexes (GaHL and GaL) absorb at 299 nm and for 278 nm, the most protonated specie (GaH_5L) is the predominant one.

It's important to refer the higher pGa value for $NTP(PrHP)_3$, when comparing with other commercially available compounds (table 2) that are used both in chelation therapy or diagnostic imaging techniques..

Table 2: pGa values for $NTP(PrHP)_3$ and other commercial compounds.

Ligand	pGa
$NTP(PrHP)_3$	27.2
EDTA[22]	20.2
DFO[22]	22.4
DFP[8]	17.8

Biological Studies with ^{67}Ga

Biodistribution and gamma imaging studies of the radiocomplex $^{67}Ga/NTP(PrHP)_3$ were performed in order to evaluate is activity and possible application in radiodiagnostic techniques PET or SPECT. These results were kindly provided by Prof. Carlos Geraldes of University of Coimbra (Fig. 5).

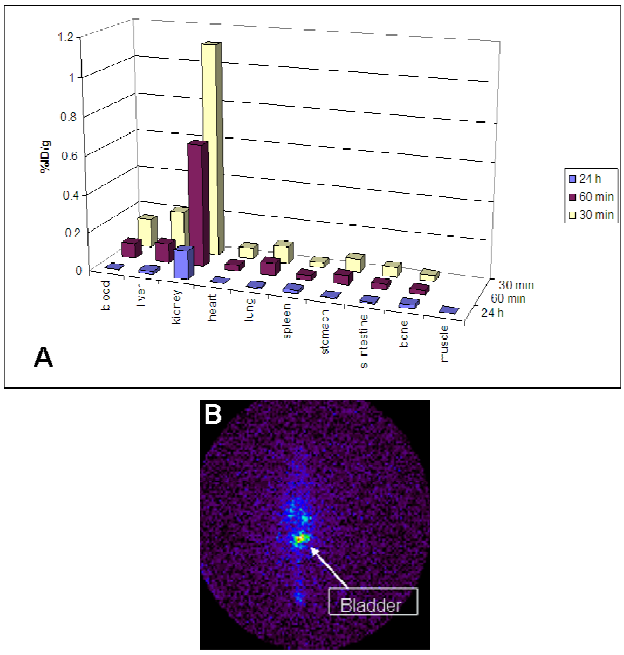

Figure 5: A – Biodistribution of **^{67}Ga-NTP(PrHP)$_3$,** 30, 60 min. and 24h after injection; **B** - Scintigraphic image obtained 24 h after injection of **[^{67}Ga]- NTP(PrHP)$_3$.**

Both biodistribution and gamma image studies made clear that the main activity is found in kidneys and bladder, which is the excretion pathway for this hydrophilic complex. The small fraction of activity located on the liver at 30 and 60 minutes is possibly related with some liver uptake together with the blood activity. In addition, almost all the radioactivity was cleared off from tissues and organs after 24 h of the injection and only very slight deposition (ca 0.02 %) in the bone and liver/spleen could be observed, which may be related to a very small degree of demetalation.

Lanthanide complexation

The overall stability constants for the lanthanide complexes with $NTP(PrHP)_3$ were determined by potentiometric titrations and are summarized in table 3. These results indicate that for all the lanthanides studied herein there was the 1:1 (metal/ligand) complex formation, resulting in the MH_iL (i=1-5) species. However, for La, Pr and Gd, there was also the

formation of 1:2 complexes, MH_3L_2 and MH_5L_2, but that didn't happen for the Er or Lu systems. This behaviour is probably related to the ionic radius of the metal ions Er and Lu (Er^{3+} - 103 pm; Lu^{3+} - 100 pm) which are smaller comparing to the other three, indicating that possibly these lanthanides don't coordinate with coordination number higher then 6.

Table 3: Global stability constants of the lanthanide complexes with $NTP(PrHP)_3$.

(m,h,l)	Log $\beta_{M_mH_hL_l}$ [1]				
	La^{3+}	Pr^{3+}	Gd^{3+}	Er^{3+}	Lu^{3+}
(1,5,1)	43.81(7)	44.1(1)	44.0(1)	43.1(1)	44.77(5)
(1,4,1)	40.27(5)	40.64(8)	40.50(7)	40.26(7)	41.28(3)
(1,3,1)	35.36(5)	36.09(9)	36.57(8)	36.3(1)	37.77(5)
(1,2,1)	29.0(3)	30.80(8)	31.91(8)	32.43(5)	33.68(4)
(1,1,1)	23.44(4)	25.31(7)	27.10(7)	27.42(5)	29.64(4)
(1,5,2)	63.60(7)	65.6(1)	66.7(1)		
(1,3,2)	50.0(1)	52.6(1)	53.5(1)		

[a] This values were determined in the inorganic chemistry laboratory of the inorganic chemistry, organometallic and analytical department of Padova University, under the supervision of Prof. P. Di Bernardo.

Figure 6 contains the potentiometric titration curves for each Ln(III)-$NTP(PrHP)_3$ system (1:1) as well as for the ligand alone. The lanthanide titration curves give the following informations: (i) the ligand has a significant affinity for the metal ions, due to the high change of the titration curves shape when comparing to the one without metal; (ii) the complex formation seems to starts at pH ~ 3 or pH ca 3.

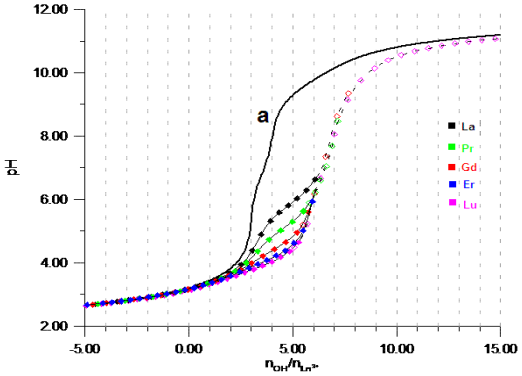

Figure 6: Titration curves for the ligand alone (curve a) and in the presence of lanthanide ions in the 1:1 molar ratio.

The distribution species diagram for lanthanum and erbium is represented in figure 7. From the analysis of the La-$NTP(PrHP)_3$ system (Fig. 7A), it can be observed that in the range of pH in study there are mainly four species in solution: MH_4L, MH_3L, MH_5L_2 and MH_3L_2. In the MH_4L specie, the metal ion is bicoordinated by one of the hydroxypyridinone units. By the other hand, in the MH_3L complex the metal becomes tetracoordinated and in MHL all the HP units interfere in the coordination to the metal ion, forming a hexacoordinated complex. From pH 5 to 7, there is the formation of the 1:2 species and at the physiologic pH, 60% of the solution is composed by MH_3L_2, in which the metal seems to be encapsulated by the cavity formed by both ligands. The behaviour found for the Pr and Gd systems is analogous to the one found for the lanthanum systems.

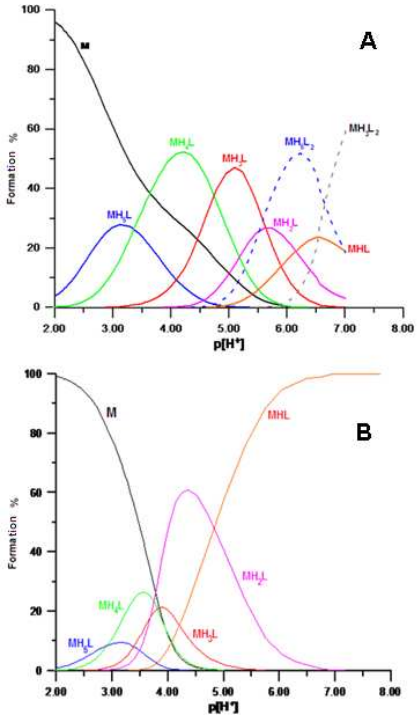

Figure 7: Distribution species diagram for the systems: A – La^{3+}/$NTP(PrHP)_3$ (1:2); B - Er^{3+}/$NTP(PrHP)_3$ (1:1).

In relation to Er and Lu, which are located in the right end of the lanthanide series, their behaviour is also very similar. Therefore, only the Er(III)/$NTP(PrHP)_3$ system will be analysed here (figure 7**B**). In this case, there is a high formation of the tetracoordinated specie MH_2L in solution at the pH range from 4 to 5, after which started the formation of the MHL species.

One of the objectives of this study with lanthanide ions, besides their application as MRI contrast agents, was the evaluation of the "lanthanide contraction". For this reason, the ions were strategically chosen taking into account their positions throughout this group of metals. Therefore, a β_{MHL} versus r^{-1} graph was created, in order to follow this lanthanides behaviour in the presence of $NTP(PrHP)_3$ (Fig. 8). The trend of the logarithm of the stability constants of the complexes MH_5L, MH_4L and MH_3L is *quasi* parallel thus indicating that a mechanism independent from the metal-ion-charge-density is responsible for the complex stability. Usually, the logβ versus 1/r paths follow a wavy trend: they increase for the first elements of the series, tend to decrease for the middle elements and increase again for the heavier ones.

Similar behaviour was already interpreted as reflecting changes in the solvation sphere of the ions during complexation. The *quasi*-parallel trends suggest that no significant changes in the solvation sphere of the metal ions occur when the above complexes form.

[1] This values were determined in the Inorganic Chemistry laboratory of the Inorganic Chemistry, Organometallic and Analytical Department of Padova University, under the supervision of Prof. Plínio Di Bernardo.

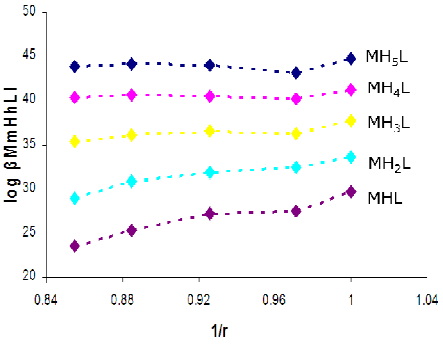

Figure 8: log β_{MHL} of the lanthanide complexes formed with $NTP(PrHP)_3$ versus r^{-1} (ionic radius of the metal).

In the case of MH_2L and MHL complexes, their stability constants increase significantly on going from La to Lu. This suggests the occurrence of a reaction, which is dependent on the acceptor charge-density, i.e., the lanthanide ion. Therefore, Lu(III) has the higher charge density and it is also the one that forms more stable complexes. This fact can be also confirmed by the potentiometric titration curves (Fig. 6), which illustrate that the lanthanide affinity for the $NTP(PrHP)_3$ ligand depends considerably of the ionic radius of the metal. For example, at pH ~4, five protons are removed from the ligand due to the formation of the Lu^{3+}, Er^{3+} and Gd^{3+} complexes, while the correspondent La^{3+} complex only exist at pH ~ 6.

It is important to refer that in this systems, as it happened with the gallium(III) system, there was a precipitate formation after pH~7, that it is thought to correspond to the neutral species ML.

Biological Studies

A series of bidentate 3-hydroxy-4-pyrydinone derivatives were biologically evaluated in terms of toxicity, DNA interaction and capacity of altering cells morphology or membrane fluidity. These ligands are metal chelating agents that eventually could have potential application in quelation therapy and diagnostic techniques.

The results are summarized in table 4. Compound **(2)** was synthesized from a DMHP unit, functionalized with an aminopropryl group in the nitrogen atom of the pyridinone ring. The main reason why he was synthesized is to serve as an intermediate in the construction of more elaborated molecules. The toxicity results in human neuroblastoma cells indicate that for all concentrations in study, the values are very close to 100% vitality, which means that this molecule doesn't induce any toxicity to the cells. It can also be seen that at the higher concentration (100µM) the % vitality is lower. Since this compound is significantly lipophilic (log P octanol/water – 0.03[9]), he can overcome the cell membrane more easily. Therefore, at the higher concentration, it invades the cell and can possibly interact with other molecules, thus decreasing the cell viability. The results of cell morphology, obtained from SEM in the presence of the compound, showed that this parameter remained unchanged and the cell culture was identical to the control cell culture.

The di-propargil derivate **(2)** was strategically synthesized in order to act as both chelating and anti-neurodegenerative agent. The propargil group is a MAO-B inhibitor[23], an enzyme responsible for the oxidation of Dopamine thus accelerating the symptoms of Parkinson disease.

The toxicity results for **(2)** indicate that it is slightly toxic for 100µM concentration, however, at 10µM the cell viability is almost 100%. In the SEM images obtained at 100µM concentration, it can be seen a considerable number of cells in apoptosis (Fig. 9A), due to the toxicity associated to this concentration value.

Compound **(3)** is a sugar derivate and it was developed taking into account a bifunctional strategy. The structures that incorporate a glucose unit are of great interest (e.g. Feralex-G) due to their reported specific interaction with hyperphosphorylated brain cell nuclear matrix[24], disturbing protein aggregation that lead to fibrilary tangles formation. In addiction to this behaviour, there is the potential remotion of Al/Fe accumulated in Alzheimer brains[25]. By the other hand, this glucose group can interact with specific glucose transporters, over-expressed in certain tumour types. Thus, the corresponding radiocomplex of **(3)** may find applications in radiodiagnostic techniques. The toxicity analysis for this compound indicates that it's non toxic. Another advantage of **(3)** is the recognition of the carbohydrate by the glucose transporters in the cell membrane thus increasing his passage into the cell.

In relation to compounds the **(4)** and **(5)**, these compounds are alkyldiethylamine derivatives, a group already reported to be potentially recognized by *sigma* receptors which are over-expressed by certain solid tumors[26]. Therefore, they can be used as radiodiagnostic agents and also as chelating agents. Both compounds didn't show any toxicity in the cells and, even at the highest compound concentration, the cell viability was ~100%, comparing to the control. However, the only problem associated to these compounds is that in physiological conditions both exist in the monoprotonated form, H_2L^+, in which the diethylamine group is protonated so there may be some resistance to the passage through membranes. No morphologic modifications were seen in the SEM images obtained.

The next compound, **(6)**, was obtained by functionalization of the HP ring nitrogen with a 1,4-arylpiperazine group. The compounds bearing arylpiperazine groups are known to target serotonin ($5\text{-}HT1_A$) receptors having an important role in psychiatric disorders[27], like Alzheimer disease[28].

By the other hand, as in the case of the diethylamine group in compounds **(4)** and **(5)**, the arylpiperazine can be also easily recognized by sigma receptors[29]. It's hydrophobic character evidenced by an high partition coefficient (1.06[19]) indicates the possibilities of favourable crossing through the blood brain barrier[14]. The vitality of the cells treated with **(6)** is quite high. Actuality, it is the highest for this series of compounds in study. This results can be confirmed by the SEM images obtained, where it can be seen an increase in cell density of the culture treated with compound **(6)**, comparing to the control (Fig. 9B).

The last compound of this series of HP derivatives under study is a sulfonamide. In this case, the toxicity results reveal some

Table 4: Cell viability results for the series of HP derivatives in study.

	Cell Viability (%) in the presence of compound							
Conc. (μM) / compounds	Control	100	10	1	0.5	0.1	0.01	0.001
(1)	100	95 ±8	103 ± 13	103 ± 5	109 ± 16	93 ± 11	103 ±13	109 ± 5
(2)	100	85 ± 4	96 ± 14	87 ± 11	93 ± 13	90 ± 14	90 ± 12	90 ± 10
(3)	100	92 ± 8	108 ± 10	108 ± 4	105 ± 6	110 ±10	99 ± 12	103 ± 8
(4)	100	104 ± 4	102 ± 10	104±10	105 ± 9	105 ± 9	108 ±13	102 ± 5
(5)	100	101 ±10	104 ± 9	109±12	108±13	102 ±14	108 ±11	105 ±10
(6)	100	99 ± 6	104 ± 10	116 ± 4	113±13	111 ± 2	117 ± 5	120 ± 5
(7)	100	70 ± 2	102 ± 3	103.0±0.3	102 ± 2	118 ± 4	113 ± 4	112 ± 3

decrease in cell viability at 100μM concentration. The correspondent vitality value is the lowest of this series, i.e., 70 ± 2. The fact that at physiological conditions the compound exists under the H_2L^+ monoprotonated form can interfere in the respective membrane crossing. This behaviour could be identified when observing the SEM images, since there was a decrease in cell density and also the presence of some cells in apoptosis.

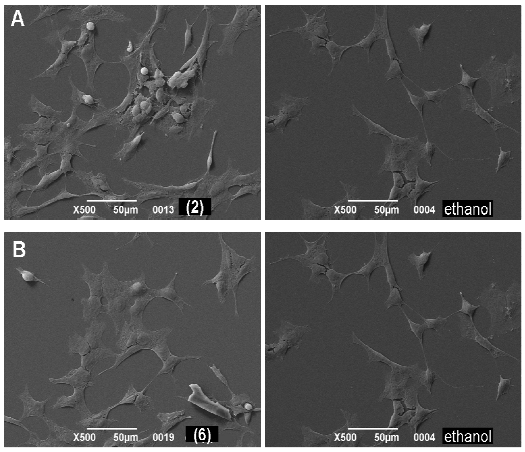

Figure 9: SEM images obtained from the neuroblastoma cells treated with: **A** – compound **(2)**; **B** - compound **(6)**. (C=100μM).

Interaction tests with DNA of neuroblastoma cells treated with the compounds in study were also performed. The results indicated that no modification of the DNA occurred, which is in accordance with the non toxicity shown in the viability tests.

In an effort to further investigate the interaction of some of the compounds (**(1)**, **(3)** and **(5)**) of the HP series under study with the membrane, anisotropy measurements were performed on liposomes (a model of cell membrane) after the addition of 100μM of compound. The results are presented in figure 10.

The liposomes were composed by L-α-DMPC phospholipids. DPH is a fluorescent probe that intercalates predominantly between the acyl chains of fatty acids in the membrane hydrocarbon core.

Fluorescent dyes represent a useful tool for the determination of membrane dynamics.

In this case, the fluorescence anisotropy values revealed no variation in the membrane fluidity after the treatment with **(1)**, **(3)** or **(5)**, when comparing with the control (liposomes with water). In fact, the lipidic transition temperature remained the same (23ºC) for all the three experiments. This temperature is specific for each type of phospholipids present in the membrane, corresponding to 23ºC for DMPH.

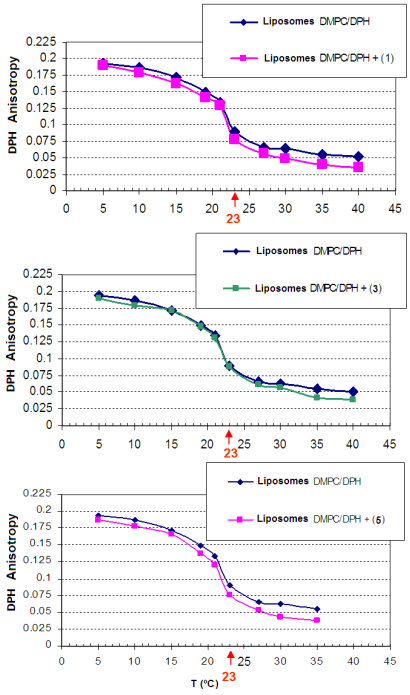

Figure 10: Fluorescence anisotropy curves of the DMPC/DPH liposomes in the presence of compounds **(1)**, **(3)** e **(5)**.

Liposomes are considered important vehicles of controlled delivery of drugs or biological active substances inside the cell. On the other hand, they have enhanced ability to incorporate a large variety of compounds, both hydrophilic and hydrophobic. Therefore, since these compounds do not interact with the liposome membrane, they may be potential candidates for this type of transport into the cell.

Final considerations

Since $NTP(PrHP)_3$ was synthesized through a "target-ligand" approach[14], it exhibits both polydenticity and N-derivatization,

in order to obtain an enhanced chelating efficacy and molecular recognition, respectively.

The complexation studies with Ga(III) and this ligand revealed the formation of GaH_5L, GaH_3L, GaHL and GaL species in aqueous solution, with stability constants considerably high. In fact, the pGa value for Ga/NTP(PrHP)$_3$ system is greater than that of other commercial compounds, including EDTA, DFO and DFP, suggesting a high chelating efficiency of this hexadentate ligand towards gallium. On the other hand, biodistribution studies of the ^{67}Ga/NTP(PrHP)$_3$ radiocomplex, showed that no accumulation occurred and besides the fact that it was excreted through the kidneys, after 24h of injection, no radiation trace of the complex was found in the organism. Therefore, these studies indicate the potential application of NTP(PrHP)$_3$ in radiodiagnostic techniques (GC and PET).

Regarding the complexation of lanthanides by this ligand, it was found that for the metal ions La, Pr and Gd not only the 1:1 complex was formed in aqueous solution, but also 1:2 complexes. However, this behaviour was not observed in Er or Lu systems, for which only 1:1 species were present. This fact is due to the smaller ionic radius of these last two metal ions, when compared to those of La, Pr or Gd. In respect to the affinity of NTP(PrHP)$_3$ by the lanthanides, it can be seen that it is smaller then in the case of gallium, since the complex formation starts at pH ~3, while in the case of Ga(III) it starts at pH less then 2. This is probably related to the fact that lanthanide ions are weaker Lewis acids, thus less competitive than the protons, for the oxygen atoms.

At physiological pH, for M/NTP(PrHP)$_3$ systems (M=La, Pr and Gd) existed the MH_3L specie while in the case of Er and Lu, the MHL specie. In these type of complexes, the metal can eventually be coordinated to water molecules, therefore improving the complex ability to act as MRI contrast agents. This is an interesting hypothesis, although more studies are necessary to prove this theory (eg. X-ray structures). The relation between the contraction effect of the lanthanides and their affinity for NTP(PrHP)$_3$ pointed to an increasment of the stability constants for the MH_2L and MHL species with the decreasing of the metal ion ionic radius. Therefore, once Lu is the smaller and also the higher density one, it is also the metal ion that forms the greater stability complexes with the ligand.

The series of biological evaluated HP derivatives are stable structures and both hydrolysis and oxidation resistant, which in combination with a high chelating efficiency, makes them potential chelating agents for hard metal ions like Fe, Al or Ga. These compounds were strategically extra-functionalized in order to obtain specific characteristics in terms of lipophilicity and protein interaction, among others. It was seen that none of the ligands studied herein were toxic for the cells or altered their morphology. However, for most of them, at the higher concentration tested, i.e. 100 µM, the cell viability decreased.

The fragmentation and interaction studies with DNA in the compounds presence, together with the fluorescence anisotropy measurements, showed no modifications in the nucleic acids and also no change in the liposomes membrane proprieties or structure.

Hence, these biological results seem to demonstrate that due to the non toxicity of this series of bidentate compounds, they could be potential chelating agents, eventually acting also as anti-oxidant and/or anti-neurodegenerative compounds.

Experimental section

Solutions

A solution of 4.16×10^{-3} M Ga^{3+} was prepared in HCl 0.1M (to prevent hydrolysis), from the respective gallium salt and standardized by inductively coupled plasma emission (ICP). The exact concentration in HCl was determined by titration with a 0.1 M HCl (Titrisol). The titrant was prepared from a carbonate-free KOH commercial solution (Titrisol) and standardized by titration with a potassium hydrogen phtalate solution. This solution was discarded whenever the percentage of carbonate, determined by the Gran method[30], was higher than 0.5% of the total amount of base.

The lanthanide solutions: La^{3+}, Pr^{3+}, Gd^{3+}, Er^{3+} and Lu^{3+} were prepared dissolving approximately 1.2 g of the correspondent lanthanide oxide (Aldrich, > 99% pure) in 7.36 mL of HCL 1M and 25 mL of distillated water. The solution was stirred until all the solid was solved and then heated. A part of the water was evaporated in order to obtain a more concentrated solution before it was filtered. To determine their exact concentrations, these solutions were titrated with a 49.7 mM EDTA solution.

Gallium complexation

Potentiometric titrations

A Crison Microph 2002 pH meter was used together with a Metrohm 6.0133.500 glass electrode and a Orion 90-00-11 Ag/AgCl reference electrode. The titrant (0.1M KOH) was added with a Crison microBU 2031 burette. All the potentiometric measurements were carried in thermostated solutions at (25.0±0.1)ºC and ionic strength 0.1M KCl. Atmospheric CO_2 was excluded from the cell, before and during the titration, by passing purified N_2 across the top of the solution inside the reaction cell. The cell ligand concentration (V_T=20 mL), in the presence or absence of the metal ion was 0.02 mmol (14.5 g), a low concentration to delay precipitation of the neutral ML species.

First, the ligand was titrated alone in order to calculate the protonation constants and 4.5 equivalents of acid were added to the medium.

The measurement of the electromotive force of the cell allowed the calculation of $[H^+]$, using the values of E^0 and Q previously determined. The value of K_w used in the computations was $10^{-13,77}$.

The complexation studies were performed in the presence of gallium 1:1 (metal:ligand), for the stability constants determination. Since complex formation started at pH<2, it was also necessary to carry out spectrophotometric titrations.

Spectrophotometric titrations

The electronic spectra of NTP(PrHP)$_3$ and Ga(III) complexes, in aqueous solution (I=0.1M KCl) were obtained in the range

250-400 nm (UV), using a Perkin-Elmer Lambda 9 UV/VIS/NIR spectrophotometer with a thermostated cell left at (25.0±0.1)ºC by using a Grant W6 thermostat and 1 cm quartz cells. The titrant (0.1M KOH) was added with a Crison microBU 2031 burette and the pH was measured with a Crison Microph 2002.

The measurements were done with a concentration of ligand in the cell of $1.22x10^{-3}$M and 0.1M KCl ionic medium. The titrant (0.1M KOH) was added to the ligand solution, so that the increase of pH was ca 0.4 units. The absorbance measurements were done in the absence of metal for 2<pH<10, after which the same procedure was done in the presence of Ga^{3+} in a 1:1 molar ratio. The measurements for pH≤2 were also performed, in the presence (1:1) and absence of the metal ion. For these titrations, the amount of acid to be added, from standard solutions (0.1 or 1M HCl), was calculated in order to adjust the pH and obtain specific pH values (0.8, 1, 1.2, 1.4, 1.6 and 1.8).

Biodistribution studies and gamma imaging

The complex $[^{67}Ga]$-NTP(PrHP)$_3$ was prepared by dissolving 1g of ligand in HEPES (200 µL, 0.1M, pH 5) and addition of 1mCi (1Ci=3.7×10^{10}Bq) of $[^{67}Ga]$citrate. The radiochemical purity of the final product was higher then 98%.

Animal experiments were carried out with groups of four to six animals (Wistar male rats) weighing approximately 200g. For the biodistribution studies, animals were anaesthetized with Ketamine (50mg/ml) /chloropromazine (2.5%) (10:3) and injected in the tail vein (biodistribution at 24 h) or in the femoral vein (early biodistribution studies and imaging) with 100-µCi of the radiochemical and sacrificed 30 min., 60 min. and 24 h later. The major organs were removed, weighted and counted in a γ well-counter. The *in vivo* gamma imaging was carried out on a gamma camera-computer system (GE 400 GenieAcq, from General Electric, Milwaukee, WI, USA). The animals were positioned in dorsol *decubitus* over the detector. Image acquisition was initiated immediately before the radiotracer injection. Sequences of 180 imagens were acquired to 64x64 matrices. In addiction static data were acquired 24 h after the radiotracer injection.

Images were subsequently processed using an IDL based program (Interactive Data Language, Research Systems, Boulder, CO, USA). In order to analyze the transport of radiotracer over time, three regions of interest (ROI) were drawn on the image files, corresponding to the thorax, liver and left kidney. From these regions, time-activity curves were obtained.

Lanthanide complexation

Potentiometric titrations

A pH AMEL model 338 pH meter was used together with a Metrohm 6.0259.100 combined electrode, incorporated with a Ag/AgCl reference electrode. The titrant (KOH) was added with a Metrohm 765 Dosimat burette. All the potentiometric measurements were carried in the same experimental conditions previously described for Ga(III) complexation studies.

The several lanthanide solutions (La, Pr, Gd, Er and Lu) were titrated with a 51.71 mM KOH solution, in order to calculate their exact concentration of acid. The ligand and metal-ligand solutions were titrated with a 51.37 mM KOH solution and a cell ligand concentration (V_t= 5 mL) of 1.040 mM. Titrations 1:1 and 1:2 metal-to-ligand molar ratios were performed.

The value of K_w used in the computations was $10^{-13,78}$.

Calculation of the equilibrium constants

The ligand protonation constants, $K_i=[H_iL]/[H_{i-1}L][H]$, were calculated by fitting analysis of the potentiometric titration curves of the ligand with the HYPERQUAD[31] programme.

In the gallium complexation studies, the spectra of the protonated species and overall metal complex (1:1) stability constants $\beta_{MmHhLl}=[MmHhLl]/[M]^m[H]^h[L]^l$, were determined by adjusting the absorbance spectra obtained from the spectrophotometric titrations at different pH values with the PSEQUAD[32] programme.

In the lanthanide systems, the overall metal complex stability constants were determined by treating the data obtained from the potentiometric titrations with the HYPERQUAD program. In this case, the stability constants of the 1:2 complexes, were determined after the calculation of 1:1 stability constants, by holding them constant and, as the last step, all the stability constants were refined together.

The hydrolytic species of the metal ions[33] were considered in the calculation of the stability constants and the speciation curves were plotted with the HYSS programme[31]. The estimated errors are the standard deviations associated to the stability and protonation constants, given by the program for the input data.

Biological studies

Chemicals

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), absolute isopropanol, chloridric acid, dimethil sulfoxide (DMSO), ethidium bromide, 1,6-diphenyl-1,3,5-hexatriene (DPH), dimyristoyl phosphatidylcholi-ne (L-α-DMPC) and TRIS buffer, were all purchased from Sigma-Aldrich.

Cell cultures

SH-SY5Y human neuroblastoma cells were purchased from ECACC (European Collection of Cell Culture, Salisbury, UK). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM/F12 (1:1); Gibco), with 15% (v/v) fetal bovine serum (FBS; Sigma-Aldrich), 0.1% of antibiotics (100units/mL penyncilin + 100µg/mL steptomicin; Gibco), 1% of non-essencial aminoacids (NEAA; Sigma-Aldrich) and maitaned at 37ºC with 5% CO_2 in a humified atmosphere (90% humidity). The medium was replaced every 2 days. 0.25% trypsin-EDTA solution and phosphate buffered saline (PBS) were obtained form Sigma-Aldrich.

Cell viability assay

MTT assay was performed with SHSY5Y cells plated onto 96-well plates (at a density of 8×10^4 cells per well and a limit of 120µL medium containing 15% FBS per well). The day after

this plating, the culture medium was replaced with the same medium with 2% FBS, containing one of the compounds studied (Fig. 2) at concentrations: 100, 10, 1, 0.5, 0.1, 0.01 and 0.001 µM. The cells were incubated for 24h and after that, 12 µL of a solution 5 mg/ml MTT was added to each well. The incubation continued for an additional 3 h, the end of which MTT solution was carefully decanted off, and formazan was extracted from the cells with 110 µL of acidic isopropanol (0.04M HCl in absolute isopropanol) in each well[34]. Colour was measured with a 96-well ELISA plate reader (Microplate SPECTRAmax® at 550 nm). All MTT assays were repeated nine times.

Scanning Electron Microscopy

SH-SY5Y human neuroblastoma cells were seeded into glass cover slips and treated with compound (Fig. 2) at 100 µM concentration for 24 h. Note that as the solvent was water , ethanol or DMSO, the % of compound in the medium was 4%, 2% and 0.2%, respectively. After this incubation, the cells on glass cover slips were fixed with formaldehyde pH 7.4 and dehydrated in a graded ethanol series. Then, the samples were critical point dried with CO_2 in an HCP-2 Hitachi 2 Critical Point Dryer and gold-coated for examination under a JEDL JSM-6490 scanning electron microscope, at the 10, 20 and 50 µm resolution. The working pressure was 10^{-4} Torr and the temperature was 5ºC.

DNA Interaction

SH-SY5Y human neuroblastoma cells were seeded onto 25 mL bottles and treated with compound (Fig. 2) at 100 µM concentration for 24h. Note that as the solvent was water, ethanol or DMSO, the % of compound in the medium was 4%, 2% and 0.2%, respectively. After this incubation, the cells were transferred into 1.5 mL ependorf´s and frozen. In the analysis laboratory, the cell membrane was destructed adding a surfactant solution, after which the cells were centrifuged over 1h30. A solution of ethanol was added and the content of each ependorf filtered, in order to obtain only de DNA molecule. The polymerase chain reaction (PCR) (Taq polymerase enzyme) was performed over the DNA to amplify the β-Globin gene, in a *Thermal Cycler model PTC-100 MJ Research* equipment programmed for: 1 minute at 90ºC, followed by three cycles of one minute each at 95, 55 and 72ºC, respectively and ending with 5 minutes at 72ºC. After the PCR reaction, an agarose gel electrophoresis was run at 90 volts of electric current. The gel was previously prepared by adding 3g of agarose, 100 mL of acetate buffer and 20 µL of ethidium bromide.

Fluorescence anisotropy

The stock solution of fluorescent probe DPH was prepared by dissolving the probe in absolute ethanol (ETOH; Sigma Aldrich) at a final concentration of 2 mM. As a membrane model, liposomes were used, produced in the laboratory and composed by L-α-DMPC phospholipids, in a TRIS buffer (0.1M, pH 7.4). The stock solution of phospholipids was prepared in aqueous solution at a final concentration of 21.1 mM. The fluorescent liposomes were formed at the temperature 35ºC by the slowly injection of DPH and DMPC in TRIS buffer. The final concentration of DPH and DMPC was 1µM and 1.6 mM, respectively. For this type of phospholipids, the CMC temperature is 21ºC. Subsequently, the liposomes were treated with the compounds **(1)**, **(3)** and **(5)** at a final concentration of 100 µM. The fluorescence intensity of the solutions in the coveut was measured in a Perkin-Elmer LS-50 spectrophotometer, incorporated with a HAAKE DC3 thermostat and an automated polarization accessory (Perkin-Elmer, Monza, Italy). The fluorescence anisotropy was followed over a growing scale of temperatures and the lectures done at 5, 10, 15, 18.5, 19, 21, 23, 27, 30 e 35ºC. The excitation and emission wavelengths for DPH were 360 and 540 nm, respectively. The fluorescence anisotropy *(r)* was obtained from the fluorescence intensities parallel (I_{vv}) and perpendicular (I_{vh}) to the direction of polarization of the excitation light using the following equation (Van der Meer)[35]: $r = (I_{vv} - I_{vh}G) / (I_{vv} + 2I_{vh}G)$, where *G* is an instrumental correction factor.

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