A novel tripodal tris-hydroxypyrimidinone sequestering agent for trivalent hard metal ions: synthesis, complexation and in vivo studies†

Sílvia Chaves,a Anabela Capelo,b Laurinda Areias,b Sérgio M. Marques,a Lurdes Gano,c M. Alexandra Estevesb and M. Amélia Santos*a

A new tripodal hexadentate ligand, NTP(PrHPM)₃, having three hydroxypyrimidinone (HPM) chelating units attached to a nitrilotripropionic acid (NTP) has been prepared and studied in terms of thermodynamic stability of the complexes with iron, aluminium and gallium and it has been subsequently in vivo assayed for its capacity to remove hard metal ions from an animal model overloaded with ⁶⁷Ga. The anchoring of the HPM units to the NTP scaffold revealed to be an interesting alternative to the reported hexadentate tris(3-hydroxy-4-pyridinone) analogue, NTP(PrHP)₃, because the new tris-HPM ligand still keeps high chelating capacity for hard metal ions and presents better water-solubility (log P = -1.51). The in vivo studies show that NTP(PrHPM)₃ induces a faster clearance from main organs and an enhancement of overall excretion, as compared with the commercial drug, DFP, or the bidentate HPM compound (HOPY-PrN), albeit slightly lower than the tris-hydroxypyridinone analogue, NTP(PrHP)₃. The solution and in vivo results herein presented encourage further studies envisaging the potential clinical applications of hexadentate HPM derivatives as metal sequestering agents.

Introduction

Despite the biological importance of iron for all living cell, when in excess, it may become very toxic to the human body. In fact, humans have very little capacity for excretion of excess iron, which can result from its administration, as in beta-thalassemia patients, or from excess absorption, as in hemochromatosis.¹,² Also, the nonessential ubiquitous aluminum (Al³⁺), through different processes of exposure, can accumulate in specific organs leading to serious problems of toxicity,³,⁴ namely bone disorders (vitamin-D-resistant osteomalacia) and neurological diseases, such as dialysis encephalopathy syndrome, and eventually Alzheimer’s disease (AD).⁵,⁶

The therapeutic strategies to minimize the adverse effects of metal overload and misplaced related illnesses have led to the development of new chelating agents, which, by far, has been more focused on iron(III) than any other hard metal ions.⁷ However, the physicochemical analogies between Fe³⁺ and other Lewis acid metal ions (e.g. Al³⁺, Ga³⁺) have orientated the potential use of many iron-chelators as Al-sequestering drugs or as radionuclide Ga-chelates for diagnostic drug.⁸,⁹ The main Al-binding protein in plasma is transferrin (Tf) which is only 30% saturated with Fe in normal serum, thus still keeping significant chelating capacity for other trivalent metal ions, such as Al.¹⁰ Thus, in principle, Fe/Al-chelators must be able to compete with transferrin for those metal ions.

The first iron-chelating drug (since ca. 5 decades ago) for beta-thalassemia patients was the hexadentate tri-hydroxamate Desferrioxamine or Desferal (DFO).¹¹ However, its drawbacks led likewise to the search for alternatives.¹² Concerning the aluminum chelators, DFO was also the first chelator introduced in clinical therapy, namely for treatment of osteomalacia and encephalopathy associated with severe dialysis Al-intoxication,¹³ but its drawbacks led likewise to the search for alternatives.⁸

The need of stronger and orally active chelators for the decorporation of iron or other specific toxic hard metal ions such as aluminum or actinides has been worldwide recognized, and so, along the last 2 decades, we have assisted to an
intensive search for new metal sequestering agents, namely hydroxypyridinone (HP)-based compounds with mono- or poly-chelating capacity, in view of single or combined drug administration protocols.

As a step forward on the discovery of new strong iron chelators, we have decided to explore new ligands with hexadentate capacity, to guarantee the metal-full coordination, absence of ligand dilution effects and so iron-scavenging capacity at very low concentration. Thus, by paralleling a design strategy recently used in our group for tripodal hexadentate ligands, we have substituted the chelating units, 3,4-HP by hydroxypyrimidinones (HPM), a kind of heterocyclic hydroxamate analogue with high hydrolytic stability, adequate hydrophilicity and chelating capacity, lying between those of the hydroxamic acids and the hydroxypyridinones.

In particular, we describe herein the study of a novel tripodal tris-chelator, NTP(PrHPM)₃, having three HPM units appended to nitrilotripropionic acid as the anchoring skeleton (Scheme 1). After the synthetic methodology, we present the results of assessing its capacity for complexation with Fe³⁺, Al³⁺ and Ga³⁺ in water, as well as the molecular modeling calculations (DFT) on the iron-complex to get some insight into its structure. The results from the study of the capacity of this new chelator for metal mobilization in mice pre-administrated with a radiotracer (⁶⁷Ga), as an animal model of metal-overload, are also analyzed. The discussion of all set of results is made in comparison with the reported properties for other synthetic chelators and bioligands.

Results and discussion
Synthesis of the ligand, NTP(PrHPM)₃
The hexadentate compound (NTP(PrHPM)₃) was obtained through the attachment of three amino-bearing O-benzyl-hydroxypyrimidinone units (O-benzyl HOPY-PrN) to the tris-carboxylic scaffold (nitrilotripropionic acid, NTP) via the formation of amide linkages and final O-benzyl deprotection through standard methodologies, as described below.

The general procedure for the synthesis of NTP(PrHPM)₃ (3,3′,3′′-nitrolotris(N-(3-(1-hydroxy-2-oxo-1,2-dihydropyrimidin-4-ylamino)propyl)propanamide) is outlined in Scheme 2.

In the first step, 1-(benzoyloxy)-4(1′,2′,4′-triazol-1′-yl)-2-(1H)-pyrimidinone was reacted with 3-((tert-butoxycarbonyl)-amino)propylamine to give 4-3-((tert-butoxycarbonyl)amino)propylamino)-1-(benzoyloxy)-2(1H)-pyrimidinone 2 in 74% yield. Removal of the Boc group involved treatment with HCl in 1,4-dioxane and provided 4-(3-aminopropylamino)-1-(benzoyloxy)-2(1H)-pyrimidinone hydrochloride in quantitative yield. The coupling of this amine-bearing HPM side arm to the backbone 3,3′,3′′-nitrilotripropanoic acid (NTP) involved a pre-activation of this tris-carboxylic acid with TBTU, under anhydrous conditions, followed by addition of compound 3, after its neutralization with N-methylmorpholine (NMM), affording the pre-final product 4 (37% yield). The final ligand (NTP(PrHPM)₃) was obtained in almost quantitative yield (91%), by O-benzyl deprotection using a standard hydrogenolysis with 10% Pd/C as a catalyst.

Solution equilibrium studies
The physico-chemical characterization of the tris-HPM ligand, NTP(PrHPM)₃, was carried out in aqueous solution, namely the study of the acid–base properties and the lipo-hydrophilic character, as well as its complexation ability towards hard metal ions (Fe³⁺, Ga³⁺ and Al³⁺). Discussion of the results is made on the basis of comparison with previously reported tripodal hexadentate tris-HP compounds (NTP(PrHP), and NTA(BuHP)₃) and also with bidentate compounds, such as the HPM derivative (HOPY-PrN) or the commercial drug DFP (see Scheme 1).

Acid–base behavior
The acid–base properties of NTP(PrHPM)₃ were mainly studied by fitting analysis of the potentiometric titration data, which provided the protonation constants; a ¹H NMR spectroscopic titration was used to check the protonation sequence; a UV spectrophotometric titration of the ligand was also performed to determine the spectral parameters of the various protonated species to be subsequently introduced in the equilibrium model of the Ga³⁺/NTP(PrHPM)₃ system.

The compound was isolated in its neutral form (H₃L), whereas, when fully protonated, it has seven dissociable protons (H₇L⁷⁺). The stepwise protonation constants, obtained from pH-potentiometric studies for NTP(PrHPM)₃, are summarized in Table 1, which also includes the corresponding reported values for some other HPM and HP analogues.

Analysis of calculated stepwise protonation constants (log Ki) for NTP(PrHPM)₃ evidences the existence of a first set
of four values in the range 6.0–8.2, corresponding to three hydroxyl groups of the HPM moieties plus the anchoring backbone amine group; the second set of values is in the range 2.0–3.3, being attributed to the 4-imine groups of the side chains. These results are in accordance with the protonation constants hitherto obtained for the hydroxyl (log $K_2 = 6.84$)
and 4-imine (log $K_3 = 2.21$) groups of HOPY-PrN. The protonation constants calculated for the HPM hydroxyl groups are lower than those obtained for the corresponding HP analogues (9.1–9.9 for the tris-HP, 9.77 for DFP), which can be due to the electron withdrawing and resonance effects of the aromatic ring, as well as to the electron-withdrawing effect of a second ring-nitrogen, contributing to the stabilization of the negative charge of the conjugate base. Moreover, the very acidic character of the 4-imine groups, requiring the addition of acid excess to guarantee the ligand full protonation, must be due to the existence of enamine/imine equilibrium together with the above referred electron-withdrawing and resonance effects of the aromatic ring.

The set of $^1$H NMR titration curves (Fig. 1) presents downfield shifts of the non-labile protons, namely in the ranges pH 6–9 (protons a, b, e, f, g) and pH 2–3.5 (protons a, b, c, d), which give some support to the attributed protonation sequence. The protonation of the hydroxyl and the backbone amine groups is practically simultaneous, as evidenced by a detailed analysis of these curve profiles, namely the occurrence of large downfield shifts on protons g and f (due to the nearby amine group) close to the pH range of hydroxyl group protonation (unfortunately, not clearly evidenced in this figure because the curve profiles for protons a and b are quite smooth).

Though the attribution of the four calculated macroconstants in the higher pH range to individualized protonation processes renders difficult, analysis of Fig. 1 seems to suggest that log $K_3$ may be mainly ascribed to the protonation of the backbone amine, taking into account the isotopic correction. The value obtained for NTP(PrHPM)$_3$ (6.78) would be quite close to the corresponding value for NTP(PrHP)$_3$ (6.77), thus indicating that for these tripodal ligands, sharing the same backbone, the protonation of the apical amine is quite independent of the appended chelating arms.

Though the acid–base properties of NTP(PrHPM)$_3$ were mainly assessed by potentiometry and $^1$H NMR (see above), we have also included herein the spectrophotometric titration of this ligand (see Fig. 2a), a requirement for the subsequent UV spectrophotometric study of the gallium complex formation (see below), as well as a combination of spectral data with the species distribution curves (Fig. 2b).

Analysis of Fig. 2b evidences that in the range $3.2 < \text{pH} < 6$, the major protonated species is $H_4L^+$; at the physiological pH, $H_3L^-$ (8%), $H_2L^-$ (33%), $HL_2^-$ (51%) and $L_3^-$ (7%) coexist, while for pH above 8.3 the ligand is essentially deprotonated ($L_3^-$). This figure also shows that the species $H_2L^{2+}$, $H_4L^+$, $H_3L$, $H_2L^-$ and $HL_2^+$ absorb at 251 nm, while $H_2L^{3+}$ absorbs at 290 nm; the absorbivity values at 318 nm reveal that the main species absorbing at this wavelength are $HL_2^-$ and $L_3^-$. Therefore, 290 nm and 318 nm seem to correspond to the absorption maximum wavelengths for the chromophores of the completely protonated ($H_4L^{3+}$) and deprotonated forms ($HL_2^-$ and $L_3^-$) of the hydroxypyrimidinone, respectively. At 251 nm, it is evidenced an equilibrium between species with different partially protonated hydroxyl/imine moieties present.
An identical spectrophotometric study was also performed for the ligand HOPY-PrN, spectra of which presented absorption maximum at 251, 285 and 318 nm (Fig. S1†).

**Lipo-hydrophilic character**

The lipo-hydrophilic character of NTP(PrHPM)₃ and HOPY-PrN was assessed via the corresponding partition coefficients (log P) between 1-octanol and a TRIS-buffered aqueous solution at the physiological pH. The value obtained for NTP(PrHPM)₃ (−1.51) is quite close to others previously reported for analogous compounds, namely hexadentate HP derivatives with similar anchoring groups.¹⁹ This value is slightly higher than those obtained for NTP(PrHP)₃ (−1.24) or NTA(BuHP)₃ (−1.40), which seems according to the higher water solubility of HPM as compared with HP derivatives. This difference is also supported by the fact that, at pH 7.4, NTP(PrHPM)₃ is mostly negatively charged (ca. 91%, as H₂L⁻, HL²⁻ and L³⁻) while NTP(PrHP)₃ is predominantly in the neutral form (80% H₃L) and only 20% as a monoprotonated species (H₄L⁺).

The HOPY-PrN compound is also slightly more hydrophilic (log P = −1.43) than the HP bidentate analogues, such as the drug DFP (log P = −0.83) or 3,4-HP-PrN (log P = −1.25). These differences can be partially rationalized on the basis of distribution curves. At pH 7.4, HOPY-PrN presents as 22% monoprotonated species (H₂L⁺), while DFP as a 100% neutral form (HL) and 3,4-HP-PrN as 98% H₂L⁺ species. Of course, besides the species charge, other factors are also determinant for the lipo-hydrophilic character, namely the solute–solvent interaction. In fact, at pH 7.4, DFP is in the neutral form but presents some hydrophilic character.

**Metal chelating capacity**

The complexation ability of NTP(PrHPM)₃ towards Fe³⁺, Ga³⁺ and Al³⁺ was studied in solution by determination of the global stability constants of the complexes which allowed the assessment of species distribution under different pH conditions. Those studies involved potentiometric and UV/Vis spectrophotometric titrations with subsequent fitting analysis of the experimental data with HYPERQUAD 2008²⁷ and PSEQUAD²⁸ programs.

The iron complexation was studied by UV/Vis spectrophotometry because at pH = 2 two protonated complex species (FeH₅L and FeH₃L) were already formed, thus precluding the use of potentiometry. This study was performed in two stages: for pH ≤ 2, a batch titration was used, in which calculated amounts of HCl and KCl were added in order to obtain the desired pH value and to keep constant the ionic strength; for pH > 2, a standard automatic titration was carried out. The first titration stage allowed the determination of the stability constants corresponding to the species FeH₅L and FeH₃L; these values were kept constant and introduced in the equilibrium model for pH > 2, which allowed the calculation of β₁ FeHL. The determination of β₁ FeHL became impossible, since under our experimental conditions, precipitation occurred above pH 6.5, probably due to the formation of the neutral FeL species or even mixed hydroxo-ligand complexes. Analysis of the UV-Vis absorption spectra registered at different pH values for the Fe³⁺/NTP(PrHPM)₃ system in Fig. 3a shows one isosbestic point at ca. 500 nm, corresponding to the interconversion of the tetrachelate complex (FeH₅L) to the hexachelate complex (FeHL). The species distribution curves associated with the proposed equilibrium model for the Fe⁵⁺/NTP(PrHPM)₃ system combined with the absorbivity at two wavelengths (466 and 510 nm) (Fig. 3b) show that the maximum absorption at 466 nm (4659 M⁻¹ cm⁻¹) corresponds to the ligand to metal charge-transfer (CT) band of the bischelate (FeHL) complex, while the band at 510 nm (3801 M⁻¹ cm⁻¹) corresponds to the CT band of the bischelate (FeHL) complex. The spectral parameters obtained for the trischelate are quite close to the reported values for the CT bands of ferric trischelated complexes with hydroxypyrimidinone analogues such as the hexadentate 3HOPY₅ (465 nm, 4550 M⁻¹ cm⁻¹)²⁹ or the bidentate HOPY-PrN (465 nm, 5624 M⁻¹ cm⁻¹).¹⁹ Thus, these studies evidenced the high iron chelating capacity of this ligand, since the trischelate (FeHL) species, formed through the {O,O} chelation of the adjacent N-hydroxy and keto-oxygen groups of the three arms, is predominant at pH above 3.5.

![Fig. 3](image-url)
Regarding the Ga\(^{3+}/\)NTP(PrHPM)\(_3\) system, since the gallium-complex formation also started below pH 2, we have also performed a two-stage spectrophotometric titration under 1 : 1 metal–ligand stoichiometry, using the same methodology cited above for the iron complexation (Fig. 4a). The batch titration (pH \(\leq 2\)) allowed the determination of the global stability constants and spectral data of the species GaH\(_5\)L and GaH\(_3\)L, while the second stage titration (pH > 2) enabled the determination of \(\beta\)GaHL and \(\beta\)GaL. Analysis of the speciation diagram for this system (Fig. 4b) discloses the presence of the bischelate GaH\(_3\)L as a major species for pH between 1.7 and 3 and above pH 3 the predominance of the trischelate as a monoprotonated (GaHL) or a non-protonated (GaL) species. Fig. 4b shows also that the fully protonated complex (GaH\(_5\)L) absorbs at 293 nm, while the absorbance at 250 nm is mostly due to the hexacoordinated gallium complexes (GaHL and GaL).

The 1 : 3 Ga\(^{3+}/\)HOPY-PrN system was studied by potentiometry, keeping constant the value of \(\beta\)GaHL previously determined by spectrophotometric titration under 1 : 1 M–L stoichiometric conditions, and the obtained Ga\(^{3+}\) complexation model is presented in Table 1.

The study of Al\(^{3+}\) complexation was performed by potentiometry using a 1 : 1 metal-to-ligand stoichiometric ratio (Fig. S2†). The Al\(^{3+}/\)NTP(PrHPM)\(_3\) titration curve shows an inflexion at \(a = 2\) (\(a\) is the ratio between the number of millimoles of a base and that of a ligand), which means that, as expected, the backbone amine is not involved in the metal coordination. Moreover, analysis of that titration curve also suggests that the deprotonation of that amine group may be followed by the formation of hydroxo species above pH 7. Nevertheless, once more the high chelating capacity of this ligand for Al\(^{3+}\) seems evidenced in Fig. 5, since the distribution curves show that the main forms of the aluminium complex above pH ca. 3.3 are tris-chelated species.

Comparison of the metal chelating capacity of ligands with different acid–base behaviour and denticity is usually made on the basis of the pM parameter (\(pM = −\log[M^{n+}]\), \(C_L/C_M = 10\), \(C_L = 10^{-6} \text{ M}\) at pH = 7.4). Unfortunately, the pFe value could not be calculated herein due to solubility limitations above pH 6.5, under our experimental conditions. This rendered impossible the determination of \(\beta\)FeL, which would result in a calculated pFe value at pH 7.4 with no real meaning. In order to try to make an adequate comparison of the iron chelating ability of this ligand with that of other ligands contained in Table 2, the pFe value was determined at pH 6.0. It was found that NTP (PrHPM)\(_3\) (pFe 19.7) is a stronger iron chelator than the drug.
DFP (pFe 15.4), although it is weaker for iron than the hexadentate tris-HP (pFe 23.7–24.5), the tetradentate bis-HP (pFe 21.6–22.5) or the commercial chelators EDTA, DTPA and DOTA. Concerning the pGa and pAl values determined for NTP(PrHPM)3, they are considerably high, albeit lower than those for the hexadentate HP analogues (NTP(PrHP)3 and NTA(BuHP)3). This is according to the reported trend of the bidentate HPM derivatives which usually present lower pM values than HP analogues (e.g. HOPY-PrN and DFP)16 (see Fig. 6).

Moreover, Table 2 shows that NTP(PrHPM)3 presents pM values in the range of the previously calculated values for bis-HP compounds (IDA(HP)2 and EDTA(HP)2), but evidencing higher affinity than other currently used drugs for Al decorporation (DFP, EDTA, DTPA, DOTA) or even for diagnostic probe containing68Ga (DFP, DFO, EDTA, DTPA, DOTA).

Table 2 also suggests that NTP(PrHPM)3 is able to compete, from the thermodynamic point of view, with transferrin for the complexation with those hard metal ions. Of course that this is a somehow limited view of the problem, since computational models predicted already significant time-dependent non-equilibrium binding of Al by ligands in competition with transferrin,36 which undoubtedly demonstrates the importance of both kinetic and thermodynamic control for metal distribution in biological systems.

In general, it can be concluded that NTP(PrHPM)3 is a better chelator for the hard metal ions studied than the drug DFP, being also more efficient for gallium and aluminium complexation than several currently used chelators, such as EDTA, DTPA or DOTA.

Molecular modeling of the Fe3+ complex

It is widely accepted that one of the best techniques to disclose the lowest energy structure of a molecule is by X-ray diffraction of single crystal. Unfortunately, until now all our efforts to obtain good crystals and get the 3D conformation of the ferric complex with NTP(PrHPM)3 revealed unfruitful. Hence, we have decided to use molecular simulations based on Density Functional Theory (DFT) to get the optimized structure for the Fe3+-NTP(PrHPM)3 complex.

The DFT modeling studies were carried out using Gaussian 03W software37 in two steps with the B3LYP hybrid functional. The first step was aimed mainly at optimizing the geometry of the ligand, while the second one, involving a more complex basis set (LANL2DZ), was more appropriate to treat the Fe atom. In order to perform the structural optimization of the Fe3+-NTP(PrHPM)3 complex, two structures were designed. The first one, in a so-called “in” conformation, displays the central N atom with its free orbital pointing inwards the complex structure, and the second one, with an “out” conformation, has the N atom pointing outwards. The two calculation processes converged and resulted in two different structures, which still maintained the original “in” and “out” conformations of the apical N atom. However, these two structures presented a difference in their global formation enthalpy of 0.0080008 Hartree (5.02 kcal mol⁻¹), the “out” conformation being the most stable (see the final structure in Fig. 7). Interestingly, from previous modeling (DFT calculations) of the corresponding Fe3+-NTP(PrHP)3 complex the “out” conformation also appeared as slightly more stable than the “in” conformation.19 This structure is slightly twisted (see Fig. 7, the right and left arms are drawn out and flattened over the back arm), which necessarily results in some energy penalty from this symmetry break and structure strain. However, such energy loss must be largely compensated by the stabilization energy afforded with the formation of three H-bonds between the three amide NH groups with one N atom and one carbonyl O atom of the HPM rings (2.48 and 2.18 Å, respectively), and with a carbonyl O atom of one arm (1.91 Å; see Fig. 7, black solid lines). Regarding the metal ion coordination sphere, the optimized structure maintained, as expected, the original

![Fig. 6 Metal complexation strength, reported as pGa versus pH, for some selected ligands.](image)

![Fig. 7 DFT-minimized structure of the complex Fe3+-NTP(PrHPM)3. The O–Fe metal coordination bonds are represented as purple lines and H-bonds as black lines. C atoms are grey, H atoms white, N atoms blue, O atoms red and Fe atom orange.](image)
input octahedral geometry with the ferric ion being chelated through the six HPM-O atoms of the ligand, and Fe–O bond lengths ranging between 1.92–1.97 Å. Concerning the final “in” geometry structure (not shown), this structure displayed an even more distorted and asymmetric geometry. It displayed two H-bonds between the NH atoms of two amide groups with one another and with one ring N atom, obviously not stabilizing enough to make it the lowest energy conformation of the Fe\(^{3+}\)-NTP(PrHPM)\(_3\) complex.

**In vivo assays**

The ability of the new hexadentate chelating agent, NTP (PrHPM)\(_3\), for *in vivo* mobilisation of \(^{67}\)Ga was assessed in mice as an animal model of metal-overload pathologies, owing to the well-known similarities between Ga\(^{3+}\) and other hard metal ions associated with those diseases (*e.g.* Fe\(^{3+}\) and Al\(^{3+}\)). In spite of the fact that the suitability of Ga as a substitute for Al in biological systems seems to be controversial, since some authors agree\(^38\) while others indicate different behaviours,\(^39,40\) the \(^{67}\)Ga radionuclide appears to be quite convenient for the bioassays and it is herein expected to give some clue about the *in vivo* efficacy of the ligand to mobilize those metals.

Biodistribution studies, at specific post-injection times, were carried out by i.v. administration of the radiotracer immediately followed by i.p. injection of the ligand solution, as previously reported by us.\(^23\) The \(^{67}\)Ga tissue distribution was compared to its pattern without simultaneous administration of any chelator (Fig. 8) and the most representative tissue distribution data are presented in Table 3.

Analysis of the biodistribution profiles clearly shows that the co-administration of the ligand NTP(PrHPM)\(_3\) and the radiotracer interferes in the usual tissue distribution of the radioactive metal, inducing a faster clearance from main organs and highly enhancing the overall excretion rate of radioactivity from whole animal body. Furthermore, no significant uptake was found in any major organ, except those related with excretory routes. The high rate of excretion is a relevant sign of the good capacity of this hexadentate ligand for the *in vivo* Ga\(^{3+}\) chelation. Hence, this favourable *in vivo* behaviour points to the potential usefulness of this ligand as a de-corporating agent of hard trivalent metal ions.

Comparison between the biodistribution data, due to the administration of the citrate radiotracer followed by injection of the ligand and due to the administration of the \(^{67}\)Ga–NTP-(PrHPM)\(_3\) complex (previously prepared with high yield and radiochemical purity) in the same animal model, at 4 h after injection (data not shown) evidences a very good analogy, especially on the uptake and clearance from main organs as well as the excretion rate. Thus, these findings indicate a rapid kinetics of *in vivo* complex formation and high affinity of the ligand to the metal ion.

The effect of this ligand on the metal uptake and clearance from the main organs of our animal model is illustrated in the histogram of Fig. 8 (at 1 h and 24 h after administration), in comparison with the corresponding effect due to the similar administration of analogues (the hexadentate NTP(PrHP)\(_3\) as well as the bidentates HOPY-PrN and the drug DFP).

Although the administration of both hydroxypyrimidinone-based compounds alters the metal biodistribution, the capacity for the \(^{67}\)Ga removal from tissues is obviously higher for the hexadentate ligand NTP(PrHPM)\(_3\) than the bidentate

![Fig. 8 Biodistribution data in the most relevant organs, expressed as % I.A./organ for \(^{67}\)Ga-citrate (i.v injection) and \(^{67}\)Ga-citrate with simultaneous intraperitoneal injection of the ligands NTP(PrHP)\(_3\), NTP(PrHPM)\(_3\), HOPY-PrN and DFP, 1 and 24 h after intravenous administration in female mice (n = 3–5).](image)

**Table 3** Biodistribution data in the most relevant organs, expressed as % I.A./organ for \(^{67}\)Ga-citrate and \(^{67}\)Ga-citrate with simultaneous intraperitoneal injection of the ligand NTP(PrHPM)\(_3\) at 15 min, 1 h, 4 h and 24 h, after intravenous administration in female mice (n = 3–5).

<table>
<thead>
<tr>
<th>Organs</th>
<th>(^{67})Ga-citrate</th>
<th>(^{67})Ga-citrate + NTP(PrHPM)(_3)</th>
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<tbody>
<tr>
<td>Blood</td>
<td>15 min</td>
<td>1 h</td>
</tr>
<tr>
<td>Blood</td>
<td>10.2 ± 2.3</td>
<td>3.2 ± 1.9</td>
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<tr>
<td>Liver</td>
<td>2.7 ± 1.3</td>
<td>1.8 ± 0.2</td>
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<tr>
<td>Intestine</td>
<td>4.0 ± 0.4</td>
<td>9.8 ± 1.6</td>
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<tr>
<td>Kidney</td>
<td>1.5 ± 0.4</td>
<td>0.9 ± 0.1</td>
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<tr>
<td>Muscle</td>
<td>20.2 ± 2.3</td>
<td>11.3 ± 3.7</td>
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<tr>
<td>Bone</td>
<td>13.8 ± 3.5</td>
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<tr>
<td>Excretion</td>
<td>6.7 ± 2.9</td>
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analogue HOPY-PrN. In fact, the administration of HOPY-PrN led to slower clearance on blood and soft-tissue, but higher bone retention and no improvement in the overall radioactivity excretion, thus excluding its potential use for chelating therapy.

A comparative analysis of the $^{67}$Ga mobilization induced by the hexadentate ligands shows that NTP(PrHPM)$_3$ led to slightly higher bone radioactivity accumulation than the 3,4-HP analogue. A further comparison between the biodistribution data obtained upon administration of the new ligand and of the chelating drug (DFP) evidences the ability of the new compound for a faster metal clearance from the main organs, especially from blood and muscle, as well as an enhancement of the overall metal excretion.

In summary, the high in vivo ability of NTP(PrHPM)$_3$ to complex with Ga, promoting its rapid clearance from main organs and fast overall excretion rate, anticipates its potential usefulness as a drug candidate for metal chelation therapy.

Conclusions

A new tripodal hexadentate hydroxypyrimidinone derivative – NTP(PrHPM)$_3$ – has been synthesized and studied in solution and in vivo in order to evaluate its capacity as a metal sequestering agent. The developed compound revealed high chelating capacity towards trivalent hard metal ions (Fe, Al, Ga), with pM values in the same order of magnitude of the tetradeutate bis-HP compounds and higher values than those of the clinically used drug DFP. Concerning the capacity of in vivo metal mobilization, NTP(PrHPM)$_3$ presents a favorable biodistribution profile, a high in vivo chelating efficiency and a faster clearance from main organs, especially from blood and muscle, as well as an enhancement of overall excretion, when compared with the drug DFP. The herein collected data give support to the potential interest of this chelator in detoxification of hard metal ions.

Experimental

General information and instrumentation

The chemicals were of analytical reagent grade, being used without further purification. Whenever necessary, the organic solvents were dried according to standard methods. Chemically pure solvents were dried according to standard methods. The chemicals were of analytical reagent grade, being used without further purification. Whenever necessary, the organic extracts were washed successively with 5% citric acid solution, H$_2$O, brine and dried over anhydrous magnesium sulfate. Evaporation of the solvent, followed by recrystallization of the residual solid from ethyl acetate, gave product 2 as a white amorphous solid (0.52 g, 74%). M.p. 162–164°C; IR (KBr pellets): $\nu$ max/cm$^{-1}$ 3375, 3259, 3127, 3031, 1711, 1643, 1502, 1170. $^1$H-NMR (400 MHz, CDCl$_3$): $\delta$ (ppm) 2.23 mmol) in dry THF (15 mL) was stirred for 16 h at reflux temperature, under a nitrogen atmosphere. The solvent was evaporated and H$_2$O added to the residue. The aqueous layer was extracted with CHCl$_3$ (5 × 20 mL) and the combined organic extracts were washed successively with 5% citric acid solution, H$_2$O, brine and dried over anhydrous magnesium sulfate. Evaporation of the solvent, followed by recrystallization of the residual solid from ethyl acetate, gave product 2 as a white amorphous solid (0.52 g, 74%). M.p. 162–164°C; IR (KBr pellets): $\nu$ max/cm$^{-1}$ 3375, 3259, 3127, 3031, 1711, 1643, 1502, 1170. $^1$H-NMR (400 MHz, CDCl$_3$): $\delta$ (ppm) 1.43 (9 H, s, OC-$\text{CH}_3$), 1.69 (2 H, q, $J = 6.4$ Hz, $\text{CH}_2\text{C}$($\text{H}_3$)$_2$), 3.20 (2 H, t, $J = 6.6$ Hz, $\text{CH}_2\text{C}$($\text{H}_3$)$_2$), 3.51 (2 H, t, $J = 7.0$ Hz, $\text{CH}_2$NHC(O)$_2$), 5.20 (2 H, s, PhCH$_2$), 5.35 (1 H, d, $J = 6.0$ Hz, NCHC(=CH$_2$)), 6.89 (1H, d, $J = 9.0$ Hz, NHC(=CH$_2$)), 7.38 (5 H, s, Ph-H); $^{13}$C-NMR (400 MHz, CDCl$_3$): $\delta$ (ppm) 28.4 (C($\text{CH}_3$)$_3$), 29.7 ($\text{CH}_2$CH$_2$CH$_2$), 30.94 (CH$_2$NHC(O)$_2$), 37.0 (PhCH$_2$), 78.0 ($\text{CH}_2$CH$_2$), 95.6 ($\text{C}$(CH$_3$)$_3$), 93.6 (NHC(=CH$_2$), 128.7 (Ph-C2 and Ph-C6), 132.5 (Ph-C5), 136.5 (Ph-C4), 145.7 (Ph-C3), 172.0 (NHC(O)$_2$), 181.8 (NO$_2$).
129.2 (Ph-C4), 130.1 (Ph-C3 and Ph-C5), 134.3 (Ph-C1), 142.5 (NCH=CHC), 153.2 (NC(O)N), 157.1 (NHC(O)O), 162.4 (N=CNH).

4-(3-Aminopropylamino)-1-(benzylxoy)-2(1H)-pyrimidine hydrochloride, 3. A solution of 4-[3-[(E)-3-hydroxysteroyl]amino]propylamino]-1-(benzylxoy)-2(1H)-pyrimidine 2 (0.5 g, 1.33 mmol) in 4 M HCl 1,4-dioxane (8 mL) was stirred at 0 °C under a nitrogen atmosphere until complete consumption of 1.33 mmol) in 4 M HCl 1,4-dioxane (8 mL) was stirred at 0 °C. This process was repeated 3 times to give product as a white solid (0.4 g, 98%). M.p. 169-171 °C; IR (KBr pellets): νmax/cm⁻¹ 3447, 3035, 1715, 1654, 1275. ¹H-NMR (400 MHz, CD₃OD): δ (ppm) 2.06 (2 H, q, J = 8.0 Hz, CH₂CH₂CH₂), 3.08 (2 H, t, J = 8.0 Hz, CH₂CH₂NH₂), 3.54 (2 H, t, J = 8.0 Hz, NHCH₂CH₂), 5.24 (1.50 H, s, C₆H₅CH₂), 5.27 (0.50 H, s, PhCH₃), 5.98 (0.75 H, d, J = 8.0 Hz, NCH=CHC), 6.26 (0.25 H, d, J = 8.0 Hz, NCH=CHC), 7.44-7.54 (5 H, m, Ph-H), 7.88 (0.75 H, d, J = 8.0 Hz, NCH=CHC), 8.19 (0.25 H, d, J = 8.0 Hz, NCH=CHC); ¹³C-NMR (400 MHz, CD₃OD): δ (ppm) 25.8 (CH₂CH₂CH₂), 36.6 (NHCH₂CH₂), 39.5 (CH₂CH₂NH₂), 79.2 (C₆H₅CH₂), 92.8 (NCH=CHC), 128.5 (Ph-C₂ and Ph-C₆), 129.3 (Ph-C₄), 129.9 (Ph-C₃ and Ph-C₅), 145.6 (NCH=CHC), 149.2 (NCO). 158.3 (N=CNH).

3,3′,3″-Nitrotril(N-3-(1-benzyloxy)-2-oxo-1,2-dihydropyridin-4-ylamino)propyl)-propanamide, 4. A mixture of 3,3′,3″-nitrotrilopropanoic acid (NTP, 0.097 g, 0.414 mmol), BTBU (0.43 g, 1.37 mmol) and N-methylmorpholine (NMM, 0.30 mL, 1.27 mmol) in dry DMF (15 mL) containing molecular sieves was stirred at r.t. for 1 h. Meanwhile, a solution of 3 (0.450 g, 1.37 mmol) and NMM (0.30 mL, 2.73 mmol) in dry DMF (10 mL) also containing molecular sieves, was stirred at r.t. for 1 h. The first mixture was filtrated, the solution was added dropwise to the second one and the mixture was stirred under N₂ for 6 h. The final mixture was evaporated under vacuum. The crude material was taken into 0.1 M HCl (75 mL) and it was washed with CH₂Cl₂ (4 × 50 mL). Concentrated ammonia was added until a precipitate appeared (pH ca. 4), and it was extracted with CH₂Cl₂ (4 × 75 mL); the solution was added until a precipitate appeared (pH ca. 8–9), and the solution was extracted with more CH₂Cl₂ (3 × 75 mL). The total organic phase was dried over anhydrous Na₂SO₄, and then evaporated. The residue was washed with water (3 × 5 mL), and then with acetone. After recrystallization from MeOH/acetone, the final product was obtained, as a beige solid (0.153 g, 37% yield). M.p. 171 °C; IR (KBr pellets): νmax/cm⁻¹ 914, 13447, 3033, 1735, 1654, 1275. ¹H-NMR (400 MHz, CD₃OD): δ (ppm) 1.57 (6 H, q, J = 6.8 Hz, CONHCH₂CH₂CH₂), 2.21 (6 H, t, J = 6.6 Hz, NCH₂CH₂CO), 2.61 (6 H, t, J = 6.6 Hz, NCH₂CH₂CO), 3.03 (6 H, q, J = 6.6 Hz, CONHCH₂CH₂CH₂), 3.19 (6 H, t, J = 6.8 Hz, CONHCH₂CH₂CH₂), 4.94 (6 H, s, C₆H₅CH₂), 5.41 (3 H, d, J = 7.6 Hz, NCH=CHC), 7.16 (3 H, d, J = 7.6 Hz, NCH=CHC), 7.19-7.26 (15 H, m, C₆H₅), 13C-NMR (400 MHz, CD₃OD): δ (ppm) 29.8 (CONHCH₂CH₂CH₂), 34.7 (NCH₂CH₂CO), 37.8 (CONHCH₂CH₂CH₂), 39.3 (CONHCH₂CH₂CH₂), 50.7 (NCH₂CH₂CO), 79.5 (C₆H₅CH₂), 95.7 (NCH=CHC), 129.8 (Ph-C₂ and Ph-C₆), 130.4 (Ph-C₄), 131.3 (Ph-C₃ and Ph-C₅), 135.3 (Ph-C₁), 144.0 (NCH=CHC), 155.8 (NCO), 164.4 (N=CNH), 175.0 (NCH₂CH₂CO).

Potentiometric studies

Measurements. Titrations of the ligands, alone and in the presence of aluminum (for NTP(PrHPM)_3) or of gallium (for HOPY-PrN), were performed in aqueous solution at T = 25.0 ± 0.1 °C and ionic strength (i) 0.1 M KCl. For all the titrations involving NTP(PrHPM), the total volume was 20 mL, the ligand concentration [L] was 10⁻⁴ M and C₆H₅/C₆H₄ was 0.1:1, 1:1.1 or 1:1.2. For the Ga⁳⁺/HOPY-PrN system, the total volume was 20 mL, the ligand concentration was 1.0 × 10⁻⁴ M and C₆H₅/C₆H₄ was 1:0.1–2.0 × 10⁻³ M and C₆H₅/C₆H₄ was 1:3. Each assay was done twice and the value determined for the ionization constant (pKᵢ) was 13.8.

Calculation of equilibrium constants. The stepwise protonation constants, Kᵢ = [HLᵢ]/[HLᵢ₋₁][H⁺] (i = 1–7), and the overall metal-complex stability constants, βᵢ[MₙLₙ₋₁] = [MₙHᵢLᵢ]/[M]ⁿ[H]ᵢ[L]ᵢ, were determined by fitting analysis of the potentiometric data with the HYPERQUAD 2008 program. Al³⁺ and Ga³⁺ hydroxide species were included in the equilibrium complexation model and the species distribution curves were obtained with the HYSS program.
The structure of the Fe$^{3+}$

Molecular modeling

valence double-zeta basis set (D95V) on the first row (all

(PrHPM)$_3$ and HOPY-PrN were determined by the

ent terms).52,53 Regarding the basis set, 3-21G is the simplest

Parr correlation functional (which also includes density gradi-

method,47,48 which is based on the concentration ratio of the

gallium complex stability constants of NTP(PrHPM)$_3$, as well

bands (π–π$^*$) of the compounds.

Determination of partition coefficients

The ocaption–water partition coefficients ($\log P$) of NTP-

(PrHPM)$_3$ and HOPY-PrN were determined by the “shakeflask”

method,47,48 which is based on the concentration ratio of the

compounds between 1-octanol and a TRIS buffered aqueous

phase ($pH = 7.4$). The species concentrations were assessed by

spectrophotometry, based on the absorbance of the benzenoid

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spectrophotometry, based on the absorbance of the benzenoid

bands (π–π$^*$) of the compounds.

Molecular modeling

The structure of the Fe$^{3+}$–NTP(PrHPM)$_3$ complex was opti-
mized by quantum mechanical calculations based on Density

Functional Theory (DFT) methods, using the Gaussian-03W

software.37 This energy minimization was carried out using the

functional B3LYP chemical model, and it was performed in
	wo steps. In the first step the 3-21G basis set was used with a

direct self-consistent field method (SCF) and SCF convergence
criterion of $10^{-5}$. The results of these calculations were

subjected to a deeper second optimization using the LANL2DZ
basis with the same SCF settings. The functional

B3LYP has been shown to be an accurate density functional

method,49 and it has proved to give reliable geometries and

energies for complexes with several metal ions, namely tran-
sition metal ions.50 The B3LYP model is a combination of the

Becke three-parameter hybrid functional51 with the Lee–Yang–
Parr correlation functional (which also includes density gradi-

tent terms).52,53 Regarding the basis set, 3-21G is the simplest

of Pople’s split-valence basis sets, and it can be used with rela-
tively good accuracy for molecules containing first and second-

row elements.54 It has been used in our first calculation step,

mostly in order to optimize the conformation of the ligand.

The LANL2DZ basis set specifies the Dunning–Huzinaga

valence double-zeta basis set (D95V) on the first row (all

the atoms of the ligand),55 and Los Alamos ECP plus DZ on

Na–Bi.56 In this way, the Fe atom is described through the Los

Alamos ECP and a double-zeta basis set including 3d orbitals

and 3d diffuse functions for the valence shell.

Biodistribution studies

In vivo biodistribution studies were carried out in groups of

3–5 female CD1 mice (randomly bred, Charles River, from

CRIFFA, Barcelona, Spain) weighing ca. 25 g. $^{67}$Ga-citr

solution was prepared by dilution of $^{67}$Ga citrate from

MDS Nordion (Ottawa, Canada) with saline to obtain a final

radioactive concentration of 5–10 MBq per 100 $\mu$L. The $^{67}$Ga–

NTP(PrHPM)$_3$ complex was synthesized by adding $^{67}$Ga-citr

to a saline solution of NTP(PrHPM)$_3$ and the radiochemical

purity, superior to 95%, was determined by ITLC, as reported

before. Mice were intravenously (i.v.) injected with 100 $\mu$L

(5–10 MBq) of $^{67}$Ga citrate via the tail vein. In a separate group

of animals the i.v. administration was immediately followed by

intraperitoneal (i.p.) injection of 0.5 $\mu$L of the ligand in

100 $\mu$L saline solution. Biodistribution of the $^{67}$Ga–NTP–

(PrHPM)$_3$ complex was assessed by i.v. injection of 100 $\mu$L of

the complex solution previously prepared. Animals were main-
tained on normal diet ad libitum and were sacrificed by cervi-
cal dislocation at 15 min, 1 h, 4 h and 24 h post-

administration. The administered radioactive dose and the

radioactivity in sacrificed animals were measured in a dose

calibrator (Aloka, Curiometer IGC-3, Tokyo, Japan). The differ-

ece between the radioactivity in the injected and sacrificed

animal was assumed to be due to whole body excretion. Tissue

samples of main organs were then removed for counting in a

gamma counter (Berthold LB2111, Berthold Technologies,

Germany). Biodistribution results were expressed as percent of

injected activity per total organ (%L.A./organ) and presented

as mean values ±SD. For blood, bone and muscle, total

activity was calculated assuming, as previously reported, that

these organs constitute 7, 10 and 40% of the total weight,

respectively.

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