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Review

Recent developments on 3-hydroxy-4-pyridinones with respect to their clinical applications Mono and combined ligand approaches

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Contents

Abstract

There has been considerable research effort invested in the discovery and development of chelators for the treatment of serious pathological disorders associated with iron (or aluminium) overload in the past two decades. A series of 3-hydroxy-4-pyridinone (3,4-HP) iron-ligands, in particular bis-(3,4-HP)s and mono-(3,4-HP)s were developed by exploring their polydenticity and/or bifunctionality. These compounds were assessed for their physicochemical and biological properties such as the chelating ability for M^{3+} *hard* metal ions (M = Fe, Al, Ga), the lipophilicity (log *P*) and the metal-clearing efficiency (MCE) from most organs in mice pre-loaded with ^{67}Ga, as a model of Fe-overload. Although the solution chemistry and the *in vivo* studies have been performed with individual ligands, a ligand combination strategy with the bis- and mono-(3,4-HP) was adopted to improve the scavenger power based on differences on their cellular-compartment accessibility. The results lead to key recommendations useful in chelator design strategies because there are significant differences in chelating affinity and MCE in mice between the bis-HP and the mono-HP ligands. The extra-functional groups of the compounds have a profound effect on log *P*, MCE and organ distribution, and so they can be targeted to organs compromised in iron-overload disease, for example, the liver or the brain. The coadjuvation or synergistic effects of the ligand combination is supported by the observed improvements on metal excretion in bioassays.

Keywords: 3-Hydroxy-4-pyridinones; Iron chelators; Chelating agents; Combined chelation

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1. Introduction

The development of metal chelators for clinical applications has been an important research area, either as chelating agents, to target specific metal ions in the body, or as metal-carriers for therapeutics or imaging purposes. The 3-hydroxy-4-pyridinones (3,4-HP) are a class of orally active therapeutic iron chelators, whose major importance emerged after the identification in 1982 of one of its members, the Deferiprone (DFP) [1], as an alternative to desferrioxamine in the treatment of iron-overload associated diseases. The 3,4-HPs are mono-anionic *N*-heterocyclic bidentate $\{O,O\}$-chelators with high affinity for *hard* metal ions, and they can be easily extrafunctionalized to modify the bioavailability of the drugs or to assist the targeting of specific tissues. A large number of bidentate or polydentate chelators have been developed, mostly for the decorporation of toxic amounts of iron [2,3], but selected analogues were also used for the removal of other hard metal ions such as Al [4,5], Pu [6]. The complexes of 3-hydroxy-4-pyridinones with several metal ions in a number of studies are used aimed at the delivery of metal ions into the body for diagnostic or therapeutic purposes [7].

This review, will include a summary of recent developments on 3,4-HP derivatives with potential clinical applications, mostly as chelating therapeutics for metal unbalance related diseases, namely those due to disorders of metal metabolism, such as beta-thalassemia, hematochromatosis (Fe), neurodegenerative diseases (Fe and Al), but also for protection against insult toxicity due to metallodrugs or environmental exposure (Al, Pu). Some references will be also made to recent developments on 3,4-HP metal complexes as potential metal-based diagnostic pharmaceuticals (Ga, In). Special emphasis will be given to aspects related with design strategies of the compounds (ligand-based and target-based strategies) and their relevance for the physicochemical properties (e.g. metal chelating affinity and lipophilic character), metal biodistribution and clearance kinetics. Insights on structure–activity relationships are included as well as on new developments simulating combined-ligand therapy approaches.

2. Metal ions in biology and medicine

All organisms and cells require essential elements, such as the transition metal ions iron, zinc and copper, for their normal growth and development. Iron is the major trace element in the body, whose functional roles include the provision of a specific binding site for oxygen in hemoglobin and the cofactor of important enzymes such as cytochrome *c* and ribonucleotide reductase [8]. There are many zinc-dependent enzymes, as for example the matrix metalloproteinases involved in the extracellular matrix degradation, and the superoxide dismutase, an important antioxidant enzyme that requires Zn and Cu for catalytic function. In healthy tissue, free metal ion concentrations are maintained at very low level by efficient homeostatic mechanisms and buffers, controlling metal compartmentalization, regulating metal release and trafficking. Any disturbance of the transition metal ion homeostasis in the body, specially the appearance of "free" non-protein-bound (NPB) iron in blood and tissues, can promote the formation of reactive oxygen species (ROS) and initiate the metal-catalysed protein and lipid oxidation and aggregation, causing various diseases.

There are also many non-essential metal ions which can be introduced in the body either by environmental exposure (Al, Pu) or as metallodrugs administered for therapeutical or diagnostic purposes. If accumulated, they can be very toxic due to competition with essential metal ions.

2.1. Iron overload and toxicity

Iron overload may result from excess of iron uptake, storage and accumulation and it can lead to iron related toxicity and damage of various organs, namely liver and heart [8,9]. Iron overload is characterized by an excess in total body iron (normal 35–45 mg/kg of body weight [10]). Chronic overload occurs mostly in patients with genetic defects, either associated with the increase of gut iron absorption (hemochromatosis) or with the impaired synthesis of hemoglobin (thalassemia major). Regular red blood cell transfusions are required. Since humans cannot effectively excrete iron, this leads to a progressive iron loading (transfusional hemosiderosis). In conditions of iron overload, there is an increase in transferrin iron saturation in plasma, which is accompanied by increase of non-transferrin-bound-iron (NTBI) and its deposition in cells and several organs (e.g. liver, pancreas and heart) resulting in their dysfunction, and death ensues, unless some treatment is made for the removal of excess iron from the body.

The appearance of "free" non-protein-bound iron (NPBI) in blood and tissues has been associated with its participation in redox reactions (by Fenton reaction) which increases the generation of reactive oxygen species (ROS) such as the hydroxyl radical. This radical species can initiate cell injury (e.g. oxidation of sugars, lipids, DNA damage, protein oxidation and misfolding) and cause various pathological disorders [11]. In normal individuals the generation of ROS is controlled by defence antioxidant systems (SOD, catalase, GPX [12]) (see Fig. 1), but the same does not apply in iron-overload patients. There are other disease situations related with misplaced iron in specific cells and tissues [13], for example, cancer [14] and neurodegenerative disorders such as Parkinson's (PD) and Alzheimer's (AD) diseases [15]. Iron chelation therapy can give a determinant contribution to the prevention of the free radical damage, responsible for several pathological disorders.

2.2. The Group 13 metal ions (Al, Ga, In): disease and medicine

Unlike iron, the trivalent Group 13 metal ions are redox-inactive and non-essential, but also of biological interest. Besides the differences between $Fe^{3+}$and these M^{3+} cations (M = Al, Ga, In) they are considered as *hard* metal ions [16], exhibiting essentially similar chemical behaviour in aqueous solution and binding to mammal proteins, namely the human transferrin (Tf) which plays a central role in the transport of the ferric ion between sites of uptake, utilization and storage. Since

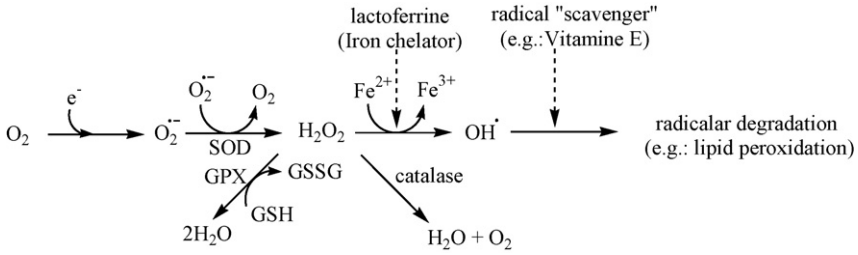


Fig. 1. Reactive oxygen species: generation and destruction (from Ref. [12]).

normal blood contains ca 30 μM serum Tf, which is only ca 30% iron saturated [10], it still has a substantial capacity for chelating other trivalent metal ions, such as the Group 13 cations. In this context, the Al, Ga and In cations can be of large biological importance.

Although aluminium plays no essential role, it is harmful if accumulated in the organism [17–19]. Its threat to human health can result from environment because, due to its large abundance in earth crust, acid rain may interfere in the biogeochemical cycle of Al, making it bioavailable namely in drinking water and the food chain [20]; or from therapeutic-base contamination (e.g. dialysed solutions and anti-acid drugs) [21,22]. Aluminium has been associated with the etiopathogenesis of several neurological disorders, such as dialysis encephalopathy syndrome [23,24] and eventually Alzheimer's disease [25,26].

In spite of the controversial link between aluminium and AD [26], the Al^{3+} content in these patient's brains is considerably increased and it has been recently assumed that it can contribute (or promote) the iron induced neuronal oxidative damage [27,28]. In fact, apart from other reasons, Al^{3+} can compete with Fe^{3+} for the same ligands in biofluids, thus influencing the availability of the ferric ion, the redox cycling of iron and its homeostatic mechanisms, and so its concomitant oxidative neurotoxicity.

Gallium and indium radionuclides, when chelated with adequate ligands, have important applications in medicine namely in diagnostic radiopharmaceuticals, due to their emitting properties (gamma and positron emission) and to their suitable half-lives [29]. The main gallium interest is associated with two radiodiagnostic imaging, namely ^{67}Ga (γ, $t_{1/2} = 3.25$ d) for SPECT (single photon emission computed tomography) and, specifically, ^{67}Ga-citrate for imaging of soft tissue tumors and skeletal disordors [30]; ^{68}Ga (β^+, $t_{1/2} = 68$ m) for PET (positron emission tomography) [31]. Ga(III) nitrate has also been used for the treatment of malignance-associated hypercalcemia [32]. ^{111}In (γ, $t_{1/2} = 2.85$ d) is widely used as radiopharmaceutical for SPECT imaging and also for blood cell (leucocyte) labelling [33].

3. Chelating drugs for Fe^{3+}/Al^{3+}- past and present

In iron-overload situations, namely when the body burden of iron increases beyond normal levels, the function of vital organs is impaired and can lead to death, unless some artificial removal of iron is accomplished. While phlebotomy (periodical blood removal) is still a usual treatment for idiopathic hemochromatosis, chelating therapy is the only method for the removal of excess iron in transfusional hemosiderosis (thalassemia major) or of misplaced iron in specific cells and tissues, promoting hepatic diseases, cardiomyopathy, neoplasia and a series of neurodegenerative diseases. Although less usual, Al can also accumulate in the body and it has been associated with a number of human diseases in connection with neurological dysfunctions, such as dialysis encephalopathy and eventually AD.

In any case, either due to a genetic disorder or an excessive external exposure, the excess of any of these *hard* metal ions requires chelating therapy. Furthermore, chelators of *hard* metal ions have been associated with many other relevant roles in disease and medicine (Fig. 2).

3.1. Chelating agents for iron overload

Desferioxamine (DFO) is a microbial tris-hydroxamate siderophore (isolated from *Streptomyces pilosus* [34]) which, for decades (since 1960), was the only chelating agent for decorporation of iron in thalassemic or other iron-excess related diseases. However, the drawbacks associated with the use of DFO, such as high cost, toxicity and low patient compliance (due to needs of prolonged subcutaneous infusion to achieve adequate plasma concentrations) [35], lead to intensive research on orally active and non-toxic chelators over the past 30 years [2–4,12]. Major developments in the design of new chelators occurred on 3-hydroxy-4-pyridinones (HP) derivatives, namely after the disclosure of 1,2-dimethyl-3-hydroxy-4-pyridinone (Deferiprone, DFP) [1] and the first report of its use in humans for the treatment of thalassemia [1]. Unlike DFO, an hexadentate ligand able to form very stable 1:1 iron complexes (pFe = 26.5 [36] see Table 1), DFP is a bidentate chelator but also forms quite stable neutral 3:1 (DFP:Fe^{3+}) iron complexes at the physiological pH

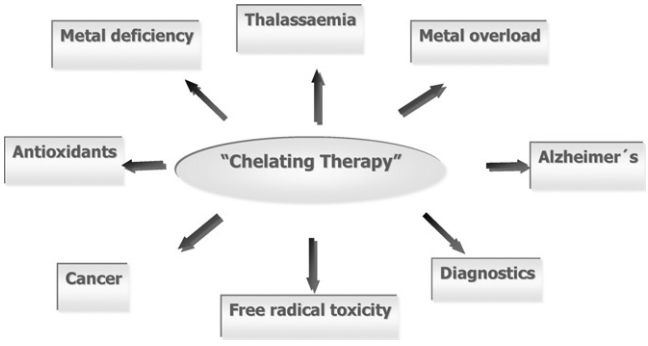
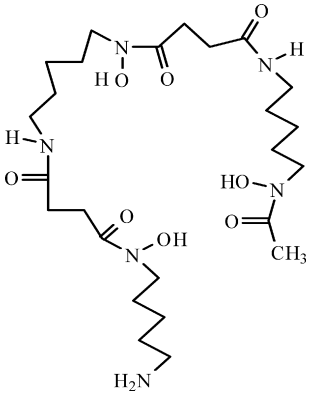
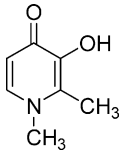
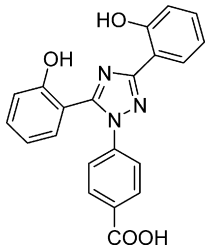


Fig. 2. Chelation of *hard* metal ions: roles in disease and medicine.

Table 1
Structure of iron(III) selective ligands used in clinical use and summary of relevant data

		
DFO, Desferioxamine-B (Desferal, NOVARTIS)	DFP, Deferiprone (Ferriprox, APOTEX)	ICL670 (Deferasirox, NOVARTIS)
Hexadentate ligand: FeL Oral inactivity Used over last 30 years Intravenous administration High cost Poor compliance	Bidentate ligand: FeL_3 Oral activity Commercialized (10 years) (Europe 2003)	Tridentate ligand: FeL_2 Oral activity Approved by FDA 2005 Intermediate cost
Log $\beta = 30.6$; pFe = 26.5[a]	Log $\beta_3 = 36.3$; pFe = 19.4[b]	Log $\beta_2 = 38.6$; pFe = 22.5[c]

[a] Ref. [36].
[b] Ref. [37].
[c] Ref. [39].

(pFe = 19.4 [37]). It was recently approved in several countries, including EU (1999), for the treatment of chronic iron overload, [38]. Many other iron chelators have also been reported in the literature [2–4,12], including tridentate ligands able to form 2:1 (chelator: Fe^{3+}) complexes [14], but only ICL670 (deferasirox, pFe = 22.5 [39]) was recently approved (2005) by FDA (phase IV clinical testing) [40]. However, the still existence of some adverse effects, which have been associated with the use of DFP [41] or ICL670 [42], has led to pursuit of newer chelators and treatment protocols but also combinations of new or already known chelators aimed at possible cross or synergistic actions [43].

The combined use of DFO and DFP was first used in some specific cases of iron-overload patients who have no response to normal therapies with those chelators [32]. Since both chelators may interact either in an additive or synergistic manner, when they are coadministered, and eventually shuttle iron from DFP to DFO [44], the combination therapy has become a regular treatment for transfused iron-overload patients [45,46].

3.2. Chelators for aluminium decorporation

Concerning aluminium accumulation in the brain, the similarities between Fe^{3+} and Al^{3+} (ionic radios 64 and 50 pm, respectively) also justify the utilization of Deferrioxamine or Deferiprone as individual drugs [5,47,48] for the mobilization and the promotion of aluminium excretion. The combination of these two drugs also showed promising results in preclinical tests with Al-loaded rats [49,50]. Other binary or ternary combinations, such as ascorbate (AS) and DFO and/or FG (Feralex-G, a glycosyl 3,4-HP derivative), have also been recently tested *in vitro*, and the results suggested they could reverse Al-induced neurological disorders [51,52].

3.3. Iron chelators for other potential utilities

Selected iron chelators, including hydroxypyridinone and hydroxypyrone derivatives, have been studied as complexes with other metal ions for potential diagnostic imaging or therapeutic purposes [7]. For example, chelates with γ- or β^+-emitting radionuclides (e.g. ^{67}Ga/^{68}Ga [5,53], ^{111}In, ^{99m}Tc [54]) for radiodiagnostic imaging); or chelates with the hard paramagnetic lanthanides (Gd) are used as contrast agents in magnetic resonance imaging (MRI), due to the short longitudinal relaxation time of the bound water molecules in tissues [55]; the Zn [56], Mo [57,58] and V [59,60] chelates have been studied for potential therapeutic application as insulin-mimetics.

4. Hydroxypyridinones as chelating drugs. General properties and methodologies

4.1. Determinant characteristics of 3,4-HPs

The hydroxypyridinones (HP) are monoionic ligands with an *alpha*-hydroxy ketone group, enabling a $\{O,O\}$ bidentate metal-coordination with formation of neutral 3:1 complexes with M^{3+} cations. Among the three classes of hydroxypyridinones, 1-hydroxy-2-pyridinones (1,2-HP), 3-hydroxy-2-pyridinones (3,2-HP) and 3-hydroxy-4-pyridinones (3,4-HP), the 3,4-HP are by far the most studied class of HPs. This is due to the highest

basicity of the hydroxyl group (pK_a ca 9–9.5) and with highest electronic density at the coordinating atoms, making them neutral at the physiological pH and also with the highest affinity for *hard* metal ions [2].

The 3,4-HPs have been widely accepted as iron (or aluminium) chelating drugs, because they are *hard* bases with high specificity for *hard* trivalent metal ions and will not deplete other *soft* biologically relevant bivalent metal ions (e.g. iron, zinc, copper). They can also be easily modulated to satisfy several important requirements, such as: (a) adequate lipo-hydrophilic balance; (b) oral activity) [61]; (c) efficient bioavailability at the disease sites; (d) chelate excretion by urine and/or bile; (e) easy incorporation of molecular vectors to specific biological targets and (f) availability at a reasonable cost.

4.2. Methodologies for the development and the evaluation of 3,4-HPs

As mentioned above, in iron excess related diseases (due to chronic iron overload or misplaced iron), the metal ion can be accumulated in different sites, namely in: extracellular compartments (e.g. serum transport proteins, Tf, or NTBI), intracellular storage proteins (e.g. ferritin in cytoplasm); brain within microglia [62]. Accordingly, different types of 3,4-HP chelators have been rationally designed and developed to meet some specific bio-targeting and chelating needs. Although, at a first stage, only bidentate DFP analogues were developed, more recently, polydentate chelators, namely with three or two bidentate moieties, have also been studied, aimed at increasing their metal affinity, while trying to keep under control some other important properties (see below). In some cases molecular modelling is used (molecular mechanics or molecular dynamics) to assist the design of the chelators or to foresee the structure of the metal complex (in absence of X-ray crystal data) to get an insight in the coordination modes and in the rationalization the experimental results.

The preparation of these compounds involves the usual organic synthesis procedures. The preparation of mono-(3,4-HP) derivatives, invariably, results from aminolysis of the corresponding *O*-protected pyrone with a primary alkylamine derivative, the *O*-deprotection being the last step. Concerning the polydentate compounds, bidentate units are attached to selected backbone scaffolds, usually aided by coupling activation reagents [4,53]. Standard spectroscopic and potentiometric techniques are used for the assessment of the physicochemical properties in solution [5], such as the pK_a values, octanol/water partition coefficients (log P) and metal-complexation capacity, log β or preferably pM (pM = $-\log [M^{3+}]$), typically calculated at pH 7.4 for conditions of micro molar concentration of the metal ion and 10-fold ligand excess. The pM values are used for comparison of chelating capacity of the ligands because, unlike the stability constant (log β) values, they take into account the effects of ligand protonation, denticity and differences in metal-ligand stoichiometries.

Regarding the cellular and animal models for metal mobilization, radio-labelling is the most usual methodology, such as radioisotope-proteins or -carriers for mice metal-overload (e.g. ^{55}Fe, [62], ^{59}Fe [63] or ^{67}Ga [5,31] with subsequent analysis of the radiometal contents in the biological samples by γ-emission counting. Non-radioemitting metal ions can also be used in some cases (e.g. Al-overload of rabbits [47] or rats [48]), but in this case the evaluation of the metal-contents in the biological samples requires much more laborious sampling and analytical procedures (e.g. ICP–MS or atomic absorption).

5. Recent developments on 3-hydroxy-4-pyridinone chelators

Among the several strategies that have been used for the rational design and development of 3,4-HP chelating agents, the first approach has been mostly based on the functionalization at the ring-*N* atom to allow the tuning of physicochemical and biological properties, aimed at guaranteeing an adequate biodistribution in specific organs and/or some ability for molecular recognition by specific biological sites (*target approach*), without compromising the thermodynamic properties of the complexes. To improve the chelating efficacy, the polydenticity of this family of compounds has also been explored by attaching two or three bidentate 3,4-HP chelating moieties to a series of backbone skeletons to afford tetradentate or hexadentate 3,4-HP ligands (*ligand approach*). The combination of these two design strategies has been recently explored either as individual ligands (*target–ligand approach*) or as a combination of two 3,4-HP ligands with complementary properties (*combined chelator approach*) (see Fig. 3).

5.1. The target approach

The target approach mostly involves the attachment of a functional group to a bidentate ligand of DFP type. The great interest in the development of new 3,4-HP iron chelators relies not only to the their recognized suitability for sequestration of iron (and related *hard* metal ions), but also for *N*-functionalization. This last criterion is particularly easy for the mono-(3,4-HP) derivatives (hereafter generally named as L^m), allowing the tailoring of desired physicochemical and biological characteristics of the ligands, namely in terms of the lipo-hydrophilic balance,

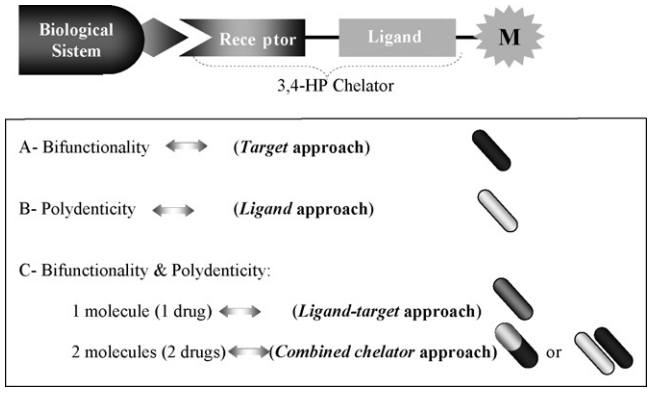


Fig. 3. Schematic strategies for metal (M) 3,4-HP chelators for biological systems.

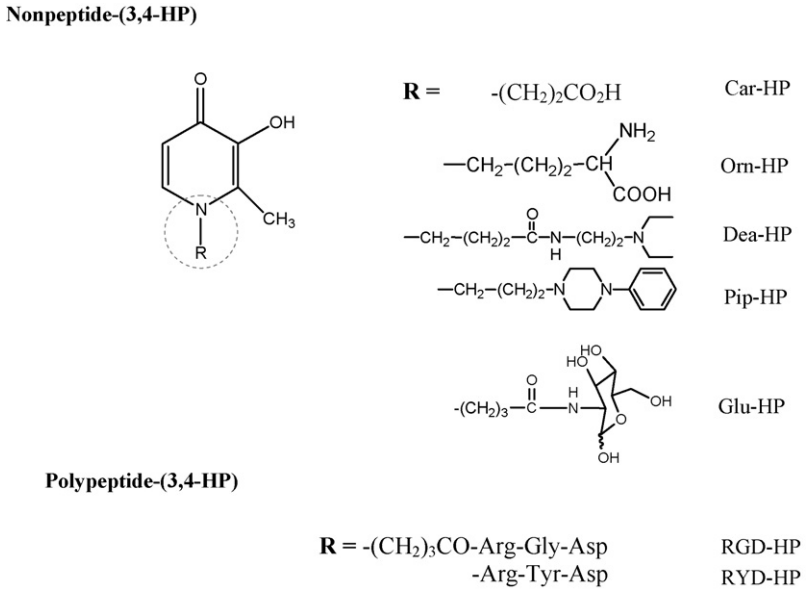


Fig. 4. Structural formula of some examples of bifunctionalized mono-(3,4-HP)s.

molecular vectorization to specific bioreceptors, endogenous BBB transporters and also organs. Although the mono-(3,4-HP)s are bidentate chelators, they still possess a considerable metal-binding affinity and have the advantage of low molecular weight, which favours the absorption from the gastrointestinal tract. Many L^m ligands have been developed by different research groups, some of them mostly designed to explore the lipo-hydrophilic balance [2,4], but others with specific targeting purposes, such as the carbohydrate and the nucleoside derivatives. The interest on the glucosylic 3,4-HPs (e.g. Feralex-G, Glu-HP) can be attributed either to the reported specific interaction with hyperphosphorylated brain cell nuclear matrix [64], disrupting protein aggregation (formation of fibrilary tangles), in combination with the potential remotion of Al/Fe accumulated in AD brains [52]; or to the carbohydrate interaction with specific glucose transporters, over-expressed in certain tumor types, and so the corresponding radiometal-complexes may find application as diagnostics [65]. Regarding the 3,4-HP nucleoside derivatives, their interest is attributed to the association of iron–chelation with anti-HIV roles of the nucleoside moiety [66].

The set of L^m analogues developed in our group, involves a diversity of *N*-attached extrafunctional groups, namely non-peptidic (e.g. alkylcarboxylic acids, alkyl-arylamines, alkyldiethylamines, alkyl-arylpiperazines, alkyl-aryl-aminoacids, alkyl-aryl-sulphonamides, alkylglucoside) monopeptidic and bioactive polypeptidic groups (see examples on Fig. 4).

Solution studies and bioassays with these non-peptidic and monopeptidic 3,4-HP derivatives showed that the *N*-bearing groups slightly affect the binding (pFe = 17–22; pAl = 14–16) but retain the high chelating efficacy; some variations were obtained on the water/octanol partition coefficient (ca three orders of magnitude; $\log P \sim [-2, +1]$), on the complex speciation around the physiological pH range as well as on the biodistribution and clearance rate of metal-load animals [5,67,68].

The considerable differences in the *in vivo* behaviour (mice) of the set of bifunctional chelators and/or the corresponding ^{67}Ga-complexes suggest different potential clinical applications. Thus, while some derivatives with pronounced specific organ uptake (e.g. Car-HP showed bone uptake) can be potentially useful as an imaging radiopharmaceutical, others, with rapid *in vivo* ^{67}Ga chelation, improved specificity on biodistribution and rapid metal clearance from most tissues, can be potentially useful as metal decorporating agents (e.g. Orn-HP). From the biodistribution data obtained for the bifunctionalized non-peptididic mono-(3,4-HP)s [5], it was possible to make some rationalization of the results and in some cases to correlate them with the physicochemical properties of the ligands. As an example, Fig. 5 shows that, for a set of ligands (H_iL^n: n = 1–4; 6–9, 11; from Ref. [5]) the liver retention of the radionuclide (expressed as the percentage of injected dose in liver, % DI/liver, upon iv administration of the ^{67}Ga–citrate and then ip adminis-

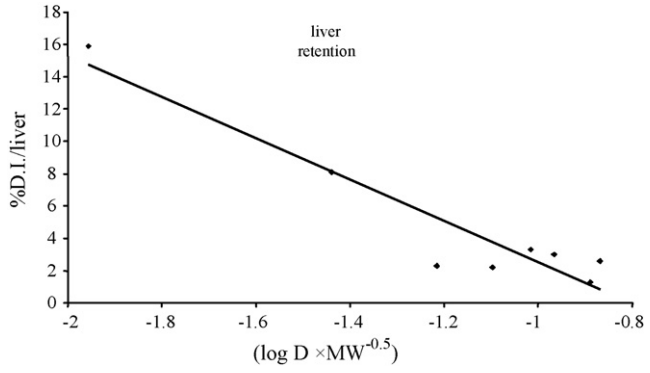


Fig. 5. Linear correlation between the $\log P$, the MW of the ligands ($\log P \times MW^{-0.5}$) and the radionuclide liver retention (%D.I./liver), at 1 h after administration of the ^{67}Ga–citrate and the ligands in mice.

tration of each ligand) is low but presents some structure–activity relationship with the partition coefficient log *P* at the physiological pH and molecular weight ($\log P \times MW^{-0.5}$).

Among the bifunctionalized non-peptidic 3,4-HPs studied, some special mention should be given to the alkyldiethylamine, ornithine, arylpiperazine [5,68] and the glucoside derivatives. The glucoside derivative (Glu-HP) is an analogue of Feralex-G but with expected improved properties (lipophilicity and chelation) because of the increase alkylic spacer size (changed from one to three methylene groups). Concerning the alkyldiethylamine derivative (Dea-HP), its interest is due to their potential recognition by *sigma* receptors which are over-expressed by certain solid tumor [69]. However, our solution and biodistribution studies did not reveal any relevant behaviour for this type of compound, probably because of some insolubility problems. The ornithine derivative (Orn-HP) ($R = (CH_2)_3CHNH_2CO_2H$) has the highest metal chelating efficacy ($pFe_{Orn\text{-}HP} = 21.9$; $pFe_{DFP} = 19.3$) and is quite hydrophilic ($\log P < 2$). It has a favourable speciation profiles in aqueous physiological conditions (pH 7.4), because the ligand is in zwitterionic form and the chelate formed is neutral and water-soluble. *L*-DOPA is a drug used to alleviate Parkinson's disease symptoms. The ornithine derivative (Orn-HP) has some structural similarities with DOPA (dihydroxyphenylalanine) and is of interest due to its eventual recognition by dopaminergic receptors. However, its high hydrophilicity limits the potential application of the compound as neuroprotective iron chelator drug in antiparkinsonism and other neurodegenerative diseases. The compounds bearing arylpiperazine groups (Pip-HP) are known to target serotomin (5-hydroxyptrypyamine, $5\text{-}HT_{1A}$) receptors with an important role in psychiatric disorders [70]. Antagonistics of $5\text{-}HT_{1A}$ receptors have found pharmacological applications in the treatment of other CNS disorders, such as the Alzheimer's disease [71].

Pip-HP is of interest because it has the highest brain retention [5,68] (Fig. 6), thus suggesting that this *N*-substituent group could provide considerable BBB transport properties to the compound, probably due to its amphiphilic character.

Other relevant bifunctional mono-3,4-HPs include *N*-bearing molecular vectors, such as bioactive tri- and tetra-peptides containing RGD and RYD aminoacid sequences [72]. Since these peptides are recognized by receptors of $\alpha_v\beta_3$ integrines (adhesion molecules of extracellular membranes which are over-expressed in angiogenesis of rapid growth tumor) [73], they may find interest for diagnostic purposes [74]. Antioxidant compounds have important protective/therapeutic roles. Therefore 3,4-HP containing *N*-bearing groups with antioxidant properties (e.g. chroman moiety of vitamin E) may serve as potential double-target therapeutics with eventual cross or synergistic effect against diseases that are multi-factorial in origin, such as the neurodegenerative diseases, [75,76].

Besides a reasonable chelation efficacy, the goal of this design strategy is focused in gaining the benefit from the interaction of the chelator with biological targets (*target approach*).

5.2. The ligand approach

Hexadentate iron(III) chelators, such as DFO, are, in principle, ideal ligands for scavenging iron and other trivalent cations, under diluted biological concentration conditions. In fact, due to the metal-ligand stoichiometry (1:1), they present an advantage over the bidentate chelators, namely in terms of kinetic inertness and thermodynamics, because the corresponding complexes present, respectively, first- and third-order dependence on ligand concentration, with the concomitant entropic contributions. Unfortunately, they usually possess molecular weight higher than 500, which reduces their membrane-crossing ability and biological activity [61,77].

Various synthetic hexadentate tris-3,2-HP chelators have been reported since 1990, aimed at iron chelation [78,79] or diagnostic purposes [55,80]. Regarding the tris-(3,4-HP) analogues, although with expected higher metal affinity, to our best knowledge, only one study has been recently published, in which

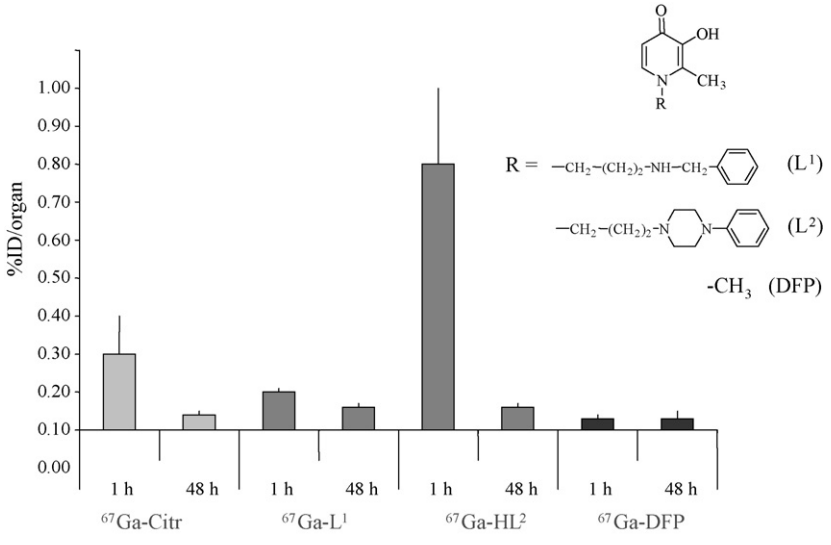


Fig. 6. Brain retention of the radionuclide (% ID/organ) after (1 h and 48 h) ip administration of the complexes of ^{67}Ga with L1, L2 (Pip-HP) and DFP.

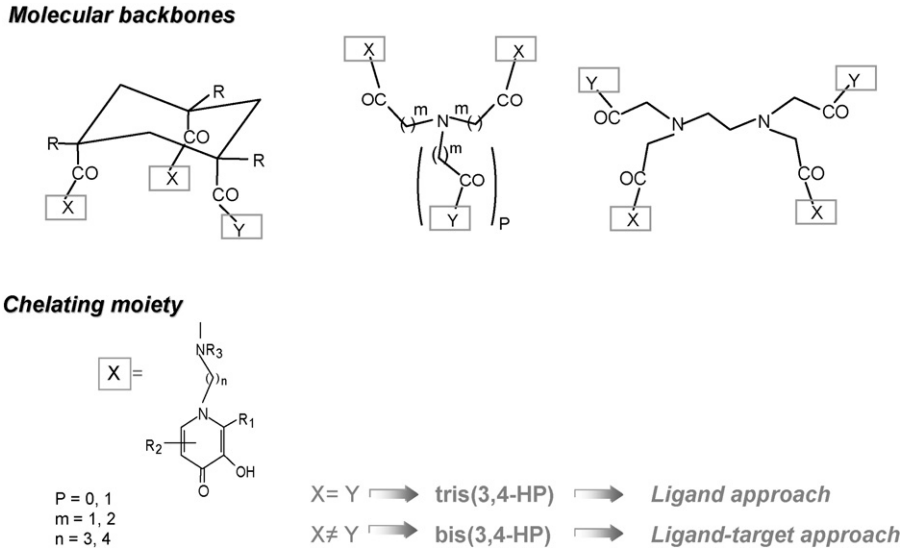


Fig. 7. Schematic representation of molecular backbones and chelating moieties for the polydentate compounds, namely tris(3,4-HP) and bis(3,4-HP) derivatives (For the already compounds studied, $R_3 = H$).

three carboxylic acid groups at the 2-position of pyridinone rings are attached to tris-amine supports [81]. However, these compounds seem to have some problems of high acidity, insolubility and some hindrance at the metal-coordination sphere, apparently due to the type of backbone, absence of spacers (between the 3,4-HP chelating moieties and the backbone) and *N*-ring substituents.

With the major goal of improving the iron-chelating efficacy of 3,4-Hp chelators, by exploring the polydenticity, a series of compounds were developed by attaching three or two chelating moieties to amino-carboxylate or cyclic carboxylate backbones (see Fig. 7), affording tris- and bis-(3,4-HP), respectively. The tris derivatives as hexadentate ligands could complete the metal coordination sphere and thus obtain the maximum ligand efficacy (*ligand approach*).

Therefore a series of hexadentate compounds, bearing three 3-hydroxy-4-pyridone-alkylamino arms attached to tris-carboxylic acid backbones, including a cyclic skeleton (Kemp acid) or two tripodal amino-tricarboxylic acids (nitrilotriacetic acid (NTA) and nitrilotripropionic acid (NTP)) has been designed with different spacers in our group [82,83]. All these hexadentate 3,4-HP chelators showed stronger affinity for the metal ion (pFe = 28–28.7) than DFO (pFe = 26.5). The chelates with the Kemp and the NTA derivatives present water-insolubility problems (the last one due to the high acidity of the backbone ammonium proton. The NTP analogue, presents the strongest affinity and the most convenient lipo-hydrophilic balance, both for the ligand and the metal complex [83] (see Fig. 8). In spite of the molecular weight limitation (>500), as compared with bidentate mono-3,4-HPs, these compounds can lead to a faster excretion of metal in metal-loaded mice, and they may have less problems of ligand toxicity because much less amount of compound is necessary to overcome the high ligand dependency of the monochelators. This type of compound is expected to have some limitations on brain–blood barrier (BBB) crossing ability, as currently happens with many others with low molecular weight if not aided by endogeneous BBB transporters [84]. These compounds may behave similarly to other drugs with high molecular weight (e.g. DFO) and may find mechanisms to bypass some membrane-crossing problem and access some cellular compartments, perhaps the extracellular ones.

5.3. The target–ligand approach

The current interest on the rational design and development of multi-target therapeutics [85] leads us to the challenging hypothesis of conjugating in the same molecule both the relevant functionalities of a good chelating agent for pharmacological application, namely the bifunctionality (*target approach)* and the polydenticity (*ligand approach*) to get a potential dual efficacy. Accordingly, a series of 3,4-HP derivatives were developed following the herein named *target–ligand approach*, which means that their architectural strategy lies on modulating and merging in the same molecular entity the advantage of polydenticity (providing higher chelating efficacy) with that of bifunctionality (enabling the tuning of physicochemical and biological properties, to improve the bioavailability and the molecular recognition by bio-receptors or -transporters). Thus, a series of tetradentate 3,4-HP chelators, namely bis-(3-hydroypyridin-4-one)-aminocarboxylic ligands (hereafter named as L^b) has been developed, by attaching two 3,4-HP moieties to aminocarboxylic skeletons, namely iminodiacetic acid (IDA) acid and ethylenediaminotetraacetic acid (EDTA), for further potential extra-functionalization (Fig. 8).

The physicochemical properties of these two compounds, $IDA(HP)_2$ [53] and $EDTA(HP)_2$ [86], confirmed their stronger chelating-affinity and higher specificity for *hard* metal ions (e.g.: Fe, Al) than for other biologically relevant metal ions (e.g. Zn), namely in comparison with mono-chelators such as DFP, or aminocarboxylic derivatives (DTPA and EDTA), which poor chelating specificity lead to Zn depletion [87,88]. As expected, these tetradentate bis(3,4-HP)s present high metal affinities, with

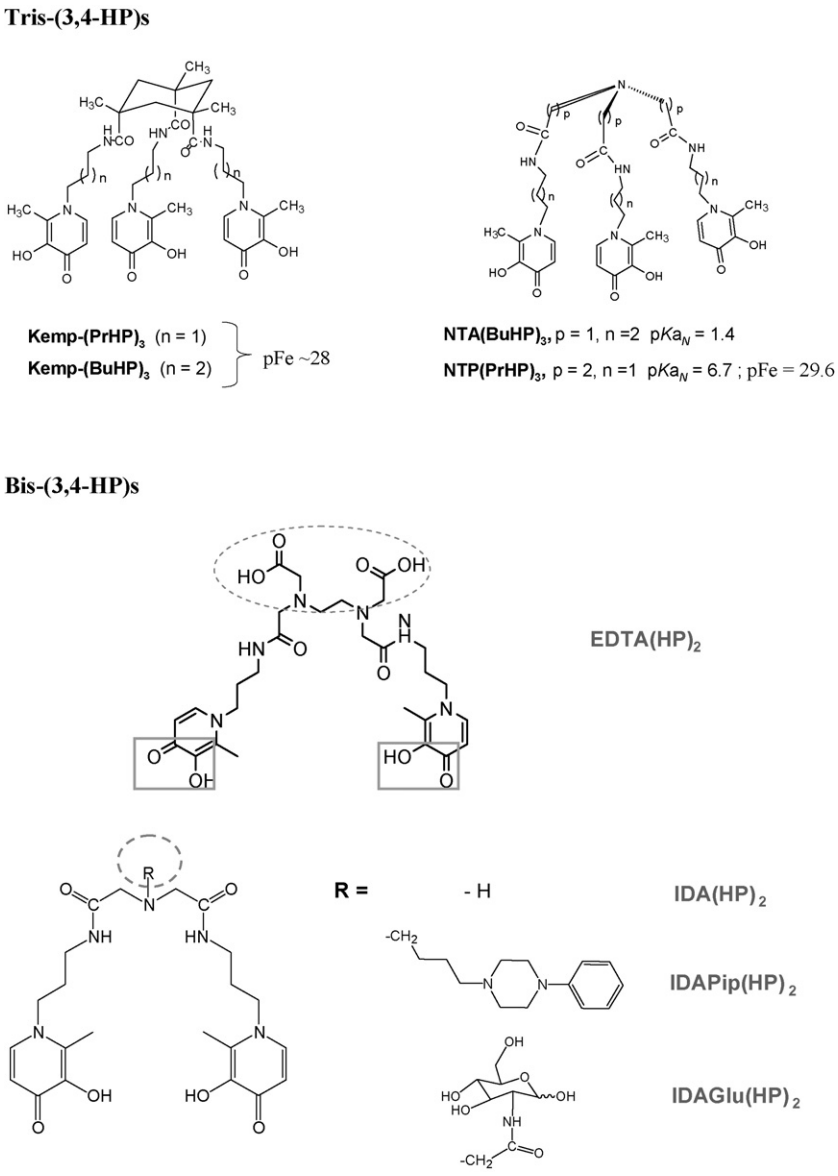


Fig. 8. Structural formula for the set of tris(3,4-HP)s and the bifunctionalized bis(3,4-HP)s (Lb) developed according to the *ligand approach* and the *ligand–target approach*, respectively.

*p*Fe values (25–26) between those of the bidentate mono-(3,4-HP)s (19–21) and of the hexadentate tris-(3,4-HP)s (28–30). However, they can still compete with transferrin [37,89], the main of the non-heme iron-transporters in plasma (see Table 2). The biological assays also showed they could compete with biological ligands such as citrate and lead to a faster metal clearance from body than DFP (see Fig. 9). Since these compounds are tetradentate, the problem of polymer formation with concomitant excretion limitations could be raised, albeit, for the *in vivo* concentration conditions, that process does not seem relevant for metal removal applications. Concerning their use as a M(III)-complex for diagnostic, the complex is quite stable, but the formation of some mixed complexes with other biological ligands can be also admitted.

EDTA(HP)$_2$ could be admitted as a bifunctional polydentate compound, due to the existence of two extra amino-carboxylic

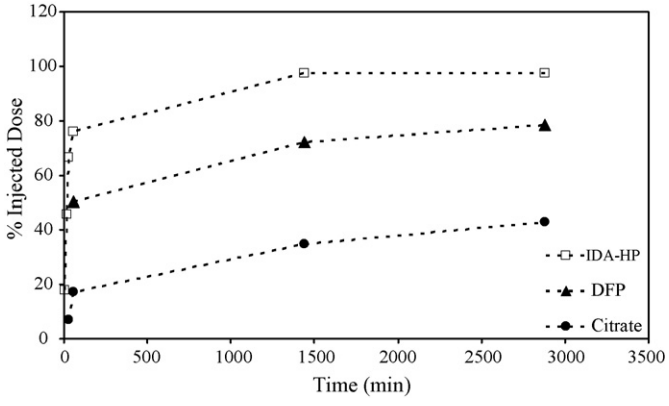


Fig. 9. Time course profile of whole body ^{67}Ga excretion after iv administration in mice.^{67}Ga-citrate and ^{67}Ga-complexes with DFP and IDA-HP.

Table 2
Metal complexation pM values ($M^{n+} = Zn^{2+}$, Fe^{3+}, Al^{+3}) and octanol/water partition coefficients (log *P*) for various synthetic and biologic ligands[a]

Compounds	*p*Fe	*p*Al	*p*Zn	Log *P*
$EDTA(HP)_2$[b]	25.4	18.8	11.6	−1.83
$IDA\text{-}(HP)_2$[c]	25.8	18.8	9.7	−1.72
$IDAPip(HP)_2$ (L^b)[d]	25.7	18.0	-	−0.31
Orn-HP (L^m) [e]	21.9	15.8	6.3	<−2
DFP[f]	19.3	16.1	6.2	−1.03
DFO[g]	26.5	19.3	6.6	−2
DTPA[h]	24.6	15.2	14.8	<−2
Transferrin[i]	20.3	14.5	–	–

[a] $pM = -\log[M]$ with $C_L/C_M = 10$ and $C_M = 10^{-6}$ mol/L at pH 7.4
[b] Ref. [86].
[c] Ref. [53].
[d] Ref. [5].
[e] Refs. [5,90].
[f] Refs. [37,90].
[g] Ref. [36].
[h] Ref. [53].
[i] Refs. [37,89].

functional groups. However, these groups are not biologically relevant (unless if further functionalized), just enabling potential extra-chelation with a second metal ion, or some extra-interactions (electrostatic or hydrogen bond) with solvent or other biomolecules.

Following the strategy of the *target–ligand approach*, two extra-functionalized $IDA(HP)_2$ tetradentate compounds were obtained by *N*-derivatization of the IDA skeleton with two different functional groups: firstly, an arylpiperazine group, $IDAPip(HP)_2$ [90,91], aimed at the ligand (or metal complex) vectorization towards neurotransmitter receptors; secondly, a glucosyl group, $IDAGlu(HP)_2$ [83], to enable its recognition by the membrane-bound cellular glucose transporters contributing for its easier internalization in nuclear compartments of brain cells which are know to have a high rate of glucose uptake and metabolism. The extrafunctional groups, besides the potential promotion of different targeting roles, also provide a quite different lipo- *versus* hydrophilic character, a relevant feature for the bioavailability and membrane crossing ability of the compounds/complexes, without interfering in the efficacy of chelating moiety (Table 2).

These extra-functionalized $IDA(HP)_2$ derivatives are representative examples of the schematic representation of Fig. 7, illustrating that when *X* does not equal *Y*, *the ligand approach* can become the *ligand–target approach*. Therefore, these extra-functionalized polydentate compounds, merging in the same molecular entity both functional groups to provide high chelating efficacy and the molecular recognition, can be considered potential drugs with dual proposals.

5.4. The combined chelator approach

The polypharmacology (combination of therapeutic mechanisms to improve efficacy over the monotherapies) [92] is of recognized growing interest. To remediate iron-overload situations, a strategy based on (DFP and DFO) combined chelation therapy has been used recently, with reported advantages over the individual administration of each compound [44,45]. This chelator combination [39,48] and others, namely ascorbate (As) and Feralex-G (FG) [50,51], were also recently bioassayed for the removal of aluminium accumulated in the body. Based on the recent polypharmacology paradigm and the recognized importance of the two functionalities explored in the *ligand–target* approach (multiple ligand drug), a further step

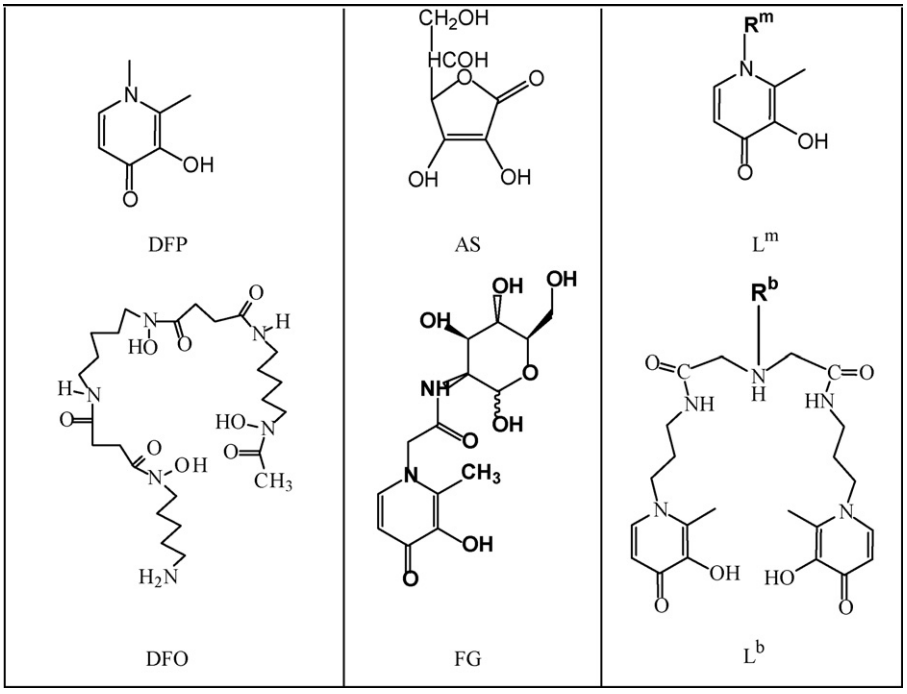


Fig. 10. Structures of iron(III)–chelator pairs for combined chelation.

was done, by combining two 3,4-HP chelators (e.g. *combined chelator* approach), each one containing a different functionality, which may be administered separately or coformulated in a single tablet (multi-component drug).

In particular, a combination of one mono-(3,4-HP) and one bis-3,4-HP (e.g. L^m and L^b) has recently been proposed (Fig. 10) [91] (also see Supplementary Information). It is conceivable that a L^m compound has lower metal-chelating efficacy than a (L^b). However, L^m has smaller size than L^b, and concomitant better achievements in terms of biological availability and accessibility to specific intracellular compartments and iron-storage sites. Therefore, L^m could complex the metal-ion and get out again, whereupon the higher size and stronger chelating agent L^b could work as a co-ligand and, eventually, make a ternary complex which could be transported to the systemic circulation for excretion. The first trial on solution and animal assays for combined chelation with 3,4-HPs, involved the Orn-HP (Fig. 4) and the IDAPip$(HP)_2$ (Fig. 8) [90,91], but there are on-going studies with other combinations. The first bioassays on the co-administration of these ligands involved a wide range of molecular ratios (from 1:1 to 1:1000 for L^b:L^m). Positive effects on metal excretion (the whole is better than the sum of the parts), were only observed for the higher concentrations of the monomeric compound. This may be interpreted in terms of a cooperative effect which demands competition between the ligands and so a large excess of the weaker ligand. Although the *in vivo* chelation results could be due to simple additive effects, further studies are need for their rationalization in terms synergistic effects or eventually trough a molecular *shuttling* mechanism [3,51].

Under the *combined chelator* approach, the combination of a tris-HP (L^t) with a L^m ligand could also be admitted but only a *shuttling* mechanism is conceivable, because of the L^t membrane-crossing limitations. Such an option could avoid eventual problems of oligomeric complexes (due to L^b) but, as stated above, under diluted conditions and great excess of L^m, the probability of their formation seems very low. Furthermore, L^b can be extrafunctionalized and improve the bioavailability and targeting facilities at sites of disease, as compared with L^t.

6. Conclusions

In the last two decades there has been a growing interest on the design and development of new chelating agents or new strategies to overcome serious disease situations associated with excess or misplaced iron (and aluminium) in body due to genetic or external insults or even ageing process. The 3-hydroxy-4-pyridinones (3,4-HP) are among the families of iron chelators that have been most studied, namely after the admission of the DFP in clinical applications for the treatment of iron-overload patients.

This review is mostly focused on the different strategies that have assisted the design and development of new 3,4-HP chelators as potential drug candidates to be used as potential mono- or poly-therapeutics. Although all these compounds possess a chelating function, their design strategy has been based on the exploitation of their bifunctionality, to improve the bioavailability and the molecular recognition by biological sites, as well as of their polydenticity, to improve the chelation efficacy. The conjugation of these two important properties has also been reported, either by jointing both the functionalities in the same molecular entity (multiple-ligand) or by ligand combination (multi-component drugs). Although the rational design of multiple-ligands can be more challenging than combining drugs, this last option may be an easier task towards the optimization of pharmacokinetic profiles. Therefore, the perspective of using polypharmacology by combination of two 3-hydroxy-4-pyridinone chelating agents with complementary properties, namely binding efficacy and accessibility to different cellular compartments, seems of high relevance for potential pharmacological applications in diseases related with iron overload or misplaced.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ccr.2008.01.033.

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