Investigation of the anti-inflammatory properties of hydroxypyridinones

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SUMMARY Synovial iron deposition associated with rheumatoid disease may result in the production of highly reactive oxygen free radicals, leading to tissue damage. This chain of events can be interrupted by iron chelation. Families of strong iron(III) chelators have been tested for their iron scavenging properties in vitro and their effects assessed in vivo using a rat model of inflammation. All the chelators competed successfully for iron with apotransferrin, and some removed up to 34% of iron from ferritin. The best anti-inflammatory effects were achieved with the most hydrophilic chelators and those which chelated iron most avidly. Activity was dependent on dose. The route of administration was also an important factor with lower affinity chelators. This work introduces a range of simple bidentate iron chelators, which under certain conditions exceed desferrioxamine in their iron scavenging abilities, and some of which, in this simple animal model, approach indomethacin in their anti-inflammatory capabilities.

Key words: rheumatoid arthritis, iron, iron chelators, iron overload, animal models, carrageenan, pleurisy, rats, transferrin, ferritin.

Disturbance of iron metabolism is a prominent feature of rheumatoid disease[1 2] as many patients with rheumatoid arthritis have increased concentrations of synovial fluid ferritin and iron deposits within their synovial tissue.[3–5] Iron deposition in early rheumatoid arthritis is associated with a poor prognosis[6] owing to the ability of ferric iron to catalyse the production of highly reactive oxygen free radicals via a Fenton type reaction.[7 8] Oxygen radicals such as superoxide and the hydroxyl ion are extremely reactive, having the power to damage all types of biomolecule.[9] Strong iron(III) chelators will remove reactive iron and therefore limit radical production and concomitant tissue damage.[10 11] The reduction of iron concentrations has also been reported to influence both acute and chronic animal models of inflammation,[12 13] iron removal being achieved by chelation or nutritional iron deficiency.

Studies of the use of iron chelators in inflammation have so far been limited to desferrioxamine. This molecule is not orally active, a characteristic

shared by many of its natural alternatives also derived from bacteria and based on hydroxamate functional groups.[14] In contrast, a family of chelators based on the hydroxypyridinone nucleus (Fig. 1) has been shown to be both orally active[15 16] and capable of iron removal from hepatocytes in both iron overloaded mice[17] and rabbits.[18] Under such conditions these chelators are superior to desferrioxamine in their ability to remove iron. In this paper we examine the iron scavenging properties of a series of hydroxypyridinones and hydroxypyrones and compare their effects in an animal model of inflammation.

Materials and methods

CHEMISTRY OF IRON CHELATORS

3-Hydroxypyridin-2-one and 3-hydroxypyridin-4-one chelators were prepared by the methods of Mohrle and Weber and Berson et al respectively.[19 20] Hydroxypyrone chelators were obtained from Aldrich and recrystallised before use. The purity of these chelators was established as greater than 99% by elemental analysis. The established chelator

Hydroxypyridin-2-ones **Hydroxypyridin-4-ones** **Hydroxypyrones** Non-chelating analogue

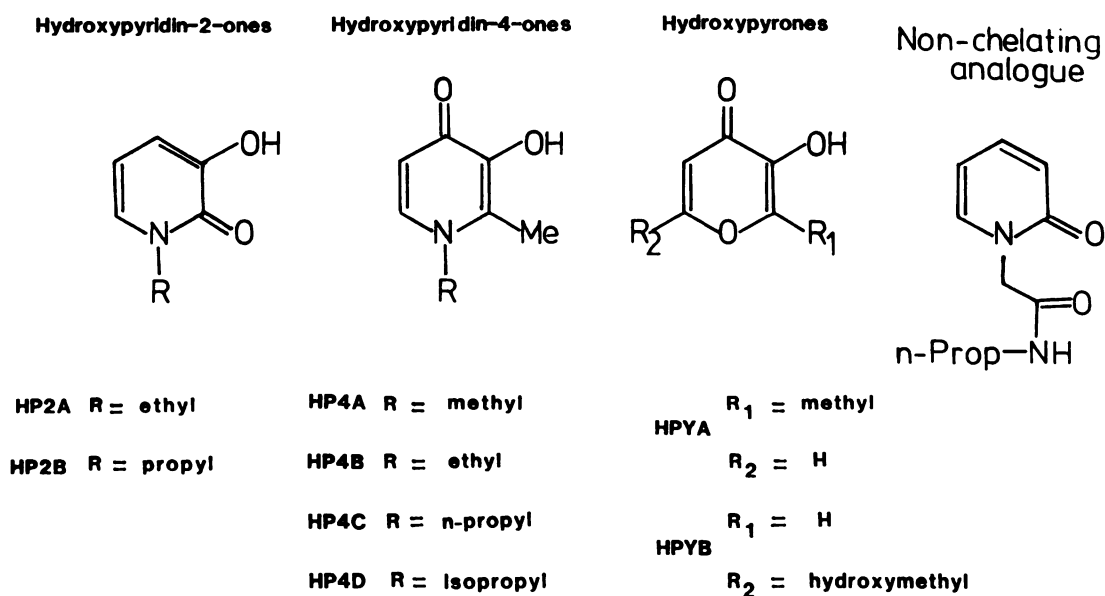|		
HP2A R = ethyl	**HP4A** R = methyl	R$_1$ = methyl
		HPYA
HP2B R = propyl	**HP4B** R = ethyl	R$_2$ = H
	HP4C R = n-propyl	R$_1$ = H
		HPYB
	HP4D R = isopropyl	R$_2$ = hydroxymethyl

Fig. 1   *Structural formulae of chelator families.*

desferrioxamine was obtained from Ciba Geigy. Apotransferrin (human) and ferritin (horse) were purchased from Sigma. Iron-59 was supplied by Amersham International.

The lipid solubility of both the chelators and their iron(III) complexes was assessed by determination of their partition coefficient between aqueous and organic solvents. Iron(III)-chelator complexes were prepared by the interaction of excess chelator with iron((III) chloride in trometamol (TRIS).HCl (20 mM, pH 7·4). Aqueous solutions of chelator alone or iron-chelator complex were shaken vigorously with an equal volume of octan-1-ol in a stoppered acid washed test tube and the concentrations of chelator or iron-chelator complex in organic and aqueous layers were measured spectrophotometrically (chelator 240–400 nm, complex 340–649 nm) after centrifugation (3000 rpm, one minute). The partition coefficient (K_{part}) was calculated from the ratio of concentrations in organic and aqueous layers.

The ability of the chelators to remove iron from ferritin was assessed after mixing of horse spleen ferritin (5 μl, 50% saturated, 180 μM) with chelator solution (3 mM in phosphate buffered saline, pH 7·4). Six and 24 hours after mixing samples were passed rapidly down a Sephadex G25 column and the amount of iron removed from ferritin was determined spectrophotometrically (350–640 nm).

Under these conditions the column bound less than 5% of the applied iron.

Iron(III)-59 transferrin was prepared by incubation of iron-59 nitriloacetic acid (1:2) with trometamol. HCl (0·1 M, pH 7·4) and apotransferrin in the same buffer containing NaHCO$_3$ (10 mM) for one hour at 20°C.[21] Iron removal from this transferrin preparation was then assessed after exposure to excess chelator for six hours and rapid fractionation of the iron containing portion by gel permeation chromatography using Sephadex G25. Under these conditions the column bound less than 2% of the applied iron. Iron associated with the high molecular weight (transferrin) and low molecular weight (chelator) fractions was determined by gamma counting.

ANIMAL STUDIES
The non-steroidal anti-inflammatory drug indomethacin was supplied by the pharmacy, Queen Elizabeth Hospital, Birmingham. Evans blue was obtained from Gurr Microscopy Materials and carrageenan from Marine Colloids Inc.

A simple irritancy test was performed using male Wistar rats (Bantin and Kingman, Hull, UK). The animals were anaesthetised using ether, and 0·5 ml of a 2% solution of Evans blue dye was injected intravenously. One hour later the abdomen was shaved and intradermal injections of 0·5 ml chelator

solutions were made. The animals were killed after one hour and the abdominal skin dissected to reveal the subcutaneous surface. Irritancy was assessed by comparing the diameter of the blue ring formed by capillary leakage with that of a buffer control.

Carrageenan pleurisy was used to assess the anti-inflammatory properties of iron chelators. Male Wistar rats were housed six in a cage with food and water freely available. After an initial settlement period the animals were anaesthetised with ether and a small incision was made on the right side between third and fourth ribs, through which 0·15 ml of a 1% solution of autoclaved carrageenan was injected into the pleural space. The wound was closed with a single suture, the animals returned to their cages, and four hours later they were killed. Pleural inflammation was assessed as follows.

Samples of blood were collected by bisection of the dorsal aorta and vena cava. The pleural space was washed out with 1 ml RPMI 1640 medium (Roswell Park Memorial Institute minimal culture medium) containing 10% chelexed fetal calf serum (Gibco). Exudate volumes were assessed by weight, smears made for differential cell counts, and samples diluted for total white cell counts (Coulter counter). After centrifugation serum and cell free exudate were stored at −20°C.

Groups of six animals were dosed with iron chelators one hour before and two hours after carrageenan administration. Chelators were dissolved in sterile water and the pH adjusted to 7 immediately before administration. A non-chelating hydroxypyridinone analogue was used as a negative control and indomethacin as the positive control. Initial screening of each chelator was by a fixed dose via the intraperitoneal route. More detailed dose response studies were then performed using the most active members of each family of iron chelators and a range of routes of administration.

Results

CHEMISTRY OF IRON CHELATORS
All three families of chelator described here combine easily with transition metals, forming neutral 3:1 complexes with trivalent cations like iron and neutral 2:1 complexes with divalent cations such as copper.[22][23] The affinity constant of the metal-chelator complexes (log β) is a measure of their chemical attraction among other things and is derived from the logarithm of the product of the equilibrium constants relating to successive additions of atoms of metal to a single chelator molecule. Thus some of the hydroxypyridinones and hydroxypyrones have a very high affinity for iron(III) with log $\beta_3 > 30$. Although all the chelators also possess

an appreciable affinity for copper, this is markedly exceeded by their affinity for iron. For example log β_2 values for the hydroxypyridin-4-ones are 27 for iron and 17 for copper, a separation of at least 10 orders of magnitude. The hydroxypyridin-4-one chelators have a higher charge distribution than the other chelator groups considered in this study and this renders them the most powerful group, with a log β_3 for iron of 37 compared with 30 for hydroxypyridin-2-ones and 29 for hydroxypyrones.

The chelator molecules have low proton dissociation constants and are therefore uncharged at neutral pH and have a low affinity for Ca^{++} (log β_2 is 5 for hydroxypyridin-2-ones and hydroxypyridin-4-ones, and 4 for hydroxypyrones).

The partition coefficients for hydroxypyridin-2-one and hydroxypyrone metal-chelator complexes are quite high (>1), which indicates a reasonable degree of lipid solubility, enabling them to cross membranes easily. Metal-chelator complexes of hydroxypyridin-4-ones, however, have partition coefficients at least one order of magnitude smaller than the corresponding hydroxypyridin-2-ones (0·65 compared with 6·2 for the n-propyl chelator for example), but even this apparently low degree of hydrophobia is sufficient to facilitate membrane permeation of the iron-chelator complex.

All three families of chelator compete successfully for iron with apotransferrin. They hydroxypyridin-4-ones are the most successful at removing iron from ferritin. This might have been expected in view of their higher log β_3 values (affinity) for iron(III). Under the conditions used they are more efficient than desferrioxamine.

The hydroxypyridin-4-ones will also remove iron from ferritin without the need for simultaneous incubation with catalytic bipyridyl or ascorbic acid, though the contrast between hydroxypyridin-4-ones and hydroxypyridin-2-ones in this respect is less marked than with transferrin (Table 1). All three families of chelator are superior to desferrioxamine in this action.

ANIMAL STUDIES
None of the chelators possessed any appreciable irritant properties as judged by the Evans blue capillary leakage test.

The pleurisy model was chosen for this investigation because it is both well established and reliable, is easily reproducible, and allows quantitative assessments to be made. Although desferrioxamine has shown anti-inflammatory properties in this model,[24] its performance was exceeded in this work by both hydroxyprones and hydroxypyridin-4-ones. As initial fixed dose screening experiments showed hydroxypyridin-2-ones to possess no appreciable

Table 1 *Iron removal from transferrin and ferritin by chelators*

Chelator*	Percentage removal of iron-59 from:		
	Transferrin† at 6 hours	Ferritin‡ at 6 hours	Ferritin at 12 hours
HP2A	12	14	24
HP2B	15	11	21
HP4A	90	13	31
HP4B	88	19	34
HP4C	91	15	26
HP4D	94	17	24
HPYA	21	7	15
Desferrioxamine	17	1·5	—

*Abbreviations explained in Fig 1.
†Human iron-59 transferrin (10^{-5} M) was incubated with a solution of chelator (4×10^{-3} M) buffered by trometamol.HCl (0·1 M, pH 7·4) at 25°C for six hours. The solution was passed down a Sephadex G25 column (1×10 cm) and the iron-59 associated with the high and low molecular weight fractions recorded.
‡Horse spleen ferritin (approx 50% saturated, 180 μM) was incubated with a solution of chelator (3×10^{-3} M) in phosphate buffered saline at 25°C. At different times the solution was passed down a Sephadex G25 column (1×10 cm), and the amount of iron removed from ferritin was determined spectrophotometrically.

Table 3 *Comparision of hydroxypyridin-4-ones and hydroxypyrones*

Chelator†	K_{part}‡	Intraperitoneal		Subcutaneous	
		Exudate	Cells	Exudate	Cells
HP4A	0·2	50**	68***	21***	74
HP4B	0·4	97	97	85	86*
HP4C	1·35	61	77	—	—
HP4D	1·45	99	92	—	—
HPYA	0·66	71*	87	81	64***
HPYB	0·14	75**	100	41****	56****

†Abbreviations explained in Fig. 1.
‡k_{part}=partition coefficient calculated from the ratio of concentrations in organic and aqueous layers.
Parameters expressed as percentages of the negative control, measured four hours after carrageenan administration. Dose=50 mg/kg, number of animals=6.
Significance assessed by Student's *t* test: *p<0·05; **p<0·02; ***p<0·01; ****p<0·001.
HP4C and HP4D were not given by the subcutaneous route owing to systemic toxicity (see text).

anti-inflammatory ability (Table 2) this family of chelators was excluded from further study. In contrast, both hydroxypyridin-4-ones and hydroxypyrones were found to reduce pleural inflammation significantly, the hydroxypyrones being active when given subcutaneously, whereas the hydroxypyridin-4-ones were active on oral and intraperitoneal application (Table 2). The inverse relation between partition coefficients (lipid solubility) of chelators and anti-inflammatory properties is highlighted by the data shown in Table 3. Thus the chelators having the lowest partition coefficients within their family (lowest lipid solubility) were the most efficient anti-inflammatory agents. Systemic toxicity was associated with the hydroxypyridin-4-ones possessing a high partition coefficient (HP4C, HP4D). The

two most hydrophilic hydroxypyridin-4-ones were studied in greater detail by the execution of dose response experiments for each route of administration. At every dose the most hydrophilic chelator (HPA4) achieved a reduction in both exudate volume and total numbers of white cells when given subcutaneously (Fig. 2). The reduction in exudate volume was significant for doses of 2 and 50 mg/kg, also exceeding the indomethacin response at these doses. In the presence of this chelator at 2 mg/kg the mean white cell count was $22 \cdot 5 \times 10^6$ (SD $3 \cdot 8 \times 10^6$), 66% of the control value of $33 \cdot 4 \times 10^6$ ($9 \cdot 8 \times 10^6$). This compares favourably with indomethacin (5 mg/kg), which reduced the white cell count to 78% of the control level. A similar trend was obtained with HP4B, which also reduced both exudate and cell numbers.

When presented via the intraperitoneal route HP4A (5–300 mg/kg) achieved reductions in exudate volume and numbers of leucocytes (Fig. 3).

Table 2 *Comparison of anti-inflammatory activity of chelator families*

Chelator†	Intraperitoneal		Subcutaneous		Oral	
	Exudate	Cells	Exudate	Cells	Exudate	Cells
HP2A	87	83	77	61***	100	92
HP4A	53***	72*	53***	94	67****	79**
HPYB	75**	100	41****	56****	85	94
Desferrioxamine	100	90	89	88	—	—

†Abbreviations explained in Fig. 1.
Parameters expressed as percentages of the negative control, measured four hours after carrageenan administration. Dose=150 mg/kg, number of animals=6.
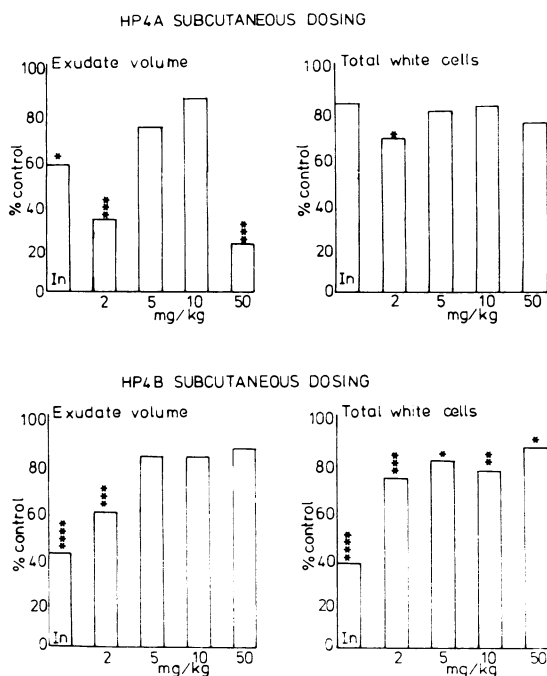Significance assessed by Student's *t* test: *p<0·05; **p<0·02; ***p<0·01; ****p<0·001.

Fig. 2   *Inflammatory parameters, measured four hours after carrageenan administration, expressed as percentage of the negative control. Data shown for subcutaneous dosing. Indomethacin was given orally. In=indomethacin. For other abbreviations see Fig. 1. Significance was calculated by Student's t test: *p<0·05; **p<0·02; ***p<0·01; ****p<0·001.*
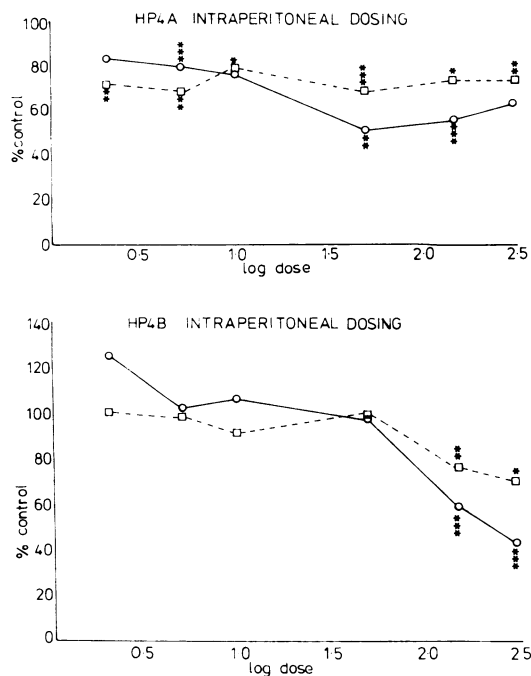

Fig. 3   *Inflammatory parameters, measured four hours after carrageenan administration, expressed as percentage of the negative control. Data shown for intraperitoneal dosing. □- - -□=white cell count; ○——○=exudate volume. Significance was assessed by Student's t test *p<0·05; **p<0·02; ***p<0·01.*

The reduction in the number of cells accumulating in the pleural cavity was significant at each dose tested. In contrast, HP4B was less effective than when given subcutaneously, achieving significant reductions in inflammatory parameters only at high doses (>100 mg/kg).

On oral presentation HP4A diminished both numbers of white cells and volumes of exudate at all doses (Fig. 4). When given in excess of 50 mg/kg this chelator triggered increased salivation in animals within 30 minutes of application. In parallel with its effects after intraperitoneal administration, HP4B failed to influence the inflammatory process when given orally even at high doses such as 150 and 300 mg/kg.

Thus HP4A possesses anti-inflammatory properties when presented by three different routes, whereas HP4B is only active when given subcutaneously.

Discussion

When undergoing a respiratory burst, eosinophils,

monocytes, macrophages, and neutrophils produce superoxide free radicals and hydrogen peroxide.[25] It is thought that these species have an important role in the phagocyte mediated tissue injury associated with inflammation,[12] although when homogeneous they are poorly reactive in aqueous solution and therefore unlikely to be directly responsible for any observed tissue damage.[26] In the presence of suitable transition metal catalysts such as copper or iron, however, superoxide and hydrogen peroxide react to produce the hydroxyl radical, a highly reactive tissue damaging species.[27] Iron in a form able to catalyse this reaction has been demonstrated in human synovial fluid.[28]

An improvement in both acute and chronic inflammation was noted on administration of desferrioxamine to animal models.[29] When given to rheumatoid patients, however, desferrioxamine caused reversible ocular abnormalities, and co-administration with phenathiazines led to loss of consciousness for up to 72 hours.[30] In contrast, lower doses of desferrioxamine administered to

HP4A ORAL DOSING
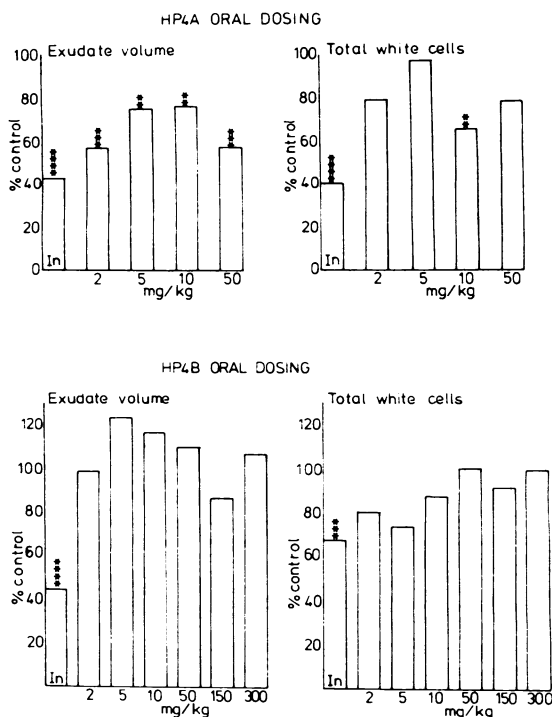

HP4B ORAL DOSING

Fig. 4 *Inflammatory parameters, measured four hours after carrageenan administration, expressed as percentage of the negative control. Data shown for intraperitoneal dosing. Indomethacin was also given orally. In=indomethacin. For other abbreviations see Fig. 1. Significance was calculated by Student's t test *p<0·05; **p<0·02; ***p<0·01; ****p<0·001.*
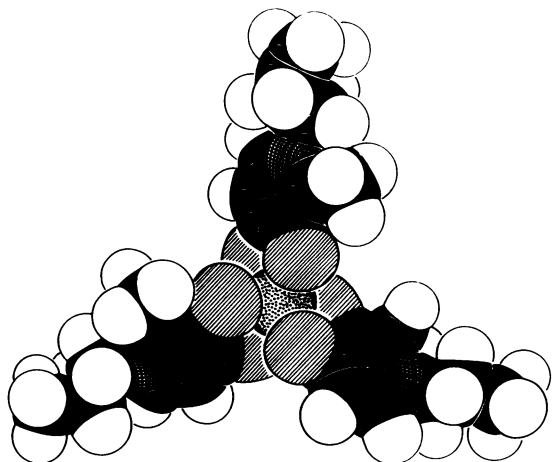

Fig. 5 *Spatial arrangement of iron(III)-chelator complex.*

anaemic rheumatoid patients had the effect of suppressing both inflammation and anaemia.[31]

The need to overcome the shortcomings of desferrioxamine (parenteral administration and lack of specificity for iron) led to the development of the chelators which have been examined in this study. Hydroxypyridin-4-ones have been shown to be capable of mobilising iron from transferrin and ferritin in vitro[32] and from iron overloaded animals in vivo, for example, mice[15-17] and rabbits.[18]

With suitable alkyl substituents these chelators and their iron complexes can be rendered both water soluble and membrane permeable. They are remarkably resistant to acid conditions, to oxidation, and to the action of proteolytic enzymes. All these properties favour both survival in and absorption from the gastrointestinal tract.

In this study the most hydrophilic member of the hydroxypyridin-4-ones family (HP4A) has been shown to possess anti-inflammatory activity in the acute carrageenan pleurisy model, regardless of the route of administration. Although the others in this family of chelators also demonstrated activity in this model (data not shown), these more lipid soluble members of the family induced much smaller changes in the various parameters of inflammation. Only HP4B was found to have equivalent activity to HP4A and then only when presented subcutaneously.

The closely related hydroxypyridin-2-ones were much less effective than the hydroxypyridin-4-ones, possibly owing to their lower affinity for iron(III) or higher partition coefficient values for both chelators and iron(III)-chelator complexes, or both. The hydroxypyrones were found to be quite active when given subcutaneously (Table 3), however when given by the intraperitoneal route they possessed anti-exudative properties only. They had no beneficial effects after oral administration, presumably because of rapid metabolism.[33]

Using a simple model of acute inflammation we have demonstrated the efficacy of iron chelation in reducing inflammation. In our tests the most effective of the bidentate chelators described here were the hydroxypyridin-4-ones. Of these, the best was the simplest, most water soluble chelator HP4A. This chelator is markedly more effective than desferrioxamine in the tests described in this work. Indeed even some of the less attractive chelators— for example, HP4B, HPYA, and HPYB, are more active than this well characterised high affinity iron(III) chelator.

The bidentate hydroxypyridin-4-ones form stable 3:1 complexes with iron (Fig. 5), which completely protect the iron(III) atom from the surrounding solvent. Consequently, once formed, the iron(III)-chelator complex has an extremely low tendency to

produce hydroxyl radicals[34] and therefore scavenge iron in a non-toxic form. Although the bidentate hydroxypyridin-4-ones possess a high affinity for iron(III), the neutral complexes dissociate at low concentrations to form the charged 2:1 complex.[22] It is conceivable that such dissociation limits the effectiveness of these molecules. Dissociation at low concentrations is much less likely with hexadentate chelators, which form 1:1 complexes with iron(III).[14] We are currently studying a range of such chelators.

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