Structure-Function Investigation of the Interaction of 1- and 2-Substituted 3-Hydroxypyridin-4-one Chelators with 5-Lipoxygenase and Ribonucleotide Reductase*

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The structural and physiochemical properties of 3-hydroxypyridin-4-one chelators (HPOs) which influence inhibition of the iron-containing metalloenzymes ribonucleotide reductase (RR) and 5-lipoxygenase (5-LO) have been investigated. HPOs with substituents at the 1- and 2-positions of the pyridinone ring have been synthesized, and their inhibitory properties compared with those of desferrioxamine (DFO). Varying the alkyl substituents does not affect the affinity constant of these ligands for iron(III), but permits a systematic investigation of the effect of hydrophobicity and molecular shape on inhibitory properties. The inhibition of RR was monitored, indirectly by measuring tritiated thymidine incorporation into DNA and directly by the quantification of the EPR signal of the enzyme tyrosyl radical. 5-LO inhibition was examined spectrophotometrically, measuring the rate of linoleic hydroperoxide formation by soybean lipoxygenase. The results indicate that the substituent size introduced at the 2-position of the HPO ring is critical for determining inhibition of both enzymes. Large substituents on the 2-position, introduce a steric factor which interferes with accessibility to the iron centers. These studies have identified chelators such as 1,6-dimethyl-2-((N-4’-N-propylsuccinimido)methyl-3-hydroxypyridin-4-one (CP358), which causes only a 10% inhibition of 5-LO after 24 h of incubation at 110 μM IBE (iron-binding equivalents) in comparison to simple dialkyl HPOs such as Deferiprone (CP20) which cause up to 70% inhibition. Using EPR spectroscopy, CP358 inhibits RR at a slower rate than CP20, while chelating intracellular iron(III) at a similar rate, a finding consistent with an indirect indication of the tyrosyl radical. However, hepatocellular iron is mobilized at a faster rate by CP358 (P < 0.001). These findings demonstrate that it is possible to design bidentate HPOs which access intracellular iron pools rapidly while inhibiting non-heme iron-containing enzymes relatively slowly, at rates comparable to DFO. It is anticipated that such compounds will possess a superior therapeutic safety margin to currently available bidentate HPOs.

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The rate at which iron chelators inhibit non-heme iron-containing metalloenzymes is predicted to have a critical influence on their cellular effects. Iron chelators in current clinical use are the hexadentate chelator desferrioxamine (DFO)1 and the smaller orally active bidentate hydroxypyridinone deferiprone (CP20) (see Fig. 1, Table 1) (1). Both are known to be antiproliferative (2) and to induce apoptosis in a variety of cell lines (3, 4). Additionally CP20 induces bone marrow hypoplasia in laboratory animals (5) and agranulocytosis in a proportion of humans (6). To limit antiproliferative activity and to have the widest possible therapeutic safety margin, such chelators must chelate excess iron without removing iron that is essential for normal cellular homeostasis.

We have shown previously that the key non-heme iron containing enzymes 5-lipoxygenase (5-LO) and ribonucleotide reductase (RR), in which the iron center is dominated by oxygen and imidazole ligands, are inhibited rapidly by bidentate hydroxypyridinone chelators such as deferiprone (CP20) but significantly more slowly by the larger hexadentate iron chelator DFO (7, 8). This work suggested that the hydrophobicity of bidentate hydroxypyridinones was important in determining the rate of inhibition of these enzymes, but the larger size of DFO might also limit inhibition of these enzymes. In these previous studies, however, variation of the substitution was only examined in the 1-position of the pyridinone ring (R1) (Fig. 1), and it was not possible to differentiate the size and shape effects from those of hydrophobicity. In the present investigation, we have therefore undertaken a detailed structure-activity investigation of a range of such inhibitors, employing substituents in both the 1- and 2-positions of the pyridinone ring, with a view to identifying the principal physicochemical properties of hydroxypyridinones that determine the rate of inhibition of 5-LO and RR. We have further sought to identify hydroxypyridinones that inhibit these enzymes as slowly as DFO but maintain their ability to mobilize intracellular iron.

Lipoxygenases are non-heme iron-containing enzymes that catalyze the site-specific oxygenation of polyunsaturated fatty acids to produce hydroperoxides. 5-LO is the principal lipoxygenase in the human neutrophil, and it catalyzes the first two steps in the conversion of its substrate arachidonic acid to leukotriene A4 which is the precursor of the potent inflamma-

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1 The abbreviations used are: DFO, desferrioxamine; 5-LO, 5-lipoxygenase; RR, ribonucleotide reductase; HPO, 3-hydroxypyridin-4-one; IBE, iron binding equivalents; EPR, electron paramagnetic resonance; shLPO, soybean lipoxygenase; PBS, phosphate-buffered saline; D40, distribution coefficient at pH 7.4; FCS, fetal calf serum; R, correlation coefficient.
tory mediator leukotriene B4 (9). 5-LO is a labile, hydrophobic 78-kDa protein consisting of 674 amino acids (10). The sequence shows homology with other lipoxygenases, including three soybean isozymes (10). Of significance is a 37-amino acid sequence containing 5 histidines that are conserved in all cloned lipoxygenases (10, 11). It has been suggested that this region may be involved in iron coordination (10, 11, 15, 16). Several groups (11–16) have investigated the geometry of the iron center in lipoxygenase using the soybean enzyme as a model due to its greater stability and availability. The various analyses are consistent with a six-coordinate iron, bound by three or four nitrogen ligands (imidazole) and one or two oxygen ligands in an octahedral symmetry. It is evident from x-ray analysis that not all six bonds to the central iron atom are from the protein and that there are possible coordination sites that may represent potential sites of coordination by metal chelators (8).

Ribonucleotide reductase (RR) is the key and rate-limiting enzyme in DNA synthesis. Class I RR, which occurs in all mammalian cells and in some prokaryotes, notably Escherichia coli, consists of two subunits as follows: a large subunit termed R1 and a smaller subunit termed R2. Each of the two subunits consists of two identical polypeptide chains. An equimolar complex of both subunits is necessary for activity (17). The small subunit R2 contains (bound to each polypeptide chain) an oxygen-linked diferric metal center which, via a redox cycle, gen-
erates and stabilizes a tyrosyl radical during the steady state turnover of the enzyme (17). The two octahedrally coordinated iron atoms in each center are doubly bridged by an oxo ligand and coordinated by a single glutamate carboxylate. The coordination about each iron is completed by histidine, aspartate, glutamate, and water (18). The tyrosyl radical is located in a hydrophobic pocket and is buried within the protein matrix at a depth of at least 10 Å from the surrounding solvent (17, 18). The E. coli protein R2 possesses conformational flexibility that may permit small molecules access to the interior of the protein (19, 20). Furthermore, several observations (21–25) have demonstrated that the mouse R2 protein has a more exposed active site topology in comparison to that of the E. coli R2 protein. Indeed, the three-dimensional structure of mouse R2 shows the existence of a hydrophobic channel that reaches about 10 Å from the surface to the proposed oxygen reaction site close to the tyrosine radical (26). Furthermore, the iron center of mouse R2 has a less hydrogen-bonded environment that makes it more open and accessible. Iron chelators can inhibit RR by forming a ternary complex with the enzyme-bound iron, chelating the iron center in the active site of the enzyme or by depriving the precursor iron from the newly synthesized enzyme pool.

This study reports a systematic investigation whereby we attempt to determine the structural and physiochemical requirements for the inhibitory actions of the 3-hydroxypridin-4-one (HPO) chelators on both the metalloenzymes RR and 5-LO. Such an investigation will generate an improved understanding of the mechanism of RR and 5-LO inhibition by these chelators. It will also provide the opportunity to identify an HPO chelator with minimal inhibitory properties toward these enzymes. Such a chelator should have potential for the clinical treatment of diseases of iron overload such as β-thalassemia. To achieve these goals, we synthesized a series of HPO chelators with different substituents at the R1 and R2 positions of the pyridinone ring (Table I), and we studied their inhibitory properties. Substituents introduced at these positions do not markedly influence the affinities of these ligands for iron(III).

### MATERIALS AND METHODS

**Reagents**—The 1- and 2-substituted 3-hydroxypridin-4-ones were synthesized as described previously (27, 28), and their purity was confirmed by H NMR and elemental analysis. Lipophilicity was quantified by the measurement of the distribution coefficient (D) of the chelators between n-octanol and Tris buffer (pH 7.4) (27). Desferrioxamine (Desferal®) was purchased from Ciba-Geigy. Human transferrin (98% pure), DNaI (type III), RNase (type I), soybean lipoygenase, soybean trypsin inhibitor, penicillin, gentamicin, and insulin were from Sigma. Collagenase (type I) was from Roche Molecular Biochemicals. All radiochemicals were from Amer sham Biosciences. Aquasol was from PerkinElmer Life Sciences. RPMI 1640 medium and fetal calf serum were from Life Technologies, Inc., and PBS was from Oxoid (UK). All other chemicals were of analytical grade.

**Statistical Analysis**—The Student's t test (unpaired) was used to identify significant differences between sample means.

**Determination of the Molecular Width of the HPO Chelators**—The three-dimensional mode using the ACD/ChemSketch program. The distance (Å) between the methyl carbon of the R2 substituent (Fig. 1 and Table I) or the carbon on the 6th position of the aromatic ring was applicable, and the final carbon of the R2 substituent was then calculated automatically by the same program.

| HPO chelators | R1 | R2 | R3 | D_{48816} |
|---------------|----|----|----|-----------|
| CP358         | CH₃| CH₃| CH₃| 0.20      |
| CP357         | CH₃| CH₃| CH₃| 0.02      |
| CP354         | CH₃| CH₃| CH₃| 0.19      |
| CP355         | CH₃| CH₃| CH₃| 0.45      |
| CP351         | CH₃| CH₃| CH₃| 8.0       |
| CP353         | CH₃| CH₃| CH₃| 1.05      |
| CP350         | CH₃| CH₃| CH₃| 2.15      |
| CP352         | CH₃| CH₃| CH₃| 0.39      |
| CP38         | CH₃| CH₃| CH₃| 0.52      |
| CP60         | CH₃| CH₃| CH₃| 0.25      |
| CP20        | CH₃| CH₃| CH₃| 0.21      |
| CP21        | CH₃| CH₃| CH₃| 0.49      |
| CP24        | CH₃| CH₃| CH₃| 5.05      |
| CP77        | CH₃| CH₃| CH₃| 17.40     |
| CP55        | CH₃| CH₃| CH₃| 79        |
| CP357       | CH₃| CH₃| CH₃| 750       |

This study reports a systematic investigation whereby we attempt to determine the structural and physiochemical requirements for the inhibitory actions of the 3-hydroxypridin-4-one (HPO) chelators on both the metalloenzymes RR and 5-LO. Such an investigation will generate an improved understanding of the mechanism of RR and 5-LO inhibition by these chelators. It will also provide the opportunity to identify an HPO chelator with minimal inhibitory properties toward these enzymes. Such a chelator should have potential for the clinical treatment of diseases of iron overload such as β-thalassemia. To achieve these goals, we synthesized a series of HPO chelators with different substituents at the R1 and R2 positions of the pyridinone ring (Table I), and we studied their inhibitory properties. Substituents introduced at these positions do not markedly influence the affinities of these ligands for iron(III).
Lipoxygenase and Ribonucleotide Reductase Inhibition

50 mg of collagenase, 5 mg of DNase, 5 mg of RNase, 10 mg of soybean trypsin inhibitor, and 200 μM CaCl₂. The collagen perfusate was recirculated for 15 min before lightly minced the liver in a sterile beaker. The mixed liver cell suspension was filtered through 250-, 85-, and 63-μm nylon meshes after each of three sedimentation steps at 400 rpm for 5 min to remove nonparenchymal cells. Two further sedimentations were performed in sterile culture medium before plating cells for primary culture. Culture medium was RPMI 1640 containing 10% FCS, 100 units/ml penicillin, 50 μg/ml gentamicin, and 12 μg/ml insulin. The resulting hepatocyte suspensions were greater than 95% pure as judged by light microscopy (34).

Cell viability following centrifugation was estimated by fluorescence microscopy using ethidium bromide and acridine orange. Suspensions containing less than 80% viable cells were not used. Cell were suspended in culture medium to a final concentration of 0.5 × 10⁶/ml, and 3 ml of suspension added to each 60-mm collagen-coated plastic plate (Primera Falcon 3802, Falcon Labware, Oxnard, CA). Plates were cultured at 37 °C in an atmosphere of 5% CO₂, 95% air. After primary adherence for 4 h, the plates were washed four times with RPMI to remove nonviable, nonadherent cells. The viability of adherent cells was >95%. Cell viability could generally be maintained at this level for over 48 h (34).

After the removal of nonadherent cells, 2 ml of culture medium containing ⁵⁷Fe human derrif ferr transferrin at a 100 μg/ml final concentration was added (34, 35). The cells were pulsed with this medium for 16 h before decanting the medium and washed four times over a known time period with RPMI, and fresh medium with or without the compound to be tested for its iron-releasing properties was added at 110 μM IBE. To measure the time course of ⁵⁷Fe release, medium was removed for ⁵⁷Fe counting and replaced with fresh medium (2 ml) after 2 and 6 h. At the end of the release experiment, the medium was decanted, the adherent cells removed from the plate, and the radioactivity measured on an LKB Compugamma 1282 counter (Amersham Biosciences). Cell damage was monitored by measuring lactate dehydrogenase in the incubation medium. Cell viability was determined at the end of the experiment by fluorescence microscopy.

**K562 Cell Culture and Freezing for EPR Analysis—**As human K562 leukemia cells give a sufficiently strong tyrosine radical signal for the kinetics of inhibition and regeneration to be studied in intact cells (7), we have therefore used this cell line to investigate the rate of suppression of the tyrosyl radical of intracellular ribonucleotide reductase by different HPO chelators.

**TABLE II**

| Chelator name | % 5-LO inhibition at 30 min | % 5-LO inhibition at 60 min | % DNA synthesis inhibition at 1 h |
|---------------|----------------------------|---------------------------|---------------------------------|
| CP358         | 2.07 ± 1.56                | 10.74 ± 3.20              | 17.71 ± 2.28                    |
| CP357         | 3.82 ± 1.60                | 13.10 ± 2.90              | 23.70 ± 3.74                    |
| CP354         | 13.48 ± 3.92               | 50.49 ± 3.55              | 24.10 ± 2.87                    |
| CP355         | 21.00 ± 2.58               | 39.25 ± 1.90              | 36.83 ± 1.53                    |
| CP351         | 29.60 ± 3.16               | 79.45 ± 1.95              | 26.60 ± 0.74                    |
| CP353         | 18.50 ± 2.47               | 60.28 ± 2.45              | 30.80 ± 3.49                    |
| CP350         | 30.00 ± 4.35               | 70.70 ± 2.75              | 24.06 ± 3.09                    |
| CP352         | 24.92 ± 2.45               | 65.51 ± 2.86              | 34.25 ± 0.48                    |
| CP80          | 48.50 ± 4.36               | 71.10 ± 3.10              | 36.80 ± 1.95                    |
| CP60          | 59.35 ± 3.90               | 82.00 ± 3.76              | 39.40 ± 3.38                    |
| CP20          | 39.40 ± 3.92               | 68.05 ± 2.49              | 40.10 ± 1.61                    |
| CP21          | 58.35 ± 4.78               | 90.00 ± 4.16              | 42.92 ± 1.22                    |
| CP26          | 80.00 ± 2.50               | 96.47 ± 1.10              | 45.61 ± 1.12                    |
| CP77          | 98.00 ± 0.91               | 100.00                    | 49.67 ± 1.17                    |
| CP25          | 93.20 ± 2.18               | 100.00                    | 54.98 ± 1.60                    |
| CP26          | 98.65 ± 1.00               | 100.00                    | 56.89 ± 2.00                    |
| DFO           | 0.00 ± 0.00                | 0.00                      | 17.22 ± 2.05                    |

K562 cells were cultured at 37 °C in RPMI 1640 medium with 5% (v/v) FCS under CO₂/air (1:19). Cell stocks were maintained at 2–6 × 10⁶ cells/ml. To ensure that cells were in exponential growth, media was changed 24 h prior to each experiment; cell viability as determined by ethidium bromide/acridine orange fluorescence microscopy was greater than 95% for all experiments. During experiments cells were grown at 4–5 × 10⁶ cells/ml (200 ml/flask). HPO chelators were added from a 10× concentrated stock solution in PBS to give a final concentration of 330 μM IBE, and an equivalent volume of PBS was added for the control sample. Cells were prepared for EPR analysis by centrifuging at 500 × g for 3 min at 0 °C, removing the supernatant, and vortex-mixing the cell pellet. 200-μl aliquots were frozen in EPR tubes. The tubes containing the cells were rapidly frozen in liquid nitrogen. The overall time taken for spinning and freezing was less than 6 min.

**EPR Analysis of Cells—**K562 cells were stored in liquid nitrogen and analyzed using a high quality spherical resonator (SP9703) on a Bruker EMX EPR spectrometer with an Oxford Instruments liquid helium cryostat. Each tube contained 80–100 million cells (0.5 ml total volume). The spectra were recorded at 30 K under nonsaturating conditions as follows: 20 milliwatts for the iron complexes (g = 4.31) and 2 milliwatts for the tyrosyl radical (g = 2). To show the structure of the radical, spectra in the g = 2 region were recorded at 0.5-millesias modulation amplitude, whereas those at g = 4.3 were recorded with 1-millesias modulation amplitude. There are significant signals from other metalloproteins in these cells that can obscure the tyrosyl radical signal. To remove these signals, a varying amount of a background spectrum was subtracted from the experimental signal until the interference was minimized. This background was obtained by incubation of K562 cells with the relevant chelator (at 330 μM) for 24 h. No tyrosyl radical was observable in the background spectra.

The relative concentration of the tyrosyl radical under different conditions was obtained by measuring the peak minus the trough value of the signal. To account for background species, the spectra were compared relative to control and radical-free (background) spectra. The signal in the absence of chelator addition was arbitrarily set to 1, and the signal in the radical-free background was set to 0 (7). Absolute quantification of the intracellular high spin ferric chelator complex was obtained by comparing the signal size with a standard curve obtained from the EPR signals of saturated iron chelator complexes of known concentration. These iron complexes were prepared by adding iron (ferrous ammonium sulfate dissolved in 0.05 M H₂SO₄, 0–80 μM dropwise to the chelator in PBS. The pH was adjusted to 7.4 immediately after the addition of iron. The iron:chelator ratio adopted for the preparation of these HPO-iron complexes was 1:3.1 (36).
RESULTS

Effect of Hydroxypyridinone R1 Substituents on 5-Lipoxygenase and DNA Synthesis Inhibition

The HPO chelators (CP20–CP26) investigated were all found to be potent inhibitors of both 5-LO and DNA synthesis, because without exception they caused >20% inhibition of DNA synthesis after only 1 h of incubation with K562 cells at a concentration of 11 μM IBE (Fig. 2) and ≥40% inhibition of 5-LO after 30 min of incubation at 110 μM IBE (Table I). In contrast to the large variation observed by this series of chelators in terms of their percentage 5-LO inhibition (Table II), there is only a slight variation between this series of chelators in terms of their percentage inhibition of DNA synthesis. The HPO currently in clinical use, CP20 was found to be the least potent inhibitor of the series, causing 22% inhibition of DNA synthesis, at 11 μM IBE, compared with 32% inhibition caused by the more hydrophobic CP26, the most potent of the series (Fig. 2). As the concentration (μM IBE) of the HPO chelators increased from 11 μM IBE to 330 μM IBE, a further increase in the inhibition of DNA synthesis was observed. CP20 and CP26 caused 60 and 66% inhibition respectively (p < 0.05) (Fig. 2). A linear correlation, at 110 μM IBE, was found to exist between the logD7.4 values and the inhibition of DNA synthesis (R = 0.96) (Fig. 3A). There is also a clear relationship between logD7.4 of HPO chelators (CP20–CP26) and the percentage inhibition of 5-LO, the more hydrophobic chelators being the most potent inhibitors (Fig. 3B).

Effect of Hydroxypyridinone R2 Substituents on 5-Lipoxygenase and DNA Synthesis Inhibition

5-Lipoxygenase—HPOs with different substituents at the 2-position show variable inhibitory properties toward 5-LO, ranging from CP60, which caused nearly 60% inhibition of the enzyme activity after 30 min of incubation at 110 μM IBE, to CP358, which caused only 2% inhibition of the enzyme activity under the same conditions (Table II). The inhibitory properties of this latter compound approach those of DFO, which does not inhibit the enzyme activity even after 24 h of incubation at a concentration of 110 μM IBE. In contrast, CP20 is a relatively potent inhibitor, causing 40% inhibition of the enzyme activity after 30 min of incubation. There is no simple correlation between the distribution coefficient of these chelators and their ability to inhibit 5-LO at both times of incubation (Table I and II). However, a correlation does exist between the size of the 2 substituents on the HPO ring and inhibitory properties, inhibition decreasing with increasing size of the substituent (Table II). In fact, Fig. 4 shows a linear relationship (R = 0.92) existing between the percentage inhibition of 5-LO after 24 h of incubation and the molecular width of the chelators. This explains why CP60, which only has a hydrogen at the 2-position, caused the maximum inhibition observed with this series, whereas CP358 which has the largest substituent was found to cause the minimum inhibition at all concentrations investigated.

DNA Synthesis—HPO chelators with different substituents at the 2-position again show variable inhibitory properties toward DNA synthesis. Thus, CP358, CP357, CP354, and CP351 cause minimum inhibition of DNA synthesis, in contrast to CP20 and CP60 (Table II). The full range of inhibitory activity falls between CP20 (68% inhibition at 330 μM IBE) to
CP358 (28% DNA synthesis inhibition). There is no clear correlation between the distribution coefficient of these HPO chelators and their ability to inhibit DNA synthesis. In contrast, however, it is clear that a correlation ($R^2 = 0.91$) does exist between the size of the substituents introduced at the 2-position of the HPO ring expressed as molecular width and inhibition of DNA synthesis, inhibition decreasing with increasing substituent size (Fig. 5). Thus CP20, which only has a methyl at the 2-position, induces the maximum inhibition observed with this series, whereas CP358 which has the largest substituent, namely $N$-$(4',N$-propylsuccinamido)methyl, causes the least inhibition, again behaving like DFO. Indeed, CP358 and DFO display a similar time course and concentration dependence on inhibition (Fig. 6, A and B), both lacking inhibitory activity at 30 min.

Iron Mobilization from Hepatocyte Monolayer Cultures

The percentage of $^{59}$Fe released from hepatocytes incubated with the chelator CP358 for 6 h at 110 $\mu$M IBE, as compared with control cells, was found to be 289 ± 30%. Also, $^{59}$Fe release was 175 ± 20% with CP20 and 251 ± 25% with DFO in the same system. Both CP358 and DFO mobilize $^{59}$Fe more efficiently than CP20 ($p < 0.001$) at 6 h. Furthermore, all three chelators mobilized iron from hepatocytes without affecting the integrity of the hepatocyte membrane as judged by lactate dehydrogenase release compared with control cells.

EPR Analysis of the Time Course of Tyrosyl Radical Removal and Iron Complex Formation by CP358 and CP20—The main features of the tyrosine radical of RR appear at $g = 2$ (Fig. 7A) (7, 37–39). This feature was nearly 70% decreased after 1 h of treatment with 330 $\mu$M IBE CP20 (Fig. 7E). In contrast, there was a slower decay of the RR tyrosine radical in the presence of CP358 (330 $\mu$M IBE), since after 1 h of treatment, the main features of the signal were still apparent and only decreased in size by ∼10% (Fig. 7C); however, after 4 h the tyrosyl radical signal was completely removed by both chelators (Fig. 7, B and D). In an attempt to account for the difference in the radical decay rates observed for these two chelators, we also monitored the rate of intracellular ferric-chelate complex formation. Low molecular weight iron(III) chelates have relatively sharp EPR signals at $g = 4.3$ (7, 40, 41). The cellular $g = 4.3$ signal was quantified by comparing it with that of either Fe(CP20)$_3$ or Fe(CP358)$_3$ complexes. The intracellular EPR-detectable iron was calculated to be 1.60 ± 0.66 $\mu$M (Fig. 8a). The addition of either CP20 or CP358 increased the size of the intracellular $g = 4.3$ signal by the same rate (Figs. 8 and 9), indicating that both chelators possess similar rates of iron-chelate complex formation ($p > 0.05$), which contrasts to the difference in the tyrosine radical decay rate ($p < 0.001$) (Fig. 7 and 9).

In an attempt to correlate the percentage inhibition of DNA synthesis to the tyrosyl radical decay rate, we have measured the rate of tyrosyl radical decay after a 1-h incubation with the HPO chelators, CP354 and CP352 in addition to CP20 and CP358 at 330 $\mu$M IBE (Fig. 10). It can be seen that there is a significant correlation ($R = 0.99$) between the inhibition of
DNA synthesis and the decrease in the tyrosyl radical signal at 1 h (Fig. 11). Furthermore, there is a strong correlation between the decrease of the tyrosyl radical signal at 1 h and the molecular width (R/H11005 0.95) (Fig. 12A) but not the lipid solubility of the HPO chelators tested (Fig. 12B). These findings support the view that the molecular size and not the lipid solubility of the chelators determine the rate of inhibition of DNA synthesis and the decrease of the tyrosyl radical. It must be noted that all four HPO chelators increased the size of the intracellular g/H11005 4.3 signal by the same rate (Fig. 13), indicating that the chelators tested possess similar rates of iron-chelate complex formation.

DISCUSSION

Like naphthols, catechols, and hydroxamic acids that also inhibit 5-LO, HPO chelators become increasingly inhibitory as their hydrophobicity increases (Fig. 3B) (31, 42, 43). However, whereas this relationship holds for hydroxypyridinones where the size of the alkyl substitution is increased in the 1-position of the pyridinone ring (Fig. 3B), this is not the case for HPO chelators with large substituents in the 2-position. In fact, the variation in the inhibitory properties of the HPO chelators possessing different R2 substituents are again not directly related to their hydrophobicity but are more closely related to the size of the 2-substituent (as expressed by molecular width), with inhibition decreasing with increasing molecular width of the chelator (Fig. 5). CP358, which has the largest substituent at the 2-position, causes only 10% inhibition of 5-LO activity after 24 h of incubation at 110 M IBE compared with 68% with CP20 (Table II). The former chelator behaves very much like DFO. Abeysinghe et al. (8) found that the inhibition of 5-LO by HPO chelators, as monitored spectrophotometrically and by using EPR spectroscopy, is associated with the removal of the enzyme active site iron center signal with concomitant formation of a hydroxypyridinone Fe(III) complex signal. Hence, Abeysinghe et al. (8) suggested that direct access and chelation of the iron center is the mechanism for lipoxygenase inhibition by HPO chelators. The hydrophobicity-dependent 5-LO inhibition observed in the results obtained in this study are consistent with this mechanism of action and with the findings of Boyington et al. (12) who demonstrated the existence of two large internal channels that are lined by hydrophobic amino acids and that connect the iron center to the protein outer surface. The strong dependence of the inhibition of 5-LO activity on the size of the 2-substituent can also be explained by the existence of these access channels.

With regard to the inhibition of DNA synthesis, the results indicate that the variation in the inhibitory properties of HPO chelators possessing different substituents at the R2 position are again not directly related to their hydrophobicity but are more closely related to the size of the 2-substituent (as expressed by molecular width), with inhibition decreasing with increasing molecular width of the chelator (Fig. 5). CP358
caused only 28% inhibition of DNA synthesis, in contrast to CP20 which caused 68% inhibition, after 1 h of incubation with K562 cells at 330 μM IBE thus behaving very much like DFO (Fig. 6A). Indeed, the DNA synthesis inhibitory properties of CP358 and DFO are almost identical (Fig. 6, A and B). In an attempt to correlate the percentage inhibition of DNA synthesis to the rate of decrease of the RR tyrosyl radical, we have measured, using EPR spectroscopy, the rate of tyrosyl radical decay after 1 h of incubation with four of the HPO chelators tested. Fig. 11 shows a strong correlation between the inhibition of DNA synthesis and the decrease of the tyrosyl radical signal at 1 h, with the rate of tyrosyl radical decrease being dependent on the molecular width and not the lipophilicity of the chelators (Fig. 12). There are at least two interpretations of these findings. First, access to intracellular iron pools is determined by molecular size more than by lipid solubility for R2 substituents, unlike R1 substituents. Second, molecular size does indeed affect the interaction of HPO chelators with the iron center in the enzyme, RR. However, consideration of the iron complex (g = 4.3) signals for the different chelators at 1 h compared with the tyrosyl radical inhibition makes the first possibility less likely (Figs. 10 and 13). For example CP358 shows relatively little tyrosyl radical decrease compared with CP20 but shows a higher concentration of intracellular iron complex (Fig. 9). The high concentration of CP358 iron complexes is unlikely to be due to their accumulation intracellularly, because the iron release experiments show more rapid release of intracellular iron with CP358 than with CP20. The findings thus suggest that molecular dimensions influence the inhibition of the tyrosyl radical without affecting access to chelatable intracellular iron. A possible mechanism is that the regeneration of the tyrosyl radical, which requires iron initially to be present in the reduced form, could be abrogated by the formation of ternary complex between the chelator and the iron center of the radical. The tendency to form such complexes with the iron center of RR would be predicted to depend on molecular size.

In summary, the findings in this paper suggest that the inhibition of 5-LO and RR by hydroxypyridinones can be limited more by the size and shape of the molecules than by their hydrophobicity. This finding can be exploited in the design of an HPO chelator with minimum inhibitory properties toward 5-LO by introducing a hydrophilic substituent at the 1-position of the HPO ring and a bulky substituent at the 2-position. Such an HPO chelator will still inhibit RR but at a much slower rate than that observed by smaller HPOs such as CP20 (deferoxiprone). One such HPO, CP358, causes minimal inhibition of 5-LO activity, RR activity, and DNA synthesis while maintaining appreciable iron mobilization from hepatocytes. It is likely, therefore, that the in vivo toxicity of CP358 will be lower than that of the more simple dialkyl HPO chelators while maintaining the same ability to mobilize excess body iron.

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Structure-Function Investigation of the Interaction of 1- and 2-Substituted 3-Hydroxypyridin-4-ones with 5-Lipoxygenase and Ribonucleotide Reductase
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