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Structure–activity investigation of the inhibition of 3-hydroxypyridin-4-ones on mammalian tyrosine hydroxylase

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Abstract

3-Hydroxypyridin-4-ones are currently one of the main candidates for the development of orally active iron chelators. Small bidentate ligands tend to inhibit iron-containing metalloenzymes and therefore can cause undesirable side effects. A range of 3-hydroxypyridin-4-ones with different R_2 substitutents was selected for the investigation of the structure–activity relationship between the chemical nature of the ligand and the inhibition of mammalian tyrosine hydroxylase. Results indicated that lipophilicity was the dominant factor in controlling the ability of this class of chelator to inhibit mammalian tyrosine hydroxylase. Ligands with hydrophilic R_2 substitutents tended to be weak inhibitors. No significant correlation was found in this study between iron-binding affinity, extended R_2 chain length, and enzyme inhibitory activity. In contrast, both the LogP values of the entire molecule and of the R_2 segment correlated well with inhibitory activity.

Keywords: Iron chelators; Hydroxypyridinone; Tyrosine hydroxylase; Lipophilicity

1. Introduction

The design of orally active, non-toxic iron chelators has been a goal for medicinal chemists for the past 25 years. Naturally occurring siderophores are good models for such molecules, but due to their physico-chemical properties, they are not well absorbed from the mammalian intestine [1]. Desferrioxamine has proved to be useful in the treatment of transfusion-induced iron overload of thalassaemic patients, but has to be administered via parenteral routes [2]. In order to achieve good oral bioavailability, bidentate [3] and tridentate [4] ligands have been investigated and both classes show potential.

3-Hydroxypyridin-4-ones (Fig. 1) are currently the optimal bidentate candidates for the development of orally active iron chelators [5]. Indeed, the 1,2-dimethyl derivative (deferiprone, L1, CP20) (**1**) (Fig. 1) is the only orally active iron chelator available for clinical use (marketed by Apotex Inc. as Ferriprox™). One disadvantage associated with the use of these smaller molecules is that they have greater access to the active sites of metalloenzymes, and thus tend to be more potent inhibitors than hexadentate siderophores [5–7]. In general, iron(III)-chelating agents do not inhibit haem-containing enzymes or iron–sulphur cluster proteins, although they do interfere with enzymes containing mono-iron and bi-iron centres co-ordinated to oxygen ligands, including lipoxygenases and aromatic amino acid hydroxylases [7,8]. Lipoxygenases are generally inhibited by hydrophobic chelators [9] and consequently the introduction of hydrophilic characteristics into a chelator tends to minimise its inhibitory potential. No such effect has been reported with the amino acid hydroxylases; for instance, the extremely hydrophilic mimosine (**2**) (Fig. 1) is a potent inhibitor of tyrosine hydroxylase [10,11] and is generally toxic [12]. As a result of a preliminary investigation with the plant enzyme tyrosinase, it was proposed that the bulk of the 2-substituent reduces the ability of hydroxypyridinone chelators to cause inhibition [10]. In the present study, we investigated the influence of size and hydrophobicity of the R_2 substitutent of a range of 3-hydroxypyridin-4-ones on their ability to inhibit mammalian tyrosine hydroxylase. The

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Abbreviations: L-DOPA, L-3,4-dihydroxyphenylalanine; MOPS, 4-morpholinepropane sulphonic acid; and DHBA, 3,4-dihydroxybenzylamine.

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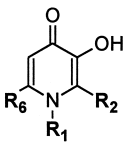

Deferiprone (CP20, L1) (**1**): $R_1 = R_2 = CH_3$, $R_6 = H$

Mimosine (**2**): $R_1 = CH_2CH(NH_2)COOH$, $R_2 = R_6 = H$

Fig. 1. General structure of 3-hydroxypyridin-4-ones.

brain enzyme was utilised in this work, as this enzyme is involved in the production of L-DOPA, a key chemical transmitter. Interference with the activity of this enzyme is likely to lead to undesirable side effects [13].

2. Materials and Methods

2.1. Iron chelators

The 3-hydroxypyridin-4-ones used in the present study were synthesized as previously described [3,14–16]. The compounds of initial interest are those which have a variable chain length at the R_2 position (Table 1). As modification of the chemical nature of the R_2 substitutent will influence the metal-binding ability of the ligand [3,16], some of these hydroxypyridinones were subjected to physico-chemical characterisation.

2.2. Determination of physico-chemical properties of ligands

Distribution coefficients ($D_{7.4}$) of the chelators between 1-octanol and MOPS buffer (pH 7.4) were determined at 25 ± 0.5°, using an automated continuous flow technique that provides accurate and reproducible measurements [14–16]. The compound to be examined was dissolved in a known volume (normally 25–50 mL) of MOPS buffer (saturated with octanol) so as to give an absorbance of between 0.5–1.5 absorbance units at a preselected wavelength (~280 nm). After equilibrium was reached, an aliquot of octanol (saturated with MOPS buffer pH 7.4) was added and re-equilibration assessed. This cycle was repeated until a predefined total volume of added octanol was reached. The aqueous phase was separated from the two-phase system (1-octanol/MOPS buffer, pH 7.4) by means of a hydrophilic cellulose filter (5-μm diameter, 589/3 Blauband filter paper, Schleicher and Schuell). The flow rate of the aqueous circuit was limited to 1 mL/min. The distribution coefficient was calculated for each octanol addition.

Equilibrium constants of protonated ligands were determined using an automated system consisting of a Metrohm 665 dosimat, a Perkin Elmer Lambda 5 spectrophotometer, a Corning Delta 225 pH meter, combined Sirius electrode, and a 286 Opus PC as controller of the integrated system. This system is capable of undertaking simultaneous potentiometric and spectrophotometric measurements [14–16]. Titrations were carried out in a jacketed cell maintained at 25 ± 0.5° under an argon atmosphere fitted with a recirculatory solution path through a 1-mm flow-through cell in the spectrophotometer. Solutions were titrated with 0.3 mL of 0.2 M KOH using 0.01-mL increments dispensed from the dosimat. A blank titration of 0.1 M KCl (25 mL) acidified with 0.15 mL of 0.2 M HCl was carried out to determine the electrode response slope and zero using Gran's plot method. The titration was repeated in the presence of ligand. The data obtained from titration were subjected to non-linear least-squares regression analysis [17].

Iron(III) stability constants of the ligands were determined from the spectrophotometric titration of the metal–ligand system using the pK_a values, electrode slope, and electrode zero determined above [16]. The analytical equipment used was similar to that employed for pK_a determination but in order to maintain sufficient sensitivity, a 10-mm path UV flow cell was utilised. After electrode calibration, the solution was re-acidified to pH 1.5–2.0 by adding concentrated hydrochloric acid. Iron(III) stock solution (atomic absorption standard, Aldrich) and the test ligand were then added to give a final ligand:iron(III) ratio of about 10:1. A preadjusted, programmed, autoburette was used for the addition of a 0.2 M KOH solution. The resulting spectrophotometric titration curve was then subjected to non-linear least-squares regression analysis for the determination of stability constants.

2.3. Determination of the effect of iron chelators on tyrosine hydroxylase activity

Male Wistar rats (250 g; Bantin and Kingman) were housed in the Biological Service Unit, King's College London. The animals were maintained at a temperature between 20 to 23°, with food and water *ad lib*. Rats were killed by cervical dislocation, with the brain removed and placed immediately on ice-cold 0.5 M potassium phosphate buffer. The striata were immediately removed, weighed, and homogenised in 0.32 M sucrose in a 1:10 v:v ratio using a Microson ultrasonic tissue disruptor at 4°.

Tyrosine hydroxylase assays were performed in 1.5-mL Eppendorf tubes. The reaction medium (100 μL) contained striatal homogenate (equivalent to 3 mg of tissue), potassium phosphate buffer (0.15 M, pH 6.0), *m*-hydroxybenzylhydrazine (NSD 1055) (99 μM), 6,7-dimethyl-5,6,7,8-tetrahydropterine ($DMPH_4$) (1 mM), and mercaptoethanol (0.1 M), to which was added the test iron chelator. The reaction was initiated by the addition of L-tyrosine (final concentration 0.4 mM) and the tubes were incubated at 37° for 20 min in a shaking water bath. The reaction was terminated by the addition of 100 μL perchloric acid (0.4 M) containing 5 μM DHBA (internal standard) and placed on ice. The reaction mixture was centrifuged at 4600 *g* for 10 min at 4° in a Sorvall RC5B plus. L-DOPA and DHBA were extracted onto alumina from 150 μL of the resultant supernatant. Tris buffer (pH 8.6, 1 mL) and 140–150 mg acid-washed alumina were added to the supernatant, shaken vigorously for

Table 1
Physico-chemical properties and molecular descriptors of selected 3-hydroxypyridin-4-ones

Ligands	R_1	R_2	R_6	MW	$D_{7.4}$[a]	LogP[b]	pK_a[c]	logβ_3[d]	pFe^{3+}[e]	LogP(R_2)[f]	R_2 length (Å)	% inhibition (1×10^{-4} M)
CP60	CH_3	H	H	125	0.25 ± 0.04	−0.58	3.29, 8.79	35.1	21.5	0.000	1.0	43.6
CP20	CH_3	CH_3	H	139	0.17 ± 0.01	−0.77	3.62, 9.78	36.4	19.4	0.500	1.51	75.3
CP69	CH_3	H	CH_3	139	0.20 ± 0.01	−0.70	3.69, 9.13	35.6	21.0	0.000	1.0	62.0
CP94	C_2H_5	C_2H_5	H	167	1.78 ± 0.01	0.25	3.81, 9.93	36.8	19.7	1.810	2.51	87.4
CP374	CH_3	$CH(OH)C_2H_5$	CH_3	197	0.73 ± 0.01	−0.14	3.78, 8.98	35.0	21.0	0.294	3.66	70.1
CP502	CH_3	$CONHCH_3$	CH_3	196	0.04 ± 0.01	−1.36	2.77, 8.44	34.3	21.7	−0.974	3.73	21.2
CP507	CH_3	$CONHCH_2CH_2OH$	CH_3	226	0.02 ± 0.01	−1.65	2.67, 8.27	33.3	21.2	−1.525	6.01	33.1
CP357	CH_3	$CH_2NHCO(CH_2)_2NHCOCH_3$	CH_3	281	0.02 ± 0.004	−1.70	3.20, 9.21	36.7	20.8	−1.441	9.47	16.9

[a] The measured distribution coefficients of the ligands between 1-octanol and MOPS buffer (pH 7.4). [b] The logarithm of the partition coefficients of the ligands between 1-octanol and MOPS buffer (pH 7.4). [c] The negative logarithm of the equilibrium constants of the protonated ligands. [d] The logarithm of the cumulative iron(III) stability constants for the ligands. [e] The negative logarithm of the concentration of the free iron(III) in solution, calculated for total [ligand] = 10^{-5}M, total [iron] = 10^{-6}M at pH 7.4. [f] The calculated LogP values of the R_2 chain of the ligands.

30 min at room temperature, and then centrifuged at 3000 *g* for 30 sec at 9°. The supernatant was aspirated and the alumina washed twice with distilled water (2 mL), centrifuging between each wash as above. Following the second wash, L-DOPA and DHBA were eluted by addition of 200 μL perchloric acid (0.4 M), shaking vigorously for 10 min and centrifuging at 1000 *g* for 10 min at 9°. The resultant supernatant was used for HPLC determination of the concentration of L-DOPA and DHBA. All assays were performed in triplicate. Non-enzymatic formation of L-DOPA was determined by the addition of D-tyrosine (0.4 mM) and 3-iodotyrosine (0.2 mM) instead of L-tyrosine.

The HPLC system used for the determination of L-DOPA and DHBA consisted of a Waters 6000A pump, a Spectraphysics 8770 autosampler, and a BAS LC 4B electrochemical detector. The potential set across the glassy carbon electrode was 0.8 V versus an Ag/AgCl reference electrode. The mobile phase was 0.1 M sodium phosphate (pH 3.0–3.2) containing EDTA (1 mM), octane sulphuric acid (0.65 mM), and 15–18% methanol. The flow rate was 1 mL/min and the column was a Spherisorb ODS2 (Waters, 4.6 × 250 mm, 5-μm particle size). The resulting peaks were integrated using a Unicam 4880 integrating recorder, and calibration achieved using aliquots (20 μL) of L-DOPA and DHBA (5 μM) extracted onto alumina as described above. Quantitation was performed using peak height ratio with the internal standard (DHBA). Results were expressed as L-DOPA formed/20 min/3 mg tissue.

2.4. Molecular properties and statistical analysis

The ACD ChemSketch program, version 4.01 [18], was used to calculate the extended R_2 chain length. Structures were 3D-optimised and distances for the R_2 chain length were measured from the optimised structures. The LogP values of the equivalent isolated R_2 chain were calculated using the CLOGP program [19]. Statistical calculations were performed with the Minitab program, version 11 [20].

3. Results

The measured physico-chemical properties ($D_{7.4}$, pK_a, and β_3) of the ligands and the calculated molecular descriptors (extended R_2 length and LogP value of the R_2 chain) are summarised in Table 1. Since the modification of R_2 substitutent can influence the pK_a values of the 3-hydroxyl group (pK_{a_2}) (Table 1), the degree of ionization of this functional group at pH 7.4 becomes appreciable with some of the compounds under investigation. The neutral fraction (Fn) of the molecule can be calculated from equation 1 by employing the determined pK_{a_2} values. The partition coefficient (P) of each compound was calculated from equation 2:

$$\text{Neutral fraction}(F_n) = \frac{1}{1 + 10^{pH - pKa_2}} \quad (1)$$

$$P = \frac{D_{7.4}}{F_n} = D_{7.4} - (1 + 10^{pH - pKa_2}) \quad (2)$$

Another comparator for ligand-binding ability, the pFe^{3+} value, can be calculated by using the determined pK_a values of the ligands and the stability constants (β_3) of the iron complex (Table 1). pFe^{3+} is defined as the negative logarithm of the concentration of the free iron(III) in solution, typically calculated for total [ligand] = 10^{-5}M, total [iron] = 10^{-6}M at pH 7.4. The comparison of ligands using this parameter is useful since pFe^{3+}, unlike the corresponding stability constants, takes into account the effects of ligand basicity, denticity, and degree of protonation, as well as differences in metal–ligand stoichiometries. The introduction of a specific substituent at the 2-position of 3-hydroxypyridin-4-ones leads to an appreciable variation of pFe^{3+} values, ranging from 19.4 (CP20) to 21.7 (CP502) (Table 1).

The inhibitory activity of the compounds when tested against rat tyrosine hydroxylase was investigated *in vitro*, and enzyme inhibitions, expressed as percentage of the corresponding control, are summarised in Fig. 2. The inhi-

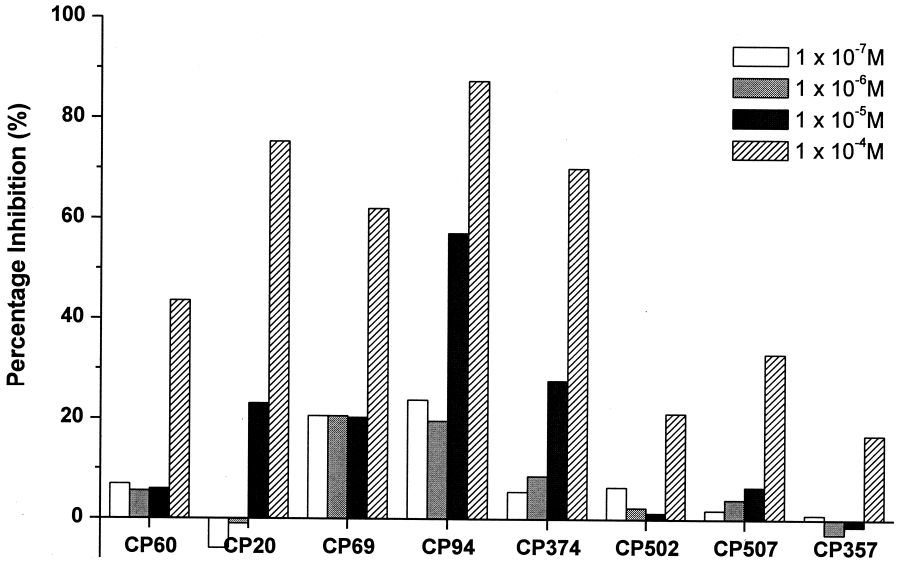

Fig. 2. Percentage inhibition of rat tyrosine hydroxylase induced by 3-hydroxypyridin-4-ones at different concentrations. The control values of L-DOPA (nmol) for each compound were: CP60, 0.450 ± 0.094 (N = 4); CP20, 0.493 ± 0.071 (N = 3); CP69, 0.739 ± 0.155 (N = 4); CP94, 0.795 ± 0.107 (N = 3); CP374, 0.733 ± 0.084 (N = 4); CP502, 0.482 ± 0.051 (N = 6); CP507, 0.652 ± 0.032 (N = 5); CP357, 0.439 ± 0.047 (N = 6). Values are expressed as means ± SD.

bition of mammalian tyrosine hydroxylase by hydroxypyridinones was found to be dose-dependent and most of compounds, except CP94 and CP69, caused very little inhibition at low ligand concentrations ($\leq 1 \times 10^{-6}$M). The most potent inhibitors were found to be CP94, CP20, and CP374 in that order, while CP502 and CP357 were the least inhibitory. The percentage inhibition at the highest concentration (1×10^{-4}M) was taken as the biological response at a fixed dose for the QSAR studies.

The influence of individual descriptors (Table 1) was assessed through simple regression analysis between the corresponding descriptors and percentage inhibition. Goodness of fit of models was determined from the r^2 values and ranked according to their goodness of fit. These are shown in Table 2 along with the fitted parameters of the equation $Y = A + BX$. At the 5% level of significance (approximately equivalent to $r^2 > 0.7$), only the two lipophilicity predictors were significant. The calculated lipophilicity of the R_2 side chain LogP(R_2) showed a better correlation with the biological response than the experimentally determined LogP values (Fig. 3).

4. Discussion

In principle, iron-binding affinity, molecular dimensions, and lipophilicity are all likely to play an important role in controlling the ability of a ligand to inhibit tyrosine hydroxylase. However, it is clear from the current study that once the pFe^{3+} value is above 20, ligands have little further influence on the inhibitory activities of this molecular class (Table 2). In contrast, bidentate ligands with pFe^{3+} values appreciably less than 20 are generally much weaker inhibitors of this class of enzyme, two such examples being maltol ($pFe^{3+} = 15$) and *N*-hydroxypyridin-2-one ($pFe^{3+} = 16$), both possessing relatively weak inhibitory ability [10].

Although no significant correlation was found in this study between the extended R_2 chain length and enzyme inhibitory activity (Table 2), a clear correction was observed with lipophilicity of the molecule, particularly the calculated LogP value of the R_2 side chain (Fig. 3). Thus CP94, with $R_2 = CH_2CH_3$, was found to be a more potent inhibitor than CP507 with $R_2 = CONHCH_2CH_2OH$; the measured

Table 2
Single regression analysis of the influence of individual molecular descriptors on mammalian tyrosine hydroxylase inhibitory activity

	LogP(R_2)	LogP	pFe^{3+}	R_2 length	MW
r	0.922	0.883	−0.711	−0.646	−0.615
r^2	0.85	0.78	0.504	0.417	0.378
P	0.001	0.004	0.05	0.08	0.10
A[a]	54.82	78.7	524.4	72.24	107.7
B[a]	21.67	33.1	−22.8	−5.83	−0.31

[a] Percentage inhibition(Y) = A + B × molecular descriptors(X).

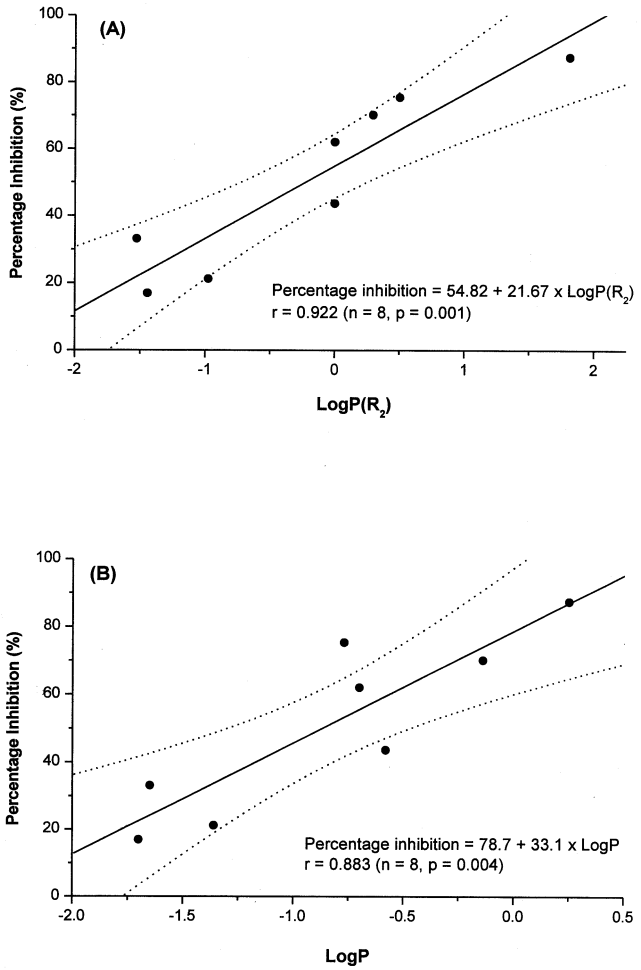

Fig. 3. Correlation between the calculated LogP values of the R_2 chain of 3-hydroxypyridin-4-ones (A) and the measured LogP values with percentage inhibition of tyrosine hydroxylase at [ligand] = 1×10^{-4}M (B). Dotted lines indicate 95% confidence bands.

LogP values of the two chelators were 0.25 and −1.65 and the calculated LogP(R_2) 1.81 and −1.525, respectively. This finding is in contrast to a previous publication describing the inhibition of a plant tyrosine hydroxylase by bidentate ligands, where it was proposed that the presence of R_2 groups irrespective of hydrophobicity would decrease the inhibitory potential against tyrosinase [10]. Tyrosinase is widely distributed throughout the bacterial, plant, and animal kingdoms [21] and in most of these organisms it catalyses two reaction types, the hydroxylation of monophenols and the oxidation of *ortho*-diphenols to *ortho*-quinones, both using molecular oxygen [22]. The active site incorporates a coupled binuclear copper centre that binds oxygen [23] and the substrates [24]. The plant enzyme possesses a rather broad selectivity [24], unlike the more specialised mammalian enzyme. Thus, the protein pocket that contains the binuclear copper site is likely to differ between plant and mammalian enzymes. The marked difference in behaviour reported in this study indicates the importance of monitoring mammalian enzymes in such work.

Significantly, a lead orally active iron chelator (CP502 [3]) was found to be a much weaker inhibitor than deferiprone (CP20) (**1**), the only orally active iron chelator currently available for clinical use. The relatively tight relationship between LogP(R_2) and inhibitory activity provides useful information for chelator design. However, it is important to note that there is also a reasonable relationship between LogP and inhibitory activity (Fig. 3B). This is important because the absorption and distribution of the chelators will be strongly dependent on this latter parameter.

In summary, in addition to the lipoxygenase family of enzymes, the mammalian brain tyrosine hydroxylase was found to be more susceptible towards inhibition by hydrophobic chelators, hydrophilic chelators (LogP ≤ -1.0) being relatively weak inhibitors. This finding will facilitate the design of non-toxic, orally active iron chelators.

Acknowledgments

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