Novel cytotoxic chelators that bind iron(II) selectively over zinc(II) under aqueous aerobic conditions

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Abstract

To achieve cellular iron deprivation by chelation, it is important to develop chelators with selective metal-binding properties. Selectivity for iron has long been the province of certain oxygen-donor chelators such as desferrioxamine, which target Fe(III) and exploit the strength of a relatively ionic Fe-0 interaction. We have been studying novel chelators that possess mechanisms to selectively chelate +2 biometals, particularly tachpyr [N,N',N'-tris(2-pyridylmethyl)-1,3,5-cis,cis-triaminocyclohexane] and derivatives from N,N,N'-trialkylation and pyridine ring alkylation. Metal-exchange and metal-binding competition reactions have been conducted at pH 7.4, 37 °C and time periods until no further change was observed (generally 24–48 h). Under anaerobic conditions, tachpyr is strongly selective for iron, binding 95±5% Fe(II) versus 5±5% Zn(II) in the forms [Fe(tachpyr)]²⁺ and [Zn(tachpyr)]²⁺, respectively. Under aerobic conditions, tachpyr complexes Fe(II) more effectively than Fe(III), forming iminopyridyl complexes [Fe(tachpyr-ox-n)]²⁺ (n = 2, 4) by O₂-induced and iron-mediated oxidative dehydrogenation. Complexes [Fe(tachpyr-ox-n)]²⁺ are also strongly bound forms of iron that are unaffected by an excess of Zn(II) (75 mol zinc: 1 mol iron complex). The preference of tachpyr for iron over zinc under aerobic conditions appears to be hindered by oxidation of Fe(III) to Fe(III), such that the proportions bound are 44±10% Fe(II) versus 56±10% Zn(II), in the respective forms [Fe(tachpyr-ox-n)]²⁺ and [Zn(tachpyr)]²⁺. However, upon addition of the reducing agent Na₂S₂O₄ that converts Fe(III) to Fe(II), the binding proportions shift to 76±10% Fe(II) versus 24±10% Zn(II), demonstrating a clear preference of tachpyr for Fe(II) over Zn(II). Iron(II) is in the low-spin state in [Fe(tachpyr)]²⁺ and [Fe(tachpyr-ox-n)]²⁺ (n = 2, 4), which is a likely cause of the observed selectivity. N-methylation of tachpyr [giving (N-methyl)tachpyr] results in the loss of selectivity for Fe(II), which is attributed to the steric effect of the methyl groups and a resulting high-spin state of Fe(II) in [Fe(N-methyl)tachpyr]²⁺. The relationship of chelator selectivity to cytotoxicity in the tach family will be discussed.

Introduction

We are studying the complexation chemistry of biometal ions, including Fe(II), Fe(III), Zn(II) and Cu(II), by novel tripodal hexadentate chelators. The seminal compound tachpyr [N,N',N'-tris(2-pyridylmethyl)-1,3,5-cis,cis-triaminocyclohexane] has been found to bind Fe(II) to form [Fe(tachpyr)]²⁺, which under aerobic conditions undergoes iron-mediated oxidative dehydrogenation of the aminomethylpyridyl arms, to afford Fe(II)-imino complexes [Fe(tachpyr-ox-n)]²⁺ (n = 2, 4, 6) (Scheme 1). In all the complexes of Scheme 1[1] the electron configuration of Fe(II) is d⁸-low-spin, a state that is associated with high inertness and affinity of Fe(II) binding [2]. By contrast, the N-methylated derivative of tachpyr, (N-Me)_₃tachpyr (where Me is methyl), has an overall weaker interaction with Fe(II) and no oxidative dehydrogenation activity, consistent with the observation that (Fe[(N-Me)_₃tachpyr])₂(ClO₄)₄ is a complex of high-spin Fe(II) [1].

Iron chelators, including desferrioxamine (DFO), pyridoxal isonicotinoyl hydrazones (PIHs) and tachpyr, are under consideration as antitumour agents [3,4]. Cellular iron depletion by these chelators may play a role in their activity. Tachpyr is strongly cytotoxic towards cultured MBT2 (mouse bladder tumour) cells, IC₅₀ ≈ 5 μM [4]. Further studies have shown that cell death occurs by an apoptotic process and that...
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Scheme I
Structures of tachpyr and \((N\text{-Me})_3\text{tachpyr}\) and the reaction of Fe(II) with tachpyr

![Diagram of structures](image)

Fe\(^{2+}\)(aq) + tachpyr \(\text{pH 7.4, 37 °C} \quad [\text{Fe(tachpyr)}]^{2+}\)(aq) \(\text{O}_2 \quad [\text{Fe(tachpyr-ox-2)}]^{2+}\)(aq) \(\text{and} \quad [\text{Fe(tachpyr-ox-4)}]^{2+}\)(aq)

while p53 protein levels increase in cells treated with tachpyr, the mechanism of cell death is p53-independent [3]. A further indication of the relationship of iron-chelating ability with cytotoxicity comes from \((N\text{-Me})_3\text{tachpyr}\), whose weaker Fe-binding properties are reflected in a complete absence of cytotoxicity.

Because of the striking connections of the biological effects of tachpyr and derivatives to their metal-binding chemistry, we continue to elaborate their inorganic chemistry and chelator design aspects. Herein we describe the outcomes of several reactions designed to probe comparative interactions of tachpyr with iron and zinc ions, and the significance of these to selective chelation of iron into the +2 state in \textit{vivo}.

**Biological chelation of Fe(II) as an alternative to Fe(III)**

We pursue the thesis that the interaction of Fe(II) with a suitable chelator may possess sufficient affinity and inertness to be of biological use. Because treatment of iron overload has been a major objective in chelator design, the development of iron chelators for medicinal use has largely focused on chelators with a preference for Fe(III), such as hydroxamates, hydroxypyridinones, catecholates and phenolates. For example, DFO (Figure 1) chelates Fe(III) specifically over other biologically available metal ions, and into a safe form with respect to injurious redox reactivity. Because the ionic bonding properties of Fe(III) are far stronger than those of Fe(II) and other potential competing biometal ions, such as Zn(II), Cu(II) or Ca(II), chelators such as DFO that favour Fe(III) are felt to be good choices as selective and safe biological iron-sequestering agents.

The properties of Fe(II) are sharply different from Fe(III). Iron(II) binds more covalently; with the appropriate chelator (generally a nitrogen donor) strong covalent binding to iron in the +2 state occurs. The strength of the interaction is
demonstrated by the formation constant (log $K_f$) of the Fe(II)-tris(o-phenanthroline) $\{[\text{Fe}(\text{o-phen})_3]^{2+}\}$ complex (Figure 1), which is considerably higher than that of the corresponding Zn(II) complex (log $K_f$ of $[\text{Zn}(\text{o-phen})_3]^{2+}$ is 17.1) [6]. The electronic state of strongly bound Fe(II) is d$^6$-low-spin, which promotes inertness of the complex [2].

Some Fe(II) complexes promote cellular oxidative damage, which argues against Fe(II) chelation in iron-overload therapy. But redox activity that creates injurious radical species may be a desirable strategy in chemotherapy.

The tach framework is effective in metal chelation and allows adjustment of metal-binding properties by variation of pendant groups in tach derivatives

Tachpyr is built by addition of 2-pyridylmethyl groups to each N of tach (Scheme 2). The tach framework has two particular advantages: with a single cyclohexyl ring flip, it attains the metal-binding conformation (thus, a large entropy decrease is not needed to attain the needed conformation, which benefits stability and inertness; the property of predisposition) [7], and secondly, +2 biometals readily fit into the pocket created by the three tach nitrogens. Indeed, tachpyr readily complexes many metals, but the fit of Fe(II), Ni(II), Cu(II), Zn(II) and Ga(III) is better than larger metals such as Hg(II) [1,8-10].

Tachpyr prefers Fe(II) to Zn(II)

In order to investigate the selectivity of tachpyr for Fe(II) relative to Zn(II), we conducted two displacement reactions in buffered pH 7.4 media under anaerobic conditions. The citrate$^{3-}$ ligand was used as a carrier of Fe(II) and Zn(II) because $\{[\text{M(citrate)}]^{-}\}$ are labile substances and have similar formation constants [log $K_f$ ([M(citrate)]$^{-}$) ≈ 4.8] [6]. The formation of $[\text{Fe(tachpyr)}]^{2+}$ was quantitated using its visible absorption, $\varepsilon_{338} = 6530 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

\[
[Zn(\text{tachpyr})]^{2+} + \text{Fe(II)} \rightarrow \text{Zn(II)}(\text{aq}) + \text{Fe(II)}(\text{aq})
\]

(1)

Zinc(II) was entirely ($\approx 96 \pm 5 \%$) displaced by iron, as shown by eqn (1). As expected, an attempted displacement reaction with excess zinc ion ($75 \text{ mol Zn(II)}: 1 \text{ mol } [\text{Fe(tachpyr)}]^{2+}$) under otherwise identical conditions to eqn (1) showed that $\approx 94 \pm 5 \%$ of $[\text{Fe(tachpyr)}]^{2+}$ remained after 24 h. The findings suggest that the formation constant of $[\text{Fe(tachpyr)}]^{2+}$ is considerably larger (> 2 log units) than that of $[\text{Zn(tachpyr)}]^{2+}$, as expected based on the affinity constants of o-phenanthroline for Fe(II) and Zn(II).

Adding steric hindrance to tachpyr decreases the preference for iron(II)

We examined the binding preferences of the N-alkylated tachpyr derivative (N-Me)$\times$tachpyr (Scheme 1). We found that (N-Me)$\times$tachpyr readily bound Zn(II) in pH 7.4 media, but Fe(II) was not bound. It has been possible to prepare $[\text{Fe}\{\text{N-Me}\}_{3}\text{tachpyr}\} \cdot \text{ClO}_4^-$ in methanol, probably because this medium lacks $\text{H}_2\text{O}^+$ and $\text{OH}^-$ that compete with (N-Me)$\times$tachpyr for iron [1]. However, upon addition of this substance to pH 7.4 media, decomposition and precipitation occur. Further studies of metal exchange are in progress.

We obtained the X-ray structure of $[\text{Zn}\{\text{N-Me}\}_{3}\text{tachpyr}\} \cdot \text{ClO}_4^-$ for comparison to the structure of $[\text{Zn(tachpyr)}] \cdot \text{ClO}_4^-$ that we previously reported [8]. Consistent with a steric effect,
the Zn-N(amine) bond distances in (Zn[(N-Me),tachpyr])·(ClO,)4 are lengthened. They are 2.228(2) Å [C1–H2–Cl2N–0–Zn, M = 708.89, trigo-
nal, space group P3 monoclinic, a = 11.2539(2), c = 13.5750(4) Å, V = 1490.37(6) Å3, D, (Z = 2) = 1.58 g · cm–3, μMo = 10.64 cm–1, specimen 0.12 × 0.12 × 0.3 mm, T, min max = 0.8, 0.95, N, = 9116, N, = 2300 (R, = 0.0373), N, = 1986, R (all data) = 0.0371, wR2 (all data) = 0.057, |Δρmax| = 0.295e Å–3; full details will be reported separately; the numbers in parentheses refer to the estimated standard deviation of the last reported decimal place], substantially longer than the distance of 2.160(3) Å of [Zn(tachpyr)].

Stability constants of high-spin Fe(II) are considerably lower than low-spin Fe(II) and closer to those of Zn(II). For example, log K, for the Fe(II) high-spin complex (Fe2-(2-pyridyl)-imidazole)2+ = 11.6, while log K, of (Zn2-(2-pyridyl)-imidazole)2+ = 12.07 [6]. Thus, the observed loss of selectivity in (N-Me),tachpyr is consistent with the spin-state change in Fe(II) caused by steric effects.

**Zn(II) is displaced from tachpyr by Fe(III) and Fe(II) under aerobic conditions**

In a competition reaction under aerobic conditions, tachpyr was reacted with equal amounts of Fe(II)-citrate and Zn(II)-citrate (eqn 2),

\[
\text{Fe(II)} + \text{Zn(II)} + \text{tachpyr} \xrightarrow{\text{0.01 M}} \text{Fe(II) + Zn(II) + tachpyr} \\
\text{1 M Hepes pH 7.4, 37 °C, citric acid 0.01 M.}
\]

\[
x[\text{Zn(tachpyr)}]^{2+} + (1 - x)\text{[Fe(tachpyr-ox-n)]}^{2+} + x\text{Fe(II) + (1 - x)Zn(II)}
\]

where DSS is sodium 2,2-dimethyl-2-silapentane-5-sulphonate [Me3Si(CH3)2SO3Na]. [Zn(tachpyr)]2+ was roughly quantitated by NMR spectroscopy, through integration of cyclohexyl Hs on the C-z to the amino groups (δ ≈ 3.4 p.p.m.; Figure 2A). Integrals were referenced to an internal standard, disuccinyl suberate at δ(CH2-Si) ≈ 0 p.p.m. Initially, [Zn(tachpyr)]2+ formed in ≈ 90% (nominal error ± 10%) conversion referenced to tachpyr concentration.

The amount of [Zn(tachpyr)]2+ decreased continuously over 2 days at 37 °C, to ≈ 56 ± 10% remainder relative to the initial tachpyr concentration, accompanied by formation of a mixture of [Fe(tachpyr-ox-n)]2+ (n = 2, 4) complexes. The presence of [Fe(tachpyr-ox-n)]2+ was confirmed by the formation of NMR signals at δ 2.9–3.3.

Although these species could not be quantitated due to the complexity and breadth of the signals, their growth and approximate concentration relative to [Zn(tachpyr)]2+ was monitored by HPLC. Using a Waters 600E/486/746 dual-pump system with UV detection at 254 nm, a Beckman Ultrasphere 4.6 x 25 cm RP-18 column was eluted with a gradient of 100% 0.05 M triethylammonium

**Figure 2**

Proton NMR spectra showing formation of metal-tachpyr complexes initially (left) and after ≈ 2 days (right) at 37 °C, pH = 7.4, in air.

(A) Competition of Zn(II) and Fe(II) for tachpyr. (B) Formation of [Zn(tachpyr)]2+. (C) Formation of [Fe(tachpyr-ox-n)]2+, n = 0, 2, 4. Spectra are scaled relative to sodium 2,2-dimethyl-2-silapentane-5-sulphonate at δ ≈ 0 p.p.m.

**t ~ 10 min:**

- **[Zn(tachpyr)]2+**
- **[Fe(tachpyr-ox-n)]2+, n=0,2,4**

**t ~ 2 days:**

- **[Zn(tachpyr)]2+**
- **[Fe(tachpyr-ox-n)]2+, n=0,2,4**

0.6 M Hepes, pH 7.4, 37 °C, citric acid 0.01 M.

**Chemical shift (δ)**

- 3.5
- 3.0
- 2.5
- 2.0
- 1.5
- 1.0
- 0.5
- 0.0
- -0.5
- -1.0
- -1.5
- -2.0
- -2.5
- -3.0

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Abstract
Iron loading of alveolar macrophages in vivo significantly altered their ability to respond to various inflammatory stimuli. This was exemplified by reduced synthesis of inducible nitric oxide synthase after stimulus with lipopolysaccharide and interferon γ, and an enhanced activation of nuclear factor κB in the absence of tumour necrosis factor α stimulation, and enhanced production of reactive oxygen species after activation with activated zymosan and PMA. Such results may indicate an imbalance in the production of reactive oxygen and reactive nitrogen species generated by

The influence of iron homoeostasis on macrophage function
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Key words: reactive nitrogen species, reactive oxygen species. Abbreviations used: LPS, lipopolysaccharide; IFNγ, interferon γ; TNFα, tumour necrosis factor α; NfκB, nuclear factor κB; iNOS, inducible nitric oxide synthase; NF-IL6, nuclear factor interleukin 6. To whom correspondence should be addressed (e-mail ward@biocudc.ac.be).