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Enzymatic oxidation of ansa-ferrocifen leads to strong and selective thioredoxin reductase inhibition in vitro

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ABSTRACT

This paper reports the inhibitory effect on the cytosolic thioredoxin reductase (TrxR1) in vitro by the ansa-ferrocifen derivative (ansa-FcdiOH, **1**). We found that **1** decreased only slightly enzyme activity (IC_{50} = 8 μM), while **1***, the species generated by enzymatic oxidation by the HRP (horseradish peroxidase)/H_2O_2 mixture, strongly inhibited TrxR1 (IC_{50} = 0.15 μM). At the same concentrations, neither **1** nor **1*** had effect on glutathione reductase (GR). The most potent TrxR1 inhibitor did not appear to be the corresponding quinone methide as it was the case for ferrocifens of the acyclic series, or the stabilized carbocation as in the osmocifen series, but rather the quinone methide radical. This hypothesis was confirmed by ab-initio calculations of the species generated by oxidation of **1** and by EPR spectroscopy. BIAM (biotin-conjugated iodoacetamide) assay showed that **1*** targeted both cysteine and selenocysteine of the C-terminal redox center of TrxR1.

1. Introduction

As part of the search for new metallodrugs, ferrocene (Fc) derivatives have been widely explored and display contrasting antiproliferative effects while relatively few mechanistic pathways have as yet become clear [1–3]. We have ourselves synthesized derivatives of phenols, collectively called ferrocifens bearing an Fc-type redox antenna and an intramolecular electronic connection afforded by an olefin group (Chart S1) [3,4]. In the acyclic series the IC_{50} values obtained on cancer cells for ferrocenyl mono, diphenol and tamoxifen like are in the order of 0.5 μM on MDA-MB-231 human breast cancer cells [5,6].

Complementary studies showed that oxidation of the [ferrocene-ene-phenol] motif gives rise to relatively stable yet electrophilic quinone methide (QM) metabolites via a mechanism involving the abstraction of 2 electrons and 2 protons [7–9]. These molecules were shown to react with thiols via a 1,8-Michael addition [10,11]. With the aim of enhancing the cytotoxic character of ferrocifens, the ansa-FcdiOH **1** was prepared [12,13] and formulated for in vivo studies [14]. It is three to five times more active than the ferrocifens of the acyclic series with IC_{50} value down to 0.09 μM on MDA-MB-231 cell line. Up to now we have not been able to settle whether the complexes of the acyclic and ansa series acted via the same mechanism of action or not [15,16].

Many cytotoxic compounds, and in particular organometallic complexes such as *N*-heterocyclic carbene (NHC) gold complexes, are strong inhibitors of thioredoxin reductases (TrxRs), selenoenzymes involved in cellular redox regulation [17]. Both cytosolic and mitochondrial thioredoxin reductase isoforms possess a C-terminal active site containing a selenocysteine residue with a low pKa [18,19] therefore endowed with enhanced nucleophilicity. Consequently, many compounds, including anticancer agents, are considered to effectively target TrxR. Knowing the strong inhibiting properties of both electrophiles and metal complexes, we recently investigated the inhibitory properties of ferrocifens and osmocifens (Chart S1) and their QMs towards both isoforms of TrxR [10,20]. As expected, the QMs were much stronger inhibitors than the parent compounds (IC_{50} around 2.5 and 15 μM, for the ferrocifens and osmocifens respectively) [10]. We also showed that mild enzymatic oxidation of the ferrocifens by the HRP/H_2O_2 system generated species with the same spectral features as the authentic QMs. On the contrary, enzymatic oxidation of the osmocifens gave rise to different species that were identified as quinone methide cations and that displayed higher inhibitory properties towards TrxR than the QMs [20]. Thus, the species responsible for TrxR inhibition seems associated with different structures related to QMs. This prompted us to study the inhibitory effect of **1** on cytosolic TrxR [16]. Conversely to

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the acyclic ferrocifens and osmocifens, no quinone methide could be isolated from chemical oxidation of **1** [12]. This finding was ascribed to the constraints associated with its ansa structure that made the QM too unstable. Therefore, in the present paper, the enzymatic oxidation of **1** by the HRP/H_2O_2 system was investigated as well as the inhibitory effect of the resulting species on TrxR1. This hypothesis was substantiated by ab-initio calculations and EPR spectroscopy studies of the intermediate species generated by oxidation allowing assignment of the active species to a radical.

2. Materials and methods

Ansa-FcdiOH **1** was synthesized as previously described [12]. Stock solution (1×10^{-2} M) was prepared in DMSO and was stable for at least two months if kept at 4 °C. Horseradish peroxidase (HRP) and yeast glutathione reductase were purchased from Sigma Aldrich.

2.1. Enzymatic oxidation of *1* by the HRP/H_2O_2 system

Enzymatic oxidation of **1** (50 μM) by HRP (46 nM) and H_2O_2 (200 μM) was performed at pH 8.1 (0.2 M Tris HCl, 1 mM EDTA) or pH 5.0 (48.5 mM citric acid and 103 mM Na phosphate dibasic) containing 10% DMSO. HRP (40 μl of 1.14 μM solution) and H_2O_2 (20 μl of 10 mM solution) were pre-incubated for 5 min then added to the solution of **1** (940 μl). The solution was immediately transferred to a cuvette and the UV–Vis spectrum was recorded between 250 and 700 nm every 30 s on a Cary 50 spectrometer (Varian- Agilent, Santa Clara, CA, U.S.A.). Rate constants (k_{obs}) and half-lives ($t_{1/2}$) were calculated by fitting the $OD_{560\ nm}$ (at pH 8.1) or $OD_{413\ nm}$ (at pH 5) versus time data according to the first order law Eq. (1) with Kaleidagraph software.

$$OD = C_0 + C_1 \times \exp(-k_{obs} \times t) \quad (1)$$

2.2. Computational details

All calculations were performed using the Gaussian09 software (cf. Supplementary information). All compounds reported in Scheme 1 were optimized in the gas phase at Density Functional Theory level using the global hybrid PBE0 functional [21] and a double zeta basis set (including the corresponding pseudopotential to describe the core electrons of the metal atom [22]). The nature of each stationary point (i.e. minima) was confirmed by subsequent harmonic frequency calculations performed at the same level of theory. Calculations for open shell species were performed with an unrestricted formalism and spin polarization was found to be negligible. Vertical electronic transitions were computed using the optimized structures at TD-DFT level of theory using the same functional (PBE0) and a more extended basis (i.e. the 6–31 g(d) basis) set for all atoms but iron.

2.3. EPR spectroscopy studies

X-band EPR spectra were recorded on a Bruker Elexsys 500 spectrometer equipped with an Oxford Instrument continuous-flow liquid helium cryostat and a temperature control system.

A series of samples of **1** (50 μM) was mixed with HRP and H_2O_2 in buffer pH 8.1 as indicated in Section 2.1, transferred in quartz tubes and incubated for different times (1, 2, 5, 10 min). At the end of the incubation the tubes were frozen in a liquid nitrogen bath and introduced in the EPR cavity. EPR experiments were performed at 10 K, using a microwave power of 0.159 mW (non saturating conditions) with a modulation of 1 G.

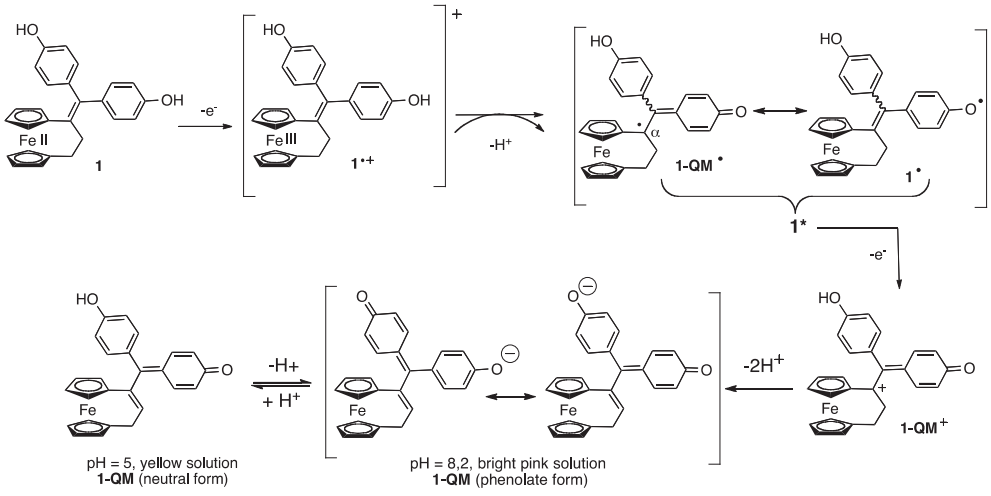
2.4. Thioredoxin reductase and glutathione reductase activity

Complex **1** (0.25 μM) was pre-incubated for different times (0.5–60 min) with HRP/H_2O_2 (22 nM/0.1 mM) mixture giving **1***. At the end of the incubation period, aliquots of highly purified TrxR1 (60 nM), prepared as described previously [23], were preincubated with the compound for 5 min at 25 °C in 0.2 M Tris-HCl buffer (pH 8.1), 1 mM EDTA, and 0.25 mM NADPH. The reaction was started with 1 mM DTNB and followed spectrophotometrically at 412 nm for about 10 min.

GR activity (yeast glutathione reductase) was measured in 0.2 M Tris-HCl buffer (pH 8.1), 1 mM EDTA, and 0.25 mM NADPH after 5 min of preincubation with the various compounds (**1** or **1***). The assay was initiated by addition of 1 mM GSSG and followed spectrophotometrically at 340 nm.

2.5. BIAM (biotinylated iodoacetamide) assay

TrxR (1 μM), pre-reduced with NADPH (60 μM) in 50 mM Tris-HCl buffer (pH 7.4) containing 0.2 mM NADPH and 1 mM EDTA, was incubated for 30 min with 50 μM of **1** or 2 μM of **1*** (prepared as described in Section 2.1). Then, aliquots (8 μl) of the reaction mixture were added to 50 μM biotinylated iodoacetamide (BIAM) in either buffer at pH 6 (0.1 M HEPES-Tris) or pH 8.5 (0.1 M Tris-HCl) and incubated at room temperature for 30 min to alkylate the remaining —SH and/or SeH groups. Then the samples were subjected to SDS-PAGE onto Bis-Tris Gel NUPAGE (12%) (Life Technologies Corporation, Carlsbad, CA,



Scheme 1. Proposed oxidation sequence of **1** involving abstraction of 2 electrons and 3 protons and leading to the quinone methide (**1-QM**) followed by acid-base equilibrium of **1-QM**.