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Toxicology

Desferrioxamine and desferrioxamine-caffeine as carriers of aluminum and gallium to microbes via the Trojan Horse Effect

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ABSTRACT

Iron acquisition by bacteria and fungi involves in several cases the promiscuous usage of siderophores. Thus, antibiotic resistance from these microorganisms can be circumvented through a strategy of loading toxic metals into siderophores (Trojan Horse Effect). Desferrioxamine (dfo) and its cell-permeant derivative desferrioxamine-caffeine (dfcaf) were complexed with aluminum or gallium for this purpose. The complexes Me(dfo) and Me(dfcaf) (Me = Al^{3+} and Ga^{3+}) were synthesized and characterized by mass spectroscopy and cyclic voltammetry. Their relative stabilities were studied through competitive equilibria with fluorescent probes calcein, fluorescein-desferrioxamine and 8-hydroxyquinoline. Me(dfo) and Me(dfcaf) were consistently more toxic than free Me^{3+} against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans*, demonstrating the Trojan Horse Effect. Wide spectrum antimicrobial action can be obtained by loading non-essential or toxic metal ions to microbes via a convenient siderophore carrier.

1. Introduction

Iron is the fourth most abundant metal in the Earth's crust and the most important transition metal for all life forms on the planet. It is central to various biochemical processes such as in the reduction of oxygen for ATP synthesis, ribonucleotide synthesis, oxygen transport, among others. For aerobic microorganisms, the difficulty of obtaining iron stems from the extreme insolubility of iron minerals in aqueous media ($Fe^{3+}_{(aq,equilibrium)} < 10^{-18}$ M) and in biological fluids. Accordingly, iron homeostasis in organisms is tightly controlled and its excretion is limited [1].

In order to meet their dietary iron requirements, higher animals rely on the biochemical machinery present in some plants and unicellular life forms that provide them with the ability to absorb the metal and make it bioavailable. Several microbes synthesize low molecular weight ligands with high affinity for iron, called siderophores. Siderophores form usually octahedral complexes with Fe(III), promoting its solubilization from highly insoluble minerals through an entropic-driven thermodynamic stabilization characteristic of multidentate ligands [1,2].

Consumption of iron from its complexed form is specific and dependent on outer membrane receptor proteins in each microorganism. However, the evolutionary pressure for more efficient ways to absorb iron under an oxidant atmosphere led to a relentless competition for the metal, giving to several bacteria and fungi the ability to capture even siderophores that themselves did not produce. *Escherichia coli* has siderophore receptors for enterobactin homologs and aerobactin, however it can also absorb iron through *e.g.* ferrichrome or ferrioxamine [3]. The fungus *Candida albicans* produces no known siderophore [4] but it can also obtain iron complexed in desferrioxamine (dfo), a siderophore produced by *Streptomyces pilosus* [5] and clinically used to remove excess iron [6].

It is possible to explore medically this kind of promiscuity. One strategy involves the conjugation of a drug to the complex [Fe(siderophore)] by means of an appropriate linker, promoting the accumulation of the drug in the target organism by the active internalization of the iron complex [7,8]. This first example of Trojan Horse Effect is a new pharmacological alternative to overcome

Abbreviations: 8-HQ, 8-hydroxyquinoline; CAFe, calcein-iron(III) complex; CFU, colony-forming unit; Dfcaf, desferrioxamine-caffeine; Dfo, desferrioxamine; DHR, dihydrorhodamine hydrochloride; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; FAS, ferrous ammonium sulfate; FLDFO, fluorescein-desferrioxamine; HBS, hepes buffer saline; NTA, nitrilotriacetic acid; TSB, tryptic soy broth.

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antibiotic resistance mechanisms posed by microbes like *Pseudomonas aeruginosa* or *Staphylococcus aureus* [7,9].

A variation of this strategy is to incorporate other (preferentially toxic) metal ions instead of iron into the siderophore, thus dispensing with linkers or conjugated drugs, as demonstrated by the $[Cd(dfo)_2Cl_4]$ complex [10]. Al^{3+} and Ga^{3+} are toxic ions with no known biological effect [11,12] and hard Pearson acids, which makes them excellent chemical substitutes for iron [13] as evidenced by their metal-dfo stability constants (logβ): 36.11 (Al^{3+}), 38.96 (Ga^{3+}) and 42.33 (Fe^{3+}) [14].

In previous works, we developed the artificial siderophore desferrioxamine-caffeine (dfcaf; Fig. 1 [15]) as a cell-permeant alternative to dfo, hypothesizing that it could make it able to deliver toxic metals even to those microrganisms that lacked dfo-binding receptors. In this work we proposed to synthesize and characterize the complexes of Al^{3+} and Ga^{3+} with both dfo and dfcaf in aqueous media and to evaluate their influence on the growth of *E. coli*, *S. aureus*, *P. aeruginosa* and *C. albicans*.

2. Materials and methods

2.1. Materials

Desferrioxamine mesylate was donated by Cristália (Brazil). Anhydrous $AlCl_3$ was purchased from Fluka (USA); calcein (CA), ferrous ammonium sulfate (FAS), 8-hydroxyquinoline (8-HQ) and $Ga(NO_3)_3$ were from Sigma Aldrich (USA). Dfcaf was synthesized according to a previous report [15] with DMF, DMSO, *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide, *N*-hydroxybenzotriazole and theophylline-7-acetic acid from Sigma Aldrich (USA), diethyl ether from Vetec (Brazil) and methanol from Synth (Brazil). Fluorescein-dfo (FLDFO) was prepared according to previously described methods [16].

2.2. Synthesis of Al(dfo), Ga(dfo), Al(dfcaf), Ga(dfcaf)

Dfo complexes: 0.5 mol L^{-1} solutions were prepared with either Al or Ga salts in HBS (Hepes Buffered Saline; hepes 20 mM, NaCl 150 mM, Chelex® 1 g/100 mL; pH 7.4) and then mixed for 10 min with a stock dfo solution in the same buffer to reach a 1:1 (metal:ligand) molar ratio at 25 °C.

Dfcaf complexes: dfcaf (2.5 mmol L^{-1}) was dissolved in DMSO and mixed for 10 min with aliquots of Al or Ga salts in HBS to reach a 1:1 (metal:ligand) molar ratio at 25 °C.

2.3. Characterization of Al(dfo), Ga(dfo), Al(dfcaf), Ga(dfcaf)

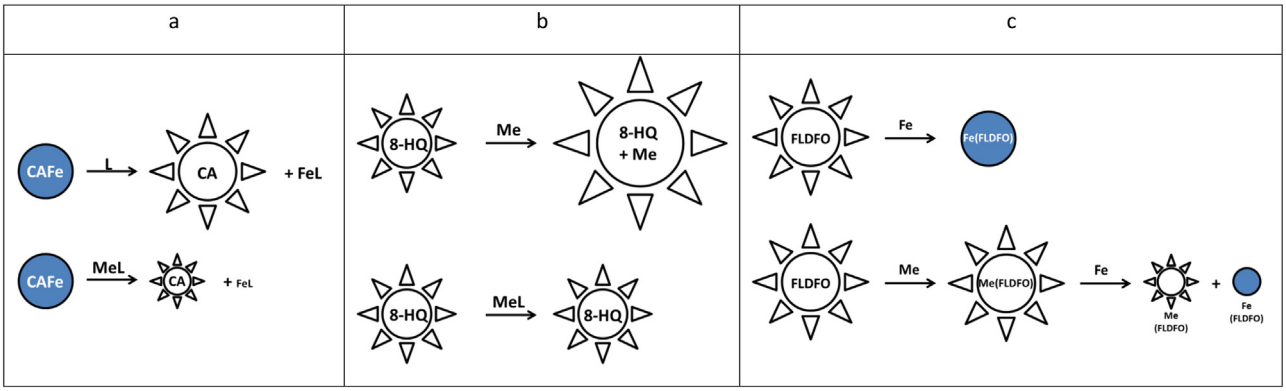
Total metal contents in the working solutions of the complexes were determined by ICP-OES in a Spectro Arcos equipment. Molecular masses were recorded in an Amazon Speed Mass Spectrometer ETD (Bruker Daltonics). Cyclic voltammograms were obtained in an Autolab potentiostat controlled by the GPES 4.9 software, using a glassy carbon (d = 1.0 mm), a platinum wire and Ag/AgCl (KCl 3 mol L^{-1}) as a working, counter and reference electrodes, in a compartment cell of 10 mL. The working electrode was polished with 2 μm alumina (Arotec, Brazil) on a metallographic felt supported on glass plaque. The supporting electrolyte solution was DMSO containing 0.1 M tetrabutylammonium tetrafluoroborate for measurements in solutions containing dfcaf complexes and 0.1 M NaCl for measurements in solutions containing dfo complexes.

2.4. Competitive equilibria with fluorescent probes

In contrast to Al^{3+} and Ga^{3+}, Fe^{3+} is a paramagnetic ion able to quench the fluorescence of molecules such as calcein or FLDFO. This suppression phenomenon is explained by the influence of the unpaired *d* electrons present in the ferric ion, which modify the energy of the electronic transitions associated with relaxation of electrons. Strong chelators such as dfo or dfcaf can revert this quenching, and this principle has been used for the quantification of physiological pools of labile iron [17]. Therefore, studying the combined effect of iron and free or (Al,Ga)-bound siderophores on the extension of quenching or de-quenching of these probes gives an indirect proof that the Al and Ga complexes were formed (Scheme 1).

Three different fluorescent probes were used to demonstrate indirectly the formation and relative stabilities of the Al and Ga complexes.

a) *Me(dfo) or Me(dfcaf) against calcein-iron (CAFe)* [17]. CAFe is a non-fluorescent, stable (logβ = 33.9 [18]) complex of Fe^{3+} and a convenient reporter of the affinity of other iron chelators such as dfo or dfcaf, which sequester iron and regenerate the fluorescence of the probe. However, if dfo or dfcaf are loaded with Al or Ga, some delay in the reaction with Fe may be expected, which is translated into a decreased fluorescence (Scheme 1a). CAFe was prepared by dissolving an appropriate mass of FAS in 1 mL of 10 mM calcein in HBS buffer, in order to obtain a final 1:1 (iron:calcein) molar ratio. The fluorescent yellow solution quickly changes to deep red and it was left to react for 1 h at



Scheme 1. Indirect fluorimetric assays to determine the formation of MeL (Me = Al, Ga; L = dfo, dfcaf). (a) Iron-calcein complex (CAFe) has its fluorescence fully recovered by means of strong chelators L, however when complexed to Me (in MeL) this recovery is limited, therefore indicating the stability of MeL. (b) Free trivalent Me enhance the natural fluorescence of 8-HQ, however when complexed (in MeL) this enhancement is hampered. This lowered enhancement is also proof of formation of MeL. (c) FLDFO has strong affinity for Fe, being quickly quenched by its coordination. However, addition of Me forms the non-fluorescent Me(FLDFO), which is less prone to react with Fe within the timeframe of the experiment. This is a way to demonstrate the formation of Me(FLDFO) (and, by extension, of Me(dfo)) in solution.