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Toxicology

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

Jesus Antonio Alvarado Huayhuaz[a], Hector Aguilar Vitorino[a], Othon Souto Campos[a], Silvia Helena Pires Serrano[a], Telma Mary Kaneko[b], Breno Pannia Espósito[a,*]

[a] Department of Fundamental Chemistry, Institute of Chemistry, University of São Paulo,Av. Prof. Lineu Prestes, 748, 05508-000, São Paulo, SP, Brazil
[b] Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, SP, Brazil

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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.

* Corresponding author.
E-mail address: breno@iq.usp.br (B.P. Espósito).

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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)

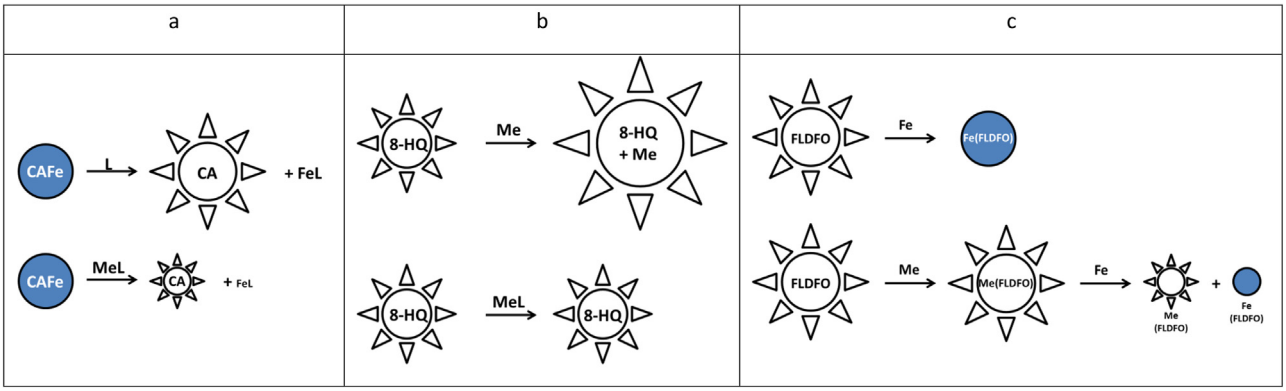
Total metal contents in the working solutions of the complexes were determined by ICP-OES in a Spectro Arcos equipment. Molecular masses were recorded in an Amazon Speed Mass Spectrometer ETD (Bruker Daltonics). Cyclic voltammograms were obtained in an Autolab potentiostat controlled by the GPES 4.9 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.

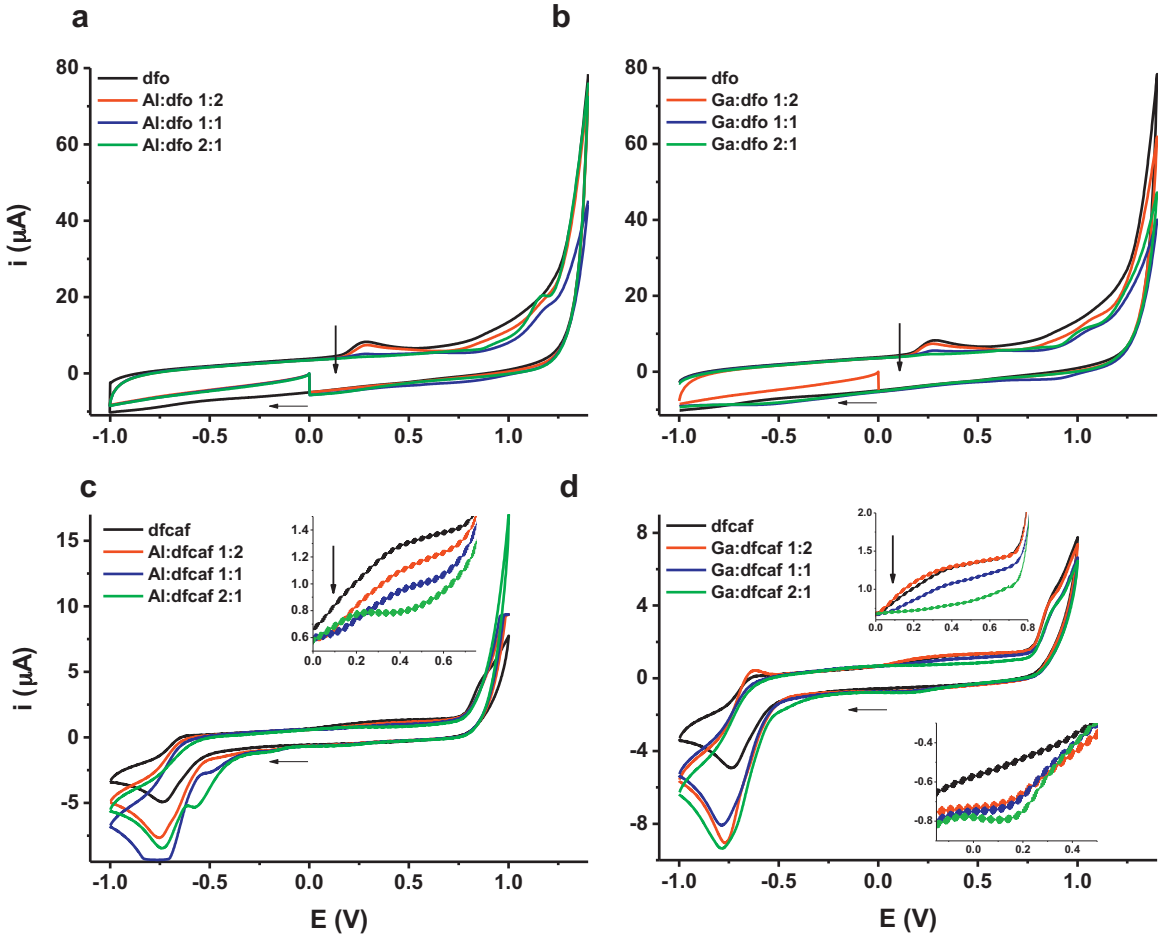


Fig. 1. Cyclic voltammograms of MeL (Me = Al^{3+} or Ga^{3+}; L = dfo or dfcaf) in DMSO containing 0.1 M tetrabutylammonium tetrafluoroborate (measurements in solutions of dfcaf complexes) and 0.1 M NaCl (measurements in solutions of dfo complexes). Ligand concentration = 100 μM; metal concentration = 0, 50, 100 or 200 μM. Vertical arrows indicate increasing metal-to-ligand mol ratios; horizontal arrows indicate the direction of the sweep potential. Experimental conditions: E_i = +0.2 V; $E_{\lambda 1}$ = −1.0 V; $E_{\lambda 2}$ = +1.4 V; E_{final} = 0.0 V and υ = 100 mV s^{-1}.

room temperature. 10 μL aliquots of the test compounds (Al^{3+}, Ga^{3+}, dfo, dfcaf, Al(dfo), Ga(dfo), Al(dfcaf) and Ga(dfcaf)) at different concentrations (0–25 μM) were transferred in triplicates to 96 well microplates and treated with 190 μL of 2 μM CAFe in HBS. The microplate was incubated at 37 °C for 10 min inside a BMG Fluostar Optima instrument and then the fluorescence was registered (λexc/λem = 485/520 nm).

b) *Me(dfo) or Me(dfcaf) against 8-HQ.* Free Al^{3+} or Ga^{3+} can enhance the natural fluorescence of 8-HQ. This phenomenon corresponds to an increase in molecular rigidity acquired by the complex with respect to the free ligand [19]. Therefore, comparing the extension of this signal enhancement promoted by Me or MeL (Me = Al, Ga; L = dfo, dfcaf) for a given total concentration of Me is another demonstration of the formation of MeL complexes (Scheme 1b). Triplicate aliquots of 10 μL of the compounds to be evaluated at different concentrations (0–12 mM) were transferred to a 96-well microplate and then treated with 190 μL of 8-HQ 750 μM in HBS. The microplate was incubated at 37 °C for 10 min inside a SpectraMax M4 instrument. The fluorescence was registered at λexc/λem = 320/520 nm.

c) *Tests with FLDFO* [17]. FLDFO is the fluorescent version of the chelator dfo, which can thus be used to prepare Al(FLDFO) or Ga(FLDFO) and have their interactions with Fe^{3+} studied. In a 96-well microplate, triplicate aliquots of 180 μL of FLDFO 2 μM in HBS were plated and the fluorescence (λexc/λem = 485/520 nm; 37 °C) started to be recorded in a BMG FluoStar equipment. At selected times, the probe was treated with 20 μL of either one of the free metals (Al, Ga, or Fe) or metal complexes (Al(dfo) or Ga(dfo)) in a 0–20 μM concentration range.

2.5. Antioxidant activity

Several iron compounds catalyze the oxidation of ascorbate in mimetic physiological medium, generating free radicals that oxidize the probe dihydrorhodamine (DHR) to its fluorescent form. Good chelators decrease the rate DHR of oxidation by scavenging iron [20]. The redox-active model iron complex Fe(nta) (ferric nitrilotriacetate) was prepared by dissolving ferrous ammonium sulfate (FAS) in aqueous NTA (10 mM) in order to reach a 1:1 (iron:ligand) molar ratio. In 96-well microplates, 10 μL of Fe(nta) 200 μM and 10 μL of the test compounds were plated together and then treated with 180 μL of a fluorogenic solution (ascorbic acid 40 μM and dihydrorhodamine hydrochloride (DHR) 50 μM in HBS). Fluorescence kinetic curves were recorded at 2 min intervals for 1 h at 37 °C in a BMG FluoStar Optima equipment (λexc/λem = 485/520 nm) and oxidation rates were obtained from the slopes of these curves after 15 min [20].

2.6. Antimicrobial activity tests

The bactericidal activity was based on methods National Committee for Clinical Laboratory Standards (NCCLS) and was applied in

Table 1
Fragments associated with molecular ion complexes studied by mass spectrometry (in g/mol)[a].

	Al(dfo)	Ga(dfo)	Al(dfcaf)	Ga(dfcaf)
Experimental	585.39	627.35	805.70	847.60
Calculated	584.73	627.45	804.88	847.60

[a] Complete fragment analysis in Table S1 (Supplementary Information).

E. coli (ATCC 8739), *P. aeruginosa* (ATCC 9027) and *S. aureus* (ATCC 6538), while the fungicidal activity it was assessed in *C. albicans* (ATCC 10231). Tryptic Soy Agar (TSA) Difco® was used to cultivate bacteria during 24 h at 37 °C and Sabouraud Dextrose Agar (SDA) Difco® to fungi during 72 h at 25 °C. Then, serial dilutions (in triplicate) of the sterile suspensions were prepared with 0.85 g/L NaCl for obtaining 10^5–10^6 colony forming units per mL (CFU/mL) for bacteria and 10^4 CFU/mL for fungi. Chloramphenicol (100,000 IU/mL), Amikacin (916 μg/mg) or Nystatin (100,000 IU/mL) were used as positive controls in solution 1 mg/mL, depending on the microorganism challenged and having 0.05 mg/mL final concentration in each well microplate, while the negative control consisted of 10 μL of solvent in 190 μL of tryptic soy broth (TSB) sterilized. TSB solutions inoculated and uninoculated correspond to growth controls and sterility, respectively, also in triplicate. The samples were incubated in 96 wells containing TSB or SDA and the dfo and dfcaf compounds further controls, at 37 °C for 24 h for bacteria and at 25 °C for 72 h for fungi. Using a microplate reader ELISA (LCG Biotechnology, Brazil), bacterial growth by absorbance of the solutions at 630 nm was determined. The average values were used to build dose-response curves (Supplementary Information) and IC50 values at 95% confidence intervals were obtained with the GraphPad Prism® v5 software.

3. Results and discussion

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

According to the 1:1 stoichiometry for Me:dfo and Me:dfcaf [15], the mass/charge ratios corresponding to a tripositive metal bound to an anionic ligand that neutralizes the charge were calculated and confirmed by mass spectrometry as 585 and 627 g/mol (for Al(dfo) and Ga(dfo), respectively) and 805 and 847 g/mol (for Al(dfcaf) and Ga(dfcaf), respectively) (Table 1 and Fig. S1).

Cyclic voltammograms obtained at glassy carbon electrode (GCE) at 100 mV/s in 0.1 M NaCl containing 100 μM desferrioxamine displays an oxidation peak at Ep,a = 0.282 V, which was attributed to the oxidation of the hydroxamate groups [21] (Fig. 1a). The metal–ligand interaction is demonstrated by the decrease in the anodic oxidation peak (ip,a) as a function of increasing Al^{3+} content in the electrochemical cell (Fig. 1a). The ligand oxidation peak, Ep,a, was completely suppressed at unitary or higher values of molar ratio metal:ligand, and the new oxidation peak observed at 1.18 V, was attributed to the Al(dfo) complex. The replacement of aluminum by gallium leads to similar results: the oxidation peak of dfo at 0.282 V is suppressed when gallium is in excess over dfo (Fig. 1b). Cyclic voltammograms for dfcaf and its complexes were obtained in DMSO containing 0.1 M tetrabutylammonium tetrafluoroborate as a supporting electrolyte. Different voltammetric profiles were obtained for dfcaf in relation to dfo. Besides the oxidation of the hydroxamate oxidation on dcaf, which occurs at the same potential as the dfo derivatives, a reduction peak (Ep,c) at −0.75 V was obtained when the cyclic voltammogram was recorded in the negative potential direction (from 0.0 to −1.0 V), which can be attributed to the ligand reduction. The addition of aluminum or gallium to the ligand seems to facilitate the reduction process and indicates the metal-ligand interaction. As the characterization of this electrochemical process is not the scope of this work, the evaluation of the influence of metal addition on the ligand oxidation process at Ep,a was done only in the positive potential range (insets on Fig. 1c and d). Suppression of the hydroxamate oxidation of dfcaf upon addition of either aluminum or gallium was also observed at ~0.28 V, suggesting that here also Al^{3+} and Ga^{3+} are forming stable complexes with the siderophore.

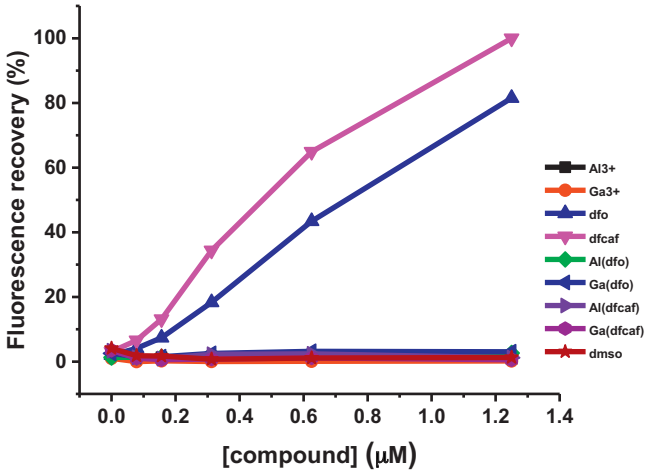


Fig. 2. Recovery of CAFe fluorescence by Me, L or MeL in HBS buffer (Me = Al^{3+} or Ga^{3+}; L = dfo or dfcaf).

3.2. Competitive equilibria with fluorescent probes

Al^{3+} and Ga^{3+} are ions from main group elements with full *p* and *d* orbitals, implying that they cannot properly stabilize complexes through ligand field contributions and that solutions of their common salts are colorless. Being dfo and dfcaf also without color, simple spectrophotometric methods are not useful to study the interaction of any of these cations with the ligands. However, there are some fluorimetric methods that can be applied to indirectly assess the formation of Me(dfo) or Me(dfcaf).

a) Me(dfo) or Me(dfcaf) against calcein-iron (CAFe)

Desferrioxamine forms a stronger (logβ = 42.33 [14]) complex with iron than calcein, and dfcaf preserves the same iron binding affinity than dfo [15]. Accordingly, we observed that free dfo or dfcaf recover 100% of calcein fluorescence, however their Al or Ga complexes do not (Fig. 2), suggesting that the MeL (Me = Al or Ga; L = dfo or dfcaf) complexes are stable enough in solution.

b) Me(dfo) or Me(dfcaf) against 8-HQ

8-HQ forms relatively stable complexes with aluminum (logβ = 14 [19]) or gallium (logβ = 36 [22]) in neutral aqueous solution, which are more fluorescent than the parent ligand. However, when Al or Ga are already part of a stable complex (Fig. 3), less metal is available to coordinate to 8-HQ (~17% of total Al and ~40% of total Ga) and thus a decreased fluorescence is observed. This is in agreement with the CAFe test and indicates the formation of MeL.

c) Tests with FLDFO

Addition of trivalent Fe, Al or Ga to FLDFO (first arrow, Fig. 4a) results in full fluorescence quenching only for the ferric ion. However, a new addition of ferric ion (Fig. 4a, second arrow) has very different effects depending upon whether Al or Ga were occupying FLDFO. Ga(FLDFO) quickly undergoes Ga^{3+} for Fe^{3+} substitution

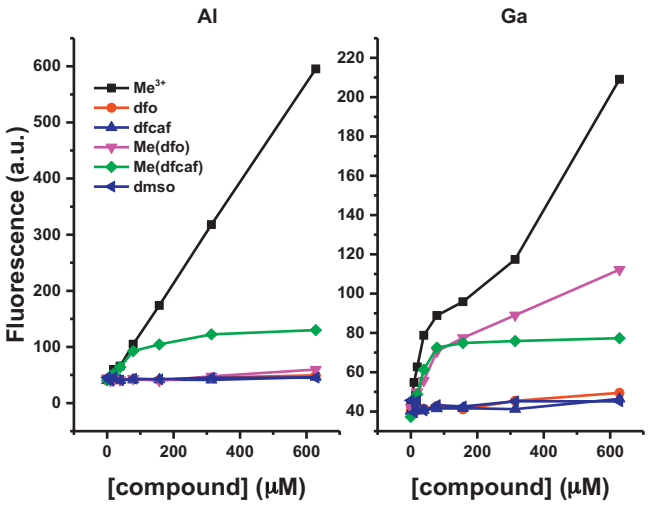


Fig. 3. Interaction of Me, L or MeL in HBS buffer (Me = Al^{3+} or Ga^{3+}; L = dfo or dfcaf) with 8-HQ 750 μM. a.u. = arbitrary fluorescence units.

accompanied by the resulting quenching. However, the fluorescence of Al(FLDFO) is unaffected within the timeframe of the experiment. The relative affinities of Al^{3+} and Ga^{3+} for dfo are approximately the same (logβ = 36.11 and 38.96, respectively [14]), however Al^{3+} is less labile than Ga^{3+} since it has a higher surface charge density [23]. Therefore, kinetic and not thermodynamic effects govern the rate of metal substitution in Me(FLDFO) when Me = Al^{3+} or Ga^{3+}.

If the above experiment is repeated but with the addition of Al(dfo) or Ga(dfo) instead of Al^{3+} or Ga^{3+} over FLDFO (Fig. 4b, first arrow), it is possible again to appreciate the relative lability differences. After adding Al(dfo) to FLDFO, further addition of iron leads to ~75% quenching, indicating that the target of iron was mostly FLDFO and only ~25% from dfo dissociated from Al(dfo). However, ferric iron has access to both FLDFO and dfo from Ga(dfo), leading to 50% quenching and suggesting again that previous gallium(III) coordination to dfo was not a hindrance for iron(III).

3.3. Antioxidant activity

Both dfo and dfcaf are efficient antioxidants against iron-catalyzed oxidation of ascorbic acid, and this property is important for efficient chelators for the treatment of iron overload [15]. In this physiologically relevant setting (considering salinity, temperature, pH and ascorbic acid concentration), Fe(nta) is a model of iron overload and dihydrorhodamine (DHR) oxidation rate responds to the generation of reactive oxygen species. Clinically approved iron chelators have been found to halt this process [20]. Here we observed (Fig. 5) that dfo and dfcaf suppress the oxidation rate of DHR under iron/ascorbate at around equivalent concentrations ($[Fe]_{total}$ = [L] = 10 μM; L = dfo or dfcaf), which is in agreement with previous reports [15]. When exploring the Trojan Horse strategy of

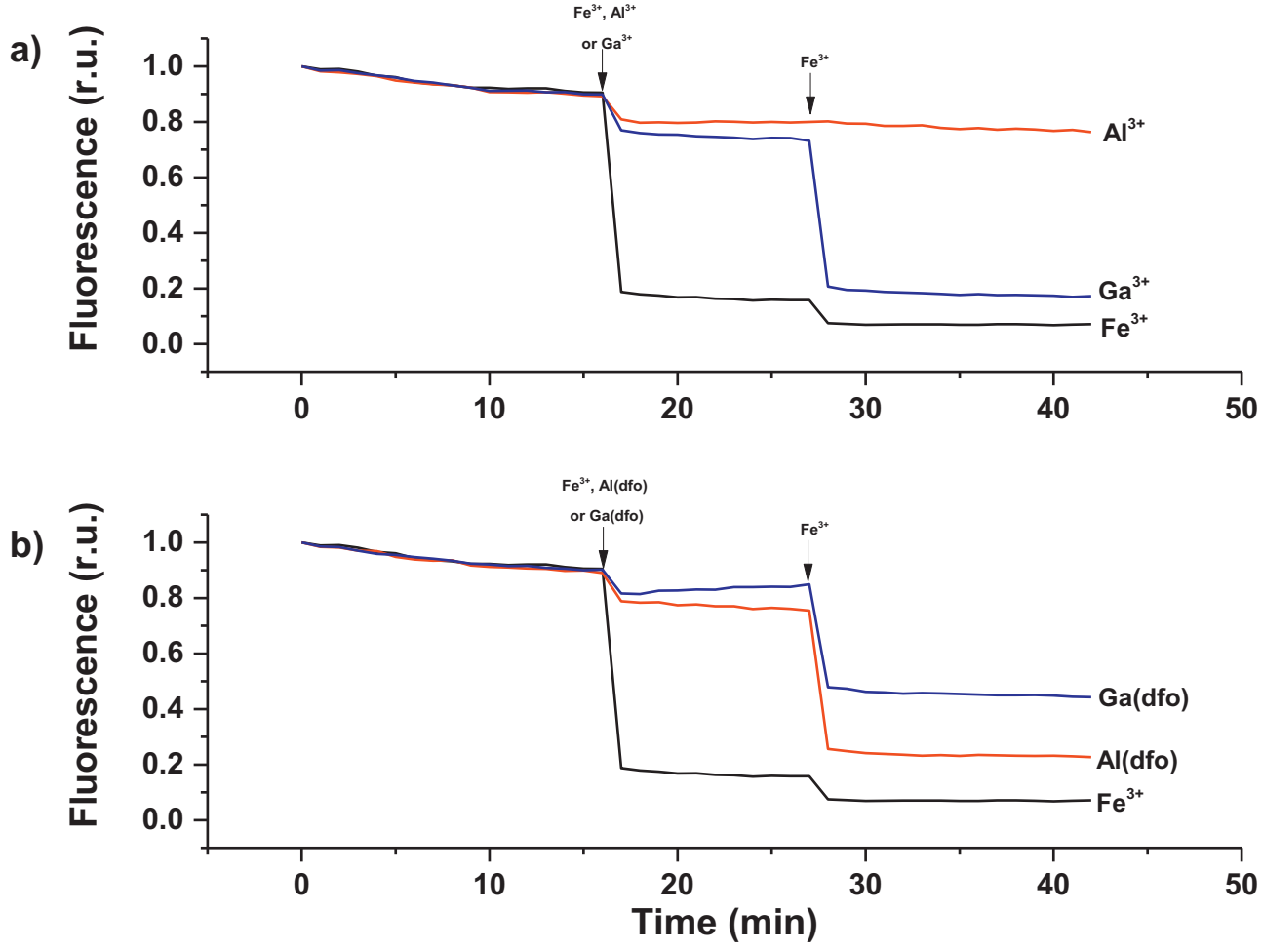


Fig. 4. Effect of (a) free Me or (b) Me(dfo) (Me = Al^{3+} or Ga^{3+}) on the fluorescence of FLDFO. Background FLDFO fluorescence (~0.3 r.u.) was subtracted for clarity. r.u. = relative fluorescence units. Final concentrations of all species was 2 μM.

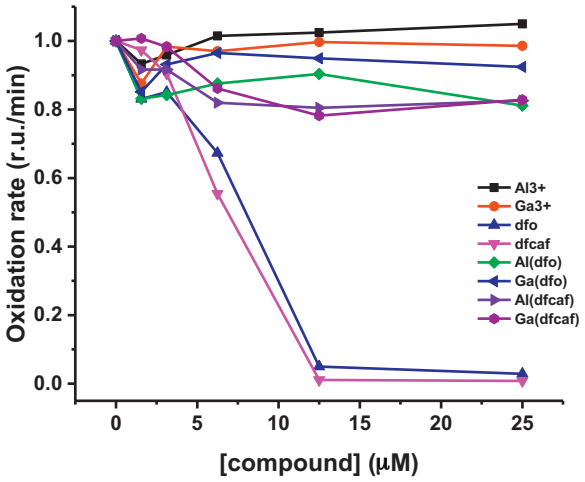


Fig. 5. Effect of Me, L or MeL (Me = Al^{3+} or Ga^{3+}; L = dfo or dfcaf) on the oxidation rate of DHR catalyzed by iron/ascorbic acid. r.u. = relative fluorescence units.

Al or Ga-loaded siderophores to kill microbes, it would be interesting that the compounds lose this antioxidant, protective effect. Accordingly, it was observed (Fig. 5) that MeL (Me = Al^{3+} or Ga^{3+}; L = dfo or dfcaf) have no appreciable antioxidant effect, as increasing their concentrations does not decrease the rate of DHR oxidation. This is because the hydroxamate groups from dfo or dfcaf are already involved in metal coordination and thus unable to acquire iron form the stable Fe(nta) ($\log\beta = 11$ [24]) oxidant standard.

3.4. Antimicrobial activity tests

Hydroxamate chelators such as dfo are among the best studied examples of microbial internalization of siderophores. Several Gram-positive bacteria (*S. aureus* [25]) and Gram-negative bacteria (*E. coli* [26,27]; *P. aeruginosa* [28]) as well as fungi (*C. albicans* [29]) are known to use Fe(dfo) (ferrioxamine) to meet their iron requirements, even though none of them secrete this siderophore. Therefore, loading toxic ions to siderophores as a means to disrupt the metal homeostasis of target microbes is an interesting therapeutic approach. In this sense, Ga(dfo) has already been successfully applied against *P. aeruginosa* biofilms to treat corneal infections in rabbits [30], which resulted in a patent [31]. In this work, we compared dfo and its cell-permeant form dfcaf as potential carriers of Al^{3+} or Ga^{3+} to target microrganisms.

IC_{50} values for *E. coli*, *P. aeruginosa*, *S. aureus* and *C. albicans* are presented in Fig. 6 and full dose-response curves in Fig. S2 in Supplementary Materials. From them, it is possible to notice some interesting trends. Initially, the dfcaf ligand is more toxic than dfo for all the target microbes. Its IC50 is ~20% (*P. aeruginosa*) to 50% (*S. aureus* and *C. albicans*) than for dfo. This is probably related to the lipophilicity conveyed by the caffeine moiety, resulting in the cytosolic release of a very strong metal chelator with great ability to affect the homeostasis of other ions such as Zn^{2+} [32]. This observation is important as dfcaf might be effective even against organisms that lack dfo internalization routes.

Al(dfo) and Al(dfcaf) were more toxic than free Al^{3+} for *E. coli* and *P. aeruginosa*. For *E. coli*, Al(dfcaf) displayed a five-fold increase in toxicity when compared with the free ion (24 μM and 109 μM, respectively), putting this complex close to a therapeutically useful range of concentrations. For *C. albicans* and *S. aureus*, only Al(dfcaf) was significantly more toxic than Al^{3+}. For *E.coli* and *S. aureus*,

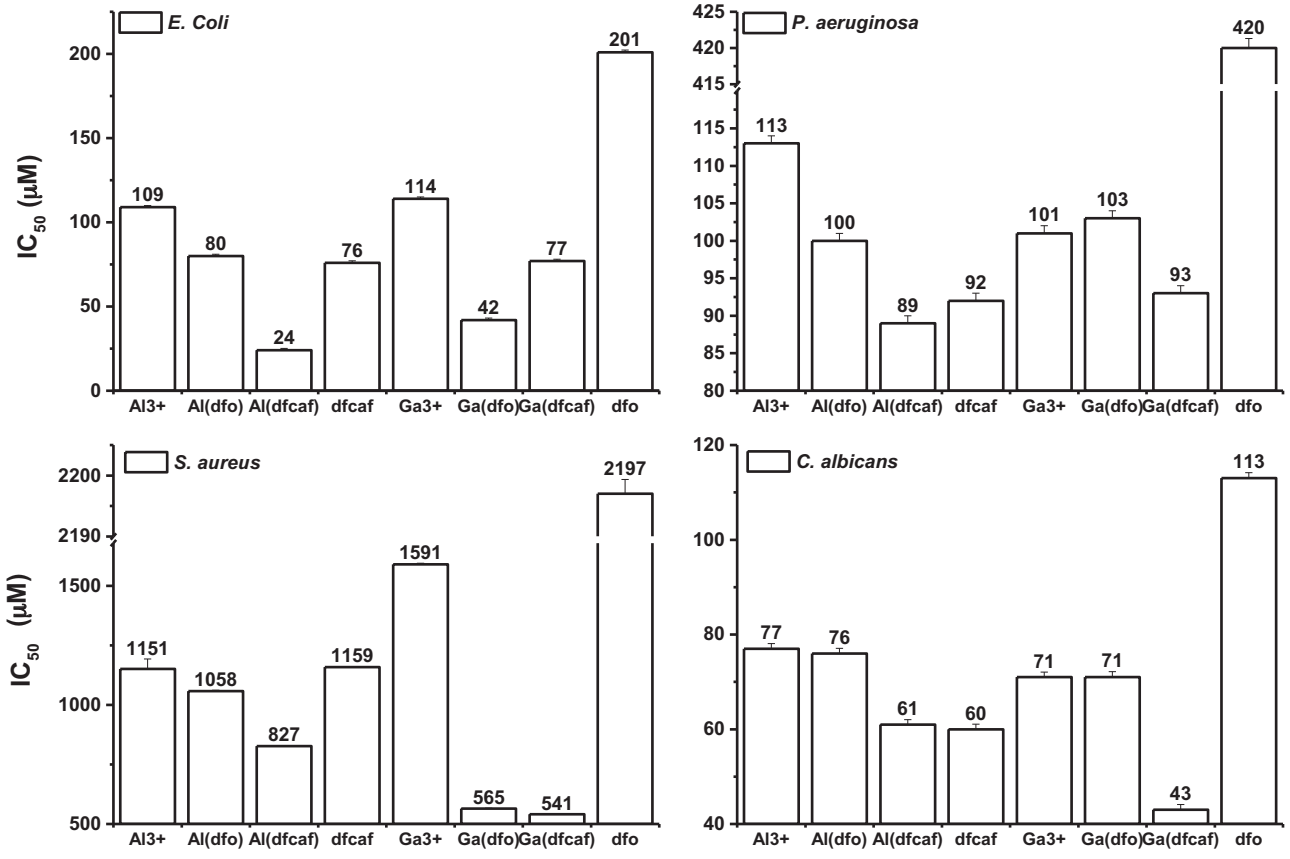


Fig. 6. IC_{50} (μM) values for the compounds in the antimicrobial activity test.

Al(dfcaf) is 1.5–3 × more toxic than free dfcaf, demonstrating that in this case a synergy between Al and dfcaf toxicities may be at work.

GaL (L = dfo or dfcaf) has higher or at least the same toxicity than free Ga^{3+}, and Ga(dfcaf) is also more toxic than Ga(dfo), except for *E. coli*. Increased internalization of Al^{3+} or Ga^{3+} and eventual replacement of them at iron-dependent active sites could disrupt the catalytic function of enzymes, due to their very low reduction potentials (E^0, mV: $Fe^{3+}/Fe^{2+} = +770$; $Al^{3+}/Al = -1680$; $Ga^{3+}/Ga = -530$) [33]. Other mechanisms for Al^{3+} or Ga^{3+} toxicity may involve DNA damage [34] or competition of these hard cations with Mg^{2+} for phosphate binding in ATP synthesis [35]. There is no obvious trend in the activities of Al(dfcaf) and Ga(dfcaf). The former is the most active in *E. coli* and the latter is the most active in *S. aureus* and *C. albicans*, while for *P. aeruginosa* both compounds displayed similar toxicity.

Gram-positive *S. aureus* was resistant to all the treatments in this evaluation (IC50 > 500 μM), which may be attributed to a thicker peptidoglycan layer and the consequent difficulty of permeabilization even for dfcaf. It was encouraging to observe that *C. albicans* was sensitive to the complexes, which can be useful for the topic treatment of fungal infections. Toxicities might be expected to be even higher for organisms cultivated in iron-restricted environments (which would lead to enhanced acquisition of metal-bound siderophores).

4. Conclusions

Aluminum and gallium ions are toxic to most microorganisms. Their complexes with dfo or dfcaf are easily prepared and stable in aqueous solution, and they have been found to enhance or maintain the toxicity of both free metal ions and free ligands against *E. coli*, *P. aeruginosa*, *S. aureus* and *C. albicans*. While the toxicities to target organisms are still low, a Trojan Horse synergy is possible and therefore other metal complexes may be useful for the topic treatment of microbial infections.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jtemb.2017.01.006.

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