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Antioxidant activity and cellular uptake of the hydroxamate-based fungal iron chelators pyridoxatin, desferriastechrome and desferricoprogen

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Abstract The hydroxamate class of compounds is well known for its pharmacological applications, especially in the context of chelation therapy. In this work we investigate the performance of the fungal hydroxamates pyridoxatin (PYR), desferriastechrome (DAC) and desferricoprogen (DCO) as mitigators of stress caused by iron overload (IO) both in buffered medium and in cells. Desferrioxamine (DFO), the gold standard for IO treatment, was used as comparison. It was observed that all the fungal chelators (in aqueous medium) or PYR and DAC (in cells) are powerful iron scavengers. However only PYR and DCO (in aqueous medium) or PYR (in cells) were also antioxidant against two forms of iron-dependent oxidative stress (ascorbate or peroxide oxidation). These findings reveal that PYR is an interesting alternative to DFO for iron chelation therapy, since it has the advantage of being cell permeable and thus potentially orally active.

Keywords Iron · Overload · Desferriastechrome · Pyridoxatin · Desferricoprogen

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Introduction

Iron overload (IO) diseases are generally the result of a hereditary dysfunction in one of the mechanisms of iron regulation and utilization (e.g., hereditary hemochromatosis, Friedreich ataxia), or of the treatment of a condition unrelated to iron metabolism (e.g., blood transfusions in thalassemia patients). IO is considered a health threat due to the ability of labile forms of iron to catalyze the formation of reactive species (free radicals) when in contact with biological oxidants such as molecular oxygen, peroxides and superoxide (Halliwell and Gutteridge 2007). IO is usually treated by chelation therapy, which involves the administration of an organic ligand as a drug to form a stable, soluble and inert coordination compound with iron within biological medium in order to accelerate its excretion. Chelators must bind excess iron in tissues such as blood, heart, liver and brain, while not interfering with the metal bound to its correct biomolecular carriers (e.g., transferrin, ferritin, hemoglobin, cytochromes).

Chelators in clinical practice for IO treatment are desferrioxamine (DFO), deferiprone and deferasirox (Fig. 1). DFO, commercialized as a mesylate salt, was the first chelator used for the treatment of IO diseases. It is a linear tris(hydroxamate) produced by the bacterium *Streptomyces pilosus*, currently the only natural product approved by FDA for the treatment of IO. Due to its extreme hydrophilicity, DFO is not

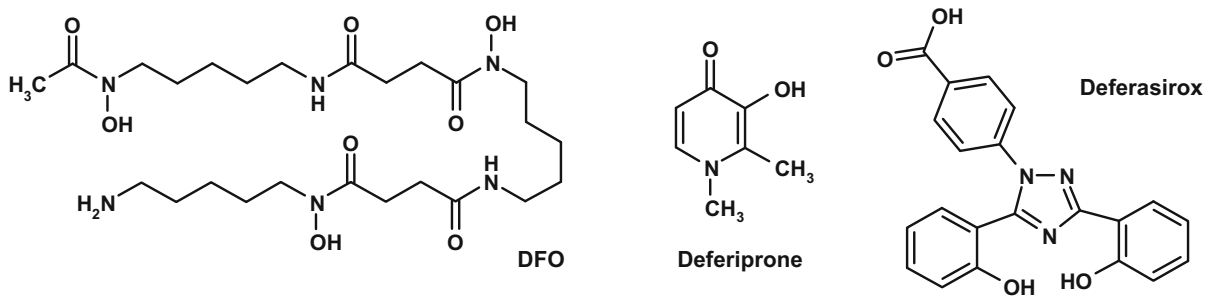

Fig. 1 Chelators approved for the treatment of iron overload (IO) diseases

absorbed orally, although the two approved synthetic chelators deferiprone and deferasirox are orally active (Kwiatkowski 2011).

Iron chelators are effective at handling IO diseases, however, as lifelong use is typically mandated for IO diseases, adverse side effects have been reported. These effects range from rashes and allergies to gastrointestinal disturbances and renal failure, and neurological symptoms and neutropenia at high doses (Kwiatkowski 2011). As side effects can sometimes prevent continuation of treatment, several other chelators have been proposed, some of which are in clinical trials (Table 1; Fig. 1).

The hydroxamic acid moiety is a common feature of several proposed (or approved) antimicrobial, antitumor, anti-neurodegenerative and anti-hypertensive drugs. The ability of hydroxamates to scavenge metals [e.g., essential metal ions such as Cu(II) or Zn(II) from metalloenzymes] on one hand, and to generate nitric oxide in vivo on the other, are at the basis of this versatility (Bertrand et al. 2013). Hence, hydroxamates from other natural sources could in principle keep the best of DFO properties for the treatment of IO diseases (formation of stable, inert complexes with iron), while overcoming some of its drawbacks, mainly the lack of proper cell absorption.

In this work, we report the performance of three previously described hydroxamate-containing natural products [pyridoxatin (PYR), desferricoprogen (DCO) and desferriastechrome (DAC); Fig. 2] in the mitigation of chemical signals of IO toxicity, and study their ability to stabilize cytosolic IO.

Materials and methods

Chemicals

The following reagents were procured from the indicated sources and used without further purification: ascorbic acid (Vetec, Brazil); calcein, Chelex® sodium form, DMSO, ferrous ammonium sulfate (FAS), NaCl, HEPES, sodium nitrilotriacetate (NTA) (Sigma-Aldrich); dihydrorhodamine hydrochloride (Biotium). Desferrioxamine mesylate (Cristália, Brazil) was a generous gift from ABRASTA (Brazilian Association for Thalassemia). HBS buffer was HEPES 20 mM, NaCl 150 mM, pH 7.4 washed with Chelex (1 g 100 mL^{-1}) to remove traces of contaminant iron. PYR (Shang et al. 2015), DCO (Kalansuriya et al. 2017) and DAC (Shang 2016) were isolated and characterized during microbial biodiscovery research (lead by author RJC).

Iron binding

The ability of the chelators to scavenge iron was studied in a competitive equilibrium assay with the fluorescent probe calcein. Upon coordination to iron, the fluorescence of calcein is quenched in a stoichiometric fashion after formation of the complex ferric calcein (CAFe). The fluorescence may be regenerated after treatment with other high affinity chelators (Baccan et al. 2012). In flat, transparent 96 well microplates, it was added 190 µL of 2 µM CAFe in HBS and 10 µL aliquots of the chelators in DMSO with final concentration up to 50 µM. Fluorescence ($\lambda_{exc}/\lambda_{em}$ = 485/520 nm) was registered after 2 h in a FluoStar Optima equipment (BMG).

Table 1 Registered trials of treatments for IO diseases and of new applications of iron chelators (www.clinicaltrials.gov, April 2019)

Drug	Phase	Identifier	Status (date)	Strategy
Amlodipine	Phase 3	NCT01395199	C (02/2015)	Calcium pump inhibition
Arginine, decitabine, sildenafil	NA	NCT00000623	C (07/2006)	Anti-hypertensive (accelerated iron excretion)
Deferatizole (FBS0701, SPD602, SSP-004184)	Phase 2	NCT01186419	C (01/2013)	Iron chelation
Deferitrin (GT56-252)	Phases 1, 2	NCT00069862	C (11/2005)	Iron chelation
LJPC-401 (synthetic human hepcidin)	Phase 2	NCT03395704, NCT03381833	R	Synthetic hepcidin (hormonal regulation of iron export)
Nifedipine	Phase 1	NCT00712738	C (09/2010)	Anti-hypertensive (accelerated iron excretion)
Osveral	NA	NCT01369719	C (05/2011)	Iron chelation
Pantoprazole	NA	NCT01524757	U	Proton pump inhibition
Pyridoxal isonicotinoyl hydrazone	Phase 2	NCT00000588	C (NA)	Iron chelation
Silymarin	Phases 2, 3	NCT00999349	U	Iron chelation
SP420	Phase 2	NCT03801889	NYR	Iron chelation
Spirulina, amlodipine	NA	NCT02671695	C (04/2018)	Iron chelation
Triapine (3-AP)	Several	Several	Several	Antitumor iron chelator
VLX600	Phase 1	NCT02222363	T (no efficacy, 12/2016)	Antitumor iron chelator

C completed, *R* recruiting, *NYR* not yet recruiting, *T* terminated, *U* unknown, *NA* not available

Antioxidant studies

The effect of the chelators on the iron-dependent autoxidation of ascorbate was studied as previously described (Esposito et al. 2003). Ferric nitrilotriacetate [Fe(nta)] standards were prepared by the 1:1 (mol:mol) reaction of FAS and NTA in water for 1 h at 37 °C. Aliquots of 10 µL of 10 µM Fe(nta) were transferred to 96-well flat bottom transparent microplates, followed by 10 µL of chelator solutions (final concentration 0–50 µmol L^{-1}). Then, each well received 180 µL of a solution containing 50 µM DHR and 40 µM ascorbic acid in HBS. Fluorescence ($\lambda_{exc}/\lambda_{em}$ = 485/520 nm) was registered during 1 h at 1 min intervals at 37 °C in a FluoStar Optima equipment (BMG) and the slopes (fluorescence min^{-1}) from 15 to 40 min were calculated for each kinetic curve.

Cell permeability studies

Human cervical carcinoma cell line HeLa was obtained through donation. Human hepatoma cell line HepG2 was purchased from Banco de Células do Rio de Janeiro (BCRJ Catalogue Number 0103). Both were cultivated under 5% CO_2 atmosphere at 37 °C in DMEM (Dulbecco's Modified Eagle's medium containing high glucose), supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% antibiotics (penicillin 10,000 U.I. mg mL^{-1}, streptomycin 10 mg mL^{-1} and amphotericin B 1 mg mL^{-1}). Medium and supplements were purchased from Vitrocell/Embriolife, Brazil. After attaining 80–90% confluence, the cells were trypsinized (trypsin/EDTA 250 mg L^{-1}) and seeded (5000 per well) in 96-well microplates coated with 2% clear commercial source gelatin (Sigma-Aldrich 2008) to improve adhesion. The microplates were kept for 48 h in the incubator, prior to the experiments. Then, the medium was removed mechanically from the microplate and the wells were washed twice with 100 µL HBS in order to remove traces of the phenol red indicator. Cells received 100 µL of acetomethoxy calcein (CAL-AM; 3 µM in indicator-free growth medium) and were kept for 20 min in the incubator. The wells were washed once with 100 µL HBS and treated with 100 µM of warm probenecid (0.5 mM in HBS) to reduce cell leakage of the fluorescent probe. The microplate was

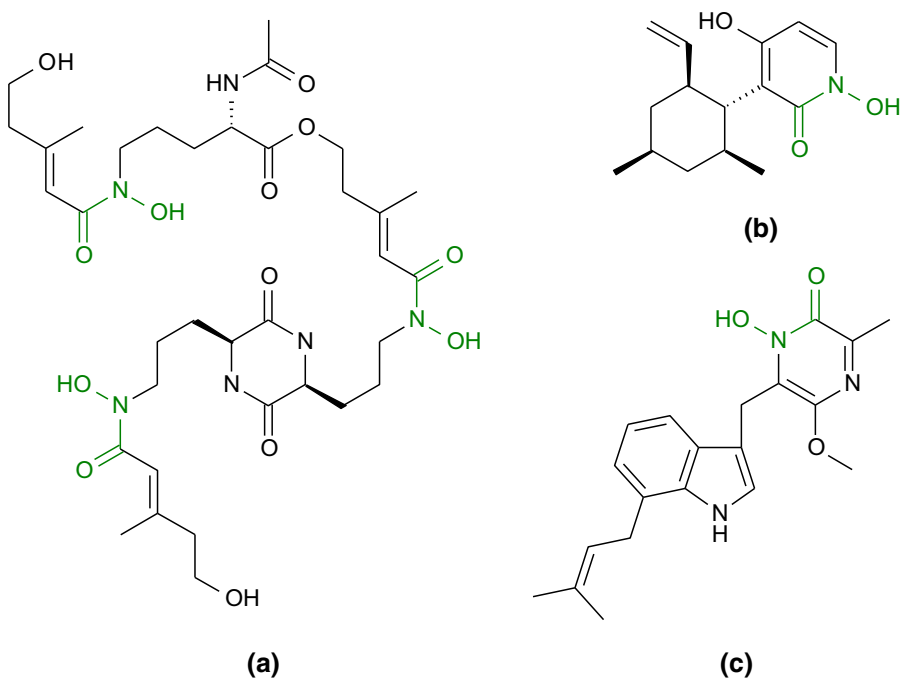

Fig. 2 Candidate fungal hydroxamates for the treatment of iron overload. The hydroxamic acid chelation site is highlighted. **a** Desferricoprogen (DCO), **b** pyridoxatin (PYR) and **c** desferriastechrome (DAC)

carried into the BMG reader and fluorescence was registered for ~ 60 min (37 °C; $\lambda_{exc}/\lambda_{em}$ = 485/520 nm). At specified times, 2 µL of stock solutions the iron compounds were added, and fluorescence reading was continued until signal stabilization. DTPA (non-permeant chelator; 1 µL of a 10 mM solution) was then added to scavenge iron from the extracellular medium. Finally, 2 µL of the chelators were added to attain a final concentration of 20 µM. This experiment was performed in duplicates (Shvartsman et al. 2010).

Intracellular antioxidant activity

Cells received 100 µL of HBS and 6.4 µL of the solution of ferric hydroxyquinoline attain the final concentration of 7.5 µM. These plates were incubated for 20 min, and during this time it was prepared a fresh H_2O_2 solution (50 µM in HBS supplemented with 10 mM glucose). Plates were washed once with DTPA 100 µM to remove excess iron and treated sequentially with 100 µL of the peroxide solution and 2 µL of DCFDA 1 mM in DMSO. The microplate was carried into the BMG reader and fluorescence was registered (37 °C; $\lambda_{exc}/\lambda_{em}$ = 485/520 nm). After 20 min, 2 µL of the chelators was added (final concentration 20 µM) to stop the generation of redox-active species catalyzed by iron. The slopes (fluorescence rate/iron concentration) before and after chelator addition were obtained. This experiment was performed in duplicates (Sohn et al. 2011).

Cell viability studies

HeLa cells were cultivated in 96 well microplates as described above, except for the medium (D10), allowing 24 h for adhesion. Cells were washed once with fresh medium. In 100 µL final volume, 1 µL of the test substances dissolved in DMSO were added, to attain a final concentration of 10 µM. Cells were incubated again for 24 h and then washed with PBS. Cell viability was assessed by the MTT method (5 mg mL^{-1}) (Präbst et al. 2017).

Results and discussion

There are few references about DAC and its iron binding abilities. DAC was first described in 1980 as a ferric complex in *Aspergillus terreus*. The

demetallated form was only obtained after the reduction of the metal to iron(II), when relatively strong chelators such as 8-hydroxyquinoline had no effect, which attests to the stability of the ferric complex (Arai et al. 1981). In *Aspergillus fumigatus*, a variant form of this iron complex was identified, the hexahydroastechrome (Yin et al. 2013).

DCO used in this study was a metabolite of *Talaromyces* sp. fungi, whose association to iron was preliminarily studied by us before (Kalansuriya et al. 2017). Similarly to DAC, it was isolated initially as a ferric species (coprogen) in 1970, in *Penicillium citrinum*. Similarly to DFO, DCO was recognized as a linear hydroxamate able to form 1:1 complex with the metal (Keller-Schierlein and Diekmann 1970). It also occurs in exudates of other species such as *Epicoccum purpurascens* (Frederick et al. 1981).

PYR is a better known substance, isolated from cultures of *Acremonium* sp. fungi, where it was recognized as a free radical scavenger (Teshima et al. 1991). In our case, it was recognized as an efficient chelator with an affinity to iron similar to deferiprone (Shang et al. 2015). PYR forms complexes with other metal ions, and one of them, $Cu(PYR)_2$, was instrumental in the resolution of the ligand structure (Du et al. 2014).

Stability against ferric calcein (CAFe)

CAFe, the non-fluorescent Fe(III) complex with calcein, has a stability constant $\log K_{ML} = 24$ (Breuer et al. 1995), hence it is a good fluorimetric indicator of the relative strength of siderophores in a given sample, which will demetallate it and render it fluorescent (Espósito et al. 2002). DFO, the gold standard of iron chelation in the treatment of IO diseases, has a $\log K_{ML} = 30$ (Poreddy et al. 2004), therefore its reaction with CAFe proceeds stoichiometrically with maximum fluorescence recovery (CAFe demetallation) at equimolar CAFe concentration (Fig. 3). The same was observed for DCO, the other hexadentate chelator. The other chelators also displayed fluorescence recovery after reaction with CAFe, although at slightly higher than equimolar concentrations. Bidentate DAC and PYR exhibited similar profiles with maxima reached at 6–12 μM corresponding to 3–4 times molar excess over calcein. DAC and PYR are more acidic than the hexadentate chelators due to the increased charge delocalizing effect facilitated by the aromatic rings. Their pK_a range from 4 to 6 in comparison to $pKa_1 \sim 9$ for DFO and DCO (Raymond 1990). Although this would suggest a facilitated interaction with cations at physiological pH, it seems that the chelate effect prompted by the hexadentate chelators is much more important, in which lower chelator concentrations are required to remove metal from calcein. In any instance, it could be said that DAC, DCO and PYR are very good chelators according to the iron binding ability criterion.

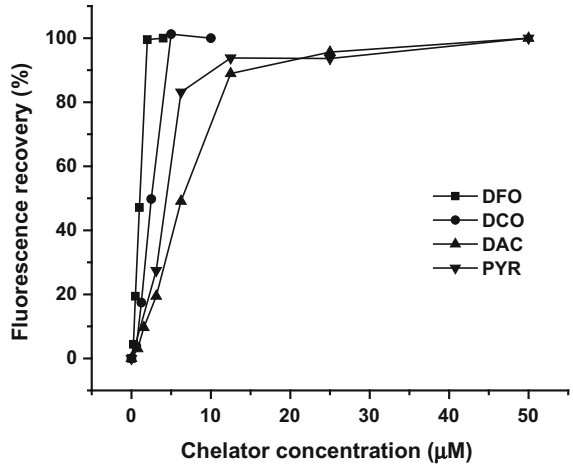

Fig. 3 Chelation ability in the ferric calcein (CAFe, 2 μM) competition assay. Data for DCO and PYR were retrieved from Kalansuriya et al. (2017) and Shang et al. (2015)

Antioxidant activity in buffer

The ability of the chelators to halt the iron-catalyzed auto-oxidation of ascorbate in physiologic medium is one of the best ways to assess the ability of a candidate molecule as a drug for the treatment of IO diseases. This antioxidant effect is easily followed in high throughput systems following the rate of oxidation of the DHR probe (Esposito et al. 2003). The chelator may prevent iron from engaging into oxidative cascades by forming a stable six coordinated complex (blocking all the coordination sites of the metal from approaching the oxidative substrates) and/or by decreasing the $E^0_{Fe(III)/Fe(II)}$ to greatly negative values (Halliwell and Gutteridge 2007). DFO and DCO had similar behavior in blocking DHR oxidation. PYR, having lower dentition, accordingly required higher concentrations to achieve the same result. However,

DAC did not display any antioxidant effect in this assay (Fig. 4).

Even though DAC was able to scavenge iron from CAFe (although at the higher mol ratio of all the chelators; Fig. 3), the bulky substituents of the indole group may make it difficult for this particular ligand to form the 1:3 (metal:ligand) complex that would be expected by the hydroxamate dentition, in our diluted solution. Lower coordination numbers, even in stable complexes, are associated to the promotion of oxidative reactions because there are still coordination sites available for the catalysis. This effect is markedly present in other stable Fe(III) complexes such as ferric edetate (Esposito et al. 2003).

Removal of iron from IO-cells

In the cell permeability assays (Fig. 5), it is clear that only DAC and PYR are able to gain access to the cytosol of both HepG2 and HeLa cells and demetallate the intracellular CAFe complex. The hydrophobicity of the substituent groups adjacent to the hydroxamate binding region is likely the reason for this behavior. Indeed, octanol–water partition coefficients (logP) for the chelators under study calculated by MarvinSketch version 19.9 (ChemAxon) in NaCl = 0.15 M were 2.61 (PYR) and 3.60 (DAC), however − 2.02 (DFO) and − 2.27 (DCO). DCO, as DFO, is a quite hydrophilic and more linear siderophore, which does not seem to have access to cells at least within the timeframe of this experiment.

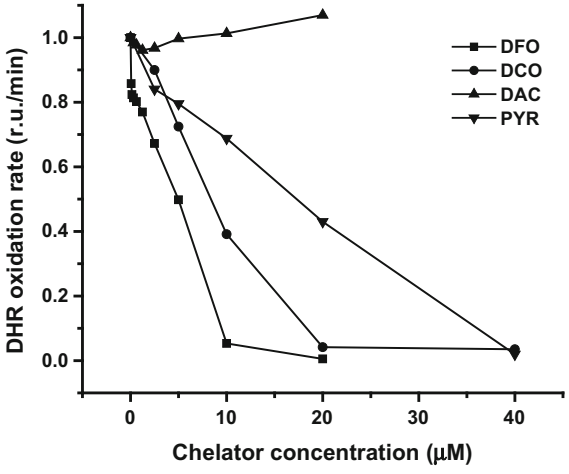

Fig. 4 Antioxidant assay in the iron/ascorbate system followed by DHR. Fe(nta) standard was 10 μM. Data for DCO retrieved from Kalansuriya et al. (2017)

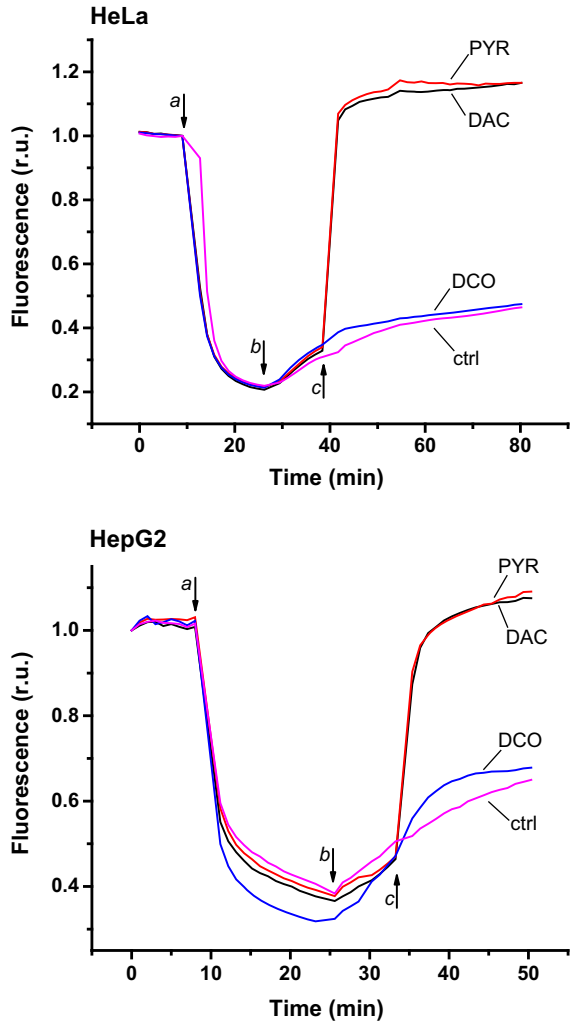

Fig. 5 Study of cell permeability of the chelators. Arrows indicate substances added at the specific time marks (see "Materials and Methods"): FeHQ (*a*), dtpa (*b*) and chelators (*c*). *r.u.* Relative fluorescence units

Intracellular antioxidant activity

Non iron-overloaded cells have resting levels of cytosolic free iron, termed *labile iron pool* (ca. 0.25–0.50 μM. Sihn et al. 2019; Zanninelli et al. 2002), that are able to oxidize probes such as DCFDA after treatment with peroxide (Fig. 6, light gray bars) and are slightly responsible to chelators (white bars). However, iron overloaded cells display increased oxidation rates (black bars) that may or may not be

controlled by externally added chelators. It was observed that only PYR displayed a significant beneficial activity (Fig. 6, dark gray bars). It was able to decrease by 30% and 50% the oxidation rate of the probe in HepG2 and HeLa, respectively. Again, DAC did not display antioxidant activity. Even though the iron source is different here, there is no reason to suspect that the rationale that explained DAC's lack of antioxidant activity in buffer would be different. Also, it should be noted that PYR has an oxidazable "phenol" group (which is lacking in DAC) that could further contribute to its antioxidant activity. DCO, not being permeable, accordingly did not display intracellular antioxidant activity.

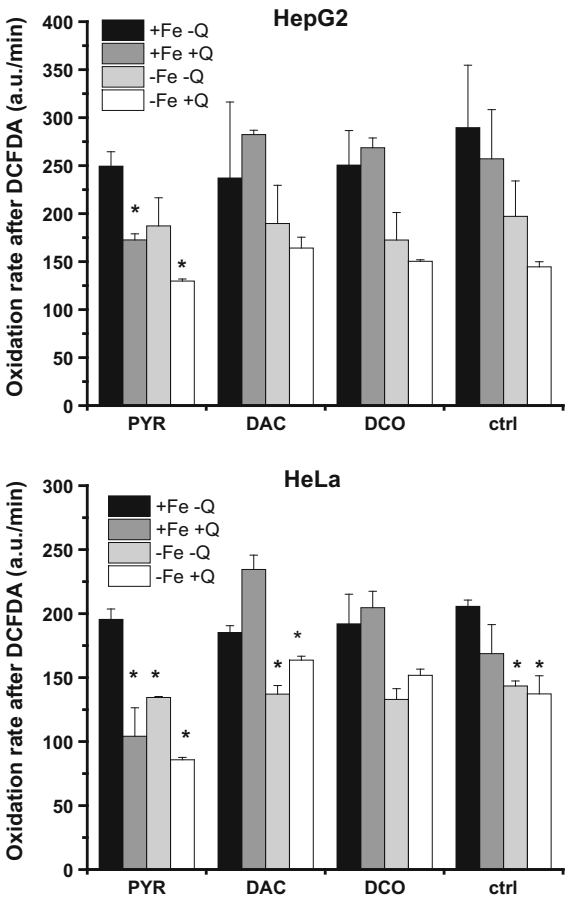

Fig. 6 Intracellular antioxidant ability of the chelators (20 μM, 1% DMSO) in HepG2 and HeLa cell lines. Cells were either loaded or not with iron or chelators. Controls received only 1% DMSO solution. $*P < 0.05$ in relation to iron loaded, non chelated cells ("+Fe-Q"). *a.u.* Arbitrary fluorescence units

Cell viability assays

A single-dose study of the toxicity of the chelators against the tumor line HeLa cells (Fig. 7) indicates that, at 10 μM concentration, all the chelators are significantly less toxic than cisplatin. The bidentate PYR and DAC are the most active.

PYR was initially recognized as a potent inhibitor of matrix metalloproteinases, with cytotoxic activity against several tumor lines (Lee et al. 1996). It is active against human colon (SW620, IC_{50} 0.3 μM), lung (NCI-H460, IC_{50} 0.35 μM), cervical (KB3-1, IC_{50} 0.4 μM) (Shang et al. 2015) and HeLa (IC_{50} 3.9 μM) (Teshima et al. 1991) carcinoma cells. These findings reinforce the notion that good iron chelators may find use in the antitumor therapy, beside their traditional use in IO diseases (Merlot et al. 2019).

Taken together, our results place PYR in the rank of the high affinity, antioxidant and permeable iron chelators. Its iron complex $Fe(PYR)_3$ also displays an increased lipophilicity (log P = 12.54 for the structure minimized with Chem3D version 17.0.0.206, Perkin Elmer), which is relevant for scavenging the metal out of the cell. Therefore, PYR is similar to other high affinity chelators such as salicylaldehyde isonicotinoyl hydrazone (SIH; Konijn et al. 1999; Murphy et al. 1985) and its analogue pyridoxal isonicotinoyl hydrazone (PIH; Link et al. 2003) (Fig. 8) as well as the approved deferiprone (Link et al. 2001) and deferasirox (Hershko et al. 2001). Our own group has already indicated that similar properties could be found in other natural chelators such as quercetin (Baccan et al. 2012) or derivatized natural molecules

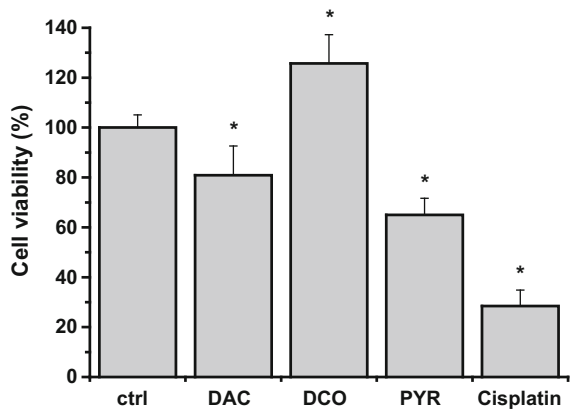

Fig. 7 HeLa viability under the studied compounds (10 μM, 1% DMSO) after 24 h exposure. $*P < 0.05$ in relation to control

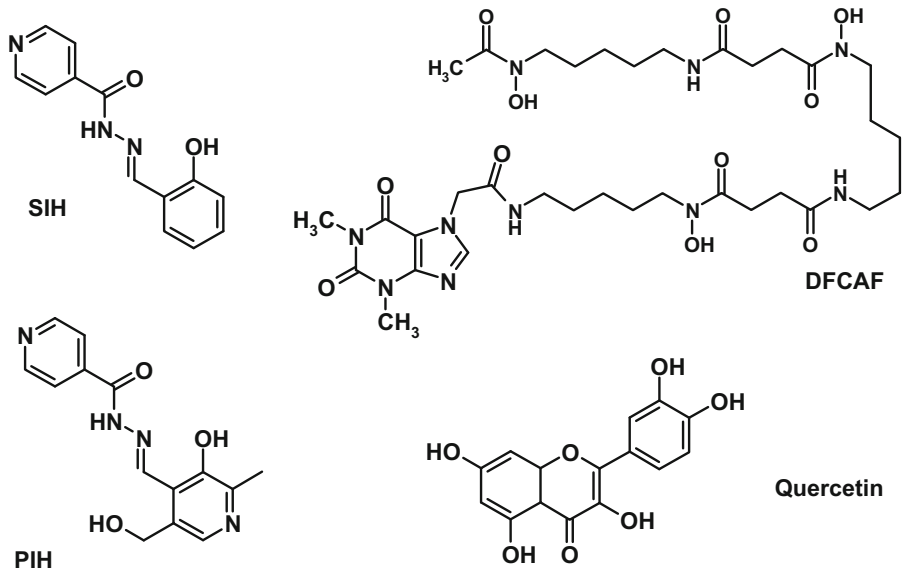

Fig. 8 Examples of antioxidant, cell permeable iron chelators

such as DFO-caffeine (DFCAF; Alta et al. 2014) (Fig. 8).

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