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ORIGINAL ARTICLE

Insights on the relationship between structure vs. toxicological activity of antibacterial rhodamine-labelled 3-hydroxy-4-pyridinone iron(III) chelators in HepG2 cells

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ABSTRACT

In the present study we investigated the *in vitro* hepatotoxicity of a set of rhodamine-labelled 3-hydroxy-4-pyridinones (3,4-HPO) that had previously demonstrated significant inhibitory effect in the intramacrophagic growth of *Mycobacterium avium*. Our aim was to establish a correspondence between the molecular structure and the *in vitro* toxicological activity of these compounds.
The impact of a set of bidentate (MRB2, MRB7, MRB8, and MRB9) and hexadentate (MRH7, MRH8, and MRH10) chelators on cellular metabolic competence and membrane integrity was investigated in HepG2 cells.
Our findings indicate that: a) hexadentate chelators are more cytotoxic than parent bidentate ligands; b) disruption of cell membrane and metabolic competence only occurred after 5 days, at the highest concentrations tested; c) strict correlation between bacteriostatic activity and *in vitro* toxicity was observed, which seems to be directly dependent on the size of the molecule and on the hydrophilic/lipophilic balance; d) among the set of bidentate ligands, carboxyrhodamine derivatives (amide linker) presented lower detrimental effects, when compared with rhodamine B isothiocyanate chelators (thiourea linker); e) contrarily, for the hexadentate series, rhodamine B isothiocyanate derivatives are less cytotoxic to HepG2 cells than carboxyrhodamine molecules; and f) for all compounds tested, when the substituents of the nitrogen atom were switched from ethyl to methyl, an increment of toxicity was observed.
Overall, all chelators seem to display suitable in vitro toxicological potential to combat fast grow bacteria. According to their *in vitro* pharmacological: toxicological potential ratio, MRH7 and MRH8 may be considered as the most suitable compounds to undergo further pre-clinical development studies.

KEY WORDS: iron chelator; 3-hydroxy-4-pyridinone (3,4-HPO); rhodamine; *in vitro* toxicity; HepG2 cells

Introduction

Considering the alarming emergence and spread of new forms of antimicrobial resistance, the development of new strategies to fight bacterial infections remains crucial to provide physicians with effective tools for bringing these pathogens under control. In this regard, our research group has been engaged in efforts to design novel effective antibiotics against mycobacterial species, specifically *Mycobacterium avium* (Fernandes *et al.*, 2010, Nunes *et al.*, 2010, Moniz *et al.*, 2013, Moniz *et al.*, 2015).

Some studies have shown a link between *M. avium* infection and increased iron deposition in tissues of infected patients (al-Khafaji *et al.*, 1997); this essential element impairs macrophage innate immunity and, consequently, favours the growth and virulence of the opportunist agent (Silva-Gomes *et al.*, 2013). An additional factor that supports the importance of this element in mycobacterial infection is that iron uptake is mediated by high-affinity chelating structures produced

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by the pathogen, the siderophores mycobactins (cell wall-associated) and carboxymycobactins (released into the extracellular medium) (Thompson *et al.,* 2006). Since iron is essential for mycobacterial growth and survival (Silva-Gomes *et al.,* 2013), iron chelation therapy, as means of removal of metal available for mycobacteria metabolism, is worth considering. Coherently, iron deprivation has proven to prevent *M. avium* proliferation in mice, although few different iron chelators have demonstrated only marginal *in vitro* mycobacteriostatic activity in macrophages (pathogen natural host cells) (Gomes *et al.,* 1999, Cronje & Bornman, 2005).

Following this research line, several 3-hydroxy-4-pyridinones (3,4-HPO) functionalized with different rhodamine moieties were synthesised (Figure 1), and their antibacterial activity against *M. avium* infection was further evaluated (Fernandes *et al.,* 2010, Nunes *et al.,* 2010, Moniz *et al.,* 2013). Carboxytetramethylrhodamine-labelled chelators (MRB8 and MRH8) were deemed capable of limiting iron supply and restricting intramacrophagic growth of *M. avium*, although in a lesser extent than rhodamine B isothiocyanate-labelled chelators (MRB7 and MRH7) (Figure 1; Table 1) (Moniz *et al.,* 2013). Since the Fe(III)-chelating competence was similar for all testing agents, we hypothesised that the pharmacological potential of these compounds was also reliant on their ability to reach relevant intracellular targets, namely by crossing membranes to reach the cytosol of the host cell or also the phagosomes. This capacity of the chelators to permeate biological membranes is dependent on a) the substituents of the amino groups of the xanthene ring of rhodamine; and b) the type of linkage between rhodamine and the chelating unit (Nunes *et al.,* 2010, Moniz *et al.,* 2016b, Moniz *et al.,* 2017).

Although some data clarifying the molecular features that are crucial for the antimycobacterial activity of those chelators already exist, no information is available regarding their eventual toxicity. Parallel to the assessment of pharmacological efficacy, the evaluation of toxic profile of new compounds is of great relevance in the process of developing new therapeutic agents, as the detrimental effects of drugs may severely compromise their clinical application. Thus, in order to evaluate the potential toxicity elicited by rhodamine-labelled chelators that have formerly demonstrated antimicrobial properties (Figure 1; Table 1) and to elucidate the main molecular features underlying those detrimental effects, we investigated the impact of several ligands on cellular metabolic competence and on cytoplasmic membrane integrity, using the human hepatoma cell line HepG2.

Materials and methods

Chemicals

The structure of the bidentate and hexadentate chelators used in the present study are shown in Figure 1. The chelators MRB2, MRB7, MRB8, MRB9, MRH7, MRH8, and MRH10 were previously synthesized by our research group. Their synthesis and characterization are described elsewhere (Nunes *et al.,* 2010, Moniz *et al.,* 2013, Moniz *et al.,* 2015, Moniz *et al.,* 2016a). Unless stated otherwise, all the cell culture reagents were purchased from Gibco (Alfagene, Lisbon, Portugal) and all other chemicals from Sigma-Aldrich (Lisbon, Portugal).

HepG2 cell culture routine

The human hepatoma HepG2 cell line was generously given by Prof. Ricardo Dinis-Oliveira (CESPU, Instituto Superior de Ciências da Saúde – Norte, Gandra, Portugal). HepG2 cells were routinely maintained in 75 cm^2 canted-neck tissue culture flasks Corning® (VWR, Lisbon, Portugal) and cultured as monolayer in cell culture medium, *i.e.* DMEM, high glucose, GlutaMAX™ (Sigma-Aldrich, Lisbon, Portugal) supplemented with 10% fetal bovine serum (FBS), 1% antibiotic (penicillin 5000 U/mL + streptomycin 10000 µg/mL), and 1% fungizone (250 mg/mL amphotericin B), in a humidified incubator with a 5% CO_2 atmosphere, at 37 °C. Cells were sub-cultured at approximately 70–80% confluence, over a maximum of 10 passages (to avoid accumulation of genetic mutations during replicative process). To promote cells detachment, the cells were washed with Hank's balanced salt solution (HBSS) after medium removal, and

Table 1. Structural features, antibacterial activity (Moniz *et al.,* 2013, Moniz *et al.,* 2015) and interaction with biological membranes (Coimbra *et al.,* 2014, Moniz *et al.,* 2016b, Moniz *et al.,* 2017) of the rhodamine-labelled 3-hydroxy-4-pyridinone iron(III) chelators.

Chelator	Structure features				Antibacterial activity against *Mycobacterium avium*	Membrane interaction
	Denticity	Type of fluorophore	*N*-substituents	Linker		
MRB2	Bidentate	Tetraethyl sulphorhodamine B	*N*-ethyl	Sulphonamide	0	nd
MRB7	Bidentate	Tetraethyl rhodamine B isothiocyanate	*N*-ethyl	Thiourea	++++	++
MRB8	Bidentate	Tetramethyl carboxyrhodamine	*N*-methyl	Amide	++	+
MRB9	Bidentate	Tetraethyl carboxyrhodamine	*N*-ethyl	Amide	++	nd
MRH7	Hexadentate	Tetraethyl rhodamine B isothiocyanate	*N*-ethyl	Thiourea	++++	++
MRH8	Hexadentate	Tetramethyl carboxyrhodamine	*N*-methyl	Amide	+++	+
MRH10	Hexadentate	Tetramethyl rhodamine B isothiocyanate	*N*-methyl	Thiourea	+++	nd

0 – no effect; + low; ++ moderate; +++ high; ++++ very high; nd – not determined (no detailed studies have been performed for these chelators regarding their capacity to interact with membranes)

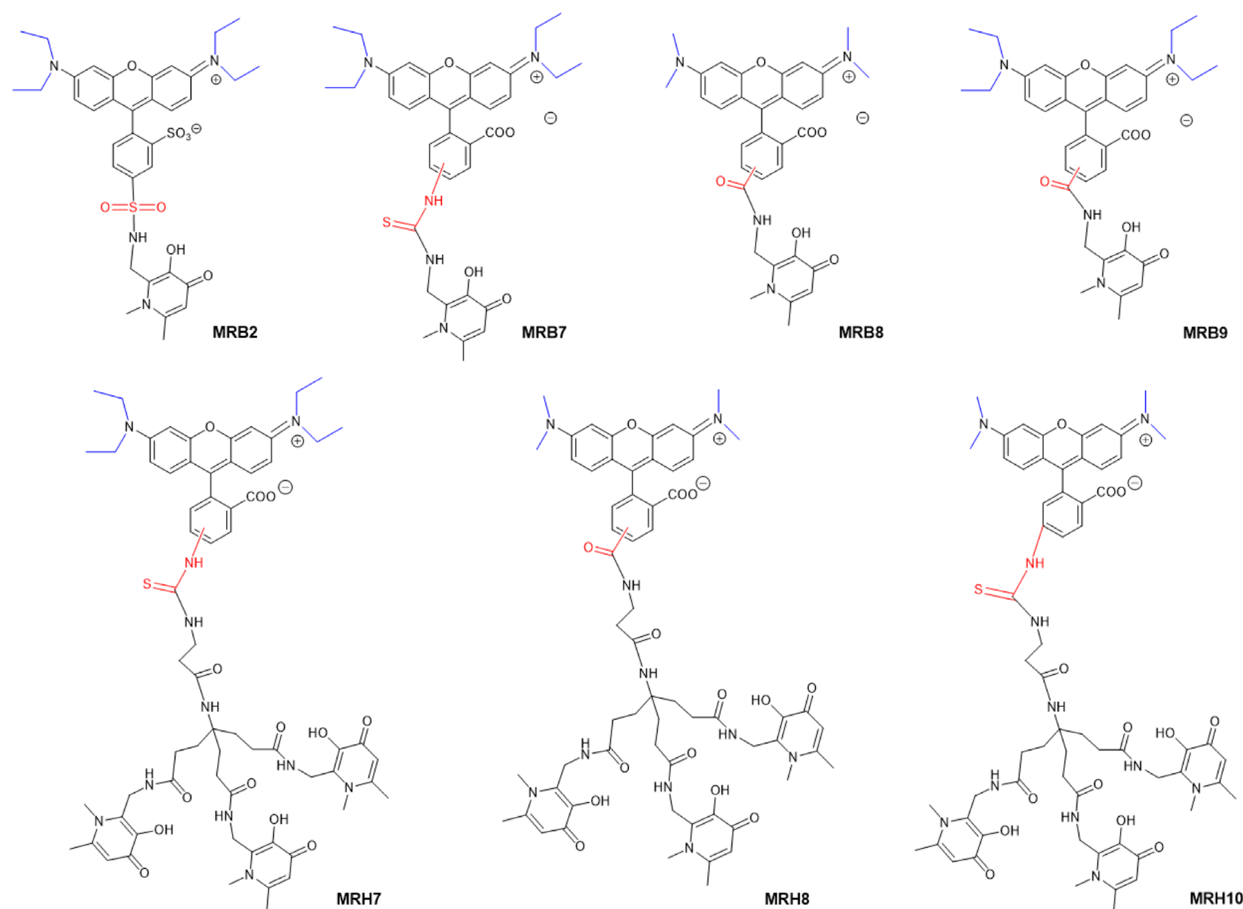

Figure 1. Structure and nomenclature of fluorescent bidentate (MRB2, MRB7, MRB8, and MRB9) and hexadentate (MRH7, MRH8, and MRH10) chelators used in the current study. In previous works, the compound MRH7 was abbreviated as CP777 (Fernandes *et al.,* 2010) and 4 (Nunes *et al.,* 2010). The molecular structure of these chelators comprise three parts: *i)* a chelating unit, *i.e.* 3-hydroxy-4-pyridinone (3,4-HPO); *ii)* a fluorophore (tetraethyl sulphorhodamine B for MRB2; tetraethyl rhodamine B isothiocyanate for MRB7 and MRH7; tetramethyl carboxyrhodamine for MRB8 and MRH8; tetraethyl carboxyrhodamine for MRB9; or tetramethyl rhodamine B isothiocyanate for MRH10); and *iii*) a linkage fragment, which is determined by the fluorophore derivative that is used in the synthesis. The chelating unit is directly coupled to the fluorophore to produce the bidentate fluorescent ligands (MRB2, MRB7, MRB8, and MRB9) or linked to a tripodal anchor to produce hexadentate chelators (MRH7, MRH8, and MRH10). The substituents on xanthene group of rhodamine are highlighted in blue. The linker between the rhodamine fluorophore and the chelating unit is highlighted in red.

added of 2 mL of 0.25% trypsin/1 mM EDTA solution. After a 5 min-incubation at 37 °C, cells were re-suspended in 10 mL complete cell culture medium and split into new flasks at appropriate ratios. For the cytotoxicity assays, cells were plated onto 96-well plates (BD Falcon, Enzifarma, Lisbon, Portugal) at a density of 5×10^4 per well (100 µL of suspension to each well) and placed in the incubator at 37 °C, for approximately 24 h, prior to the compound treatments. Peripheral wells on the plate were filled with sterile water to prevent evaporation and concentration of the test solutions (da Silva *et al.,* 2014).

Incubation of the chelators

On the day of the experiment, the medium was removed and HepG2 cells were exposed to a set of concentrations of each rhodamine-derived chelator (ranging from 1 to 40 µM and from 3 to 120 µM for hexadentate and bidentate ligands, respectively). The concentration intervals were selected on the basis of the concentrations tested in previous studies for the evaluation of the antimycobacterial effect (Moniz *et al.,* 2013, Moniz *et al.,* 2015). The concentrations tested of hexadentate chelators were three times lower than those selected for bidentate ligands; this testing design is explained by the fact that three molecules of a bidentate ligand are needed to chelate the same amount of iron(III) chelated by one molecule of a hexadentate ligand.

Stock solutions were prepared in 4% DMSO (V/V) and stored at 4 °C, whereas test dilutions were freshly prepared in complete cell culture medium on the day of the experiment. In all experiments, a solvent control (cells treated with 0.05% DMSO, V/V) was included to mimic the maximum concentration of DMSO present in the treatments. A positive control consisting of 1% Triton X-100 (V/V) and a negative control (complete cell culture medium without treatments) were also included. Each treatment condition

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was tested in triplicates by adding 250 μL of solution to each well of the 96-well plate. Cells were then incubated at 37 °C, for 5 days. No significant differences were observed between solvent and negative controls (data not shown; $p>0.05$, Student's unpaired *t*-test).

Evaluation of cytoplasmic membrane integrity by the LDH leakage assay

The LDH leakage assay was performed to access the membrane integrity of HepG2 cells in the presence of chelators. As LDH is a cytoplasmic oxidoreductase, its presence in the extracellular medium is indicative of alterations in membrane permeability and consequently in cell integrity.

The enzyme catalyses the reversible conversion of pyruvate to lactate, in the presence of β-nicotinamide adenine dinucleotide (β-NADH), which in turn is oxidized to NAD^+. So, after 1 day, 3 days, and 5 days of incubation with the chelators, a 10 μL aliquot of supernatant of each well was placed into a new 96-well plate. Then 40 μL of 0.05 M potassium phosphate buffer (pH 7.4, KH_2PO_4, Merck) and 200 μL of 0.15 mg/mL β-NADH were added. Immediately before the absorbance reading, 25 μL of 2.5 mg/mL sodium pyruvate were pipetted into each well to start the reaction. The β-NADH and sodium pyruvate solutions were freshly prepared in buffer solution. The oxidation of NADH to NAD^+ was followed by measuring the absorbance at 340 nm (Amit *et al.*, 2014), every 16 seconds, for 3 min, using an automatic plate reader Power Wave X™ (BioTek Instruments, Inc.) in a kinetic photometric mode. Since the absorbance range of the 3,4-HPO chelators does not overlap with that for NAD^+, there is no possibility of interference in the spectrophotometric assay. Data were scaled between positive (100%) and negative controls (0%) and results were graphically presented as percentage of extracellular LDH *versus* chelator concentration (μM).

Evaluation of cellular functioning by the MTT reduction assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay is a colorimetric test used to measure the activity of the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidoreductases that reduce the soluble yellow MTT into purple insoluble crystals of formazan. Cellular NADPH-dependent oxidoreductase activity is an indicator of general viability, as the redox state is a key parameter of cellular physiology, desegregating evidence on the cell catabolic efficiency, antioxidant defence, and biosynthetic potential. Since NADPH-dependent enzymes are largely ubiquitous in the cytosolic compartment, the amount of purple formazan produced by cells treated with the chelators was compared with the amount of formazan produced by untreated controls to assess the ability of the compound to change the normal metabolic competence of the cell. After 5 days of incubation at 37 °C, the medium was removed and 100 μL 0.5 mg/mL MTT (prepared in HBSS) were added to the attached cells. The plates were incubated at 37 °C, for 90 min. Then, the MTT solution was aspirated and the formed intracellular formazan crystals were dissolved with 100 μL DMSO. The plates were shaken for 15 min and, as MTT is photosensitive, all steps of the procedure were executed under light protection. The absorbance was recorded at 550 nm, using a multi-well plate reader BioTek Synergy™ HT (BioTek Instruments, Inc.) (Dias da Silva *et al.*, 2013, da Silva *et al.*, 2014). Data were scaled between negative (100%) and positive controls (0%) and results were graphically presented as percentage of MTT reduction *versus* chelator concentration (μM).

Statistical analysis

The cytotoxicity data from the MTT reduction and LDH leakage assays were obtained from four independent experiments. Eight increasing test concentrations of each chelator were tested in three replicates, within each experiment. To reduce variability between experiments, data were normalized plate-by-plate by negative and positive controls, as previously described (Rajapakse *et al.*, 2004, Dias da Silva *et al.*, 2013, da Silva *et al.*, 2014). The results are presented as mean ± standard error of the mean (SEM). Normality of data distribution was assessed by the Kolmogorov-Smirnov, D'Agostino & Pearson omnibus, and Shapiro-Wilk normality tests. Statistical comparisons were performed by Kruskal-Wallis test followed by Dunn's multiple comparison *post hoc* test. The solvent and negative control values (raw data) were compared by the Student's unpaired *t*-test and differences were considered insignificant (data not shown). Nonlinear regression analysis of all four bidentate chelators was carried out using the Hill equation. All of the nonlinear regression models describe sigmoidal concentration–response relationships. Data obtained were analysed using the software Graph Pad Prism version 6.0. Significance was accepted at $p<0.05$.

Results

Results obtained in LDH leakage assay show that disruption of cytoplasmic membrane increases over time when hepatocytes are incubated with the chelators tested. Accordingly, no effects were observed after one day (Figures 2 and 3, $p>0.05$). After three days, a tendency for chelator-induced disturbance of cell integrity was observed, however this observed augment of membrane disruption had no statistical significance ($p>0.05$). Significant differences were only detected after five days of exposure to bidentate ligand MRB8 (Figure 2, $p<0.05$; from 24.5 μM up) and to hexadentate MRH7, MRH8 and MRH10 ligands (Figure 3, $p<0.05$; from 1.5 μM up). The less cytotoxic chelators were bidentate ligands MRB2, MRB7 and MRB9, whose LDH leakage effect was not higher than 12.79±9.54%, 16.63±7.59% and 15.26±8.71%, respectively (Figure 2, $p>0.05$). With exception of MRB7, these are also the chelators displaying the lowest antibacterial activity (Moniz *et al.*, 2013, Moniz *et al.*, 2015). Results of the LDH leakage in HepG2 cells following 1, 3, and 5 day-exposures to bidentate and hexadentate chelators are presented in Figures 2 and 3, respectively.

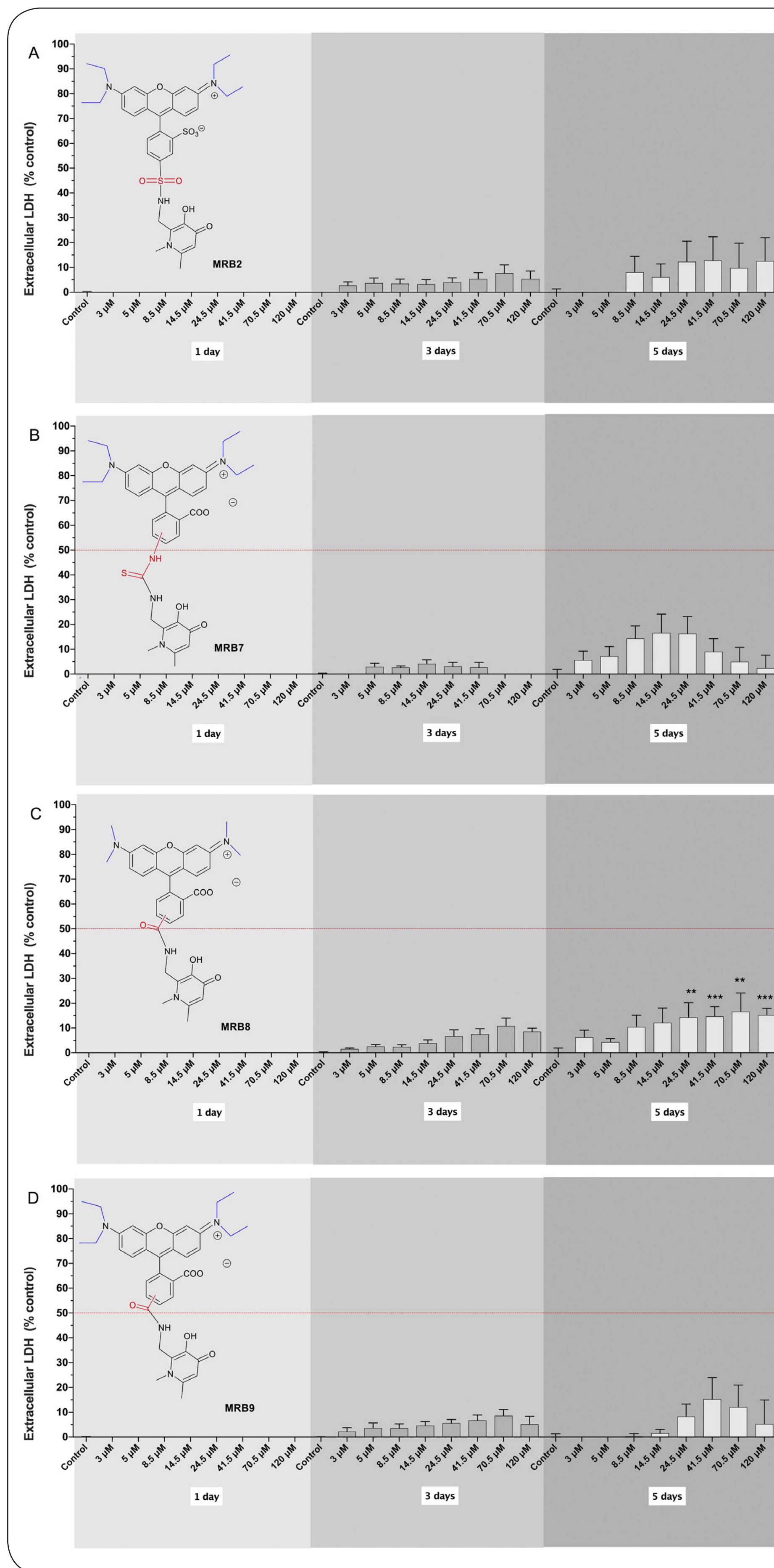

Figure 2. Cell membrane disruption assessed by the leakage of lactate dehydrogenase (LDH) to the extracellular medium, in Hep G2 cells, following exposure to bidentate chelators, *i.e.* MRB2 (A), MRB7 (B), MRB8 (C), and MRB9 (D), for 1, 3, or 5 days, at 37 °C. Each chelator was tested at concentrations ranging from 3 to 120 µM. Data are presented as mean ± standard error of the mean (SEM) of percentage of extracellular LDH, relative to the positive controls (cells treated with 1% Triton-X100), and were obtained in four independent experiments, performed in triplicates. The dashed red line represents half of the percentage of LDH leakage obtained for the positive controls (50%). Statistical analysis was performed using Kruskal-Wallis test followed by Dunn's multiple comparison *post hoc* test. *p<0.05; **p<0.01 and ***p<0.001, when compared with controls at the same time-point.

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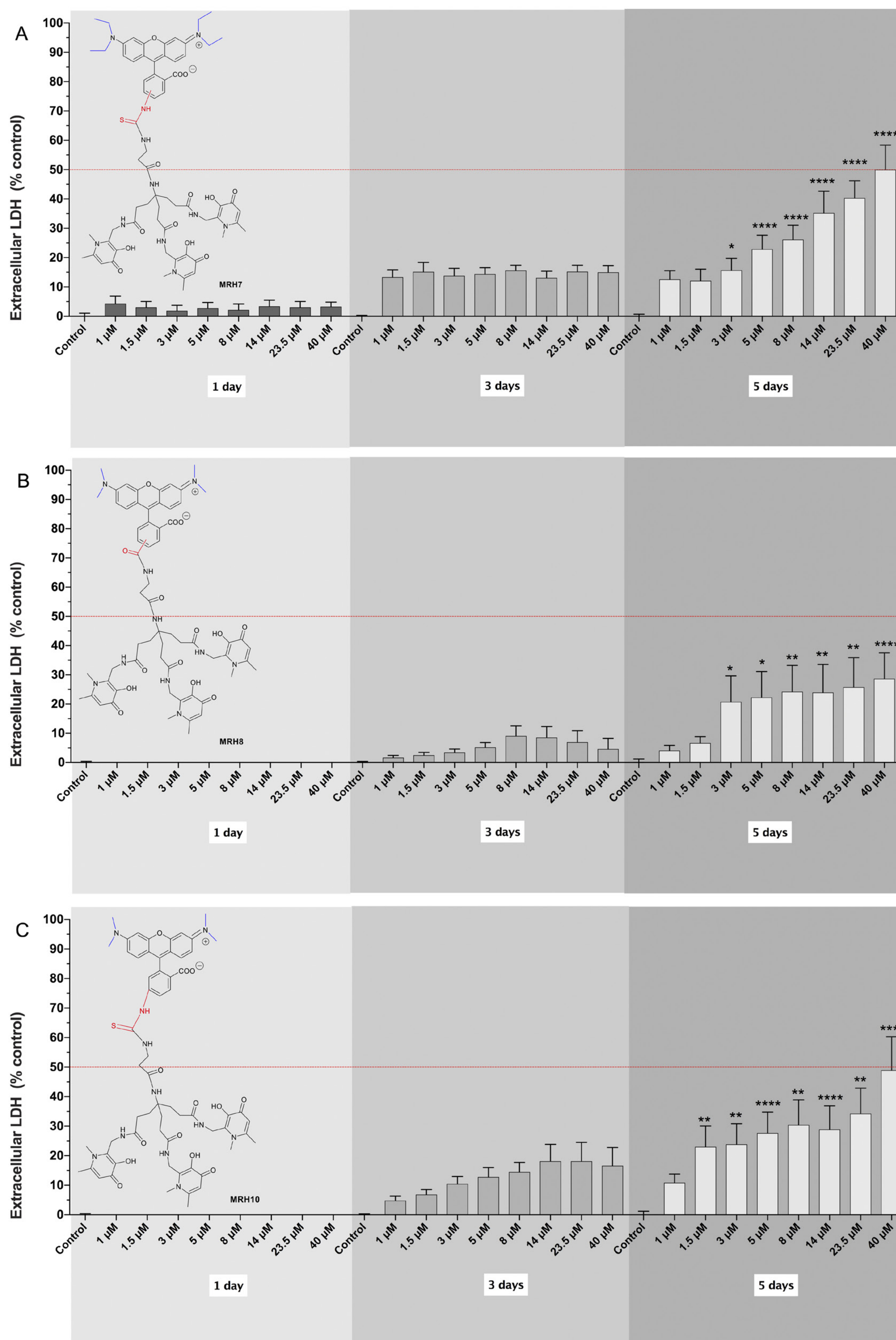

Figure 3. Cell membrane disruption assessed by the leakage of lactate dehydrogenase (LDH) to the extracellular medium, in Hep G2 cells, following exposure to hexadentate chelators, *i.e.* MRH7 (A), MRH8 (B), and MRH10 (C), for 1, 3, or 5 days, at 37 °C. Each chelator was tested at concentrations ranging from 1 to 40 µM. Data are presented as mean ± standard error of the mean (SEM) of percentage of extracellular LDH, relative to the positive controls (cells treated with 1% Triton-X100), and were obtained in four independent experiments, performed in triplicates. The dashed red line represents half of the percentage of LDH leakage obtained for the positive controls (50%). Statistical analysis was performed using Kruskal-Wallis test followed by Dunn's multiple comparison *post hoc* test. *$p<0.05$, **$p<0.01$ and ***$p<0.001$, when compared with controls at the same time-point.

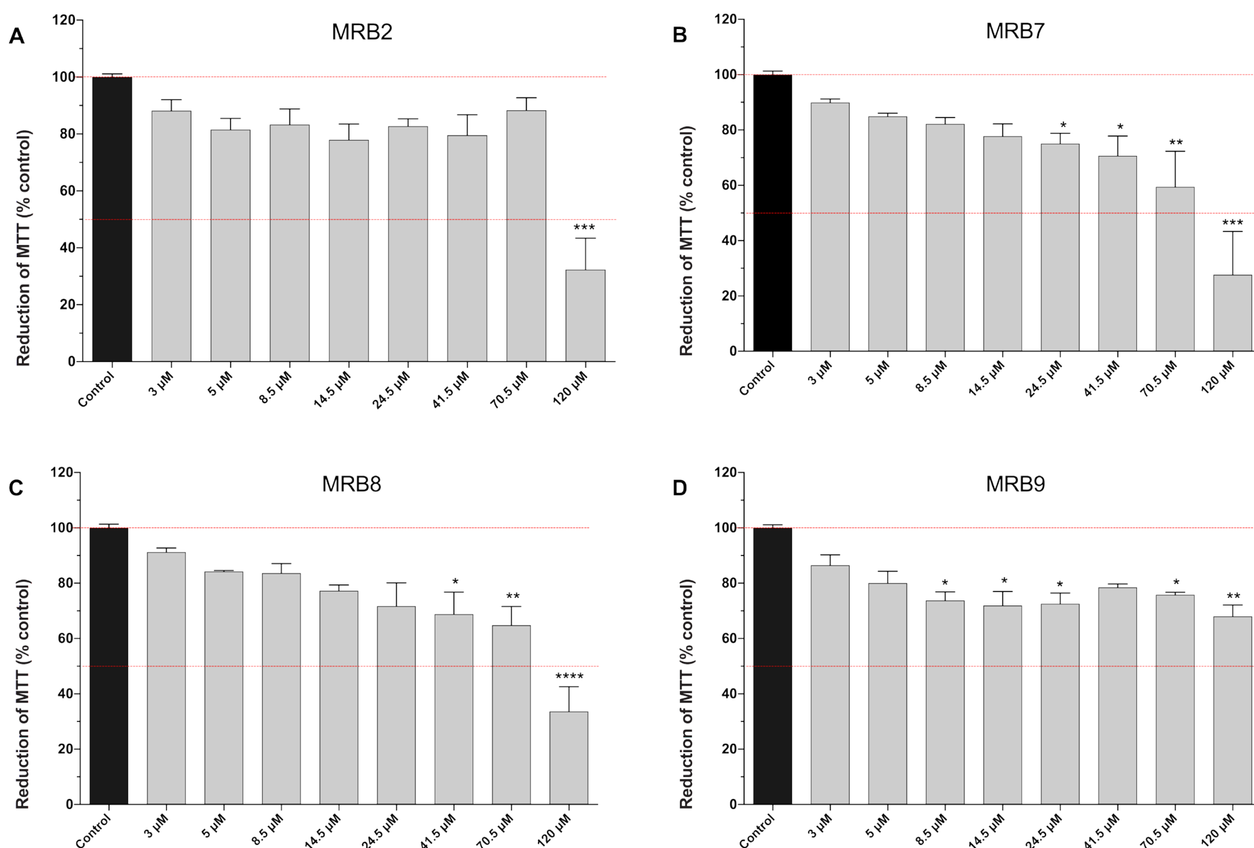

Figure 4. Cell metabolic competence assessed by the reduction of the MTT assay, following exposure of HepG2 cells to bidentate chelators, *i.e.* MRB2 (A), MRB7 (B), MRB8 (C), and MRB9 (D), for 5 days, at 37 °C. Each chelator was tested at concentrations ranging from 3 to 120 µM. Data are presented as mean ± standard error of the mean (SEM) of percentage of MTT reduction, relative to the negative controls (black bar), and were obtained in four independent experiments, performed in triplicates. The dotted red lines represent 50% and 100% effect. Statistical analysis was performed using Kruskal-Wallis test followed by Dunn's multiple comparison *post hoc* test. *p<0.05, **p<0.01, and *** or **** p<0.001, when compared with control.

Considering the rate of MTT reduction, in general, results for bidentate (Figure 4) and hexadentate chelators (Figure 6) indicate that all fluorescent compounds concentration-dependently decrease the cell ability to reduce the MTT, although the chelators presented distinct cytotoxicity profiles. When bidentate chelators where tested at 70.5 µM, mitochondrial viability ranged from 88.34±4.37% (MRB2; p>0.05) to 59.46±12.90% (MRB7; p<0.01). A substantially pronounced deleterious effect was only consistently induced by all bidentate chelators at the concentration of 120 µM (p<0.01). Accordingly, this was the only concentration tested above the respective EC_{50} values (as denoted by responses below 50% effect, Figure 4). The exception was MRB9, which presented an EC_{50} value higher than 120 µM. At this concentration of ligand, 67.98±4.14% cells were viable, compared to controls; while other bidentate chelators, profoundly disturb cell reduction ability to levels as low as 27.71±15.63% (MRB7; p<0.001). Overall, MRB9 presented the lowest cytotoxic potency in the MTT assay (as determined by the calculated EC_{50} value), showing a milder toxicity profile when compared to the other chelators (Figure 5). Although at lower concentrations (<24.5 µM) the toxicity of MRB9 is slightly higher when compared to other test chelators, these differences in toxicity are rather small and are only seen at very low levels of cytotoxicity (<20% effect; Figure 5).

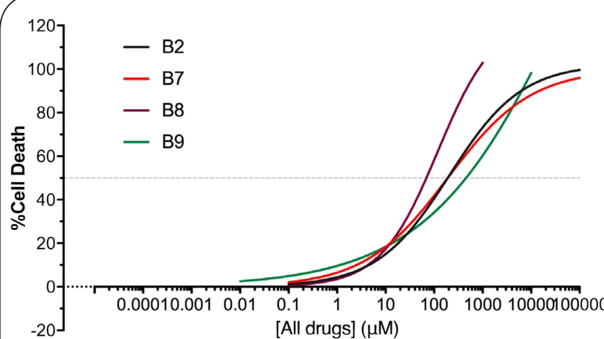

Figure 5. Regression models for the cell metabolic competence, as assessed by the reduction of the MTT assay, following exposure of HepG2 cells to bidentate chelators, *i.e.* MRB2 (black line), MRB7 (red line), MRB8 (purple line), and MRB9 (green line), for 5 days, at 37 °C. Data relative to the negative controls were from four independent experiments run in triplicate. The dotted line represent 50% effect.

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The analysis of the cytotoxic profiles obtained for hexadentate chelators by MTT assay evidenced that, in what relates to loss of metabolic competence, the two highest concentrations tested were already above the EC_{50} values for all chelators, *i.e.* from 23.5 µM up MTT reduction was below 50% of controls (Figure 6). Accordingly, MTT reduction at this concentration level ranged from 48.74±9.74% (MRH7; $p<0.001$) to 40.06±9.37% (MRH8; $p<0.01$). The MTT metabolism was further disrupted when hexadentate chelators were tested at 40 µM, with cell viability reaching values as low as 9.14±6.17% for MRH8 (Figure 3; $p<0.0001$), which was the chelator presenting the highest cytotoxic potency in the current assay.

Results of the MTT reduction assay in HepG2 cells in the presence of bidentate and hexadentate chelators are presented in Figures 4 and 6, respectively.

Discussion

Antimicrobial-resistant bacteria are a major concern of yet unpredictable proportions in public health. In this context, mycobacteria control urges the development of new drugs to circumvent the challenging mechanisms of resistance associated with poor therapeutic outcomes (Renvoise *et al.,* 2015). Aiming to provide attractive alternative therapies to control mycobacteria infection we synthesized a set of 3,4-HPO chelators and analysed their pharmacological bacteriostatic activity against *M. avium* (Fernandes *et al.,* 2010, Moniz *et al.,* 2013, Moniz *et al.,* 2015). Amongst the fluorescent chelators functionalized with different fluorophores, the rhodamine B isothiocyanate-labelled chelator MRH7 exhibited the strongest inhibitory activity of intramacrophagic *M. avium* growth (Fernandes *et al.,* 2010).

Following validation of the mycobacteriostatic activity of the new chelators, we further intended to appraise the *in vitro* safety of these bioactive derivatives, as this is a major requirement to pursue their therapeutic application. To achieve our purpose, HepG2 cells were selected, as they are vastly utilised as an *in vitro* model for the hepatocyte (Dykens *et al.,* 2008, Swiss & Will, 2011, Hynes *et al.,* 2013).

Because the liver is one of the main targets for drug toxicity, *in vitro* models representative of this organ are

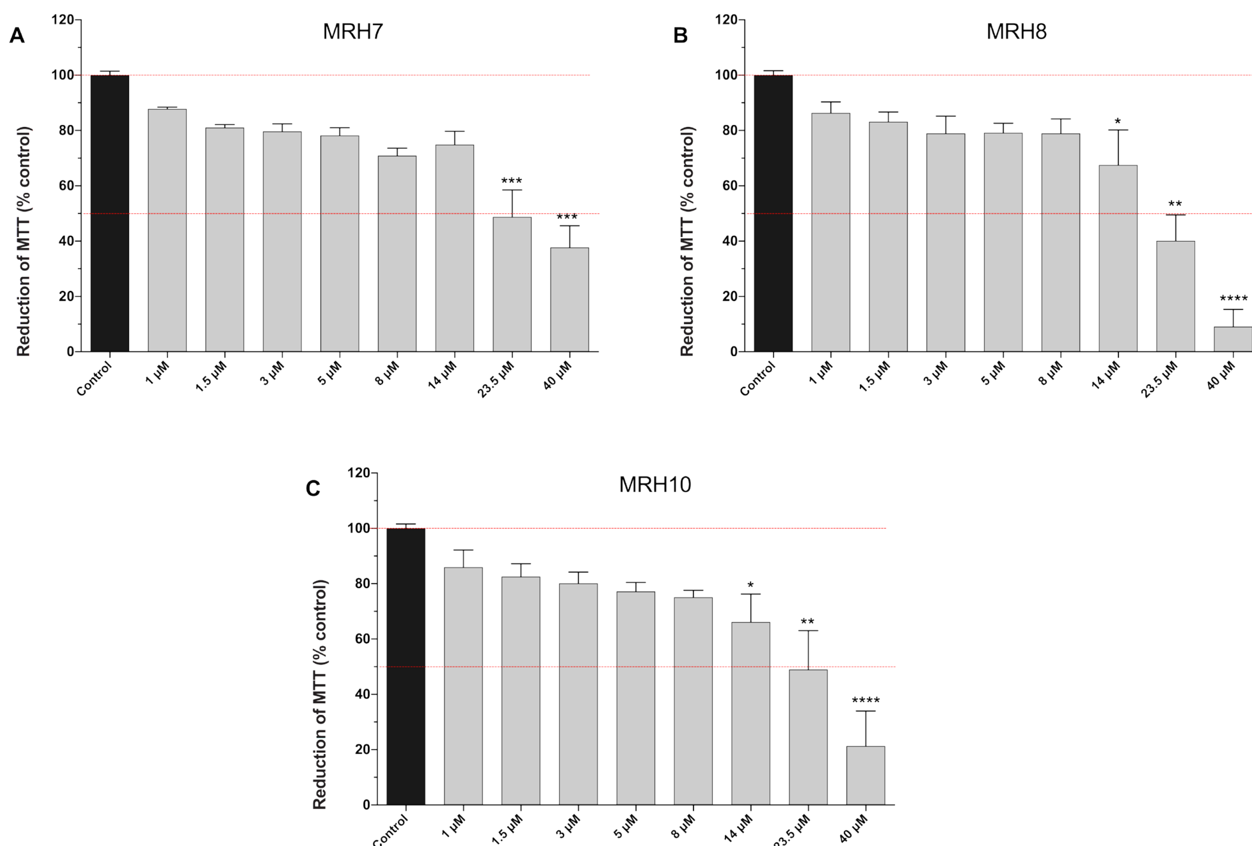

Figure 6. Cell metabolic competence, assessed by the reduction of the MTT assay, following exposure of HepG2 cells to hexadentate chelators, *i.e.* MRH7 (A), MRH8 (B), and MRH10 (C), for 5 days, at 37 °C. Each chelator was tested at concentrations ranging from 1 to 40 µM. Data are presented as mean ± standard error of the mean (SEM) of percentage of MTT reduction, relative to the negative controls, (black bar) and were obtained in four independent experiments, performed in triplicates. The dotted red lines represent 50% and 100% effect. Statistical analysis was performed using Kruskal-Wallis test followed by Dunn's multiple comparison *post hoc* test. *$p<0.05$, **$p<0.01$ and *** or **** $p<0.001$, when compared with control.

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of utmost suitability for the preliminary toxicological screening of new drugs. In this particular case, HepG2 cells preserve morphological and functional differentiation of hepatocytes *in vivo*; they are highly polarized, *i.e.* display cell asymmetry resultant of the presence of basolateral and apical poles and, in addition to the phenotypical features, they also express several enzymes involved in the metabolism and detoxification of xenobiotics, justifying the relevance of this model for the toxicological screening of our set of chelators (Sassa *et al.,* 1987, Knasmuller *et al.,* 2004, Mersch-Sundermann *et al.,* 2004, Decaens *et al.,* 2008, Gerets *et al.,* 2009, Moreira 2013).

Using HepG2 cells, the toxicity of the pharmacologically active rhodamine-labelled 3,4-HPO chelators was herein first investigated by the LDH assay, which is one of the most accepted tests for determination of toxicity of drugs *in vitro* (Weyermann *et al.,* 2005, Fotakis & Timbrell, 2006, Smith *et al.,* 2011, Brown *et al.,* 2014). According to LDH assay, the deleterious effect provoked by the chelators on cell membrane integrity was indirectly evaluated through the measurement of the extracellular LDH activity. The presence of this cytoplasmic enzyme in the extracellular medium is indicative of alterations on cell permeability; therefore, the higher the amount of enzyme released into extracellular culture medium, the higher the impairment of cell integrity triggered by chelators, which may directly result from the interaction of these compounds at the membrane, or may be a late event subsequent to the disruption of other cell organelles.

Despite the advantages of the LDH assay, such as its non-destructive nature, reliability, and quickness (Decker *et al.,* 1988, Smith *et al.,* 2011), it may also present some disadvantages. The enzyme has relatively short half-life in the medium what could introduce some artefacts on the obtained results (von Eyben *et al.,* 2001). Accordingly, an eventual degradation of the released enzyme may justify the low reproducibility observed at extended chelator exposures (5 day-incubations) for all chelators, regardless the concentration tested.

To overcome potential limitations of the LDH assay at the higher time point tested, a complementary assay was used to investigate the toxicity of the rhodamine labelled 3,4-HPO chelators. Accordingly, through the MTT assay, we measured the detrimental impact provoked at the metabolic level, after exposing HepG2 cells to bidentate and hexadentate chelators, for five days.

As cell metabolic activity is correlated with its viability, the cytotoxicity of the chelators was estimated considering the rate of MTT reduction (van Meerloo *et al.,* 2011).

Despite cytotoxicity tests such as the MTT assay being often used for the primary screening of new molecules (Berridge *et al.,* 2005, Fotakis & Timbrell, 2006), we have to consider some drawbacks. First, as the MTT assay is a destructive test (cells are lysed at the end of the experiment), hampered the possibility of evaluating the cytotoxicity at intermediate time points, along the time course of the experiment. Second, the formazans formed and the rhodamine-derived chelators have maximal absorbance in the same wavelength range (Nunes *et al.,* 2010, Moniz *et al.,* 2013, Moniz *et al.,* 2015). To preclude overestimation of the absorbance values recorded in the assay and, therefore, inaccurate conclusions of higher cell metabolic competence, controls were performed, in which absorbance readings occurred following aspiration of the treatment solutions – previous to addition of MTT solution, and no chelator-derived pigmentation was detected (purple coloration only occurred after MTT metabolism).

Regarding the conditions of our testing system, another aspect should be considered. It is well established that long incubation periods largely impact cell viability. Results obtained in MTT assay were achieved after 5 day-chelator exposures, this way allowing comparisons with the studies conducted to evaluate the antimycobacterial effects of the chelators (Moniz *et al.,* 2015). During that period, the cell culture medium was not changed and cells were not split to provide room for growth. Therefore, some level of basal stress naturally occurred, which could potentiate the toxicity induced by chelators.

From the above mentioned, it is important to highlight that the toxicity observed for chelators after 5-day exposures in both LDH and MTT assays is very likely overestimated but this provides more cautious estimations of the deleterious effects elicited by these chelators. In addition, compared to LDH assay, MTT method measures an earlier toxicological phenomenon (under most conditions, metabolic damage occurs prior to cytolysis), contributing to more conservative assumptions. Therefore, conclusions from the current study will mostly rely on the results attained in the MTT test.

Taken together, the results obtained herein shed light on the relationship toxicity-structure of the rhodamine-labelled 3,4-HPO chelators, unveiling that bidentate ligands are less detrimental to cells than hexadentate chelators, yet the test concentrations were 3x higher (*e.g.* MRB7 and MRB8 are less toxic than MRH7 and MRH8, respectively). Notwithstanding, hexadentate chelators revealed to be the most effective chelators against *M. avium* (Moniz *et al.,* 2013, Moniz *et al.,* 2015), indicating a strict correlation between this pharmacological activity and toxicity, which may be directly dependent on the size of the molecule, and/or on the hydrophilic/lipophilic balance (Moniz *et al.,* 2017). These properties may influence the ability of the chelators to interact and/or cross biological membranes and ultimately to reach their targets, namely the phagosome where *M. avium* resides. Similarly, the capacity of the chelators to interact with biological membranes may also be related to their toxicity, as it affects the availability of the drug at the toxicological targets, such as mitochondria, lysosome or endoplasmic reticulum.

Confocal microscopy studies have shown that endocytosis is not the preferred pathway for the cellular uptake of 3,4-HPO chelators (Moniz *et al.,* 2017). Therefore, the permeation thought the biological membranes must be determinant for their final toxicological effects. Previous experiments on the interaction of the chelators with membranes revealed that the compounds that herein presented higher toxicity strongly interact with the lipid

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phase, rapidly permeating into the cell, and further reaching cytosol and phagosome. Accordingly, the intracellular uptake of rhodamine B isothiocyanate derivatives MRH7 and MRB7 was more efficient than that observed for the carboxytetramethylrhodamine chelator MRH8, which is in line with the observation that the interaction with the hydrophobic region of the membrane is strengthened by the presence of the *N*-ethyl groups and the thiourea linkage (Coimbra *et al.,* 2014, Moniz *et al.,* 2016b).

Also, in what concerns to the chemical structure, our data signpost the relevance of the linker and of the substituents of nitrogen atom on the toxicity exhibited by chelators. Among the set of bidentate ligands, carboxyrhodamine derivatives (amide linker), like MRB8 and MRB9, present lower detrimental effects, when compared with rhodamine B isothiocyanate chelators (thiourea linker; MRB7). A similar relationship structure–antibacterial activity against *M. avium* was also established (Moniz *et al.,* 2013, Moniz *et al.,* 2015). Contrarily, for the hexadentate series, rhodamine B isothiocyanate derivatives (MRH7 and MRH10) are less cytotoxic than the carboxyrhodamine molecules (MRH8), but more active against *M. avium* (Moniz *et al.,* 2013, Moniz *et al.,* 2015) and other Gram positive and Gram negative bacteria (Moniz *et al.,* 2018). For all chelators tested (hexadentate and bidentate series), when the substituents of the nitrogen atom are switched from ethyl to methyl (MRH7 compared to MRH10 and MRB9 compared to MRB8), an increment of toxicity was observed.

Of note, MRB2 chelator is an exception to the former structure–bacteriostatic activity deductions; as this is the only molecule displaying both a sulphonamide liker and a sulfonate group on the position 2' of the phenyl ring, comparisons to other derivatives are precluded as differences to toxicological profiles cannot be unequivocally attributed to the presence of the linker or of the SO_3^- group. Notwithstanding, these structural properties might be responsible for the absence of bacteriostatic activity against *M. avium* and for the low toxicity displayed by the chelator.

Overall, our results indicate that an incubation time of five days might be excessively long for the majority of the testing chelators, when tested at the highest concentrations. A feasible treatment approach to fight infection of *M. avium* would be using lower concentrations of the most active chelators, such as 5–14 μM MRH7 or 5–8 μM MRH8, since at this concentration ranges pharmacological activity is already observed with no significant toxicological impact (Moniz *et al.,* 2015). Contrarily, MRB9 was the substance tested that revealed the worst benefit: risk ratio, as it only disclosed an antimycobacterial tendency at concentrations as high as 60 μM, but presented significant toxicity from 8.5 μM on (Moniz *et al.,* 2015). Other chelators, such as MRB7, MRH8, and MRB8 also displayed significant inhibition of intramacrophagic *M. avium* growth at very low concentrations but deductions on potential benefit: risk are precluded by the different experimental settings (*i.e.* incubation time) of the pharmacological and toxicological assessments (Moniz *et al.,* 2013). Furthermore, as our chelators presented no cytotoxicity at 1 day-incubation, their applicability as antibiotics may be also relevant to fight fast growth mycobacteria (*e.g.*, *M. smegmatis*) or others requiring short term antibiotic regimens. Accordingly, MRH7 already demonstrated significant antimicrobial activity against *Staphylococcus aureus, S. epidermis*, and *Escherichia coli*, after 1 day (Gomes *et al.,* 1999, Cronje & Bornman, 2005). In addition, MRH10, MRH8, MRB9, MRB8 and MRB7 were also active against *S. aureus*; and MRH10, MRB9, MRH8, and MRB8 against *S. epidermis* (Moniz *et al.,* 2018).

Overall, the integration of antimycobacterial properties with the toxicity profiles enabled ranking chelators according to their pharmacological: toxicological potential ratio and identification of MRH7 and MRH8 as the most suitable chelators to undergo further pre-clinical development studies.

Conclusion

Our results show that only after long periods of incubation, *i.e.* 5 days, the rhodamine-labelled chelators induce significant toxicity to HepG2 cells; for earlier time-points, the cytotoxicity was inexistent or low, supporting a potential clinical applicability of all chelators tested herein as bacteriostatic agents against fast growth bacteria. Bidentate chelators were the least toxic chelators, in particular MRB2, MRB7, and MRB9. Amongst the hexadentate series, MRH7, which is the most active chelator against *M. avium*, was the least toxic agent; at lower concentrations, MRH8 might also be a reasonable strategy to treat *M. avium*.

To the best of our knowledge, this work is the first report concerning the *in vitro* cytotoxic profile of these chelators, which gains particular relevance since these chelators display high potential to be clinically applied as antibacterial agents. In this regard, these present results improved our knowledge on the contribution of the different chemical groups in the molecular framework for the toxicity of the molecule, a key point to be considered in the molecular design of novel chelators.

Further investigations aiming to thoroughly elucidate the mechanisms underlying the toxicity we described herein and establish the metabolic profile of these chelators will be of utmost relevance, particularly in the case of MRH7 and MRH8.

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