Abstract Iron is an essential component for multiple biological processes. Its regulation within the body is thus tightly controlled. Dysregulation of iron levels within the body can result in several disorders associated with either excess iron accumulation, including haemochromatosis and thalassaemia, or iron deficiency. In cases of excess body iron, therapy involves depleting body iron levels either by venesection, typically for haemochromatosis, or using iron chelators for thalassemia. However, the current chelation options for people with iron overload are limited, with only three iron chelators approved for clinical use. This presents an opportunity for improved therapeutics to be identified and developed. The aim of this study was to examine multiple compounds from within the Davis open access natural product-based library (512 compounds) for their ability to chelate iron. In silico analysis of this library initially identified nine catechol-containing compounds and two closely related compounds. These compounds were subsequently screened using an in vitro DNA breakage assay and their ability to chelate biological iron was also examined in an iron-loaded hepatocyte cellular assay. Toxicity was assessed in hepatocyte and breast cancer cell lines. One compound, RAD362 [N-(3-aminopropyl)-3,4-dihydroxybenzamide] was able to protect against DNA damage, likely through the prevention of free radicals generated via the Fenton reaction; RAD362 treatment resulted in decreased ferritin protein levels in iron-loaded hepatocytes. Lastly, RAD362 resulted in significantly less cell death than the commonly used iron chelator deferoxamine. This is the first study to identify compound RAD362 as an iron chelator and potential therapeutic.
**Keywords**  Iron chelation · Fenton reaction · Catechol · Natural product · Hydroxyl radical · Ferritin

**Abbreviations**
ROS  Reactive oxygen species  
FeSO₄  Ferrous sulphate  
FeCl₃  Ferric chloride  
H₂O₂  Hydrogen peroxide  
DF  Deferoxamine mesylate  
FAC  Ferric ammonium citrate

**Introduction**
Iron is essential for many biological processes. Iron is incorporated into several proteins such as haemoglobin and myoglobin for oxygen transport, as well as proteins necessary for DNA replication and repair (Papanikolaou and Pantopoulos 2017). Excess iron can be detrimental, playing a central role in the Fenton reaction that results in the generation of reactive oxygen species (ROS) and subsequent lipid peroxidation and DNA damage (Jomova and Valko 2011). Therefore, a stable balance of iron must be maintained to ensure proper functioning of critical proteins while also preventing ROS damage.

Dysregulation of iron leads to either iron overload or deficiency, leading to health complications. Iron overload is characterised by excessive iron deposition in various organs including the liver and can result from several disorders including haemochromatosis and thalassaemia. Thalassaemia involves incomplete erythropoiesis resulting from mutations in either the alpha or beta globin genes that prevent proper globin protein production (Rund and Rachmilewitz 2005), leading to abnormal haemoglobin production and anaemia. Patients with thalassaemia typically receive regular blood transfusions to maintain red blood cell counts (Musallam et al. 2013). This often leads to iron overload in the liver, heart and endocrine glands (Rund and Rachmilewitz 2005). Thus, thalassaemic patients are also prescribed iron chelators to remove excess iron (Musallam et al. 2013). Deferoxamine mesylate (DFO) is a common iron chelator used for treating conditions such as iron loading anaemias and β-thalassaemia (Bacon et al. 2011; Brissot 2016). However, DFO is poorly absorbed within the gastrointestinal tract with a short plasma half-life and must be administered 4–5 days a week typically with injection times ranging between 8–12 h (Brisson 2016). In addition, several systemic toxicities including cardiovascular, respiratory, gastrointestinal, cutaneous, and nervous system can result from DFO treatment (Brittenham 2011). Considering these challenges other iron chelators have been developed such as deferiprone (DFP) and deferasirox (DFX) (Brisso 2016). DFP possesses a short half-life requiring multiple doses each week, however it has been associated with increased incidence of agranulocytosis in some patients (Brissot et al. 2017). DFX has the longest half-life (10–16 h) of all the approved iron chelators but it may associated with adverse effects such as gastrointestinal symptoms, renal failure, skin rashes, and increased transaminases (Brisso 2016).

Haemochromatosis is a genetic disorder characterised by inappropriate levels of the iron regulatory hormone, hepcidin, in response to body iron levels (Brissot et al. 2018). Haemochromatosis is caused by mutations in several iron regulatory proteins, HFE (the haemostatic iron regulator), hepcidin, haemojuvelin, transferrin receptor 2 and ferroportin (Brissot et al. 2018). Iron overload resultant from haemochromatosis clinically presents with several complications including hyperpigmentation, liver fibrosis/cirrhosis, arthropathy, pituitary hypogonadism, diabetes and cardiomyopathy (Piperno et al. 2020). Phlebotomy is the most common treatment option for haemochromatosis, due to its effectiveness and low cost (Kontoghiorghes et al. 2010; Piperno et al. 2020). However, in severe cases and in cases with unstable hemodynamic status due to severe congestive heart failure, advanced liver cirrhosis or anaemia, phlebotomy is not appropriate. Under these circumstances, iron chelation has also been used to remove excess iron (Kontoghiorghes et al. 2010; Piperno et al. 2020).

Excess iron has also been associated with several neurodegenerative disorders including aceruloplasminemia (Nunez and Chana-Cuevas 2018). This has led to an interest in iron chelators that are able to effectively infiltrate the blood brain barrier and remove excess iron from the brain (Nunez and Chana-Cuevas 2018). DFO and DFX have been tested in some patients with aceruloplasminemia, however the results of these studies were inconsistent (Nunez and Chana-Cuevas 2018). Lastly, the anti-cancer effects of various iron chelators have also been investigated as cancer cells display a greater...
requirement for iron due to their increased metabolism (Bajbouj et al. 2018). DFO has been shown to decrease cell viability and cell migration in MCF-7 breast cancer cells (Bajbouj et al. 2018; Head et al. 1997). The exact therapeutic properties of iron chelators in treating cancer remain controversial. Liu et al. (2016) and Chen et al. (2019a, b) have recently found that DFO treatment in the highly aggressive breast cancer cell line, MDA-MB-231, increased cell migration while not affecting migration in MCF-7 cells (C. Chen et al., 2019a, b; Liu et al., 2016).

Siderophores are secondary metabolites produced by microorganisms which possess the ability to chelate iron and have more recently been found in mammals (Khan et al. 2018). Siderophores can be broadly classified into four groups, catecholate, hydroxamate, carboxylate and mixed types based on the moiety responsible for the iron chelation (Khan et al. 2018). Catecholate containing siderophores are primarily found within bacteria (Paul and Dubey 2015). This type of siderophore binds iron through the interaction of the catecholate and hydroxyl groups with adjacent catechol or hydroxyl ends (Paul and Dubey 2015). Enterobactin is a highly studied catecholate type siderophore which displays the highest affinity towards iron of all known siderophores (Raymond et al. 2003). Lastly, catecholate siderophores have increased lipophilicity, stability and resistance to environmental pH when compared with other siderophore types (Winkelmann 2002). These properties make these compounds ideal candidates for therapeutic treatments.

The identification of new iron chelators, with more favourable biochemical and pharmacological properties than the currently available options, for the treatment of iron overload disorders such as thalassaemia is desirable. In this study we investigated the iron chelating properties of several natural products and derivatives that were selected following in silico analysis of an open access natural product-based library (Zulfiqar et al. 2017).

Materials and methods

Open access library and compound supply

The Davis open access natural product-based library consists of 512 distinct compounds, the majority (53%) of which are natural products that have been obtained from Australian natural sources, such as endophytic fungi (Davis 2005), plants (Levrier et al. 2013), macrofungi (Choomuenwai et al. 2012), and marine invertebrates (Barnes et al. 2010). Approximately 28% of this library contains semi-synthetic natural product analogues (Barnes et al. 2016), while a smaller percentage (19%) are known commercial drugs or synthetic compounds inspired by natural products. In silico analysis of the Davis open access library was performed using the open source software, DataWarrior (Sander et al. 2015). A substructure search on the catechol motif (a known siderophoric motif) identified 11 compounds that were available in sufficient quantity for biological evaluation. This motif has been previously reported to form a complex with free iron and is commonly found in iron chelators (Chen et al. 2019a, b). The 11 hit compounds (nine catechol-containing compounds and two closely related compounds) resulting from in silico analysis included endiandrin A (RAD047) (Davis et al. 2007), gallic acid (RAD085) (Souza et al. 2020), altenuis (RAD145) (Nakanishi et al. 1995), pistillarin (RAD198) (Choomuenwai et al. 2012), 3-acetoxy-7,8-dihydroxysserrulat-14-en-19-oic acid (RAD288), 3,7,8-trihydroxysserrulat-14-en-19-oic acid (RAD289) (Barnes et al. 2013), 3,19-diacetoxy-8-hydroxysserrulat-14-ene (RAD290) (Barnes et al. 2013), N-(3-aminopropyl)-3,4-dihydroxybenzamide (RAD362) (Choomuenwai et al. 2012), quercitrin (RAD382) (Yamazaki et al. 2007), rutin (RAD511) (Kreft et al. 1999) and microthecaline A (RAD685) (Kumar et al. 2018) (Table 1). All compounds have been previously reported as either natural products, semi-synthetics or synthesised compounds. Compounds evaluated during these studies were analysed for purity prior to testing and all were shown to be > 95% pure. All compounds were provided as a stock solution of 10 mM dissolved in dimethyl sulfoxide (DMSO) (34,869, Sigma Aldrich, St Louis, Missouri, USA), as such DMSO was used as the vehicle control.

Cell culture

HepG2-C3A (ATCC CRL-10741) and T47D (ATCC HTB-133) cells were originally obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). Cells were grown in 25cm² flasks (136196, Nunc Thermofisher, Rochester, New York,
USA) containing Minimum Essential Medium supplemented with 25 mM HEPES, 2 mM l-Alanyl-Glutamine (42360032, Gibco Thermofisher, Waltham, Massachusetts, USA) and 10% foetal calf serum (302220FNZ, Gibco Thermofisher) (for HepG2-C3A cells) or Roswell Park Memorial Institute Medium (11875119, RPMI) supplemented with 10% foetal calf serum (for T47D cells). All cells were housed in an incubator at 37°C with 5% CO₂.

DNA breakage assay via fenton reaction

Fenton reaction-induced DNA damage was measured using the methods described in Lee et al. (Lee et al. 2007). Briefly, 100 ng of supercoiled plasmid DNA (pmaxGFP, Lonza, Basel, Switzerland) was mixed with 10 mM Tris(hydroxymethyl)aminomethane hydrochloric acid (TRIS-HCl) (10708976.001, Roche, Basel, Switzerland) buffer, and either ferrous sulphate (FeSO₄) (F7002, 50 μM) or ferric chloride (FeCl₃) (157740, Sigma Aldrich, St Louis, Missouri, USA) (50 μM). The cells were then treated with either 250 μM of library compounds dissolved in DMSO, DFO mesylate (433,065, Hospira, Victoria, Australia) (250 μM) dissolved in DMSO (1/40 dilution) or a vehicle control of 2.5% DMSO alone. The mixture was incubated with 350 μM hydrogen peroxide (H₂O₂) (1.07209.2500, Merck, Darmstadt, Germany) for reactions with FeSO₄ or 750 μM H₂O₂ for reactions with FeCl₃ for 2 h at room temperature before adding stopping solution 4 M urea (H8637, Sigma Aldrich, 50% sucrose (10,274.413, Radnor, Pennsylvania, USA) 50 mM ethylenediaminetetraacetic acid (EDTA) (304672, Ajax Finechem, Rochester, New York, USA) and 0.1% bromophenol blue (B8026, Sigma Aldrich). The degree of DNA damage was visualised and quantitated on a 1% agarose gel by comparing the band intensity of pure plasmid against the intensity of the compound treated plasmids, as described previously by Lee et al (2011). Further DNA breakage assays were conducted using lower concentrations (range of 31.25–125 μM) of the compounds to determine the effectiveness of their chelating properties. These titration experiments were conducted with FeSO₄, as it resulted in greater DNA damage then FeCl₃ in the first screening experiments.

Western blot analysis

HepG2-C3A cells (1.5 × 10⁵ cells/well) were seeded on 12-well plates and allowed to settle before being treated with 100 μM ferric ammonium citrate (FAC) (Fluka, St. Louis, Missouri, USA) for 18 h. Cells were then treated with 100 μM of either the library compounds, DFO (100 μM) or DMSO (1%) alone for 18 h. After treatment with the iron chelators, cells were lysed with sample buffer (50 mM Tris pH 6.8, 10% β-mercaptoethanol (8.05740.0250, Merck) 10% glycerol (GA010, Chemsupply Pty Ltd, South Australia, Australia), 2% sodium dodecyl sulphate (161–0302, Bio-Rad, Hercules, California, United States) and 0.1% bromophenol blue (B8026, Sigma Aldrich)). 20 μl of denatured cell lysates were electrophoresed using a 12% sodium dodecyl sulphate polyacrylamide gel, transferred to nitrocellulose membrane, and blocked for 2 h in milk at room temperature (RT). The blots were then incubated with rabbit anti-ferritin Ab (1.64 μg/ml) (4393, Cell

| Library code | Compound name                        | Reference                  |
|--------------|--------------------------------------|----------------------------|
| RAD047       | Endiandrin A                         | Davis et al. (2007)        |
| RAD085       | Gallic acid                          | Souza et al. (2020)        |
| RAD145       | Altenusin                            | Nakanishi et al. (1995)    |
| RAD198       | Pistillarin                          | Choomuenwai et al. (2012)  |
| RAD288       | 3-acetoxy-7,8-dihydroxyserrulat-14-en-19-oic acid | Barnes et al. (2013) |
| RAD289       | 3,7,8-trihydroxyserrulat-14-en-19-oic acid | Barnes et al. (2013) |
| RAD290       | 3,19-diacetoxy-8-hydroxyserrulat-14-ene | Barnes et al. (2013) |
| RAD362       | N-(3-aminopropyl)-3,4-dihydroxybenzamide | Choomuenwai et al. (2012) |
| RAD382       | Quercitrin                           | Yamazaki et al. 2007       |
| RAD511       | Rutin                                | Kreft et al. (1999)        |
| RAD685       | Microthecaline A                     | Kumar et al. (2018)        |
Signalling, Danvers, Massachusetts, USA), and rabbit anti-actin (5 ng/ml) (A2066, Invitrogen, Waltham, Massachusetts, USA). The membranes were washed three times with Tris-buffered saline containing 10% Tween-20 (Sigma Aldrich) (TBST) for 15 min and incubated with the secondary antibody [HRP-labelled goat anti-rabbit secondary Ab (100 ng/ml) (65–6120, Invitrogen)] for 1 h at room temperature. After washing with TBST, blots were incubated with substrate [Luminata Forte Western Chemiluminescent HRP Substrate (WBLUF0500, Merck-Millipore, Burlington, Massachusetts, USA)] for 5 min before being exposed to film (Super RX, Fujifilm, Tokyo, Japan) in a dark room using a Minolta film processor (Konica Minolta, Inc., Tokyo, Japan). Band volume and density were quantitated using ImageJ software.

Immunofluorescence microscopy

HepG2-C3A (1.5 \times 10^5 cells/well) cells were seeded onto collagen-coated cover slips. Cells for immunofluorescence were treated using the same methods as described for the western blot analysis. After treatment, cells were fixed with 3% paraformaldehyde (P-6148, Sigma Aldrich) for 10 min at RT, washed with 50 mM ammonium chloride (A4514, Sigma Aldrich) before being permeabilised with 0.05% saponin (558,255, Calbiochem, Diego, California, USA) for 10 min at RT. Cells were then incubated with rabbit anti-ferritin antibody (2.05 µg/ml) in fluorescence dilution buffer (FDB) (5% donkey serum, 5% FBS and 2% BSA (Sigma Aldrich)) in phosphate buffered saline with calcium chloride (1 mM, C3881, Sigma) and magnesium chloride (1 mM, A296, Ajax Finechem) (PBSCM)) for 1 h at RT. Lastly, cells were stained with donkey Alexa 594 anti-rabbit (10 µg/ml) (A32740, Invitrogen) in FDB for 45 min at 25 °C in the dark and mounted onto slides with ProLong Gold Antifade Mounting Media (P36935, Thermofisher). After each antibody incubation step, slides were washed 3 times with PBSCM for 3 min. Images were captured using a Z2 Axio Imager (Zeiss, Oberkochen, Germany).

MTT toxicity assay

HepG2-C3A or T47D (1 \times 10^3 cells/well) cells were seeded in triplicate into a 96-well plate overnight. The cells were then treated with 31.25, 62.5 or 125 µM of DFO or library compounds. Sodium azide (0.1%) (NaN₃) (S8032, Sigma Aldrich) was used as a positive control to confirm cell death. After 24, 48 and 72 h of treatment, media was removed and replaced with media containing 0.45 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (M5655, Sigma Aldrich) reagent for 2 h. MTT was then aspirated and the cells incubated with 100 µL DMSO at 37 °C for 15 min. The absorption was measured at 570 and 690 nm using ClarioSTAR (BMG Labtech, Ortenberg, Germany). Cell viability was estimated by comparing the normalised absorbance of the untreated controls with those of the treatment groups.

Statistics

Statistical analysis was performed using GraphPad Prism 8.4.2 for Windows (GraphPad Software, GraphPad Software, California, USA). For the DNA breakage assays and western blot analysis, one-way ANOVA followed by Dunnett’s multiple comparisons test was conducted. Cell viability assays were analysed using two-way ANOVA followed by Tukey’s multiple comparisons test. Values are presented as mean ± standard deviation with p < 0.05 considered statistically significant.

Results

The compounds for this study originated from the Davis open access library that contains 512 natural or nature-inspired compounds, which is curated by Compounds Australia at the Griffith Institute for Drug Discovery (Griffith Institute for Drug Discovery 2020). In silico structural analysis of the library identified 9 compounds as potential iron chelators due to the presence of at least one catechol motif. Catecholate iron chelators are one of seven different types of natural iron chelators (Chen et al. 2019a, b) which chelate iron through the interaction of both catecholate and hydroxyl groups (Khan et al. 2018).

DNA breakage via Fenton reaction—screening

We first examined the ability of candidate compounds to chelate ferrous and ferric ions using the DNA breakage assay. All compounds except for RAD145,
RAD290 and RAD685 were able to prevent hydrogen peroxide-induced DNA damage when used at a concentration of 250 μM. This protective effect was seen when the compounds were incubated with either FeCl₃ or FeSO₄. However, not all compounds proved equally as effective at chelating both forms of iron; RAD288 and RAD289 were less effective at chelating ferric iron compared to ferrous iron (Fig. 1b, d). Based on their ability to prevent DNA breakage resultant from the Fenton reactions between iron and hydrogen peroxide (Fig. 1b, d) RAD047, RAD085, RAD198, RAD362, RAD382 and RAD511 were selected for further analysis. To determine the activity of the remaining compounds (RAD047, RAD085, RAD198, RAD362, RAD382 and RAD511) further DNA breakage assays were conducted using decreasing concentrations of the compounds (125, 62.5 and 31.25 μM) (Fig. 2a). With decreasing concentrations, all compounds displayed a significant reduction in total supercoiled plasmid DNA compared with DFO except for RAD047 and RAD511. RAD198 at the lowest concentration (31.25 μM) did not display a significant difference in total nicked DNA when compared to DMSO, these results prompted us to exclude RAD198 from further analysis.

RAD362 can chelate iron in iron overloaded human hepatoma cells

We next examined the ability of the effective compounds to chelate iron in a biological system. HepG2-C3A cells were treated with FAC to induce iron accumulation which results in an increase in expression of the iron storage protein ferritin (Fig. 3a). Iron-loaded HepG2-C3A cells treated with 100 μM DFO showed a significant reduction in ferritin expression, an indication of reduced cellular iron levels. Of the compounds tested, only RAD362 (100 μM) was able to significantly reduce ferritin protein levels in normalised to untreated control after treatment with compounds, iron and hydrogen peroxide. All other compounds were effective at chelating both forms of iron. ‘a’ indicates a significant difference (p < 0.05) between DMSO control and treatment group using an ordinary one-way ANOVA followed by Dunnett’s multiple comparisons test. N = 2 biological replicates.

**Fig. 1** Screening of potential iron chelators via a DNA breakage assay. Supercoiled plasmid DNA (100 ng) was treated with 50 μM FeCl₃ or FeSO₄, 750 or 350 μM H₂O₂, DFO (250 μM) or the library compounds (250 μM). The plasmid DNA after incubation with 750 μM H₂O₂ and 50 μM FeCl₃ (a) or 350 μM H₂O₂ and 50 μM FeSO₄ (c) was visualised on a 1% agarose gel. b and d Percentage of supercoiled DNA normalised to untreated control after treatment with compounds, iron and hydrogen peroxide. All other compounds were effective at chelating both forms of iron. ‘a’ indicates a significant difference (p < 0.05) between DMSO control and treatment group using an ordinary one-way ANOVA followed by Dunnett’s multiple comparisons test. N = 2 biological replicates.
iron-loaded cells suggesting that it could chelate iron from cells.

We also examined the effect of iron chelation on ferritin protein expression using immunofluorescence. As can be seen in Fig. 4, ferritin protein levels significantly changed (increase in red staining) in the FAC-treated cells compared to the untreated cells. Ferritin levels in cells treated with both FAC and DFO were similar to the untreated control, indicating that DFO can chelate iron. RAD362 treatment (100 μM) also resulted in a change in ferritin expression as compared to the FAC-treated cells (Fig. 4).

Fig. 2 Titration of library compounds to identify potency of iron chelation ability via a DNA breakage assay. Supercoiled plasmid DNA (100 ng) was treated with 50 μM FeSO₄, 350 μM H₂O₂, DFO or the compounds [(A) 31.25, (B) 62.5 and (C) 125 μM]. Plasmid DNA was visualised on a 1% agarose gel. a Gel visualising 100 ng plasmid DNA post H₂O₂ reaction and iron ± compounds. b Graph illustrating percentage of supercoiled DNA normalised to untreated control after treatment with compounds, iron and H₂O₂. For statistical analysis, percentage of supercoiled DNA after DFO treatment was compared with the corresponding compound treatments. ‘a’, ‘b’ and ‘c’ indicate a significant difference (p < 0.05) between 31.25 μM, 62.5 μM and 125 μM DFO controls respectively using a one-way ANOVA followed by Dunnett’s multiple comparisons test. N = 3 biological replicates.

RAD362 is less toxic than DFO

Prolonged DFO treatment is known to have several undesirable side effects such as ocular and auditory disturbances (Brittenham 2011). To investigate whether RAD362 treatment was toxic to HepG2-C3A cells, an MTT cell viability assay was performed. As can be seen in Fig. 5, DFO treatment resulted in a significant increase in cell death after 48 h at all concentrations. However, lower concentrations of RAD362 (31.25 μM) were less toxic up to 72 h as shown by less cell death. Treatment with 125 μM RAD362 resulted in a similar amount of cell death after 48 h compared to DFO, suggesting that higher concentrations may be toxic.
An emerging area of research involves the use of iron chelators for the treatment of several cancers; cancer cells display a greater requirement for iron due to their increased metabolism (Bajbouj et al. 2018). However, the exact benefit of iron chelators in the context of cancer remains controversial, as previously DFO has been shown to negatively affect proliferation in MCF-7 breast cancer cells (Bajbouj et al. 2018; Head et al. 1997). While DFO treatment results in increased migration and proliferation in MDA-MB-231 cells (Liu et al. 2016), we investigated the effect of RAD362 on cell viability in the breast cancer cell line T47D using the same MTT assay employed to determine toxicity in the HepG2-C3A cell line. At lower concentrations RAD362 (31.25 μM) displayed similar levels of cell death compared to DMSO. At the 72-h time point, using the highest concentration (125 μM), cell viability was comparable between DFO and RAD362. However, the cell viability for none of the compounds was significantly different compared with the DMSO controls, possibly due to the high toxicity effect of DMSO.

**Discussion**

In this report we investigated the effectiveness of several different catecholate containing natural products or derivatives for their ability to chelate iron. Initial studies were conducted in vitro using a DNA breakage assay to determine whether the selected compounds could prevent DNA damage through the Fenton reaction. This protective effect results from the sequestering of iron, limiting the reaction with hydrogen peroxide and thus preventing ROS generation (Lee et al. 2011). ROS interacts with the supercoiled DNA resulting in damage to the structure of the DNA. Compounds that could sequester both forms of iron (ferrous and ferric) were then tested at decreasing concentrations to determine the most effective candidate compound. This identified RAD511 as the most potent compound using the DNA breakage assay. Next the iron chelating activity of the compounds was assessed in HepG2-C3A cells through changes in ferritin protein expression levels. Only one compound, RAD362 resulted in a significant reduction in ferritin levels after treatment (100 μM), suggesting effective iron chelation properties (Figs. 3, 4). Upon identification of the compounds it was discovered the candidate compound, RAD362, is a structural derivative of the known siderophore pistillarin (RAD198) (Choomuenwai et al. 2013) called [N-(3-aminopropyl)-3,4-dihydroxybenzamide] (Fig. 6). It was generated through a two-step coupling/decoupling process with 3,4-dimethoxybenzoic acid and N-Boc-1,3-diamino-propane (Choomuenwai et al. 2013). RAD511 was the compound rutin (Aherne and O’Brien 1999; Omololu et al. 2011), which has been shown to be a nontoxic iron chelator. In addition, RAD382 was found to be the known iron chelator quercitrin which displaces iron from an iron-calcein complex in a cell free assay (Kaiserová et al. 2007) and reduces iron bioavailability from red bean digests (Hanen et al. 2009). This agrees with our results which found that quercitrin was able to chelate iron in a DNA breakage cell free assay. However, quercitrin was unable to decrease ferritin protein expression in our assay. To

![Fig. 3](image-url)
our knowledge the ability of quercitrin to chelate intracellular iron has not been assessed previously.

As mentioned previously DFO displays cytotoxic effects on several cell types. However, DFO is still currently the first line iron chelator prescribed to patients suffering several iron overload disorders. Therefore, to compare the utility of RAD362 as a therapeutic iron chelator, its effect on cell viability in a hepatocyte cell line was determined. Interestingly, RAD362 was found to be less toxic than DFO when used at the two lowest concentrations (31.25–65 μM) over a period of 72 h indicating the potential for this compound as a safer future therapeutic. At the 125 μM concentration RAD362 and DFO both displayed similar levels of cell death at all three timepoints with both the 48 and 72 h timepoint resulting in significant cell death. The finding that RAD362 did not impact cell viability agrees with previous reports by Choomuenwai et al., where RAD362 was shown to be non-toxic in neonatal foreskin fibroblast cells at concentrations up to 100 μM (Choomuenwai et al. 2013).

Due to the increased metabolism of cancer cells compared with untransformed cells, cancerous cells have developed multiple mechanisms to increase iron uptake including up-regulation of transferrin receptors (TFRs) and divalent metal ion transporter 1 as well as down-regulation of ferroportin (Wang et al. 2018). Iron overload has been associated with increased risk for the progression of multiple cancers such as breast cancer (Wen et al. 2014). The critical role iron plays in the progression of breast cancer is highlighted by the negative effect of reduced ferroportin expression on metastasis-free survival (Pinnix et al. 2010), while increased TFR1 expression is linked to poor breast cancer prognosis (Habashy et al. 2009). This presents iron chelation as a potential target in the battle against breast cancer. RAD362 displayed a trend towards increased cell death as compared with DMSO vehicle control at the 72-h timepoint at the highest concentration (125 μM), suggesting a possible use for RAD362 in targeting iron homeostasis in breast cancer cells.

In conclusion, this study identified several compounds with the ability to chelate in vitro ferrous and ferric iron. However, only one compound was found to significantly reduce cellular ferritin protein levels, RAD362. In addition, RAD362 when used at concentrations comparable to the therapeutic iron chelator, DFO, was found to be less toxic, suggesting that this compound could be considered as a future therapeutic.
for iron chelation. Future studies will involve testing the efficacy and safety of using RAD362 \([N-(3-\text{aminopropyl})-3,4\text{-dihydroxybenzamide}]\) in mouse models of genetic and secondary iron overload.

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Fig. 5 Cell viability assay. a HepG2-CA and b T47D cells were treated with 31.25, 62.5 and 125 \(\mu\)M of iron chelators (DFO or RAD362), 1.25% DMSO or 0.1% sodium azide (NaN\(_3\)) for 24, 48 and 72 h. Cell viability was assessed via metabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Values for each timepoint were normalised to their respective untreated (UT) control. For statistical analysis, UT controls were compared with compound treatments for each corresponding time point. ‘a’, ‘b’ and ‘c’ indicate significant differences \((p < 0.05)\) after 24 h, 48 h and 72 h treatments respectively, using a two-way ANOVA followed by Tukey’s multiple comparisons test as compared with UT. \(N = 3\) biological repeats with 3 technical repeats per biological repeat.

Fig. 6 Chemical structure of the siderophore RAD362 \([N-(3-\text{aminopropyl})-3,4\text{-dihydroxybenzamide}]\)

Conflict of interest The authors declare they have no conflict of interest.

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