BJP British Journal of Pharmacology

DOI:10.1111/bph.12045
www.brjpharmacol.org

RESEARCH PAPER

Deferasirox (ICL670A) effectively inhibits oesophageal cancer growth *in vitro* and *in vivo*

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Keywords
oesophageal cancer; iron chelation; cancer; deferasirox

Received
22 August 2012
Revised
9 October 2012
Accepted
15 October 2012

BACKGROUND AND PURPOSE
Growing evidence implicates iron in the aetiology of gastrointestinal cancer. Furthermore, studies demonstrate that iron chelators possess potent anti-tumour activity, although whether iron chelators show activity against oesophageal cancer is not known.

EXPERIMENTAL APPROACH
The effect of the iron chelators, deferoxamine (DFO) and deferasirox, on cellular iron metabolism, viability and proliferation was assessed in two oesophageal adenocarcinoma cell lines, OE33 and OE19, and the squamous oesophageal cell line, OE21. A murine xenograft model was employed to assess the effect of deferasirox on oesophageal tumour burden. The ability of chelators to overcome chemoresistance and to enhance the efficacy of standard chemotherapeutic agents (cisplatin, fluorouracil and epirubicin) was also assessed.

KEY RESULTS
Deferasirox and DFO effectively inhibited cellular iron acquisition and promoted intracellular iron mobilization. The resulting reduction in cellular iron levels was reflected by increased transferrin receptor 1 expression and reduced cellular viability and proliferation. Treating oesophageal tumour cell lines with an iron chelator in addition to a standard chemotherapeutic agent resulted in a reduction in cellular viability and proliferation compared with the chemotherapeutic agent alone. Both DFO and deferasirox were able to overcome cisplatin resistance. Furthermore, in human xenograft models, deferasirox was able to significantly suppress tumour growth, which was associated with decreased tumour iron levels.

CONCLUSIONS AND IMPLICATIONS
The clinically established iron chelators, DFO and deferasirox, effectively deplete iron from oesophageal tumour cells, resulting in growth suppression. These data provide a platform for assessing the utility of these chelators in the treatment of oesophageal cancer patients.

LINKED ARTICLE
This article is commented on by Keeler and Brookes, pp. 1313–1315 of this issue. To view this commentary visit http://dx.doi.org/10.1111/bph.12093

Abbreviations
5-FU, 5-fluorouracil; ALB, albumin; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; BrdU, bromodeoxyuridine; DFO, deferoxamine; DMT1, divalent metal transporter 1; Dp44mT, di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone; FPN, ferroportin; Hb, haemoglobin; HCT, haematocrit; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; MCV, mean cell volume; mTOR, mammalian target of rapamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NEUT, neutrophil count; PLT, platelets; RET, reticulocyte count; TfR1, transferrin receptor 1; TIBC, total iron binding capacity; TP, total protein; UIBC, unsaturated iron binding capacity; WBC, total white cell count

Introduction

Oesophageal cancer, and in particular, oesophageal adenocarcinoma, has seen an unprecedented rise in incidence during recent years (Devesa *et al.*, 1998). Five-year survival is less than 8% as the majority of patients have advanced, unresectable disease upon presentation (CRUK, 2012). The current standard of care is pre-operative chemotherapy followed by surgery in patients with locally advanced resectable disease and palliative chemotherapy for unresectable disease. However, response rate to standard chemotherapy regimens is poor (Grünberger *et al.*, 2007; Courrech Staal *et al.*, 2010).

While Barrett's metaplasia is the major risk factor for the development of oesophageal adenocarcinoma, lifestyle factors, including obesity and diet, are also important (Lagergren, 2005). In particular, emerging evidence suggests that dietary iron is associated with oesophageal carcinogenesis (Haggitt, 1994; Ward *et al.*, 2012). This association is supported by animal models demonstrating that increasing body iron can markedly amplify tumourigenesis (Hann *et al.*, 1988; Chen *et al.*, 1999; Chen *et al.*, 2000; Pierre *et al.*, 2003; Ilsley *et al.*, 2004; Seril *et al.*, 2005). In the context of oesophageal tumourigenesis, Chen *et al.* (1999; 2000) showed that rates of oesophageal adenocarcinoma were 10-fold higher in rodents subjected to i.p. injections of iron dextran compared with untreated controls.

Several studies suggest that during the transition from Barrett's metaplasia to oesophageal adenocarcinoma, there is a progressive increase in the expression of proteins involved in cellular iron acquisition, including transferrin receptor (TfR1) and divalent metal transporter 1 (DMT1) (Zhang *et al.*, 2007; Boult *et al.*, 2008). The acquisition of cellular iron through increased TfR1 expression is a consistent feature also found in other cancers (Trowbridge and Lopez, 1982; Kemp *et al.*, 1995). The importance of the TfR1 is highlighted by several studies showing that blockade of TfR1-mediated endocytosis can reduce cellular growth (Daniels *et al.*, 2012). This increased expression of the cellular iron import proteins together with a loss of cellular iron efflux is likely to explain the increased cellular iron deposition observed in oesophageal cancer tissue (Boult *et al.*, 2008). The resulting increased levels of cellular iron is likely to lead to a plethora of cellular processes for which iron is crucial, including oxidative phosphorylation and DNA synthesis, as well as cell cycle progression and growth (Le and Richardson, 2002). Indeed, the authors have previously demonstrated that increasing iron levels in oesophageal models results in increased cellular viability and proliferation (Boult *et al.*, 2008). Investigations from our laboratories have also shown that an excess of cellular iron can drive Wnt signalling, which is a major oncogenic signalling pathway of the gastrointestinal tract, including oesophageal cancer (Brookes *et al.*, 2008; Wang *et al.*, 2009). In addition, iron is likely to be driving tumourigenesis through Fenton reaction chemistry and the subsequent generation of reactive oxygen species (Valko *et al.*, 2006; Toyokuni, 2009). Reactive oxygen species can cause oxidative damage to lipids, proteins and DNA; the latter includes mutations of tumour suppressors and oncogenes, chromosomal rearrangements and microsatellite instability, all classic hallmarks of cancer.

The hypothesis that excessive cellular iron levels promote tumourigenesis is further supported by evidence that iron chelators possess potent anti-neoplastic properties and is illustrated by the experimental iron chelators developed by several of the authors (Whitnall *et al.*, 2006; Richardson *et al.*, 2009; Kovacevic *et al.*, 2011). For example, the chelator, di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone (Dp44mT), suppressed tumour growth in a range of murine xenograft models without inducing systemic iron depletion (Whitnall *et al.*, 2006). This is particularly pertinent since it potentially allows the administration of such compounds to cancer patients who often present with iron deficiency anaemia. In addition to its anti-tumourigenic effects, it was observed that Dp44mT could also be used to overcome multi-drug resistance (Whitnall *et al.*, 2006). These observations are supported by evidence that iron chelators can decrease the expression of multi-drug resistance genes, including MDR1 (Fang *et al.*, 2010).

However, the clinical use of experimental iron chelators requires extensive preclinical pharmacological and toxicological assessment in multiple animal models and depends on successful clinical trials. An alternative approach could be to use licensed iron chelators such as deferoxamine (DFO) or deferasirox, which have a proven safety profile (Merlot *et al.*, 2012). Both *in vitro* and *in vivo* data highlight their potential as possible anti-cancer agents (Richardson, 2002; Whitnall *et al.*, 2006; Yu *et al.*, 2006; Merlot *et al.*, 2012). The iron chelator, DFO, has shown promise as an anti-tumour agent in human clinical trials involving neuroblastoma and leukaemia (Estrov *et al.*, 1987; Donfrancesco *et al.*, 1990; 1992; 1995). However, the utility of iron chelators in treating patients with oesophageal cancer has not yet been addressed.

Therefore, the aims of this investigation were to assess whether DFO and deferasirox could inhibit iron-mediated tumour-promoting effects observed in oesophageal models and whether they possess anti-oesophageal cancer activity *in vivo*. These studies could provide a sound rationale for evaluating the usefulness of these agents in human clinical trials as treatments for oesophageal cancer and also as potential chemo-sensitizers. The latter is particularly relevant in oesophageal cancer, as the majority of patients either receive neo-adjuvant or palliative chemotherapy.

Methods

Iron chelators and chemotherapeutic drugs

DFO was purchased from Sigma-Aldrich (St. Louis, MO) and was used throughout this study at its IC_{50} value (10 μM). Deferasirox (a kind gift from Novartis, Basel, Switzerland) was used at concentrations of 0–40 μM. The ligand, Dp44mT, was synthesized as previously described and used at 1 μM, where it shows high anti-tumour activity (Yuan *et al.*, 2004). Epirubicin (Mayne Pharma Plc, Warwickshire, UK), cisplatin (TEVA UK, Eastbourne, UK) and fluorouracil (5-FU; Mayne Pharma Plc) were used at concentrations between 0 and 32 μM (IC_{50} values: 1, 8 and 8 μM respectively). The IC_{50} values above were determined over a 48 h period using the OE33, OE19 and OE21 oesophageal cell lines.

Cell culture

The oesophageal adenocarcinoma lines, OE19 and OE33, and oesophageal squamous cell carcinoma lines OE21 and TE4, were routinely cultured in DMEM with 10% FCS (Invitrogen, Mulgrave, VIC, Australia) (Rockett *et al.*, 1997; Takashima *et al.*, 2008). In addition, the oesophageal squamous cell line, TE4 (kind gift from Prof W Dinjens, University of Rotterdam), previously reported to be cisplatin-resistant, was cultured with and without cisplatin (2 μM) to maintain resistance (Takashima *et al.*, 2008).

Assessment of cellular iron uptake and efflux

Inhibition of cellular ^{59}Fe uptake by iron chelators. Cells (1×10^5 mL^{-1}) were plated in triplicate to achieve 70% confluence in culture dishes (35 × 10 mm). Cell monolayers were then incubated for 3 h/37°C with media (1 mL) containing ^{59}Fe-transferrin (^{59}Fe-Tf; 60 μg·mL^{-1}) and the iron chelators (1–20 μM) (Richardson *et al.*, 1995). At the end of the incubation, the monolayer was washed four times on ice with ice-cold PBS and then incubated on ice with Pronase (1 mg·mL^{-1}) for 30 min/4°C to remove cell surface-bound ^{59}Fe-Tf (Richardson *et al.*, 1995). The cell monolayer was detached using a plastic spatula, followed by centrifugation and collection of the supernatant (representing cell surface-bound ^{59}Fe-Tf) (Richardson *et al.*, 1995). The cell pellet was re-suspended in PBS (1 mL), and ^{59}Fe levels were quantified in the pellet and supernatant using a gamma counter (2480 Wizard^2, Perkin Elmer, Turku, Finland). Data are presented as a percentage in comparison with control cells.

Cellular ^{59}Fe efflux from cells by iron chelators after pre-labelling with ^{59}Fe-Tf. Cells were plated as in uptake experiments and pre-labelled for 3 h/37°C with ^{59}Fe using 1 mL of media per plate containing ^{59}Fe-Tf (60 μg·mL^{-1}), implementing standard methods (Richardson *et al.*, 1995). The cell monolayer was then washed four times on ice with ice-cold PBS (Richardson *et al.*, 1995). Cells were then re-incubated for 3 h/37°C with medium alone or medium containing the chelators (1–20 μM). The media was removed, and the amount of ^{59}Fe in the media and monolayer was assessed as above. Results were expressed as the percentage of ^{59}Fe mobilized from cells incubated with control medium alone.

qRT-PCR

qRT-PCR was performed as described previously (Le and Richardson, 2002; Boult *et al.*, 2008). All reactions were performed using human 18S ribosomal RNA as an internal standard (Life Technologies Ltd, Paisley, Renfrewshire, UK) and contained both human probe and primers to *TfR1*, *ferritin-H* and/or *ferroportin* (*FPN*).

Western blotting

Western blotting was performed by standard methods[30] using antibodies to TfR1 (Cat. #: ab1086 Abcam, UK; 1:500), FPN (Cat. #: ab85370 Abcam, UK; 1:300) or ferritin heavy chain (ferritin-H; Cat. #: ab65080 Abcam, UK; 1:500). To ensure normalization of protein loading, β-actin (Cat. #: ab8226 Abcam, UK; 1:2000) monoclonal antibody was employed (Yuan *et al.*, 2004). Immunoreactive bands were subjected to densitometry using NIH Image 1.62 software (National Institutes of Health, Bethesda, MD).

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cellular proliferation assay

Cells (1×10^6·mL^{-1}) were plated into 96-well plates and upon attaining 70% confluence were incubated with media (with or without chelator) for 24–48 h. At the end of the culture period, 10 μL of MTT solution (5 mg·mL^{-1} in PBS) was added to each 100 μL of culture media and incubated for 3 h/37°C. The medium was then aspirated, and the cells were solubilized using DMSO (100 μL). The absorbance at 490 nm was read using a Bio-Tek ELx800 absorbance microplate reader (Potton, Bedfordshire, UK), and the results were expressed as percentage viability with respect to the untreated control.

BrdU proliferation assay

The BrdU assay was performed according to manufacturer's instructions (Roche Applied Science, Indianapolis, IN). Briefly, cells (1×10^6·mL^{-1}) were plated into 96-well plates and upon attaining 70% confluence were incubated with media (with or without chelator) for 48 h. Cells were then labelled with BrdU, fixed, DNA denatured using 'FixDenat' solution (Roche Applied Science) and incubated with anti-BrdU. The immune complexes were detected using a TMB substrate reaction and assessed at 490 nm.

Murine xenograft model system

All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). All procedures for the animal experiments were approved by the Sydney University and Birmingham Animal Ethics Committee. Female BALB/c nu/nu mice were used at 8–10 weeks of age (90 mice in total) (Laboratory Animal Services, University of Sydney). Mice were housed under a 12 h light–dark cycle, routinely fed basal rodent chow and watered *ad libitum*. Suspensions of OE33, OE19 and OE21 cells (1×10^7 cells) were centrifuged and re-suspended in 50 μL of culture media, and cell viability was monitored using Trypan blue exclusion. Immediately prior to injection, the cell slurry was mixed 50/50 (v/v) with Matrigel (BD Biosciences, San Jose, CA). The cell suspension was then s.c. injected into the right flanks of mice. Tumour size was measured using digital Vernier callipers, and tumour volumes were calculated, as described (Whitnall *et al.*, 2006). Chelator treatment began once the tumours reached a volume of 100 mm^3 (approximately 2 weeks after injection of tumour cells). The health of the mouse was assessed by measuring weight and monitoring behaviour.

The mice were gavaged on alternate days with either a deferasirox suspension in vehicle [30% 1,2-propanediol/70% sterile 0.9% sodium chloride solution (v/v)] or vehicle alone. After 3 weeks of treatment, mice were anaesthetized and exsanguinated by direct cardiac puncture, and blood/plasma was retained for full blood count and biochemical analysis [urea, creatinine, alanine transaminase (ALT), aspartate transaminase (AST), albumin, total bilirubin, serum iron and total iron-binding capacity]. The liver, heart and spleen were removed and weighed before immediate division into tubes containing formalin or RNAlater (Invitrogen). Tumours were removed and weighed to assess tumour burden and divided

into three samples for tissue iron levels (see ferrozine assay below), qRT-PCR analysis and immunohistochemistry.

Ferrozine assay

Iron levels were assayed as previously described and iron content expressed as nmol of iron·mg^{-1} protein (Brookes *et al.*, 2008). Protein concentrations were assessed by the Bradford assay (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK).

Immunohistochemistry

Immunohistochemistry was performed as previously described on the xenografted tumours with antibodies specific to TfR1 (1/250), ferritin-H (1/200) and FPN (1/200) (Boult *et al.*, 2008). Slides were scored for intensity of immunoreactivity and the percentage of epithelial cells stained (Di Martino *et al.*, 2006).

Data analysis and statistics

All experimental errors are shown as two standard errors of the mean (representing 95% confidence intervals). To assess the ability of iron chelators to overcome drug resistance, an iron chelator (Dp44mT, deferasirox or DFO) and a chemotherapeutic agent (cisplatin, 5-FU or epirubicin) were examined in combination. Two series of cell counts were performed: one at various concentrations of the iron chelator alone and another in combination with the chemotherapy agent at a fixed concentration. The additional effect of the iron chelator was estimated from the difference between the two series of counts, and significance was assessed using a paired Student's *t*-test or Wilcoxon signed rank test. Data were considered statistically significant when $P < 0.05$.

Results

The effect of DFO and deferasirox on cellular iron uptake and efflux

The efficiency of the ligands at chelating cellular iron in the three oesophageal cell models was explored using cellular iron uptake and cellular iron mobilization assays (Figure 1). It should be noted that these assays implement highly sensitive estimation of the radioisotope ^{59}Fe using γ-counting. This enables direct measurement of the effect of the chelators on both iron mobilization and inhibition of iron uptake from ^{59}Fe-Tf. Cells were incubated with ^{59}Fe-Tf with increasing concentrations of DFO and deferasirox (1–20 μM) to assess their ability to prevent cellular iron uptake from the physiological iron donor, transferrin (Le and Richardson, 2002). Both DFO and the experimental chelator, Dp44mT, were used as positive controls, as their activities are well characterized (Richardson *et al.*, 1995; Yuan *et al.*, 2004). Of the three chelators used, Dp44mT exhibited the most profound inhibition of cellular iron uptake, limiting uptake to ~10–30% of the control in the OE33, OE19 and OE21 cell lines (Figure 1A). Deferasirox inhibited ^{59}Fe uptake to ~20–50% of the control, with DFO exhibiting the weakest inhibition (~50–90%) across all three lines (Figure 1A).

To assess the ability of the iron chelators to mobilize cellular iron, cells were pre-loaded with ^{59}Fe then incubated with increasing concentrations of chelator (1–20 μM). The amount of ^{59}Fe released was measured in the supernatant and expressed as a percentage relative to the total ^{59}Fe (cellular plus released ^{59}Fe). Across all cell lines, the most effective chelator was Dp44mT, mobilizing ~60–75% of cellular ^{59}Fe. Deferasirox and DFO were less effective and mobilized ~30–65% of cellular ^{59}Fe (Figure 1B).

Effect of DFO and deferasirox on the expression of iron metabolism proteins

Cellular iron levels are mainly regulated at the post-transcriptional level by the iron regulatory proteins 1 and 2 (IRP1 and 2) that homeostatically control iron levels (Muckenthaler *et al.*, 2008). Upon cellular iron depletion, the IRPs bind to iron-responsive elements (IREs) in the 3′ untranslated region (UTR) of the *TfR1* mRNA and 5′ UTRs of *ferritin-H* and *FPN* mRNAs to induce up- and down-regulation respectively (Muckenthaler *et al.*, 2008). Hence, the effect of deferasirox and DFO on the expression of these molecules was assessed to provide a relevant measure of cellular iron status (Figure 2A–F).

Incubation of cells with deferasirox (20 μM) or DFO (10 μM) for 48 h resulted in a significant ($P < 0.05$) increase in *TfR1* mRNA and protein expression in all cell lines (Figure 2A,B), consistent with IRP theory (Muckenthaler *et al.*, 2008). These observations were in good agreement with the ^{59}Fe efflux and uptake studies where chelator treatment led to cellular iron deprivation, resulting in TfR1 up-regulation.

In contrast to TfR1, ferritin-H and FPN are regulated post-transcriptionally by the IRPs, and this results in decreased protein expression, but not mRNA levels (Muckenthaler *et al.*, 2008). In this study, *ferritin-H* mRNA and protein levels were not significantly altered in OE19 and OE21 cells, while there was a significant reduction in ferritin-H protein expression in OE33 cells (Figure 2C,D). It is unclear why the chelators did not cause any significant alteration in ferritin-H levels in OE19 or OE21 cells. However, a possible explanation for this disparity between the cell lines is the dynamicity in which H-ferritin is modulated by intracellular iron. It may be that ferritin-H is more dynamically repressed in OE33 cells compared with the OE19 and OE21 cell lines over the 48 h incubation utilized (Figure 2). This could be the reason why that only in the long-lived xenograft model do we observe suppression of ferritin-H in all three tumour types following deferasirox treatment over 3 weeks (see results below) Expression of *FPN* mRNA was unaltered after incubation with chelators, except for a significant decrease in its levels in OE21 cells incubated with DFO (Figure 2E). The chelators significantly suppressed FPN protein expression in all three cell lines (Figure 2F), as may be expected considering its regulation by IRPs (Muckenthaler *et al.*, 2008). Collectively, these studies in Figures 1 and 2 demonstrate cellular iron depletion by deferasirox.

Effect of DFO and deferasirox on oesophageal cellular viability and proliferation

Incubation of the OE33, OE19 and OE21 oesophageal cancer cell lines with DFO or deferasirox for 48 h caused a statistically significant reduction in cellular viability and prolifera-

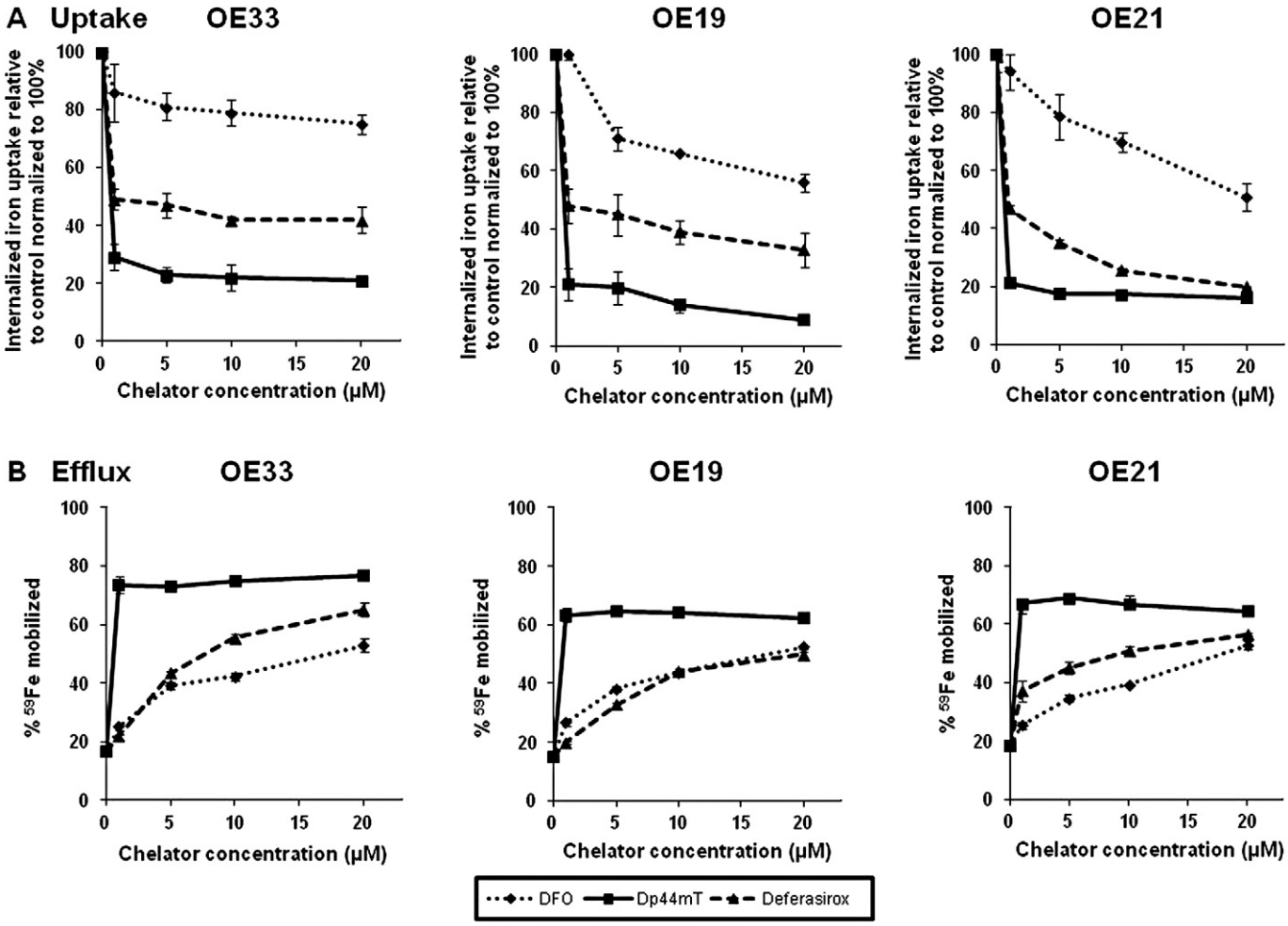

Figure 1

Effect of iron chelators on cellular iron uptake and efflux. The effect of an increasing concentration of DFO, deferasirox and Dp44mT on (A) inhibiting cellular ^{59}Fe uptake and (B) mobilizing cellular ^{59}Fe was assessed in the OE33, OE19 and OE21 cell lines. Results are expressed as mean ± SEM (three experiments).

tion as judged by the MTT assay and BrdU incorporation respectively (Figure 3A,B). Incubation of all three cell lines with iron sulphate (100 μM) resulted in statistically significant increases in cellular viability and proliferation, which could be inhibited by the addition of chelator. (Figure 3A,B). This observation demonstrates that the iron-binding ability of the ligands is important in terms of their inhibitory effects on cellular viability and proliferation.

Use of DFO and deferasirox as chemotherapy sensitizers

To assess whether DFO and deferasirox can enhance the response to standard chemotherapy, the three cell lines were cultured with or without epirubucin, cisplatin or 5-FU, at concentrations equal to their pre-determined IC_{50} values (1, 8 and 8 μM respectively). Cells were then incubated with increasing concentrations of an iron chelator (Dp44mT, DFO or deferasirox), and the effect on cellular viability was assessed (Figure 4A–C; Supporting Information Figure S1). In all three cell lines, very low doses of Dp44mT (0.25 μM) could suppress viability in the presence of cisplatin, 5-FU or epirubicin (Figure 4C, Supporting Information Figure S1C,F). Suppression of viability was also observed for both DFO (Figure 4B, Supporting Information Figure S1B,E) and deferasirox (Figure 4A, Supporting Information Figure S1A,D) with cisplatin, 5-FU and epirubicin across all three cell lines. Irrespective of the iron chelator and chemotherapy agent used, the effect of combining the two classes of drugs (iron chelator + cisplatin/5-FU/epirubicin) was significantly more cytotoxic than that seen with the drug alone (Supporting Information Table S1).

To further examine if the iron chelators, deferasirox and DFO, enhance the effect of chemotherapeutics, the cisplatin-resistant oesophageal cell line, TE-4, was employed. As expected, there was no significant loss in cellular viability when culturing cisplatin-resistant TE-4 cells with cisplatin (2 μM) relative to cells incubated with control media (Figure 5A,B). However, culturing cisplatin-resistant TE-4 cells with even a very low concentration of deferasirox (5 μM) and cisplatin (2 μM) resulted in a significant ($P < 0.05$) decrease in

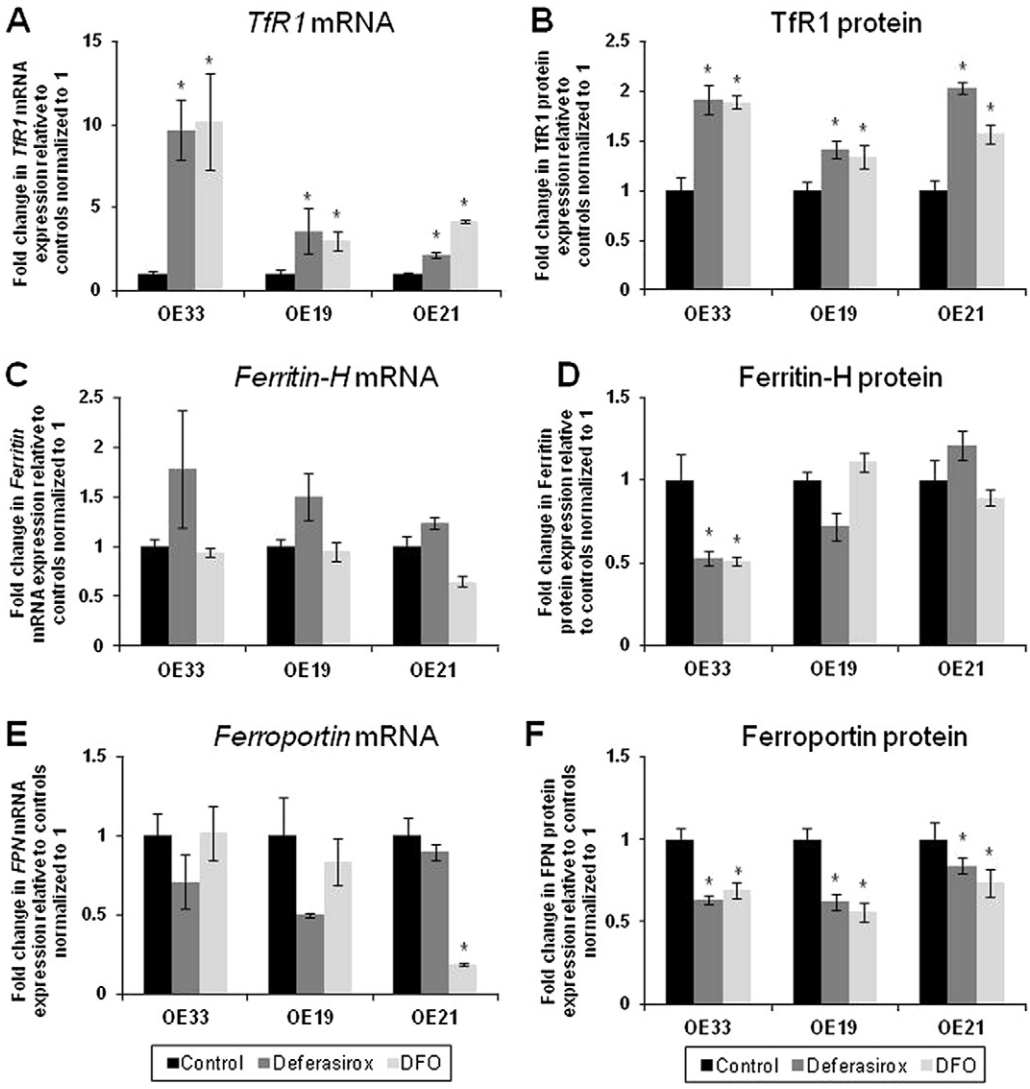

Figure 2

Effect of iron chelators on the expression levels of classical iron metabolism proteins. All three oesophageal cell lines, OE33, OE19 and OE21, were incubated with deferasirox (20 μM) or DFO (10 μM) for 48 h/37°C. qRT-PCR was employed to assess levels of (A) *TfR1*, (C) *ferritin-H* and (E) *FPN* mRNA relative to control cells. In addition, protein lysates were subject to Western blotting to assess protein levels of (B) TfR1, (D) ferritin-H and (F) FPN. Results as expressed as mean ± SEM (three experiments). * denotes statistical significance compared with control, $P < 0.05$.

cellular viability compared with cisplatin alone. Notably, this deferasirox concentration alone did not induce a significant loss of viability compared with cisplatin-resistant TE-4 cells incubated with media alone (Figure 5A). However, higher concentrations of deferasirox alone (10 and 20 μM) gave similar results as that found with the analogous concentrations of deferasirox added with cisplatin (Figure 5A). Similarly, a low concentration of DFO (2.5 μM) co-cultured with cisplatin (2 μM) resulted in a significant suppression of cellular viability compared with cisplatin alone (Figure 5B). Again, this DFO concentration did not lead to a significant decrease in cellular viability when used with TE-4 cells alone, while higher concentrations of DFO alone (5 and 10 μM) significantly reduced viability. Collectively, these results suggest that both deferasirox and DFO can inhibit the growth of cisplatin-resistant TE4 tumour cells.

Effect of deferasirox on xenograft growth

Deferasirox given orally on alternate days at 20 mg·kg^{-1} for 3 weeks significantly suppressed tumour growth by 32%, 37% and 43% in xenografts from the three cell lines (OE19, OE21 and OE33, respectively) compared with mice gavaged with vehicle alone (Figure 6A,B). Mice showed no signs of ill health during the 3 week treatment period at this relatively low dose. Average mouse and organ (liver, spleen and heart) weights did not differ statistically from mice treated with the vehicle alone (data not shown). Importantly, deferasirox treatment did not change haematological parameters, including haemoglobin, haematocrit, mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC) and reticulocyte, white cell and neutrophil counts (Table 1). No change was observed in serum iron levels, total iron binding

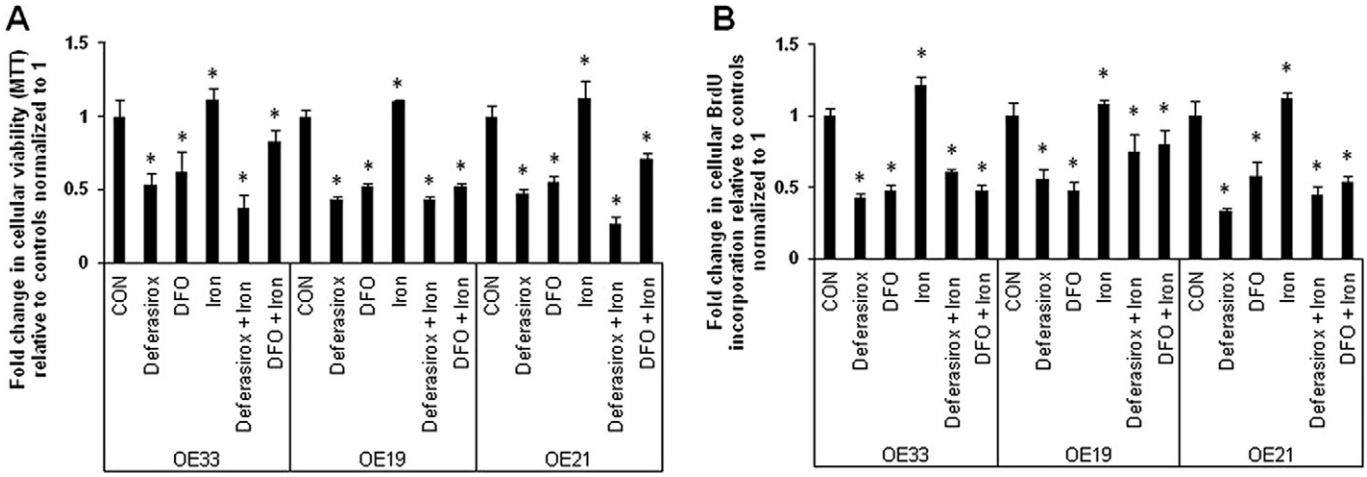

Figure 3

Effect of iron chelators on oesophageal cellular viability and proliferation. To assess the effect of DFO and deferasirox on cellular viability and proliferation, MTT (A) and BrdU assays (B) were employed. In addition, all three cell lines (OE33, OE19 and OE21) were also challenged with or without iron sulphate (100 μM). Results as expressed as mean ± SEM (three experiments). * denotes statistical significance compared with untreated control cells (CON), $P < 0.05$.

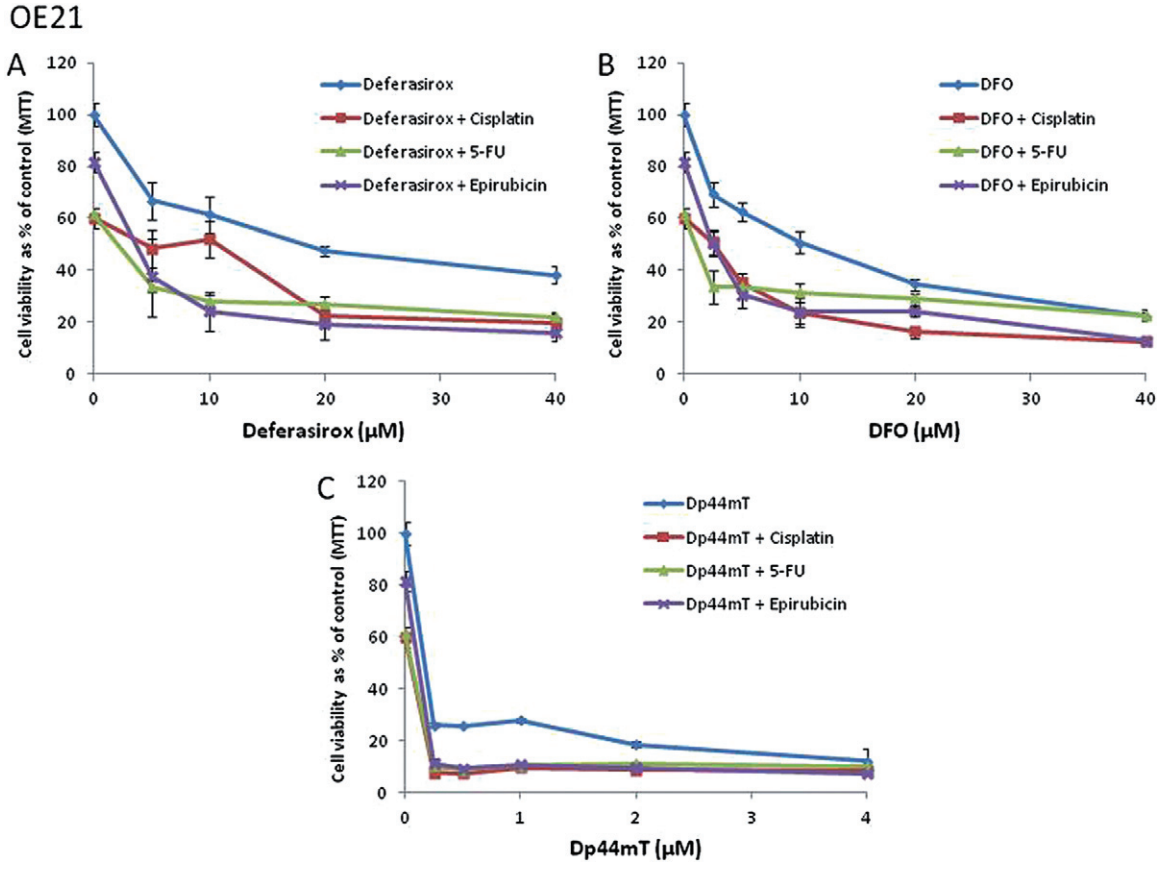

Figure 4

Effect of iron chelators and standard chemotherapeutic agents on oesophageal cellular viability. Cellular viability was assessed using the MTT assay in OE21 cell line following increasing doses of: deferasirox (A), DFO (B) and Dp44mT (C) alone or in the presence of a fixed concentration of epirubicin (1 μM), cisplatin (8 μM) or 5-FU (8 μM). Results as expressed as mean ± SEM (three experiments).

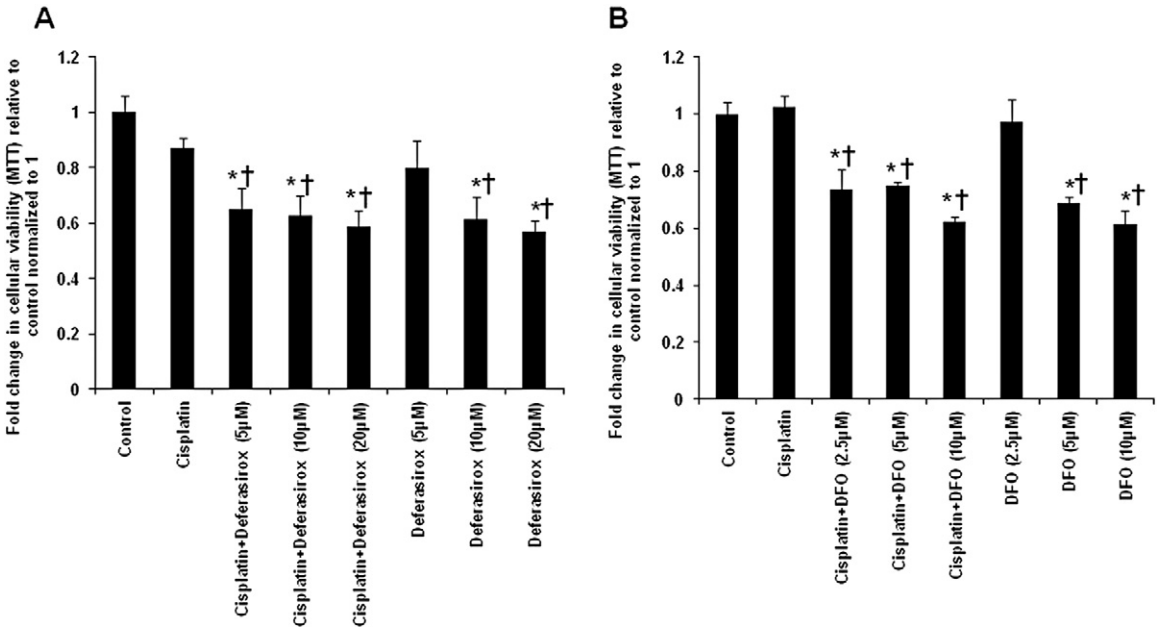

Figure 5

Effect of iron chelators on TE-4 cellular viability. TE-4 cells were incubated with cisplatin (2 μM) in the presence or absence of (A) deferasirox (5–20 μM) or (B) DFO (2.5–10 μM) and cellular viability assessed utilising the MTT assay. Results are expressed as mean ± SEM (three experiments). * denotes statistical significance compared with untreated control cells, $P < 0.05$. † Denotes statistical significance compared with cells treated with cisplatin alone.

Table 1

Measured biochemical parameters from serum of control and deferasirox-treated nude mice bearing a human OE19 oesophageal xenograft

	Control	Deferasirox
Hb ($g \cdot L^{-1}$)	153 ± 0.57	154.25 ± 1.75
HCT (%)	48.4 ± 0.25	47.7 ± 0.60
MCV (fL)	47.63 ± 0.28	46.5 ± 0.24
MCH (pg)	15.1 ± 0.07	15.1 ± 0.09
MCHC ($g \cdot L^{-1}$)	316.2 ± 1.01	323.5 ± 1.0
PLT ($10^9 \cdot L^{-1}$)	929.5 ± 102	1457.25 ± 74
WBC ($10^9 \cdot L^{-1}$)	6.65 ± 0.37	6.05 ± 0.76
RET ($10^{12} \cdot L^{-1}$)	0.59 ± 0.03	0.58 ± 0.02
NEUT (%)	19.4 ± 2.1	23.75 ± 2.3
Serum iron ($\mu mol \cdot L^{-1}$)	33.8 ± 1.1	35.054 ± 5.7
TIBC ($\mu mol \cdot L^{-1}$)	57.5 ± 0.6	56.2 ± 0.7
UIBC ($\mu mol \cdot L^{-1}$)	65.2 ± 1.1	67.9 ± 6
ALP ($U \cdot L^{-1}$)	139.8 ± 5.9	159.5 ± 3.3
ALT ($U \cdot L^{-1}$)	30.9 ± 3	34.4 ± 3.9
ALB ($g \cdot L^{-1}$)	32.5 ± 0.89	31.7 ± 0.7
TP ($g \cdot L^{-1}$)	55.1 ± 1.7	53.4 ± 1.4
Urea ($mmol \cdot L^{-1}$)	7.9 ± 0.2	7.9 ± 0.25

Deferasirox was given orally on alternate days at 20 $mg \cdot kg^{-1}$ for 3 weeks. At the end of the experiment, blood was taken from heart and sent for haematological and biochemical analysis. Results are mean ± SEM (n = 12 mice per group).

capacity (TIBC) or unsaturated iron binding capacity (UIBC). Examination of serum albumin, total protein, alkaline phosphatase (ALP), ALT and renal function tests did not show any significant alterations (Table 1).

Direct iron measurements of the excised tumours showed a marked reduction in tumour iron content (34%, 42% and 57% in the OE21, OE19 and OE33 cell lines, respectively) in mice treated with deferasirox compared with vehicle alone (Figure 6C). This was supported by qRT-PCR analyses demonstrating a significant ($P < 0.05$) increase in *TfR1* expression and a significant ($P < 0.05$) decrease in *ferritin-H* and *FPN* mRNA expression in all tumours from deferasirox-treated mice compared with vehicle alone (Figure 6D). These observations agreed with immunohistochemical studies on tumour xenografts where semi-quantitative analysis of tumour sections showed a marked and significant increase in TfR1 protein expression and significantly decreased ferritin-H and FPN protein levels in deferasirox-treated mice compared with vehicle control-treated tumours (Figure 7).

Discussion and conclusions

Mounting evidence supports a role for iron chelators in the treatment of cancer (Richardson, 2002; Whitnall *et al.*, 2006; Yu *et al.*, 2006; Richardson *et al.*, 2009; Kovacevic *et al.*, 2011; Merlot *et al.*, 2012). However, many of these studies are based on experimental iron chelators that are at the preclinical stage and are not approved for clinical use. Another approach is to use existing licensed iron chelators, commonly used for the treatment of patients with iron overload disease. One such chelator, DFO, has shown promise in clinical trials

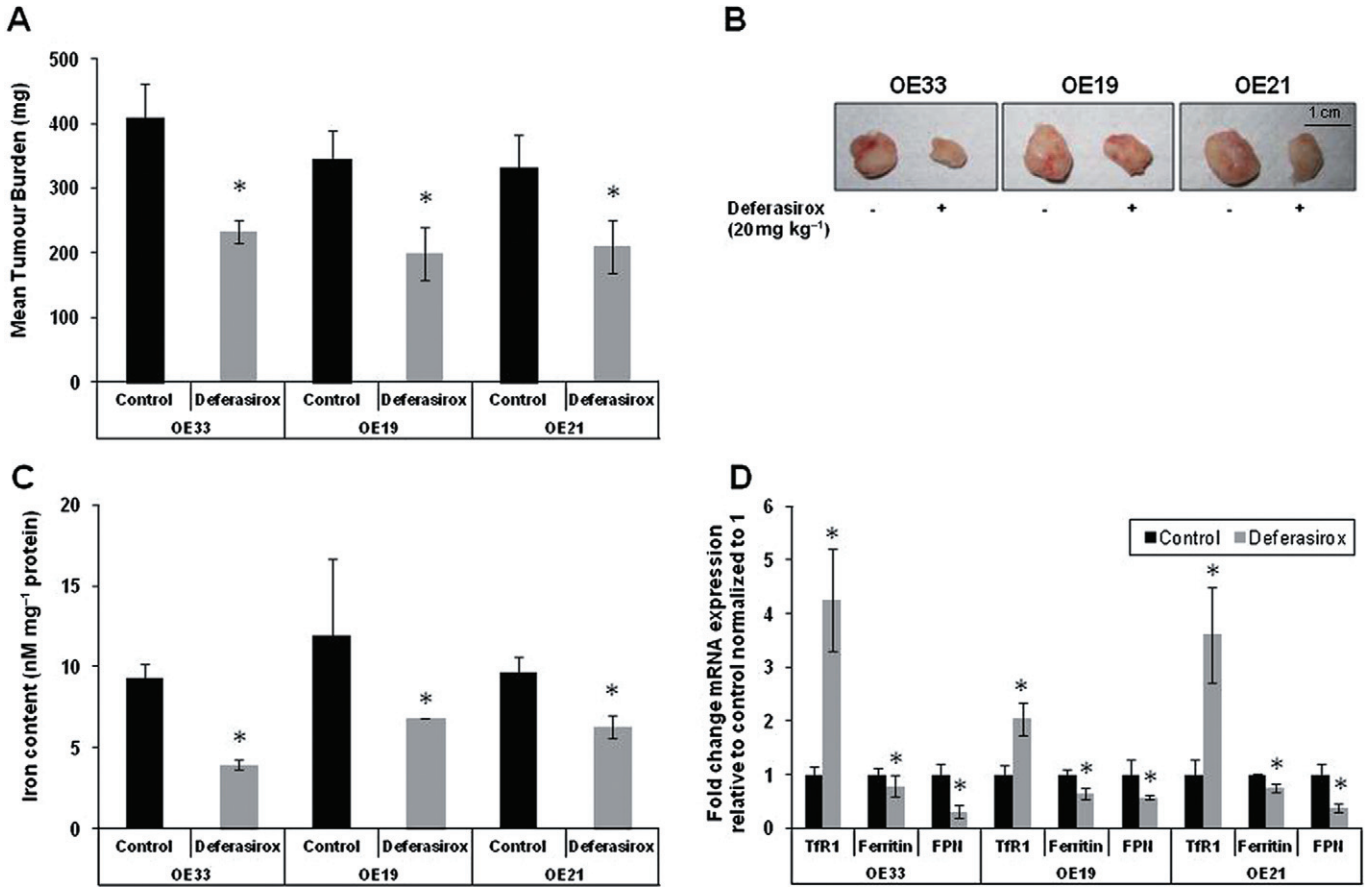

Figure 6

Effect of deferasirox on murine xenograft growth. Tumour xenografts of OE33, OE19 and OE21 cells were generated as described in the *Methods* section and were treated on alternate days with either vehicle alone or deferasirox (20 mg·kg^{-1}) for a 3 week period. Tumours were then excised and weighed to ascertain the effect of deferasirox on tumour burden relative to the vehicle control (A). Representative image of the excised tumours from mice treated with (+) or without (–) deferasirox (B). Tumours were analysed for tissue iron content by ferrozine assay, and iron levels were expressed as nanomoles of iron·mg^{-1} protein (C). In addition, mRNA expression levels of *TfR1*, *ferritin-H* and *FPN* were assessed relative to control tumour xenografts normalized to 1 (D). Results are expressed as mean ± SEM (three experiments with n = 3–5 mice/group). * denotes statistical significance compared with tumours from vehicle control-treated mice, $P < 0.05$.

(Estrov *et al.*, 1987; Donfrancesco *et al.*, 1990; 1992; 1995). However, the major drawback of DFO in clinical practice is the requirement for continuous subcutaneous infusions related to its short half-life and hydrophilic nature (Merlot *et al.*, 2012). An alternative is the licensed oral iron chelator, deferasirox, which has a half-life of 7–18 h and is administered once daily (Lindsey and Olin, 2007). However, little data are available assessing this drug as an anti-cancer agent (Chantrel-Groussard *et al.*, 2006; Lescoat *et al.*, 2007; Cappellini, 2008; Ohyashiki *et al.*, 2009; Messa *et al.*, 2010; Fukushima *et al.*, 2011). The current study is the first to provide evidence that deferasirox may be of use in oesophageal cancer treatment.

Deferasirox (like DFO) can act as an iron chelator in oesophageal cancer cell models and is able to both inhibit iron uptake and mobilize iron from cells. The resulting decrease in cellular iron in oesophageal cancer cells is reflected by increased TfR1 expression, consistent with classical IRP theory and previous studies examining iron chelators as anti-cancer agents (Whitnall *et al.*, 2006; Muckenthaler *et al.*, 2008). Reduced cellular iron is likely, in part, to account for the anti-proliferative effect of these ligands against oesophageal cancer cells. In fact, both DFO and deferasirox are able to ablate iron-mediated pro-proliferative responses (Boult *et al.*, 2008). Importantly, these *in vitro* effects were observed across all three oesophageal cell lines, suggesting the effect of modulating cellular iron levels is not cell lineage dependent. This is consistent with existing literature using experimental chelators (Richardson, 2002; Whitnall *et al.*, 2006; Yu *et al.*, 2006; Richardson *et al.*, 2009; Kovacevic *et al.*, 2011; Merlot *et al.*, 2012).

Additionally, we assessed the ability of deferasirox to inhibit tumour growth *in vivo* using a murine xenograft model. Deferasirox was chosen as this is the most attractive licensed iron chelator for future human clinical trials, given its oral delivery route and good tolerability (Lindsey and Olin, 2007; Cappellini, 2008). To mimic potential future clinical trials, 'primary' tumours were allowed to form, and then mice were gavaged with deferasirox (20 mg·kg^{-1}). This dosage was used as it is the starting dose for patients with iron

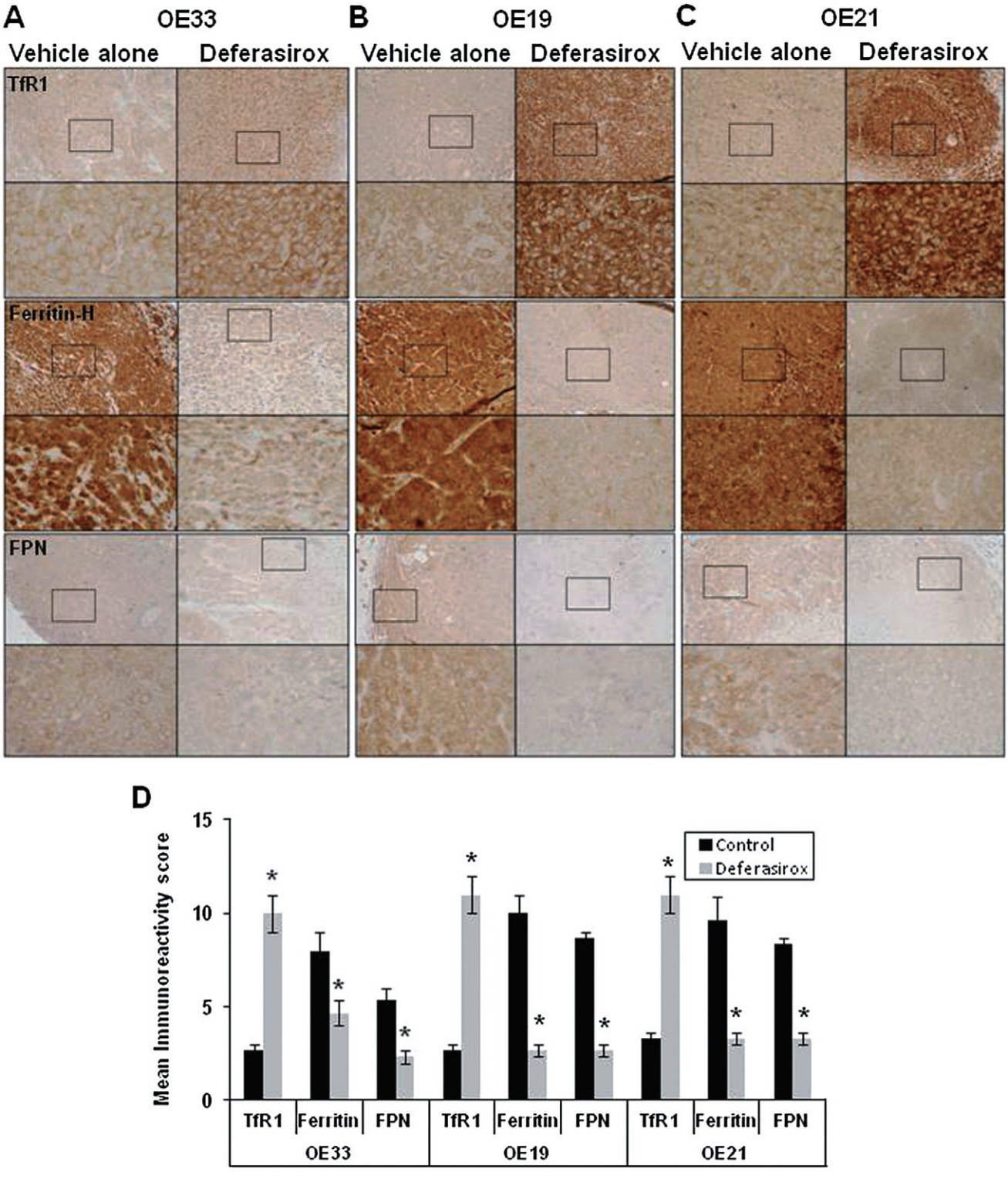

Figure 7

Effect of iron chelators on cellular localization of TfR1, ferritin-H and FPN in oesophageal-derived xenografts. Immunohistochemistry was performed on xenografts derived from OE33 (A), OE19 (B) and OE21 (C) in vehicle- or deferasirox-treated mice to assess the localization of TfR1, ferritin-H and FPN. Immunoreactivity of the trans-membrane proteins, TfR1 and FPN, was predominantly observed on the cell border in all tumours. Ferritin-H immunoreactivity was observed in all cellular compartments. Immunoreactivity was semi-quantitatively assessed, and the mean immunoreactivity score was reported for TfR1, ferritin-H and FPN in all excised tumours (D). Boxes denote field of view that is further magnified in lower panels. Results are expressed as mean ± SEM (three experiments). * denotes statistical significance compared with the mean immunoreactivity score observed in the untreated control tumours, $P < 0.05$.

overload (Nisbet-Brown *et al.*, 2003). To minimize effects on systemic iron levels, deferasirox was only administered once every second day. Importantly, 3 weeks of deferasirox therapy (10 treatments in total) resulted in a 32–43% suppression in tumour burden compared with tumours in mice treated with vehicle alone. Deferasirox treatment resulted in a marked reduction in tumour iron levels compared with the control, suggesting that its anti-neoplastic function is related to its ability to deplete tumour iron levels. Notably, the mechanism involved in the ability of deferasirox to inhibit tumour growth *in vivo* is different to that observed for Dp44mT, which does not induce iron depletion in tumour xenografts (Whitnall *et al.*, 2006). Significantly, the anti-neoplastic efficacy of deferasirox has also been reported in a leukaemic murine model (Ohyashiki *et al.*, 2009). Furthermore, deferasirox induced complete remission of a patient with chemotherapy-resistant acute monocytic leukaemia (Messa *et al.*, 2010; Fukushima *et al.*, 2011). It is important to discuss, that while the xenograft model is the most widely used murine model system to assess the efficacy of drugs on tumour burden, it does differ in several ways to oesophageal tumours in man. Most notably, the xenografted tumours are probably not as well vascularized and are not established in the presence of human stroma. However, despite poor tumour vascularity, deferasirox still has a dramatic effect on tumour xenograft burden, which clearly underlines the efficacy of this agent.

Currently, the effects of deferasirox at the molecular level are unknown, although its growth inhibitory and apoptotic functions have been described in several cell lines, including leukaemic and hepatoma lines (Chantrel-Groussard *et al.*, 2006; Lescoat *et al.*, 2007; Ohyashiki *et al.*, 2009; Messa *et al.*, 2010; Fukushima *et al.*, 2011). Suggested modes of activity include the inhibition DNA replication and cellular metabolism, notably polyamine metabolism. Previous studies suggest deferasirox may mediate its anti-neoplastic properties by modulating caspase-3, the mammalian target of rapamycin (mTOR) and NF-κB (Lescoat *et al.*, 2007; Ohyashiki *et al.*, 2009; Messa *et al.*, 2010), which are molecular targets implicated in oesophageal cancer development (Yen *et al.*, 2008; Hormi-Carver *et al.*, 2009). A recent study has also demonstrated that deferasirox is also a potent inhibitor of oncogenic Wnt signalling, a pathway described to be induced by iron (Brookes *et al.*, 2008; Song *et al.*, 2011). Additionally, it is likely that deferasirox mediates its anti-neoplastic properties through the chelation of iron and the suppression of reactive oxygen species (Valko *et al.*, 2006; Toyokuni, 2009). The latter are cytotoxic and induce lipid, protein and DNA damage, which likely contribute to mutations, chromosomal rearrangements, microsatellite instability and ultimately cancer.

In our murine tumour xenograft experiments, the most promising finding was that tumour volumes were reduced without apparent adverse effects over the relatively short treatment period. There was no loss of weight in deferasirox-treated compared with untreated controls. Iron levels were unaltered in all organs examined. Liver and renal function tests were normal, and all haematological parameters were unaltered. Collectively, this demonstrates that the deferasirox treatment regimen was very well tolerated by mice, possibly reflecting the low dose, short treatment period and/or the rest interval between doses. The marked oesophageal tumour inhibition observed, without detectable negative side effects, highlights the fact that tumour cells are more sensitive to iron chelation compared with normal cells, providing a promising platform for therapeutic intervention. This is consistent with a previous study of a leukaemic mouse model, where deferasirox suppressed tumour growth without any adverse effects (Ohyashiki *et al.*, 2009).

In addition, we demonstrated that iron chelators possess chemo-sensitizing properties. All iron chelators assessed enhanced the anti-proliferative efficacy of cisplatin, 5-FU and epirubicin (commonly used chemotherapeutic drugs in oesophageal cancer treatment) in all three cell lines examined. Furthermore, the cisplatin-resistant cell line, TE-4, became responsive in the presence of small doses of deferasirox or DFO. These low doses of deferasirox or DFO alone had no impact on cellular viability. These data are consistent with other studies utilizing Dp44mT, which has been shown to overcome both etoposide and vinblastine resistance (Whitnall *et al.*, 2006). Similar data have been generated in the leukaemic cell line, K562, where pre-incubation of cells with deferasirox and subsequent incubation with etoposide led to increased apoptosis above that observed with either drug alone (Ohyashiki *et al.*, 2009).

In conclusion, deferasirox shows promise as an anti-neoplastic and chemo-sensitising agent. This study provides a basis for assessing the utility of deferasirox in the treatment of patients with oesophageal cancer. It is of particular note that anaemia in GI cancers is common, with up to 45% of oesophageal cancer patients being anaemic (Tanswell *et al.*, 2011). The results from the current study suggest that administration of deferasirox at appropriate doses would have no effect on their systemic iron levels and thus would not compound the level of their anaemia. Thus, this treatment strategy may be applicable to oesophageal cancer patients and could provide a platform for mediating tumour suppression and increasing the responsiveness of patients to standard chemotherapeutic regimens.

Acknowledgements

This work was funded by the kind support of Cancer Research UK (Gordon Hamilton Fairley Clinical Research Fellowship to SJF). DRR thanks the National Health and Medical Research Council of Australia (NHMRC) for a Senior Principal Research Fellowship (571123) and Project Grant (632778). ZK gratefully acknowledges the NHMRC for an Early Career Fellowship (1037323). DSK and PJJ thank the Cancer Institute NSW for Early Career Fellowships.

Conflicts of interest

None declared.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 Effect of iron chelators and standard chemotherapeutic agents on oesophageal cellular viability. Cellular viability was assessed using the MTT assay in OE19 and OE33 cell lines following increasing doses of deferasirox (A, D), DFO (B,E) and Dp44mT (C,F) alone or in the presence of a fixed concentration of epirubicin (1 μM), cisplatin (8 μM) or 5-FU (8 μM). Results are expressed as mean ± SEM (three experiments).

Table S1 Assessment of statistical significance of treating oesophageal cell lines with chelator alone compared with chelator and a standard chemotherapeutic agent. Oesophageal cell lines (OE33, OE19 and OE21) were treated with an increasing dose of the iron chelator (Dp44mT, deferasirox or DFO) in the presence or absence of epirubicin (1 μM), cisplatin (8 μM) or 5-FU (8 μM) and then subjected to either MTT (Figure 4) and/or BrdU assays (data not shown) to assess viability and proliferation respectively. Mean cell counts were then assessed across each regimen, and statistical analysis was performed between different regimes and *P*-values calculated (three experiments).