The Iron Chelator, Deferasirox, as a Novel Strategy for Cancer Treatment: Oral Activity Against Human Lung Tumor Xenografts and Molecular Mechanism of Action


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ABSTRACT

Deferasirox is an orally effective iron (Fe) chelator currently used for the treatment of iron-overload disease and has been implemented as an alternative to the gold standard chelator, desferrioxamine (DFO). Earlier studies demonstrated that DFO exhibits anticancer activity due to its ability to deplete cancer cells of iron. In this investigation, we examined the in vitro and in vivo activity of deferasirox against cells from human solid tumors. To date, there have been no studies to investigate the effect of deferasirox on these types of tumors in vivo. Deferasirox demonstrated similar activity at inhibiting proliferation of DMS-53 lung carcinoma and SK-N-MC neuroepithelioma cell lines compared with DFO. Furthermore, deferasirox was generally similar or slightly more effective than DFO at mobilizing cellular 59Fe and inhibiting iron uptake from human transferrin depending on the cell type. However, deferasirox potently inhibited DMS-53 xenograft growth in nude mice when given by oral gavage, with no marked alterations in normal tissue histology. To understand the antitumor activity of deferasirox, we investigated its effect on the expression of molecules that play key roles in metastasis, cell cycle control, and apoptosis. We demonstrated that deferasirox increased expression of the metastasis suppressor protein N-myc downstream-regulated gene 1 and upregulated the cyclin-dependent kinase inhibitor p21CIP1/WAF1 while decreasing cyclin D1 levels. Moreover, this agent increased the expression of apoptosis markers, including cleaved caspase-3 and cleaved poly(ADP-ribose) polymerase 1. Collectively, we demonstrate that deferasirox is an orally effective antitumor agent against solid tumors.

Introduction

Cells require iron (Fe) for numerous important cellular processes, including energy generation, oxygen transport, and DNA synthesis (Kalinowski and Richardson, 2005; Whitnall et al., 2006). Iron is able to cycle between two stable redox states, the ferric and ferrous forms, allowing it to act as an electron donor/acceptor (Kalinowski and Richardson, 2005). Consequently, iron can participate in the generation of cytotoxic reactive oxygen species (Kalinowski and Richardson, 2005). Due to the deleterious effects of reactive oxygen species, intracellular iron levels must be tightly regulated (Chua et al., 2007).

Iron chelators for clinical use were initially developed for the treatment of iron overload (Cappellini, 2007; Porter, 2009). Examples of clinically used iron chelators include deferiprone (Ferririx®; ApoPharma, Toronto, Canada), deferasirox (Exjade®; Novartis, Basel, Switzerland; Fig. 1A), and the current gold-standard chelator desferrioxamine (DFO; Fig. 1B) (Kalinowski and Richardson, 2005). Because deferasirox is orally active and has a longer plasma half-life than DFO, it is considered to be a better alternative as it avoids the long hours of subcutaneous administration that result in poor patient compliance with DFO (Hershko et al., 2001). Deferasirox at high doses can mobilize liver iron and has been shown to be effective and safe in clinical studies (Nick et al., 2009; Finkenstedt et al., 2010).

In recent years, the potential for iron chelators in the treatment of cancer has emerged. This reflects the fact that cancer cells typically require more iron than normal cells to mediate their generally rapid DNA synthesis and growth (Whitnall et al., 2006). Hence, depriving cancer cells of iron is
a novel approach for cancer treatment (Kalinowski and Richardson, 2005). An iron chelator that has been specially developed for cancer therapy is 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP) or Triapine; Vion Pharmaceuticals, New Haven, CT; Fig. 1C), which is currently in phase II clinical trials (Knox et al., 2007). However, this agent demonstrates low efficacy and serious side effects, such as mehemoglobinemia and hypoxia (Chaston et al., 2003; Yen et al., 2004; Kalinowski and Richardson, 2005). More recently, our laboratory has developed novel thiosemicarbazones, namely, the di-2-pyridylketone thiosemicarbazone and 2-benzoylpyridine thiosemicarbazone series of iron chelators, which have shown marked antiproliferative activity both in vitro and in vivo (Kovacevic et al., 2011a; Yu et al., 2011a). One of the most effective compounds, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT; Fig. 1D), has been shown to be more effective than 3-AP at inhibiting the growth of human tumor xenografts in vivo (Whitnall et al., 2006).

The reported mechanism of action of di-2-pyridylketone thiosemicarbazone and 2-benzoylpyridine thiosemicarbazone chelators includes cellular iron deprivation, resulting in the inhibition of ribonucleotide reductase, which catalyzes the rate-limiting step of DNA synthesis (Merlot et al., 2012). More recently, chelators from these classes, namely, Dp44mT and 2-benzoylpyridine-4,4-dimethyl-3-thiosemicarbazone (Bp44mT), were shown to have an additional mechanism of ribonucleotide reductase inhibition via their effects on major thiol-containing systems (Yu et al., 2011b). Additionally, these ligands modulate the expression of a variety of proteins involved in cell cycle control, such as members of the cyclin family and cyclin-dependent kinases (Yu et al., 2007). Another significant mode of action is the upregulation of the growth and metastasis suppressor protein N-myc downstream-regulated gene 1 (NDRG1) (Kovacevic et al., 2011a), which has been shown to be critically important in the progression and outcome of a variety of neoplasms (Guan et al., 2000; Bandyopadhyay et al., 2003, 2004; Hosoi et al., 2009).

Recently, deferasirox was reported to inhibit the growth of myeloid leukemia cells in vitro and in vivo (Ohyashiki et al., 2009). Moreover, deferasirox was shown to induce a complete remission in a patient suffering chemotherapy-resistant acute monocytic leukemia (Fukushima et al., 2011). Hence, considering its oral activity, low toxicity, and demonstrated antiproliferative effects, deferasirox may have potential applications in cancer treatment. However, no studies have assessed the ability of deferasirox to inhibit the growth of solid human tumors in vivo. Additionally, its mechanism of action remains poorly understood. Hence, further in vitro and in vivo studies examining this compound are required to fully elucidate its antitumor activity.

Herein, we investigate for the first time the in vivo activity of deferasirox against solid human tumor xenografts. We also investigate the in vitro activity of deferasirox against human DMS-53 small-cell lung carcinoma and SK-N-MC neuroepithelioma cells, and dissect its molecular mechanism of action by examining its effect on the expression of molecules involved in cellular iron metabolism, tumor metastasis, and cell cycle control.

**Materials and Methods**

**Cell Culture.** The human DMS-53 small-cell lung carcinoma and SK-N-MC neuroepithelioma cell lines were obtained from the American Type Culture Collection (Manassas, VA). The DMS-53 and SK-N-MC cells were cultured in RPMI 1640 and Dulbecco's modified Eagle's media (Life Technologies, Carlsbad, CA), respectively. Media were supplemented with 10% (v/v) fetal calf serum, l-glutamine (2 mM), streptomycin (100 μg/ml), nonessential amino acids (0.1 mM), and sodium pyruvate (1 mM; all supplements from Life Technologies) using standard techniques, as described previously (Whitnall et al., 2006). All cells were incubated at 37°C in a humidified atmosphere containing 5% CO2. Experiments were performed when the cultures were approximately 80% confluent.

**Chelators.** The base of the ligand, Dp44mT, was synthesized and characterized using standard procedures (Whitnall et al., 2006; Yuan et al., 2004). DFO and deferasirox (Exjade) were obtained from Novartis (Basel, Switzerland). Both Dp44mT and deferasirox were dissolved in dimethyl sulfoxide at a stock concentration of 10 mM, and were used at the concentrations indicated under Results and figures by dilution in culture media containing 10% fetal calf serum. For in vivo studies, deferasirox was dissolved at a stock concentration of 8.2 mM in sodium chloride solution (0.9% w/v; Baxter Healthcare, Old Toongabbie, NSW, Australia). The DFO was dissolved directly in culture media.

**Cellular Proliferation Assay.** The well-established 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay was used to assess cell proliferation (Richardson et al., 1995). The cells were incubated with Dp44mT, DFO, and deferasirox for 72 hours at 37°C. Formazan product formation was shown to be directly proportional to viable cell counts (Richardson et al., 1995).

**Preparation of 59Fe-Transferrin.** Human transferrin (Tf; Sigma-Aldrich, Castle Hill, NSW, Australia) was labeled with 59Fe (PerkinElmer Life and Analytical Sciences, Boston, MA) to produce 59Fe2-Tf (59Fe-Tf), as previously reported (Richardson and Baker, 1992). The concentration of transferrin was determined by calibration with bovine serum albumin (BSA; Sigma-Aldrich). 59Fe Efflux from SK-N-MC and DMS-53 Cells. Iron efflux experiments examining the ability of various chelators to mobilize 59Fe from SK-N-MC and DMS-53 cells were performed using established techniques (Baker et al., 1992; Richardson et al., 1995). In brief, following prelabeling of cells with 8.2 mM (0.75 μM) for 3 hours at 37°C, the cultures were washed four times on ice with ice-cold phosphate-buffered saline (PBS) and subsequently reincubated for 3 hours at 37°C.
hours at 37°C with each chelator (25 and 50 μM) or the medium alone (control). The overlying media containing released 59Fe was then separated from the cells and placed into γ-counting tubes. The cells were then removed from the plate in 1 ml of PBS using a plastic spatula and placed into a separate set of γ-counting tubes. Radioactivity was measured in both the cell pellet and supernatant using a γ-scintillation counter (Wizard 1480 3G2, PerkinElmer-Wallac, Turku, Finland).

**Effect of Chelators at Preventing 59Fe Uptake from Tf.** The ability of the chelators to prevent cellular 59Fe uptake from 59Fe-Tf was examined using standard techniques (Becker and Richardson, 1999; Yuan et al., 2004). In brief, SK-N-MC or DMS-53 cells were incubated with 59Fe-Tf (0.75 μM) for 3 hours at 37°C in the presence of each of the chelators (50 μM) or the medium alone (control). The cells were then washed four times on ice with ice-cold PBS, and internalized 59Fe was determined by placing the culture plates on ice and incubating the cell monolayer with the general protease, Pronase (1 mg/ml; Sigma-Aldrich), for 30 minutes at 4°C (Richardson and Baker, 1992). The cells were then removed from the monolayer using a plastic spatula and centrifuged for 1 minute at 14,000 rpm. The resulting supernatant represented membrane-bound 59Fe-Tf that was released by the protease, and the internalized 59Fe in the cell pellet was the Pronase-insensitive fraction (Baker et al., 1992). The fractions were placed in different γ-counting tubes, and the radioactivity was measured using the γ-scintillation counter described previously.

**Tumor Xenografts in Nude Mice and Deferasirox Administration.** All animal experiments were performed according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and approved by The University of Sydney Animal Care and Ethics Committee. Female BALB/c (nu/nu) mice were purchased from the Animal Resources Centre (Canning Vale, Perth, WA, Australia) and were housed in sterile conditions. Experiments commenced when the mice were 8–10 weeks of age. Tumor cells (DMS-53) were previously described under Methods. The overlying media containing released 59Fe was then removed from the plate in 1 ml of PBS using a plastic spatula and centrifuged for 1 minute at 14,000 rpm. The resulting supernatant represented membrane-bound 59Fe-Tf that was released by the protease, and the internalized 59Fe in the cell pellet was the Pronase-insensitive fraction (Baker et al., 1992). The uptake of iron from 59Fe-labeled human transferrin (Tf) was measured using standard protocols (Yuan et al., 2004; Whitnall et al., 2006). The well described chelators DFO (Kalinowski and Richardson, 2005) and Dp44mT (Whitnall et al., 2006) were included in these studies to provide appropriate positive controls.

Deferasirox showed antiproliferative activity against DMS-53 lung carcinoma cells that was similar to that found for DFO (Fig. 2; Table 1). However, deferasirox showed slightly less antiproliferative efficacy than DFO using the SK-N-MC neuroepithelioma tumor cell lines using the MTT proliferation assay. The SK-N-MC and DMS-53 cell lines were chosen because the antiproliferative activity of the chelator Dp44mT has been well characterized in these cells both in vitro and in vivo (Yuan et al., 2004; Whitnall et al., 2006). The well described chelators DFO (Kalinowski and Richardson, 2005) and Dp44mT (Whitnall et al., 2006) were included in these studies to provide appropriate positive controls.

Deferasirox showed antiproliferative activity against DMS-53 lung carcinoma cells that was similar to that found for DFO (Fig. 2; Table 1). However, deferasirox showed slightly less antiproliferative efficacy than DFO using the SK-N-MC neuroepithelioma cell line (IC50: 14 ± 2 and 10 ± 1 μM, respectively; Fig. 2; Table 1). The activity of deferasirox in these cell lines was at least 1400-fold lower when compared with the potent thiosemicarbazone chelator Dp44mT (Fig. 2; Table 1). In agreement with previous studies, the activity (IC50) of DFO was at least 1000-fold lower than Dp44mT in SK-N-MC and DMS-53 cells (Whitnall et al., 2006). The more potent activity of Dp44mT in these cells may reflect enhanced membrane permeability relative to DFO and deferasirox (Kalinowski and Richardson, 2005).

**Iron Efflux and Uptake Studies.** Considering the link between antiproliferative activity and iron chelation (Richardson et al., 1995), we next examined the ability of deferasirox to remove iron from cells prelabeled with 59Fe and to prevent the uptake of iron from 59Fe-labeled human transferrin (Tf) using standard protocols (Richardson et al., 1995; Lovejoy and Richardson, 2002). To provide appropriate positive controls, we included the chelators DFO and Dp44mT, which have been well characterized in previous iron efflux and uptake studies (Richardson et al., 1995; Lovejoy and Richardson, 2002).

The current studies showed that in terms of iron efflux, deferasirox was slightly more effective than DFO (Fig. 3A), although both of these chelators exhibited moderate iron chelation efficacy relative to the highly active lipophilic
chelator Dp44mT. Specifically, Dp44mT (25 and 50 μM) was able to efflux approximately 50% of cellular $^{59}$Fe from SK-N-MC cells after a 3-hour incubation with $^{59}$Fe-Tf, whereas DFO and deferasirox were only able to efflux 18 and 36% cellular $^{59}$Fe, respectively (Fig. 3B). For both cell types, the chelator-mediated $^{59}$Fe mobilization was significantly ($P < 0.001–0.01$) greater than that found for the control medium alone.

Using SK-N-MC cells, deferasirox was more active than DFO in terms of preventing $^{59}$Fe uptake, limiting it to 40% of the control at 50 μM, whereas DFO was only able to limit $^{59}$Fe uptake to 80% of the control in these cells (Fig. 3C). Again, Dp44mT was most active, reducing $^{59}$Fe uptake to approximately 5% of the control at 25 and 50 μM. However, in DMS-53 cells, DFO and deferasirox reduced $^{59}$Fe uptake to a similar extent, 41% and 42%, respectively (Fig. 3D), whereas Dp44mT was able to limit $^{59}$Fe uptake to 3% of the control. These studies again confirm the greater iron chelation efficacy of Dp44mT compared with both DFO and deferasirox, and for all ligands, the decrease in $^{59}$Fe uptake was significantly ($P < 0.001–0.01$) different from the control.

**Effect of Deferasirox on the Growth of Human Lung Carcinoma Xenografts.** Considering the demonstrated in vitro antiproliferative activity of deferasirox (Fig. 2; Table 1), we next examined whether deferasirox could inhibit the growth of DMS-53 lung carcinoma tumor xenografts in BALB/c nude mice. As deferasirox is given to patients orally (in tablet form), we administered deferasirox as a saline suspension by oral gavage in accordance with previous studies (Sato et al., 2011). Initially, we attempted to define a maximum tolerated dose to determine an optimal treatment regimen. In fact, we were unable to reach maximum tolerated dose weight-loss criteria with an increasing dose, with no weight loss occurring in mice even after a 200 mg/kg dose every second day, three treatments per week for 4 weeks. However, because a 20 mg/kg per day regimen is considered effective and well tolerated in patients with iron overload (Nisbet-Brown et al., 2003), we reasoned that 20 and 40 mg/kg could be appropriate initial doses to examine in these tumor xenograft studies. Indeed, a preliminary tumor growth experiment in mice suggested that a 20 mg/kg dose markedly suppressed tumor growth (data not shown). Significantly, to minimize effects on systemic iron levels in the mice, deferasirox was only administered once every second day.

In a more comprehensive DMS-53 lung carcinoma tumor study in mice, deferasirox administered orally at 20 and 40 mg/kg (every second day, three treatments per week for 18 days) resulted in marked inhibition of tumor growth as determined by measurements of tumor volume and tumor weight (Fig. 4, A and B). After 18 days of oral treatment with the vehicle control (saline solution), the tumor xenografts reached an average volume of 1095 ± 85 mm$^3$. In contrast, tumor volume was significantly ($P < 0.01$) reduced to 424 ± 100 and 517 ± 80 mm$^3$ in mice treated with 20 and 40 mg/kg deferasirox, respectively (Fig. 4A). These final tumor volumes after 18 days of treatment were consistent with weights of excised tumors postnecropsy. In fact, control tumors weighed 1.3 ± 0.1 g, whereas tumors treated with 20 and 40 mg/kg oral deferasirox weighed significantly ($P < 0.01$) less than control tumors (0.5 ± 0.2 and 0.6 ± 0.2 g, respectively; Fig. 4B).

**Toxicological Effects of Oral Deferasirox Treatment in Tumor-Bearing Mice.** Treatment with deferasirox, given at 20 or 40 mg/kg over an 18-day treatment period, did not cause any significant ($P > 0.05$) loss of body weight in the mice (Fig. 4C), nor were weights of the liver, spleen, kidney, heart, brain, or lung significantly affected (Table 2).

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<thead>
<tr>
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<th>IC$_{50}$ (μM)</th>
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<tbody>
<tr>
<td></td>
<td>DMS-53</td>
<td>SK-N-MC</td>
<td></td>
</tr>
<tr>
<td>Dp44mT</td>
<td>0.006 ± 0.003</td>
<td>0.01 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>DFO</td>
<td>15 ± 2</td>
<td>10 ± 1</td>
<td></td>
</tr>
<tr>
<td>Deferasirox</td>
<td>12 ± 1</td>
<td>14 ± 2</td>
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</table>
Hence, we carefully assessed tissue sections for evidence that these effects were partly reversible (Yu et al., 2011a). Additionally, another study demonstrated that the chelator Bp44mT induced cytoplasmic vacuolation in the liver of mice, thereby increasing the weight of the organ, although use of a recovery group of mice showed no evidence of necrosis or fibrosis in the livers of deferasirox-treated mice compared with control hearts (Supplementary Fig. 1). All other H&E-stained sections, including those from the kidney, lung, and brain, appeared normal (data not shown). These data agreed with the lack of change in the weights of these organs in deferasirox-treated and control groups (Table 2).

**Total Tissue Iron, Copper, and Zinc Levels.** Although deferasirox binds ferric iron with high affinity and selectivity, its ability to transiently lower plasma copper and zinc has been noted (Steinhauser et al., 2004). Hence, we quantified all three of these metals using inductively coupled plasma atomic emission spectrometry in liver, kidney, and tumor tissue excised from deferasirox- and vehicle control–treated mice. No significant ($P > 0.05$) difference in iron levels between the deferasirox and control groups was observed in the liver, kidney, or tumor (Supplementary Table 1). However, deferasirox at both 20 and 40 mg/kg significantly ($P < 0.05$) increased copper levels in the kidney relative to untreated controls (Supplementary Table 1). Copper content was also significantly ($P < 0.05$) elevated in tumors from the group treated with 40 mg/kg deferasirox (Supplementary Table 1). However, copper levels in the livers of deferasirox-treated mice were similar to those of the control group.

In terms of zinc concentration, there were no significant ($P > 0.05$) changes in the livers or kidneys of deferasirox-treated mice relative to the control (Supplementary Table 1). However, zinc levels in tumors from the group treated with 20 mg/kg deferasirox were significantly ($P < 0.05$) lower than the control group. Although zinc levels were also lower in the group treated with 40 mg/kg deferasirox, they were not significantly ($P > 0.05$) lower than the control level (Supplementary Table 1).

**Deferasirox Regulates the Expression of the Iron-Regulated Proteins, TfR1 and NDRG1, and Proteins Involved in Cell Cycle Control, Namely p21$^{CIP1/WAF1}$ and Cyclin D1.** Iron is involved in cell cycle progression by modulating the expression of molecules involved in cell cycle control, including p21$^{CIP1/WAF1}$ and cyclin D1 (Fu and Richardson, 2007; Nurtjahja-Tjendraputra et al., 2007). In addition, iron depletion using potent chelators can also upregulate the expression of the well known metastasis and growth suppressor, NDRG1 (Le and Richardson, 2004; Kovacevic and Richardson, 2006). Recently, it has been shown that NDRG1 can augment the expression of p21$^{CIP1/WAF1}$ in a variety of cancer cell types (Kovacevic et al., 2011a), which...
therefore negatively regulates the cell cycle leading to a G₁/S arrest (Harper et al., 1993). Hence, studies were initiated to assess the effects of deferasirox on the expression of these critical regulators of the cell cycle in comparison with DFO and Dp44mT. As a positive control for cellular iron depletion, we also examined the expression of the TR1, which is upregulated under these conditions (Hentze and Kuhn, 1996).

In these studies, cells were incubated with deferasirox (25, 50, and 250 μM), Dp44mT (5 and 10 μM), or DFO (25, 50, and 250 μM) for 24 hours at 37°C. Notably, lower concentrations of Dp44mT were used, as this ligand shows far greater cytotoxic activity than DFO or deferasirox (Fig. 2; Table 1). In agreement with previous findings, TR1 expression was shown to increase significantly ($P < 0.05$) as compared with untreated control cells for all chelator treatments in both cell types, except for DFO at 25 μM in DMS-53 cells (Fig. 5, A and B). This effect is likely a compensatory response to the depletion of iron that is caused by these agents (Kwok and Richardson, 2002).

Assessing total NDRG1 protein expression after incubation with chelators, two bands were observed in DMS-53 cells migrating at $\sim 43$ and 44 kDa (Fig. 5C), which was similar to that previously reported in pancreatic cancer cells (Kovacevic et al., 2011b). However, depending on the chelator and its concentration, two to three NDRG1 bands were observed in SK-N-MC cells, with two bands migrating at $\sim 43$ and 44 kDa and a third band migrating at $\sim 45$ kDa (Fig. 5D). After incubation with any of the three chelators, there was generally a dose-dependent increase in the $\sim 44$-kDa band relative to the control, whereas for SK-N-MC cells, incubation with 250 μM DFO or 5 and 10 μM Dp44mT also led to the appearance of a third NDRG1 band at $\sim 45$ kDa (Fig. 5D). This third NDRG1 band could also be observed in cells incubated with deferasirox (250 μM) after overexposure of the blots (data not shown). It has been suggested that these bands may represent different phosphorylation states of the NDRG1 protein, which may play a role in its antitumor activity (Murakami et al., 2010; Kovacevic et al., 2011b). Therefore, the upregulation of NDRG1 may, in part, account for the antineoplastic efficacy of these chelators. In addition, our results demonstrate that treating both cell lines with these chelators increased the expression level of p21CIP1/WAF1 in a dose-dependent manner compared with control cells (Fig. 6, A and B).

Cyclin D1 levels have previously been demonstrated to significantly decrease in different cancer cell types when treated with iron chelators (Nurtjahja-Tjendraputra et al., 2011). Therefore, we also examined the expression of the Cyclin D1 in our cell lines. As shown in Fig. 6C, the expression of Cyclin D1 was decreased in both cell lines after treatment with deferasirox or DFO.

### Table 2

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Vehicle Control</th>
<th>Deferasirox 20 mg/kg</th>
<th>Deferasirox 40 mg/kg</th>
</tr>
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<tbody>
<tr>
<td>Body weight (% initial)</td>
<td>112 ± 2</td>
<td>108 ± 4</td>
<td>116 ± 4</td>
</tr>
<tr>
<td>Tumor (g)</td>
<td>1.3 ± 0.1</td>
<td>0.5 ± 0.2*</td>
<td>0.6 ± 0.2*</td>
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<tr>
<td>Liver (g)</td>
<td>0.86 ± 0.02</td>
<td>0.89 ± 0.03*</td>
<td>0.86 ± 0.03</td>
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<td>Spleen (g)</td>
<td>0.06 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.06 ± 0.00</td>
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<td>Kidney (g)</td>
<td>0.21 ± 0.01</td>
<td>0.21 ± 0.01</td>
<td>0.18 ± 0.03</td>
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<td>Heart (g)</td>
<td>0.081 ± 0.004</td>
<td>0.087 ± 0.003</td>
<td>0.083 ± 0.004</td>
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<td>Brain (g)</td>
<td>0.30 ± 0.02</td>
<td>0.30 ± 0.02</td>
<td>0.31 ± 0.02</td>
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<tr>
<td>Lung (g)</td>
<td>0.10 ± 0.01</td>
<td>0.11 ± 0.01</td>
<td>0.11 ± 0.01</td>
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* $P < 0.01$ versus control.

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**Fig. 4.** Orally administered deferasirox markedly inhibits the growth of human lung carcinoma xenografts in nude mice. (A) Deferasirox (20 and 40 mg/kg orally, given by gavage every second day, three treatments per week for 18 days) effectively inhibited the growth of human lung carcinoma DMS-53 tumor xenografts in vivo. (B) Average weights of excised tumors postnecroscopy from mice treated with 20 and 40 mg/kg deferasirox and mice treated with the vehicle control. For statistical analysis, each treatment was compared with the vehicle control. **$P < 0.01$ versus control.
In agreement with these studies, our results also demonstrate that DFO, Dp44mT, and deferasirox were able to significantly ($P < 0.001–0.05$) reduce cyclin D1 expression in both cell lines examined (Fig. 6, C and D).

Collectively, these results demonstrate that deferasirox, as well as Dp44mT and DFO, significantly upregulate the expression of TfR1, NDRG1, and p21$^{CIP1/WAF1}$, while reducing cyclin D1 levels in DMS-53 and SK-N-MC cell lines.

### TABLE 3

| Hematological and serum indices from nude mice bearing a DMS-53 xenograft and treated orally by gavage with either vehicle control or deferasirox (20 or 40 mg/kg every second day, three treatments per week) for 18 days. Values are expressed as the mean ± S.E.M. Statistical analysis was performed using the Student’s $t$ test ($n = 6$ mice per group) comparing each treated group to the respective vehicle control. |

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Units</th>
<th>Vehicle Control</th>
<th>Deferasirox 20 mg/kg</th>
<th>Deferasirox 40 mg/kg</th>
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<tr>
<td><strong>Hematologic indices</strong></td>
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<tr>
<td>Red blood cells</td>
<td>$10^{12}$/l</td>
<td>10.7 ± 0.2</td>
<td>10.3 ± 0.2</td>
<td>10.5 ± 0.2</td>
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<tr>
<td>White blood cells</td>
<td>$10^{9}$/l</td>
<td>3.5 ± 0.5</td>
<td>3.5 ± 0.5</td>
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<td>Hemoglobin</td>
<td>g/l</td>
<td>158 ± 1</td>
<td>151 ± 3</td>
<td>155 ± 2</td>
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<td>Hematocrit</td>
<td>%</td>
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<td>0.44 ± 0.01</td>
<td>0.45 ± 0.35</td>
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<tr>
<td>Platelets</td>
<td>$10^{9}$/l</td>
<td>740 ± 126</td>
<td>822 ± 104</td>
<td>969 ± 65</td>
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<td><strong>Serum biochemical indices</strong></td>
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<td>Serum iron</td>
<td>$\mu$M</td>
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<td>32 ± 1</td>
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<td>Total iron-binding capacity</td>
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<td>57 ± 2</td>
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<td>$\mu$M</td>
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<td>Alkaline phosphatase</td>
<td>U/l</td>
<td>78 ± 6</td>
<td>95 ± 10</td>
<td>93 ± 5</td>
</tr>
<tr>
<td>Alanine aminotransferase</td>
<td>U/l</td>
<td>58 ± 8</td>
<td>43 ± 5</td>
<td>43 ± 4</td>
</tr>
<tr>
<td>Albumin</td>
<td>g/l</td>
<td>31.6 ± 0.8</td>
<td>30.3 ± 0.6</td>
<td>30.4 ± 0.3</td>
</tr>
<tr>
<td>Total protein</td>
<td>g/l</td>
<td>53 ± 1</td>
<td>57 ± 3</td>
<td>53 ± 1</td>
</tr>
</tbody>
</table>

Fig. 5. The effect of deferasirox relative to DFO or Dp44mT on the expression of (A and B) TfR1 and (C and D) NDRG1 in DMS-53 or SK-N-MC cells. Cells were incubated for 24 hours at 37°C with DFO (25, 50, and 250 $\mu$M), Dp44mT (5 and 10 $\mu$M), or deferasirox (25, 50, and 250 $\mu$M), and then the expression levels of TfR1 and NDRG1 were examined using Western blotting. The gel photographs are representative of three to six experiments, and the densitometric analysis is the mean ± S.E.M. ($n = 3$–6 experiments). For statistical analysis, each treatment was compared with the untreated control. *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$ versus control.
Deferasirox, as well as Dp44mT and DFO, Induces Apoptosis in the DMS-53 Lung Carcinoma and the SK-N-MC Neuroepithelioma Cell Lines. Several studies have demonstrated that the iron chelators DFO and Dp44mT can induce apoptosis in a variety of cancer cell lines (Richardson and Milnes, 1997; Yuan et al., 2004). Although the mechanisms by which this occurs are not completely understood, DFO has been reported to increase the activity of caspases-3, -8, and -9 (Brard et al., 2006; Wang et al., 2006), whereas Dp44mT has been shown to increase caspase-3 activity (Yuan et al., 2004), thereby activating apoptosis via the mitochondrial/intrinsic pathway. Recent studies have also revealed that deferasirox induces apoptosis in myeloid leukemia cells by targeting caspases (Kim et al., 2011). Iron chelators have also been shown to increase the expression of PARP1, which plays a role in DNA damage detection, repair, and cell death pathways (Tang and Porter, 1996; Greene et al., 2002; Kovacevic et al., 2011b).

Considering this, we examined the effect of iron chelators on apoptosis by examining the expression levels of the apoptosis markers, cleaved PARP1 (at ∼90 kDa) and cleaved caspase-3 (at ∼17 and 19 kDa), in DMS-53 and SK-N-MC solid tumor cells following incubation with either deferasirox or DFO (25, 50, and 250 μM) and Dp44mT (5 and 10 μM), or deferasirox (25, 50, and 250 μM), and then the expression levels of p21<sup>CIP1/WAF1</sup> and cyclin D1 were examined using Western blotting. The gel photographs are representative of three to six experiments, and the densitometric analysis is the mean ± S.E.M. (n = 3–6 experiments). For statistical analysis, each treatment was compared with the untreated control. *P < 0.05; **P < 0.01; and ***P < 0.001 versus control.

**Discussion**

Iron chelators currently approved for clinical use, e.g., DFO and deferiprone, have generally demonstrated limited efficacy against tumors in vivo (Merlot et al., 2012). For DFO, this is likely due to its hydrophobic nature, short plasma half-life,
and rapid metabolism (Olivieri and Brittenham, 1997). To date, the reported antitumor activity of deferasirox has been limited to leukemia cell models and a hepatoma cell line in vitro (Lescoat et al., 2007; Ohyashiki et al., 2009). Herein, we investigated, for the first time, the in vivo activity of deferasirox against solid human tumor xenografts. We also assessed the in vitro efficacy of deferasirox and the molecular mechanism of action underlying its antiproliferative effects relative to other well described ligands.

We observed that the antiproliferative activity of deferasirox was similar to DFO in DMS-53 cells and slightly lower compared with DFO in SK-N-MC cells (Fig. 2; Table 1). Considering the link between antiproliferative activity and iron chelation, we also examined the ability of deferasirox to remove iron from cells and prevent iron uptake from Tf. We found that deferasirox was generally similar or slightly more effective than DFO at mobilizing $^{59}$Fe depending on the cell type, and that both of these chelators were far less effective than the highly cytotoxic chelator Dp44mT (Fig. 3). Similarly, deferasirox and DFO showed similar efficacy at inhibiting iron uptake from Tf, but again, were far less effective than Dp44mT. Therefore, the ability of deferasirox to inhibit solid tumor cell growth in vitro may be due, at least in part, to its ability to bind and mobilize cellular iron that is vital for replication.

Given the in vitro antiproliferative activity of deferasirox and its efficacy at chelating cellular iron, we then examined its activity on the growth of tumor xenografts in nude mice. Studies in vivo found a marked inhibition of DMS-53 tumor xenograft growth in mice treated with 20 and 40 mg/kg deferasirox by oral gavage after 18 days (Fig. 4, A and B). Considering the high efficacy of deferasirox against DMS-53 xenografts, it was important to examine any potential toxic side effects. By the last day of treatment, we noted no significant loss of body weight (Fig. 4C), nor significant alterations to white or red blood cell count, hemoglobin, hematocrit, or platelet count (Table 3). Additionally, no significant changes in biochemical indices were observed, suggesting that oral deferasirox was well tolerated.

In terms of the antitumor effect observed in vivo, there was no significant decrease in tumor iron levels, which correlated with the lack of alteration in hematologic and serum biochemical indices at the deferasirox doses implemented. Previous studies have reported that deferasirox at similar
doses [i.e., 30 mg/kg per day for 8 weeks (Nick et al., 2009) or 10 mg/kg twice every day or every other day for 7 days (Ibrahim et al., 2007)] also did not significantly alter tissue iron levels, hematologic indices, or serum biochemical indices in mice. The observations in our study showing little effect on normal tissue or tumor iron levels could be related to the low doses of the chelator (20 and 40 mg/kg) and the short treatment duration (18 days). Considering the lack of a significant effect of deferasirox on tumor iron levels, the marked effect on tumor growth was surprising. This may be explained by the formation of the deferasirox iron complex within the tumor that is not effluxed because of its increased hydrophobicity. The formation of an intracellular iron complex with deferasirox would prevent the utilization of iron for tumor cell proliferation. However, this explanation does not correlate with the effect of deferasirox in cell culture, where it induced iron efflux and inhibited iron uptake (Fig. 3). This may potentially reflect the well known differences in the tumor cell microenvironment in vitro and in vivo. Similar observations have also been reported for thiosemicarbazone chelators in vivo, where substantially lower doses markedly inhibited tumor growth, but had little effect on tumor iron levels (Whitnall et al., 2006; Yu et al., 2011a). For these latter ligands, this effect could lead to tumor cytotoxicity due to the redox activity of these complexes (Whitnall et al., 2006; Yu et al., 2011a). However, this could not explain the antitumor efficacy of deferasirox, as its iron complex is not redox-active (Bendová et al., 2010; Hašková et al., 2011).

Another important outcome of the current study was that oral deferasirox demonstrated no significant histopathology in major organs. We observed no cytoplasmic vacuolation of the liver or evidence of necrosis or fibrosis, as well as no cardiac fibrosis (Supplementary Fig. 1). This finding agreed with Perls’ stained spleen sections, which indicated no alteration in hemosiderin levels. Further, we quantified total tissue iron, copper, and zinc levels in liver, kidney, and tumor tissue and found no significant difference in iron levels between mice treated with deferasirox and those treated with the vehicle. Hence, deferasirox did not affect normal systemic or tumor iron metabolism. However, deferasirox increased kidney copper levels at both 20 and 40 mg/kg, and at the highest dose, there was almost a 2-fold increase in tumor copper. In contrast, deferasirox had no effect on zinc levels in normal tissues, but reduced zinc in the tumor at both doses, and this was significant at 20 mg/kg (Supplementary Table 1). The significant increase in copper in the kidney upon deferasirox treatment may reflect filtration of the copper-deferasirox complex and subsequent trapping within the organ, potentially due to the greater lipophilicity of the complex. The increase in copper, but decrease in zinc, in the tumor in the absence of alterations in iron levels is intriguing. These observations could reflect perturbations in metal metabolism mediated by deferasirox that could inhibit tumor growth. Indeed, similar to iron, zinc is also necessary for proliferation (Merlot et al., 2012), and its depletion could lead to the decreased tumor growth in vivo after deferasirox treatment.

To better characterize the mechanisms involved in the antiproliferative activity of deferasirox, we investigated its effects on key molecules involved in growth, metastasis, cell cycle regulation, and apoptosis. Deferasirox was observed to significantly upregulate the metastasis suppressor NDRG1 in both cell types (Fig. 5, C and D). This observation is in good agreement with findings from other studies involving other iron chelators against a variety of cancer cell types (Le and Richardson, 2004; Whitnall et al., 2006).

We recently demonstrated that NDRG1 is able to upregulate the cyclin-dependent kinase inhibitor p21CIP1/WAF1 (Kovacevic et al., 2011b), which may be important for its tumor-suppressive functions. Here, we further demonstrated that deferasirox and the other chelators also augmented p21CIP1/WAF1 expression in DMS-53 lung carcinoma and SK-N-MC neuroepithelioma cells, which may be mediated, in part, by NDRG1 upregulation. Classically, p21CIP1/WAF1 overexpression leads to G0/S arrest due to its ability to act as a cyclin-dependent kinase inhibitor (Yu et al., 2007). However, the effect of iron chelators on p21CIP1/WAF1 expression appears to be cell-type–specific or dependent on the ligand used (Fu and Richardson, 2007).

We also investigated the expression of the cell cycle regulatory molecule cyclin D1 following treatment with iron chelators (Yu et al., 2007). Both Dp44mT and DFO significantly reduced cyclin D1 levels in DMS-53 and SK-N-MC cells (Fig. 6, C and D), as observed previously (Gao and Richardson, 2001; Nurtjahja-Tjendraputra et al., 2007). Similarly, we also showed that deferasirox was able to significantly decrease cyclin D1 levels. It is notable that in a number of cancer cell types, overexpression of cyclin D1 is correlated with poor patient survival (Kornmann et al., 1998; Mishina et al., 1999). Consequently, the ability of iron chelators, including deferasirox, to reduce cyclin D1 levels could be important for their antiproliferative effects.

Considering the alterations in the expression of proteins involved in cell metabolism and cell cycle control, we then investigated the effect of deferasirox on molecules involved in mediating apoptosis, namely, cleaved PARP1 and cleaved caspase-3. We demonstrated that deferasirox was able to significantly induce cleavage of PARP1 and caspase-3 in DMS-53 and SK-N-MC cells. These observations in cells from solid tumors agree with similar results in leukemia cells (Kim et al., 2011). These findings indicate that this agent is able to induce apoptosis, further supporting the potential of deferasirox as an agent for cancer treatment. Finally, because the combination of chelators with current chemotherapeutics can significantly enhance cytotoxicity (Messa et al., 2010; Lovejoy et al., 2012), the potential for deferasirox to be used in combination with established cytotoxic drugs is also promising.

In summary, this study is the first to examine the in vivo antitumor activity of deferasirox against human cancer cells from solid tumors. Importantly, we observed that deferasirox inhibited the growth of DMS-53 tumor xenografts, while having no significant toxic side effects. We also demonstrated that deferasirox displayed antiproliferative effects in vitro in DMS-53 and SK-N-MC cells. At the molecular level, deferasirox upregulated the expression of NDRG1 and p21CIP1/WAF1 and downregulated cyclin D1, which are key molecules involved in tumor growth, metastasis, and cell cycle control. Moreover, deferasirox also upregulated the expression of the apoptosis markers, cleaved PARP1 and cleaved caspase-3. Collectively, these results indicate that deferasirox is an effective and selective iron chelator with potential as an orally active chemotherapeutic.
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Authorship Contributions

Participated in research design: Lui, Obeidey, Ford, Tselepis, Jansson, Kovacevic, Richardson, Lovejoy.

Conducted experiments: Lui, Obeidey, Sharp.

Contributed new reagents or analytic tools: Obeidey, Lovejoy, Richardson.

Performed data analysis: Lui, Obeidey, Kovacevic, Lovejoy, Richardson.

Wrote or contributed to the writing of the manuscript: Lui, Obeidey, Kalinowski, Lovejoy, Kovacevic, Richardson.

References


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