Inhibition of Malignant Cell Growth by 311, a Novel Iron Chelator of the Pyridoxal Isonicotinoyl Hydrazone Class: Effect on the R2 subunit of Ribonucleotide Reductase

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ABSTRACT

The key roles of iron and iron proteins in cell proliferation make them potential targets for cancer therapy. However, clinical trials directed toward perturbation of tumor iron homeostasis by iron chelation have been limited to the use of deferoxamine (DFO). There is thus a need to develop agents with greater efficacy. In the present study, we investigated the mechanism of cytotoxicity of 311 (2-hydroxy-1-naphthylaldehyde benzoyl hydrazone), a novel iron chelator of the pyridoxal isonicotinoyl class. We found that 311 inhibited the growth of CCRF-CEM cells in a time- and concentration-dependent fashion with an IC50 that was ~20-fold lower than that of DFO. 311 also inhibited the growth of mast, bladder, and head and neck cancer cell lines. Using electron spin resonance (ESR) spectroscopy analysis, we found that a 12-h exposure of CCRF-CEM cells to 311 inhibited the tyrosyl radical ESR signal of the R2 subunit of ribonucleotide reductase. However, overproduction of the R2 subunit in hydroxyurea-resistant CCRF-CEM cells was associated with a decrease in sensitivity of cells to 311 but not to DFO. Our studies show that 311 is a more potent cytotoxic agent than DFO, with activity against both hematopoietic and nonhematopoietic cell lines regardless of their p53 status. Furthermore, the ESR studies suggest that inhibition of the R2 subunit of ribonucleotide reductase is at least one mechanism by which 311 blocks cell proliferation.

INTRODUCTION

Iron is essential for cell viability and proliferation. Because both iron excess and iron deprivation have deleterious effects, iron homeostasis within cells is highly regulated by proteins responsible for iron uptake (transferrin, transferrin receptor) and storage (ferritin; Ref. 1). Transferrin receptor and ferritin synthes, in turn, are regulated by the interaction their respective mRNAs with cytoplasmic iron regulatory proteins (2).

Whereas iron is required for a variety of cellular functions, including the Krebs cycle and oxidative phosphorylation in the mitochondria, the importance of iron in cell division is related to the iron-dependent activity of RR,2 the enzyme responsible for the synthesis of deoxyribonucleotides (3, 4). RR consists of two protein subunits, termed R1 and R2. The R1 subunit contains effector and substrate binding sites, whereas the R2 subunit contains nonheme iron and a tyrosyl free radical that gives a characteristic signal on ESR spectroscopy (5). The activity of the tyrosyl radical has been shown to increase 3–7-fold when synchronized mouse mammary tumor cells pass from G1 to the S phase of the cell cycle (6, 7). The presence of iron is essential for R2 function, and a limitation of iron availability for R2 leads to a loss of its ESR signal and RR enzyme activity (5, 8). More recent studies have identified a p53-inducible protein with 80% homology to R2 (termed p53-R2) that appears to be important in the repair of DNA damage caused by various genotoxins (9, 10).

The critical roles of iron and iron proteins in cell proliferation make them potential targets for cancer therapy. Indeed, disruption of cellular iron homeostasis by antitransferrin receptor antibodies, gallium complexes, or iron chelators inhibits the growth of malignant cells in vitro and in vivo (11–19). Iron chelators such as DFO, parabactin, and hydroxypyridones have been shown to inhibit RR by limiting iron availability for the activity of the R2 subunit, presumably by chelating an intracellular iron pool (20, 21). However, although many iron chelators have been shown to inhibit the proliferation of tumor cells in vitro (16–19, 22, 23), the number of iron chelators that have actually been tested in clinical trials for cancer treatment is extremely limited. DFO is the only iron chelator presently available for clinical use and trials, but the use of DFO for treatment of neuroblastoma in patients has led to conflicting results (24, 25). Interestingly, however, whereas DFO failed to inhibit tumor growth in human neuroblastoma xenografts in nude mice (26), it inhibited the growth of mammary adenocarcinoma in an iron-deficient rat (27). Such studies suggest that the impact of iron deprivation on tumor growth may differ in tumors of different lineages and that there is a need to explore and develop new agents that can inhibit tumor growth by targeting iron homeostasis.

Recently, several analogues of the PIH class have been...
shown be potent iron chelators and inhibitors of malignant cell proliferation (18, 28, 29). One of the more lipophilic PIH analogues, 2-hydroxy-1-naphthylaldehyde benzoyl hydrazone (termed 311), was shown to be particularly promising in its ability to inhibit cellular proliferation when compared with PIH and DFO (29). Exposure of cells to 311 results in apoptosis and is accompanied by an increase in GADD45 and WAF1 mRNA levels (29); however, the mechanism of cytotoxicity of 311 has not been elucidated.

In an attempt to understand the mechanism of action of 311, we examined its effects on the proliferation of different malignant cell lines and on RR in human leukemic CCRF-CEM cells. Our studies show that 311 inhibits the growth of hematopoietic and nonhematopoietic cell lines regardless of their p53 status and that it decreases the activity of the R2 subunit of RR.

MATERIALS AND METHODS

Materials. The iron chelator 311 was synthesized as described previously (29). 311 was made up as a 10 mM stock solution in DMSO. Further dilutions of this stock were made in RPMI 1640 containing 10% FCS prior to use in experiments. DFO and hydroxyurea were purchased from CIBA-Geigy Pharmaceutical Co. (Summit, NJ) and Calbiochem (La Jolla, CA), respectively. MTT was obtained from Sigma Chemical Co. (St. Louis, MO).

Cells. CCRF-CEM (T-lymphoblastic leukemia), MCF-7 (breast cancer), J82 (bladder cancer), and A253 and FaDu (head and neck cancer) cell lines were obtained from American Type Culture Collection (Manassas, VA). A hydroxyurea-resistant line of CCRF-CEM cells that overexpresses the R2 subunit mRNA and protein was developed by exposing wild-type CCRF-CEM cells to incremental concentrations of hydroxyurea. Hydroxyurea-resistant subclones (HU-R cells) were selected by serial dilution and were maintained continuously in culture in the presence of 90 μM hydroxyurea. We have described these cells previously (30). Cells were grown in RPMI 1640 (CCRF-CEM, MCF-7, and J82 cells), MEM-α (A253 cells) or McCoy’s Medium (FaDu cells; Life Technologies, Inc., Grand Island, NY) supplemented with 10% (v/v) FCS in an atmosphere of 6% CO₂ at 37°C.

Cell Growth and Dose-Response Assays. The effects of DFO or 311 on the proliferation of the various cell lines were measured by MTT assay as described by Mosmann (31). Briefly, cells were plated at 0.2 × 10⁶ cells/ml in 96-well plates (100 μl/well) with various concentrations of the iron chelators and were incubated at 37°C for 24–120 h. At the end of incubation, 10 μl of MTT (5 mg/ml stock) were added to each well, and the incubation was continued at 37°C for an additional 4 h. One hundred μl of 0.04 N HCl in isopropyl alcohol were then added to each well to solubilize the cells, and the absorbance of each well was determined by spectrophotometry at the dual wavelengths 570 and 630 nm in an EL-310 microplate autoreader (Biotech Instruments, Winooski, VT). The effects of DFO or 311 on cell growth were determined by comparing the absorbance of the respective wells with that of wells in which additives were omitted (cells only; controls). The IC₅₀ was defined as the concentration of the agent that reduced cell proliferation by 50% relative to the untreated (control) cells.

RESULTS

Analysis of RR by ESR Spectroscopy. ESR studies of the tyrosyl free radical of the R2 subunit of RR were performed at the National Biomedical ESR Center at the Medical College of Wisconsin as we reported previously (32). X-Band ESR spectra were obtained by a standard Century series Varian E-100 spectrophotometer operating at X-band (9–9.5 GHz) with 100 KHz field modulation. ESR measurements were made on frozen intact cells. In preparation for ESR, CCRF-CEM cells were expanded in RPMI 1640 containing 10% FCS. Cells (10⁶ cells/ml) were incubated at 37°C for 12–24 h in the presence of 311 (0.7 μM) or DFO (30 μM) or in the absence of these additives. At the specified times, 9.0 × 10⁶ cells/sample were harvested and rapidly frozen at −196°C in quartz finger Dewar flasks. ESR measurements on frozen cells were then made as reported previously (32). Spectra from each sample were recorded 9–16 times and averaged by computer.
with 311 or DFO for the respective period was replated in fresh medium with the corresponding chelator. As shown in Fig. 3, removal of DFO or 311 from the medium after 6 or 24 h of incubation resulted in complete or near-complete recovery of proliferation. In contrast, after 48 h of incubation with DFO or 311, cell growth recovered to 40% of control (untreated cells) over the subsequent 48 h of incubation.

**Effects of 311 on Proliferation of Malignant Cell Lines with Different p53 Status.** The effects of 311 on the growth of selected nonhematopoietic tumor cell lines was also examined. As shown in Fig. 4, chelator 311 inhibited the proliferation of MCF-7 (breast cancer), J82 (bladder cancer), and A253 and FaDu (head and neck cancer) cell lines in a dose-dependent manner, indicating that its activity is not limited to hematopoietic cells. The growth-inhibitory activity of 311 was further analyzed in the context of p53, a tumor suppressor gene whose product plays a central role in the activation of cell cycle arrest and apoptosis (33, 34). Information regarding the p53 status of these cell lines has been reported previously (35–38). Differences in p53 expression or function could potentially impact on the effects of 311 in various cells if its cytotoxicity is mediated through p53. However, as shown in Table 1, the IC_{50} for 311 in cells expressing mutated or no p53 (null) ranged from being lower to higher than that in MCF-7 cells with wild-type p53. These results suggest that no correlation exists between growth inhibition by 311 and the expression of p53 (wild-type or mutant) and that 311 acts through a pathway that is independent of this protein.

**Effects of DFO and 311 on the R2 Subunit of RR.** The amplitude of the ESR signal of the R2 subunit of RR corresponds closely with RR enzyme activity (6, 7). The effects of 30 μM DFO or 0.7 μM 311 (growth-inhibitory concentrations) were examined in intact cells by ESR spectroscopy. In these experiments, cells were incubated without (control) or with the respective chelator for 12–24 h and then harvested and packed by centrifugation in a Dewar flask for measurement of the ESR signal by ESR spectroscopy. As shown in Fig. 5, both chelators produced a marked reduction in the amplitude (Fig. 5, vertical arrows) of the ESR signal of the R2 subunit compared with untreated control cells. After 24 h of incubation, the ESR signal was diminished by ~48% in DFO-treated cells and by ~58% in 311-treated cells relative to their corresponding controls (Fig. 5). Importantly, incubation of cells with 311 for 12 h produced a decrease in the ESR signal similar to that seen with the 24-h exposure, indicating that a relatively brief exposure of cells to 311 is sufficient to inhibit RR activity (Fig. 5B).

**Differential Effects of DFO and 311 on R2-overproducing HU-R Cells.** The hydroxyurea-resistant, R2-overproducing CCRF-CEM cell line described previously by us is an important tool for investigating the action of agents on the components of RR (30). As shown in Fig. 6A, the IC_{50} of hydroxyurea was increased ~6-fold for HU-R cells compared with wild-type CCRF-CEM cells. Because both DFO and 311 inhibited the ESR signal of the R2 subunit, we examined whether HU-R cells would display similar sensitivity to both iron chelators. As shown in Fig. 6B, HU-R and wild-type CCRF-CEM displayed relatively similar dose-response curves to DFO with 10–20% maximum differences between the two concentrations.
curves. In contrast, HU-R cells were markedly less sensitive to 311 than wild-type cells: 35–41% differences between the dose-response curves were seen with 311 concentrations of 0.3–0.5 μM (Fig. 6C).

**DISCUSSION**

Cellular iron deprivation, whether produced by removal of iron from the environment or by the use of iron chelators, leads to inhibition of cell proliferation and apoptosis in a variety of different cell types (39–41). Whereas the steps by which disruption of iron homeostasis induces apoptosis have not been elucidated, prior studies have indicated that one consequence of cellular iron deprivation is the inhibition of deoxyribonucleotide synthesis resulting from blockade of RR activity (17, 20, 39, 42). In the present study, we show that the chelator 311 is a potent inhibitor of the proliferation of malignant cells of different lineages. The demonstration that 311 decreases the ESR signal of the R2 subunit of RR in CCRF-CEM cells, similar to the reduction seen with the iron chelator DFO, strongly suggests that one mechanism by which 311 exerts its cytotoxicity is the inactivation of the iron-dependent R2 subunit of RR.

Comparison of the dose-response curves for DFO and 311 provides additional insights into factors important for the cytotoxicity of different iron chelators. The IC₅₀ for 311 is ~20-fold lower than that for DFO, indicating that 311 is a far more potent inhibitor of CCRF-CEM cell proliferation than is DFO. Assuming that both DFO and 311 act on the same intracellular iron pool, an important determinant of efficacy appears to be the ability of either agent to effectively enter the cell and perturb this iron pool. Because 311 is a lipophilic chelator (whereas

![Fig. 4 Effects of 311 on the proliferation of solid tumor cell lines. Cell lines representing breast (MCF-7), bladder (J82), and head and neck (A253 and FaDu) cancers were incubated with increasing concentrations of 311. Cell growth was determined by MTT assay after a 3-day (MCF-7 cells) or 5-day (other cell lines) incubation. Values shown represent means ± SE (bars) of representative experiments performed in quadruplicate.](image)

![Fig. 5 Inhibition of the RR R2 subunit ESR signal by DFO and 311. ESR studies were performed on frozen intact cells after a 12- or 24-h incubation with or without the chelator. A, cells incubated without (control) or with 30 μM DFO for 24 h. B, cells incubated without (control) or with 0.7 μM 311 for 12 or 24 h. The amplitudes of the individual ESR signals from cells are represented by the vertical double-ended arrows drawn at the left of each spectrum. Note that the amplitude of the ESR signal for chelator-treated cells is diminished compared with controls, signifying a decrease in the activity of the R2 subunit of RR.](image)

### Table 1  Lack of correlation between p53 status and cell sensitivity to 311

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Lineage</th>
<th>IC₅₀a (μM)</th>
<th>p53 status (Reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCRF-CEM</td>
<td>T-lymphoblastic leukemia</td>
<td>0.28</td>
<td>Mutated (35)</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Breast cancer</td>
<td>0.65</td>
<td>Wild-type (36)</td>
</tr>
<tr>
<td>J82</td>
<td>Bladder cancer</td>
<td>1.3</td>
<td>Mutated (37)</td>
</tr>
<tr>
<td>A253</td>
<td>Head and neck cancer</td>
<td>2.4</td>
<td>Null (38)</td>
</tr>
<tr>
<td>FaDu</td>
<td>Head and neck cancer</td>
<td>2.3</td>
<td>Mutated (38)</td>
</tr>
</tbody>
</table>

*IC₅₀ were determined from the dose-response curves shown in Figs. 2 and 4.*
DFO is a hydrophilic chelator), its greater cytotoxicity may relate, in part, to its ability to cross membranes, penetrate cells, and chelate the intracellular iron pool more rapidly than DFO. This mechanism is supported by an earlier study in which 311 was shown to increase the release of iron from BE-2 neuroblastoma and SK-N-MC melanoma cells to a greater extent than an equimolar concentration of DFO (29).

Although both DFO and 311 inhibit the R2 subunit, they differ in their ability to inhibit cell growth when the R2 subunit is overexpressed in HU-R cells. DFO inhibited the growth of HU-R and wild-type cells to a similar extent, whereas 311 inhibited the growth of HU-R cells to a lesser extent than wild-type cells. A possible explanation for this finding is that although both 311 and DFO are iron chelators, they may not be similar with respect to their action on intracellular targets. That overexpression of the R2 subunit in CCRF-CEM cells results in greater resistance to 311 than to DFO raises the possibility that 311 may target the R2 subunit to a greater extent than does DFO. However, these experiments provide only indirect evidence supporting this possibility, and confirmation of potential differences in the relative potencies of 311 and DFO against RR will require more detailed studies of enzyme kinetics in cell-free assays. Nonetheless, a potential important implication of the observed differences between 311 and DFO in HU-R versus wild-type cells is that iron chelators may differ with respect to their predominant mechanisms of cytotoxic action. Hence, observations regarding the potential antineoplastic efficacy of a given iron chelator cannot be readily extrapolated to another chelator.

Whereas our studies have identified RR as a target for 311, it is becoming increasingly clear that other molecules involved in cell-cycle progression are affected by iron chelators. Indeed, a comprehensive analysis by Gao and Richardson (46) has recently shown that incubation of cells with DFO or 311 produces a decrease in the phosphorylated form of the retinoblastoma susceptibility gene product (pRb); a decrease in the expression of cyclins D1, D2, D3, A, and B1; a decrease in cyclin-dependent kinase 4; and an increase in WAF1 and GADD45 mRNA. Because RR is a key rate-limiting enzyme for DNA synthesis (3), it remains an important target for the action of iron chelators; however, its inhibition is likely to be one of several steps involved in the arrest of cell proliferation produced by iron chelators. Furthermore, it is likely that cellular targets for iron chelators may vary among cells of different lineages, depending on inherent differences in iron metabolism.

In addition to 311, other chelators, such as Tachpyr, O-Trenso, and Desferri-exochelin, that display potent antiproliferative activity against a variety of malignant cell types have recently been developed (19, 22, 23). Collectively, these agents represent a group of novel iron chelators that warrant further critical evaluation of their effects on other cell-regulatory molecules and on tumor growth in animal models. In the past decade, there have been tremendous strides in our understanding of cellular iron metabolism with the discovery of novel proteins responsible for maintaining iron homeostasis in cells (47). The continued development of new agents with enhanced specificity for critical iron-dependent steps in tumor growth is clearly warranted because it will, undoubtedly, increase our armamentarium in cancer therapy.

**REFERENCES**


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