The effect of potent iron chelators on the regulation of p53: examination of the expression, localization and DNA-binding activity of p53 and the transactivation of WAF1

S.X. Liang and D.R. Richardson

Children’s Cancer Institute Australia for Medical Research, Iron Metabolism and Chelation Program, PO Box 81, High Street, Randwick, Sydney, New South Wales 2031, Australia and The Heart Research Institute, Iron Metabolism and Chelation Group, 1/2 Missenden Road, Camperdown, Sydney, New South Wales 2050, Australia

Iron (Fe) chelators induce a G1/S arrest and several of these are undergoing clinical trials as anticancer agents. Despite this, little is known concerning the precise function of Fe in cell cycle progression and the role of p53 in this process. The aim of this study was to assess the effect of Fe chelators on p53 and the mechanism involved in the chelator-mediated increase in mRNA levels of the universal cyclin-dependent kinase inhibitor p21WAF1. Cells were incubated with the potent Fe chelator 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone (311) and the results compared with those from cells treated with actinomycin D (Act D), which induces p53. Following incubation with 311, a 3- to 5-fold increase in nuclear p53 protein was observed in cells with wild-type p53. In addition, 311 increased p53 DNA-binding activity 2-fold, while Act D increased it 3- to 5-fold in cells with native p53. To determine the role of p53 in WAF1 transcription, a reporter construct was used consisting of a WAF1 promoter containing the p53-binding site. In cells with wild-type p53, chelators had no effect on luciferase activity, while the positive control, Act D, caused a significant increase. Hence, despite increased p53 protein expression and p53 DNA-binding activity following chelation, these latter results suggested it had no role in up-regulating WAF1 mRNA. Our experiments demonstrated: (i) that the elevated WAF1 mRNA expression after Fe chelation was due to increased transcription and also to a post-transcriptional mechanism that was sensitive to cycloheximide; and (ii) that Fe-chelation increased WAF1 expression through a p53-independent pathway.

Introduction

Iron (Fe) plays a key role in many metabolic reactions, including the rate limiting step in DNA synthesis that is catalyzed by ribonucleotide reductase (for reviews see 1,2). In the absence of Fe, cells cannot progress from the G1 to the S phase of the cell cycle, which eventually leads to apoptosis (1,2). Therefore, understanding the role of Fe in cell cycle progression and division is essential.

The importance of Fe in tumor cell growth is demonstrated by many in vitro studies in cell culture and in clinical trials (1). These investigations showed that the clinically used Fe chelator desferrioxamine (DFO) (Figure 1) can markedly inhibit the growth of a variety of tumors (1). The ability of DFO and other chelators to prevent tumor growth is probably due to the high Fe requirement of neoplastic cells that is necessary for DNA synthesis (2). Up to the present, most studies in vivo have examined rapidly growing or aggressive neoplasms (e.g. leukemia and neuroblastoma) that could be expected to be affected by Fe deprivation (1,2). The effect of chelators on more slowly growing tumors, e.g. some carcinomas or sarcomas, has not yet been extensively assessed. The fact that studies with DFO and other chelators have demonstrated selective antitumor activity in animal models and clinical trials suggests an exploitable therapeutic window does exist (1,2). However, Fe chelation using DFO suffers from its requirement for long infusions and poor membrane permeability that moderates its efficacy as an antitumor agent (2).

Apart from DFO, a variety of chelators have been developed that exhibit antitumor activity, including thiosemicarbazones, e.g. Triapine (3), Tachpyr (4) and Trensox (5). Indeed, several of these agents have entered clinical trials as effective anticancer agents (1,2). Over the past 7 years we have developed potent Fe chelators of the pyridoxal isonicotinoyl hydrazone (PIH) class as antitumor agents (6–8). These studies identified the 2-hydroxy-1-naphthylaldehyde benzoyl hydrazone group of Fe chelators as potential antiproliferative agents. This group

Abbreviations: Act D, actinomycin D; BSA, bovine serum albumin; BSS, Hank’s balanced salt solution; cdks, cyclin-dependent kinases; CHX, cycloheximide; DFO, desferrioxamine; EMSA, electrophoretic mobility shift assays; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HIF-1α, hypoxia-inducible factor-1α; MEM, Eagle’s modified minimum essential medium; PBS, phosphate-buffered saline; PIH, pyridoxal isonicotinoyl hydrazone; TBS, Tris-buffered saline; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; 1,10-P, 1,10-phenanthroline; 311, 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone.

Fig. 1. Illustration of the structures of (A) DFO compared with (B) 311.
of tridentate ligands are highly membrane permeable and selectively bind Fe(III) (9,10). One of the most active chelators identified was 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone, also known as 311 (Figure 1) (6).

Recent studies showed that 311 and other chelators markedly increased the mRNA levels of the p53-transactivatable genes WAF1 and GADD45 (7,11). This was interesting, since WAF1 encodes p21(3p2/3p21), which is a universal inhibitor of cyclin-dependent kinases (cdks) and induces a G1/S arrest (12). The GADD45 gene encodes a molecule critical for DNA repair and cell cycle arrest at G1/S (12). The ability of 311 and DFO to inhibit proliferation and increase WAF1 expression was dependent on their ability to bind Fe, as their Fe complexes had no effect (6). Moreover, the effects of Fe chelators on increasing WAF1 mRNA levels was reversible after Fe supplementation (11). Considering these results in cells with wild-type p53, chelators may increase p53 DNA-binding activity to transactivate its target genes. However, WAF1 may also be transactivated by AP2, Sp1 or the p53 homologs p63 and p73 (13,14). Furthermore, the transactivation of WAF1 after Fe chelation has been demonstrated to occur in cell types with (7,15) or without p53 (11,16). These latter experiments suggest, but do not prove, that a p53-independent mechanism was involved in increasing WAF1 expression. Thus, it is important to assess the role of p53 in transactivation of WAF1 after exposure of cells with wild-type or mutant p53 to Fe chelators.

Very few studies have investigated the effect of potent Fe chelators on the DNA-binding activity of transcription factors. Like many cysteine-containing transcription factors, such as API, AP2, Sp1 and NFkB (17,18), p53 was found to be subject to redox regulation (19). It is known that cysteines and zinc(II) are essential for p53 DNA-binding activity (19) and disruption of these by reductants or metal chelators can abolish DNA-binding activity. In fact, in vitro, the chelator 1,10-phenanthroline (1,10-P) and oxidants result in a decrease in p53 DNA-binding activity (20,21). In contrast, using intact cells, 1,10-P increased p53 transcriptional activity without increasing p53 protein, while DFO was shown to increase p53 levels via activating the hypoxia-inducible factor-1α (HIF-1α) (15).

In the current investigation, we examined the mechanism involved in the chelator-mediated increase in WAF1 expression in cells with mutant or wild-type p53. Our work suggests that a p53-independent pathway responds to Fe chelation to induce WAF1 mRNA levels by a transcriptional and post-transcriptional mechanism. The identification of this p53-independent pathway could be important for discovering specific molecular targets that inhibit cancer cell growth by chelators and other drugs.

Materials and methods

Cell treatments and reagents

The DFO was purchased from Novartis Ltd (Basel, Switzerland), and 311 was synthesized and characterized using standard procedures (22). The Fe(III) complexes of DFO and 311 were prepared as described previously (11,22). Actinomycin D (Act D) was obtained from Sigma Chemical Co. (St Louis, MO).

Cell culture

The human SH-SY-5Y neuroblastoma cell line was provided by Mr Hiroki Nishimura (Queensland Pharmaceutical Research Institute, Brisbane, Australia). Human SK-N-MC neuroepithelioma cells, human MCF-7 breast cancer cells and human MRC-5 fibroblasts were obtained from the American Type Culture Collection (Rockville, MD). All cell lines were cultured in Eagle’s modified minimum essential medium (MEM) (Gibco, Rockville, MD) containing 10% fetal calf serum (FCS) (CSL, Melbourne, Australia), 2 mM l-glutamine (Sigma), 1% (v/v) non-essential amino acids (Gibco), 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco) and 0.028 μg/ml fungizone (Squibb Pharmaceuticals, Montréal, Canada) at 37°C in a humidified atmosphere of 5% CO2 in air. Cells were grown and viability assessed using standard techniques described previously (23).

Preparation of 59Fe-transferrin

Human apotransferrin (Sigma) was labeled with 59Fe (Du Pont NEN, Boston, MA) to produce 59Fe2+/transferrin, as previously described (24). Unbound 59Fe was removed by exhaustive vacuum dialysis against 0.15 M NaCl buffered to pH 7.4 using 1.4% NaHCO3 (23). Fully saturated diferric transferrin was used in all experiments.

Iron efflux experiments

To assess the ability of the chelator to permeate cell membranes and bind intracellular Fe pools, cells were prelabelled with 59Fe-transferrin (0.75 μM) for 3 h at 37°C using established methods (6). The cells were then placed on ice and washed four times with ice-cold Hank’s balanced salt solution (BSS) and then reincubated for 3 h at 37°C in the presence of medium alone (control) or medium containing DFO (5–50 μM) or 311 (5–50 μM). The cells were then reincubated with MEM for incubation periods of 3 h at 37°C. The overlying medium was then removed and placed into 7-counting tubes. The cells were then removed from the Petri dishes in 1 ml of BSS using a plastic spatula and transferred to a separate set of 7-counting tubes. The radioactivity in the samples were then quantitated using a 7-scintillation counter (Wallace 1282 CompuSamm; LKB, Turku, Finland).

Antibodies used in western blot analysis

The mouse monoclonal anti-human β-actin antibodies were from Sigma (clone AC-15) and were used at 1/5000. The rabbit polyclonal anti-human p53 (catalog no. sc-6243) was used at 0.0025 μg/ml, respectively, and was from Santa Cruz Biotechnology (Santa Cruz, CA).

Western blot analysis

Cells were collected and incubated for 20 min at 4°C with the lysis buffer [20 mM HEPES, pH 7.6, 20% glycerol, 10 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM diithiothreitol, 10 mM NaCl, Complete™ protease inhibitors (Roche; Mannheim, Germany)] and centrifuged at 5000 r.p.m. for 5 min at 4°C. For nuclear extracts, the pellet containing nuclei was vigorously resuspended in lysis solution containing 500 mM NaCl and gently rocked for 1.5 h at 4°C. The lysates were then centrifuged at 10 000 r.p.m. for 15 min at 4°C and the supernatant containing the nuclear proteins collected. The protein concentration of the lysate was assessed with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

The lysates were then mixed with loading buffer containing 20% β-mecaptoethanol and loaded at 10 μg per sample onto an SDS–PAGE gel consisting of a 4% stacking and 15% resolving gel. After electrophoresis, the proteins were transferred onto polyvinylidene difluoride membranes (Amersham Biosciences, Piscataway, NJ) overnight at 4°C. These membranes were then soaked in methanol and immediately blocked with 5% skimmed milk in Tris-buffered saline (TBS) for 2 h at room temperature. Following blocking, the membranes were incubated with the primary antibodies diluted to working concentrations in 5% skimmed milk in TBS for 3 h at room temperature. Membranes were then washed four times in TBS containing 0.1% Tween-20 (Sigma) for 10 min each. After washing, anti-mouse (0.03–0.1 mg/ml, Sigma) or anti-rabbit (0.05–0.1 mg/ml, Zymed) antibodies conjugated with horseradish peroxidase were incubated with the membranes for 1 h at room temperature. After washing, the membranes were developed using the ECL Plus™ western blot detection reagent (Amersham Biosciences). The films were scanned and montages assembled in Microsoft Word®. To ensure even loading of proteins, membranes were probed for β-actin. All densitometric data were normalized to β-actin.
temperature. The cells were then washed four times in PBS for 10 min and the anti-rabbit antibody conjugated to fluorescein isothiocyanate (FITC) (20 μg/ml) (Sigma) was added. After the secondary antibody incubation, the cells were again thoroughly washed with PBS and mounted onto a glass slide with Prolong® antifade mounting medium (Molecular Probes, Eugene, OR). For each experiment, 10 fields were assessed containing a total of more than 500 cells. The experiment was then repeated three times.

**Electrophoretic mobility shift assays (EMSA)**

Nuclear protein extracts of all cell types were prepared by previously described methods (24). Protein- and DNA-binding reactions were incubated at 4°C overnight and contained 15 μg of nuclear extract in a volume of 20 μl containing 2 mM Tris (pH 7.5), 10 mM NaCl, 1 μg poly(dI-dC), 0.2 mM dithiothreitol, 0.2 mM EDTA and 1% glycerol. All double-stranded oligonucleotides (p53, AP2 and Sp1) were end-labeled with [32P]ATP and ~105 c.p.m. was used per reaction. Sequences of all the oligonucleotides are shown in Table I. The double-stranded oligonucleotide for detecting p53 corresponded to the p53 cis-element of the ribosomal gene cluster promoter (25, 26). For the analysis of p53 DNA-binding, 100 ng of the p53-specific polyclonal antibody (FL-393; Santa Cruz) was added. After the secondary antibody incubation, the cells were ther treated with 2 μl proteinase K (10 mg/ml) and 5 μl yeast RNA (10 mg/ml) at 37°C for 30 min and the RNA extracted three times with a 1:1 mixture of phenol/chloroform. The labeled RNA was precipitated using isopropanol. The [32P]-labeled RNA pellet was resuspended in 10 mM N-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES) (pH 7.4), 0.2% SDS, 10 mM EDTA and 150 μCi of [α-32P]UTP at 37°C for 45 min. Nuclei were digested with 10 μl DNase I (10 μg/ml; Gibco) at 30°C for 15 min. The reaction mixture was further treated with 2 μl proteinase K (10 mg/ml) and 5 μl yeast RNA (10 mg/ml) at 37°C for 30 min and the RNA extracted three times with a 1:1 mixture of phenol/chloroform.

**Northern blot analysis**

Northern blot analysis was performed by isolating total RNA using the Total RNA Isolation Reagent from Advanced Biotechnologies Ltd (Epsom, UK), as described previously (11). The membranes were hybridized with probes specific for human WAF1 and β-actin. The WAF1 probe consisted of a 1 kb fragment from pSVX (ATCC catalog no. 79928).

**Results**

The effect of chelator concentration on iron mobilization from normal MRC-5 fibroblasts compared with SK-N-MC neuroepithelioma cells

Our previous studies using neoplastic cell types have shown that 311 is a far more active chelator than the ligand in current clinical use, DFO (6, 11, 34). In the current investigation it was important to examine the Fe chelation activity of DFO and 311 in both normal and neoplastic cell types. As a normal cell type we used MRC-5 human lung fibroblasts, which are mortal and senesce after limited passage in vitro (18). For comparison, we examined the SK-N-MC neuroepithelioma cell type, which...
is immortal. There have been no investigations to date comparing the chelation efficacy of DFO and 311 in normal and neoplastic cells. This was important for the current study where the effect of the chelators on p53 were assessed in both cell types after Fe chelation.

To investigate the ability of the chelators to bind intracellular Fe pools, cells were labeled with $^{59}$Fe-transferrin (0.75 μM) for 3 h at 37°C. The cells were then washed and reincubated for 3 h at 37°C in medium containing DFO or 311 (5–50 μM). For both cell types, 311 was more effective than DFO at mobilizing internalized $^{59}$Fe at all chelator concentrations (Figure 2). Comparing the ability of the chelators to mobilize $^{59}$Fe from the two cell types, DFO was less effective at mobilizing $^{59}$Fe from SK-N-MC than from MRC-5 cells (Figure 2). The low activity of DFO at removing $^{59}$Fe from SK-N-MC cells has been noted previously when comparing these cells with other neoplastic cell types (e.g. BE-2 neuroblastoma cells; 11). Hence, the effect observed for DFO in MRC-5 fibroblasts was not unique. Further, it is important to note that we cannot claim any differences in the activity of the chelators between normal and neoplastic cells using such a small number of cell types. In contrast to the results found for DFO, 311 was more effective at mobilizing $^{59}$Fe from SK-N-MC cells than MRC-5 fibroblasts (Figure 2). The high activity of 311 as a function of concentration probably reflects its considerable lipophilicity and marked membrane permeability (6,11).

In contrast, DFO is quite hydrophilic and does not readily permeate the plasma membrane (11; for a review see 2).

The effect of DFO and 311 on p53 expression, cellular localization and p53 DNA-binding activity in normal and neoplastic cells

Previous investigations have shown an increase in p53 protein levels after exposure to DFO (15,30) which may be attributable to direct stabilization of the protein by HIF-1α (15). Our own studies are in accord with this observation, as incubation with 311 did not result in any significant increase in p53 mRNA levels (7), suggesting a post-transcriptional alteration after Fe chelation. In addition, both DFO and 311 increased the mRNA levels of downstream targets of p53, namely WAF1 and GADD45 (7,11,35). However, as far as we can determine, there have been no studies assessing whether specific Fe chelators such as DFO or 311 can increase p53 DNA-binding activity. This was relevant to examine since p53 protein can exist in a latent and active DNA-binding form (37,39). Moreover, it has been suggested, although not experimentally proven, that incubation of cells with chelators (i.e. DFO or Tachpyr) results in a latent form of p53 that has poor transcriptional activity (16,30). This was vital to directly assess in normal and neoplastic cells using DFO and 311, as it may have consequences in the use of these compounds as antimutagenic agents.

The effect of the chelators on p53 protein expression

Western analysis was used to assess nuclear p53 levels following incubation of MRC-5, MCF-7, SH-SY-5Y and SK-N-MC cells with DFO (150 μM) or 311 (25 μM) for 24 h at 37°C (Figure 3A–D). As a positive control, cells were incubated with the DNA-damaging agent Act D (9 nM). At this concentration Act D does not prevent transcription and increases p53 protein levels (30). Both DFO and 311 increased p53 2- to 3-fold in MRC-5 cells, while Act D resulted in a marked 6-fold increase (Figure 3A). Similar results were found when treating MCF-7 cells with DFO, 311 or Act D, which resulted in 2-, 5- and 9-fold increases in p53 protein levels, respectively (Figure 3B). For the SH-SY-5Y cell type (Figure 3C), 311 and Act D markedly increased p53 levels to 7- and 15-fold that of the control, while DFO was less effective, increasing it to four times the control value. Hence, in both normal and neoplastic cell types with wild-type p53, chelators could increase p53 protein expression in a similar way. In contrast, when examining SK-N-MC cells, DFO or 311 only slightly increased nuclear p53 compared with the control, while Act D markedly increased it to 4-fold that of the control (Figure 3D).

Considering this, it is of interest to note that SK-N-MC cells have mutant p53 (29) while the other cell types all have wild-type protein (30,31). The mutation of p53 in SK-N-MC cells results in a shorter transcript (7,29), indicating a relatively large scale disruption in the gene. In fact, the deletion encompasses a 200–300 bp fragment that includes exons 2 and 3 (29). Collectively, our data indicate that DFO or 311 could only increase nuclear p53 protein levels in cells with wild-type but not the mutant gene.

The effect of the chelators on the intracellular distribution of p53

Considering that p53 must enter the nucleus to act on its target genes (for a review see 12), we used immunofluorescence to examine the effect of the chelators on the intracellular distribution of p53 compared with the positive control, Act D. This was done as there is some evidence suggesting defective transport of p53 to the nucleus after incubation with DFO in immortal neoplastic cells (MCF-7), compared with the mortal cell type MRC-5 (30). Comparing the normal MRC-5 cell type and the neoplastic MCF-7 cells, DFO (150 μM), 311 (25 μM) and Act D (9 nM) all induced nuclear accumulation of p53 protein (Figure 4), while incubation with control medium did not (Figure 4). Similar results were also observed for the
As found using western analysis for SK-N-MC cells (Figure 3D), nuclear p53 staining was only markedly enhanced after incubation of these cells with Act D, the chelators having little effect on p53 distribution compared with the control (Figure 4).

**The effect of the chelators on p53 DNA-binding activity**

Considering that the chelators induced an increase in p53 protein levels in cells with wild-type (MRC-5, SH-SY-5Y and MCF-7) or mutant p53 (SK-N-MC), cells were incubated for 24 h at 37°C with DFO (150 μM), 311 (25 μM) or Act D (9 nM) and the protein levels of p53 assessed using western analysis of nuclear lysates (see Materials and methods for details). Densitometric analysis of the bands is shown below each blot. The results are typical experiments from at least three performed.

**The effect of DFO and 311 on the DNA-binding activity of other redox-sensitive transcription factors in normal and neoplastic cells**

Considering the results showing an increase in DNA-binding activity for p53 after exposure to the Fe chelators (Figure 5), we thought it prudent to examine other redox-sensitive transcription factors that could be affected by intracellular Fe levels and play roles in cellular proliferation. Examining the literature, AP2 and Sp1 are cysteine-containing transcription factors that are subject to redox regulation (17,18) and these molecules play critically important roles in differentiation, proliferation and cell cycle control. Furthermore, the WAF1 promoter has one AP2 and six Sp1 DNA-binding sites (13), and these transcription factors have previously been shown to...
play important roles in the expression of WAF1. Hence, an increase in DNA-binding activity of AP2 and/or Sp1 to the WAF1 promoter could possibly explain the p53-independent increase in WAF1 mRNA expression seen in SK-N-MC and K562 cells after exposure to chelators (11).

**AP2 DNA-binding activity.** Incubation of control cell lysates with the AP2 oligonucleotide (Table I) resulted in three major bands (Figure 6A, lane 2) that were not present when the probe alone was run (Figure 6A, lane 1). Addition of a 50-fold excess of specific competitor (i.e. non-labeled AP2 oligonucleotide) totally ablated the top two bands and slightly reduced the intensity of the bottom band (Figure 6A, lane 3). A non-specific competitor oligonucleotide (i.e. Sp1) at a 50-fold excess had little effect on AP2 DNA-binding (Figure 6A, lane 4) when compared with the control (Figure 6A, lane 2). The mutant AP2 probe (Table I) showed that the bottom two bands became bound to this oligonucleotide (Figure 6A, lane 7), indicating that the top band represented specific AP2 DNA-binding activity.

The DNA-binding activity of AP2 could not be detected in MRC-5 fibroblasts in contrast to all of the neoplastic cell types examined (Figure 6A). The reason for the lack of AP2 DNA-binding activity in MRC-5 cells remains unclear at present. The AP2 DNA-binding activity in the neoplastic cell types was variable. After incubation with DFO, in MCF-7 cells, there was no effect on AP2 DNA-binding activity, while in SH-SY-5Y and SK-N-MC cells, it increased to 196 ± 2 and 217 ± 8% of the control, respectively (Figure 6A). Incubation of all neoplastic cell types with 311 resulted in
an increase in AP2 DNA-binding activity that varied from 203–272% of the control (Figure 6A).

**Sp1 and Sp3 DNA-binding activity.** Addition of the Sp1 oligonucleotide (Table I) to control lysates resulted in two bands (Figure 6B, lane 2) that were consistent with binding of the transcription factors Sp1 and Sp3 (13). These latter two bands were not found when the probe alone was run (Figure 6B, lane 1). An excess of specific competitive oligonucleotide (unlabeled Sp1) totally inhibited DNA binding (Figure 6B, lane 3), while the non-specific competitor (unlabeled AP2) had no significant (*P* > 0.05) effect (Figure 6B, lane 4) over three experiments. Mutant probe (Table I) did not result in any DNA-binding activity (Figure 6B, lane 7).

The incubation of SK-N-MC cells with DFO or 311 resulted in a marked increase in Sp1 DNA-binding activity to 328 and 439% of the control, respectively, while the increase in Sp3 DNA-binding activity was less marked but still significant (*P* < 0.005) compared with the control (Figure 6B). In the other neoplastic cell types, Sp1 and Sp3 DNA-binding activity in the presence of DFO or 311 was also significantly (*P* < 0.005) increased above the control and ranged from 190 to 258% of the control (Figure 6B). In contrast to neoplastic cells, DFO had no significant effect on either Sp1 or Sp3 DNA-binding activity in MRC-5 fibroblasts, while 311 caused significant (*P* < 0.005) 219 and 231% increases, respectively, compared with the control (Figure 6B). Collectively, the DNA-binding results from neoplastic cells suggested that AP2, Sp1 or Sp3 may play a role in the p53-independent increase in WAF1 expression.

**Fig. 5.** The iron chelators DFO and 311 increased p53 DNA-binding activity, but to a lesser extent than the DNA-damaging agent Act D. (A) EMSA examining p53 DNA-binding activity after incubation of MRC-5 fibroblast cells for 24 h at 37°C with DFO (150 μM), 311 (25 μM) or Act D (9 nM). Results are representative of three experiments that showed similar results. (B) Densitometric analysis of the p53 DNA-binding activity shown in (A). (C) The effect of the chelators on p53 DNA-binding activity in four different cell types as measured by EMSA. Results are means ± SD (three experiments).

**Table 1.** Effect of Chelators on p53 DNA-Binding Activity in Different Cell Types

<table>
<thead>
<tr>
<th>Cell type</th>
<th>DFO (% of control)</th>
<th>311 (% of control)</th>
<th>Act D (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRC-5</td>
<td>202% ± 11</td>
<td>211% ± 11</td>
<td>352% ± 12</td>
</tr>
<tr>
<td>MCF-7</td>
<td>208% ± 10</td>
<td>295% ± 11</td>
<td>464% ± 11</td>
</tr>
<tr>
<td>SH-SY-5Y</td>
<td>203% ± 9</td>
<td>196% ± 8</td>
<td>-</td>
</tr>
<tr>
<td>SK-N-MC</td>
<td>194% ± 8</td>
<td>197% ± 10</td>
<td>200% ± 12</td>
</tr>
</tbody>
</table>

The effect of chelators on WAF1 promoter activity

To determine the possible role of p53, AP2, Sp1 or Sp3 in the chelator-mediated increase in WAF1 expression, experiments were performed using a WAF1 promoter construct containing functional p53, AP2 and Sp1 DNA-binding sites linked to the luciferase reporter gene (Figure 7, closed bars) (27). As a negative control, cells were also transfected with the same vector that did not contain the WAF1 promoter (Figure 7, open bars). These constructs were transiently transfected into SK-N-MC cells that have mutant p53 (negative control) (29), or MCF-7, SH-SY-5Y or MRC-5 cells that have wild-type p53 (positive controls) (30,31) (Figure 7A–D).

In all studies the vector without the WAF1 promoter (Figure 7, open bars) did not respond to any of the treatments and luciferase levels were at least 10-fold less than those found with vector containing the promoter (Figure 7, closed bars). Act D, DFO or 311 had no effect on luciferase levels compared with the control in SK-N-MC cells (Figure 7A). Higher chelator concentrations up to 25 μM also had little effect (data not shown). This clearly indicates that the mutant p53 in SK-N-MC is non-functional in terms of its transactivation of WAF1. In contrast, Act D increased luciferase activity in all cell types with wild-type p53, while chelators had no effect (Figure 7B–D). Collectively, and to our surprise, while Fe chelators increased p53 protein expression (Figure 3A–D), nuclear localization (Figure 4) and DNA-binding activity (Figure 5) in all cell types with wild-type p53, this did not result in an increase in transactivation of the p53 target gene, WAF1 (Figure 7B–D). These results indicate that for cells with wild-type or mutant p53, the increase in WAF1 mRNA levels (7) was via a p53-independent mechanism. In addition, despite the fact that EMSA demonstrated an increase in AP2, Sp1 and Sp3 DNA-binding activity after incubation with DFO and 311 in most cell types (Figure 6A and B), there was no increase in luciferase activity after exposure to these chelators (Figure 7). These observations indicate that the increased DNA-binding activity found in EMSA after incubation with chelators does not result in increased WAF1 promoter activity using intact cells.

Chelators increase WAF1 mRNA levels via a transcriptional mechanism

Our current studies using northern blotting demonstrate that 311 markedly increases WAF1 mRNA levels in MCF-7 and SK-N-MC cells (Figure 8A). These experiments confirmed our previous investigations using five different cell types (7,8,11,35). While we showed that p53, Sp1, Sp3 or AP2 were not involved in the transactivation of WAF1 (Figure 7), it was essential to determine whether the increase in WAF1 mRNA levels was due to a transcriptional mechanism. To examine this, MCF-7 and SK-N-MC cells with wild-type or mutant p53, respectively, were incubated with either control...
medium or 311 and nuclear run-on assays performed (Figure 8A and B). The 311 ligand was chosen for these assays due to its marked effect on the expression of WAF1 (11).

After incubation with 311, WAF1 mRNA levels were increased while expression of the controls (β-actin and transferrinR1) remained unchanged. The WAF1 vector, pSXV, without an insert, was used as another negative control and showed no binding in control or 311-treated cells (Figure 8A and B). The levels of nuclear GADD45, which can also be transactivated by p53-dependent or p53-independent pathways (11,12), were increased after incubation with 311 compared with control medium in both MCF-7 and SK-N-MC cells (Figure 8A and B). These studies suggested that the increase in WAF1 and GADD45 mRNA observed after incubation with chelators was at least partially mediated through a transcriptional mechanism.

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**Fig. 6.** The Fe chelator 311 and, to a lesser extent, DFO increased the DNA-binding activity of the AP2, Sp1 and Sp3 transcription factors. (A) An EMSA examining AP2 DNA-binding activity after incubation of SK-N-MC cells for 24 h at 37°C with DFO (150 μM) or 311 (25 μM). The gel illustrated is representative of three experiments that showed similar results. Shown below the gel photo is a table detailing the effect of the chelators on AP2 DNA-binding activity in four different cell types as measured by EMSA. Results are means ± SD (three experiments). (B) An EMSA examining Sp1 and Sp3 DNA-binding activity after incubation of SK-N-MC cells for 24 h at 37°C with DFO (150 μM) or 311 (25 μM). The gel shown is representative of three experiments that showed similar results. Shown below the gel photo is a table detailing the effect of chelators on Sp1 and Sp3 DNA-binding activity in four different cell types as measured by EMSA. Results are means ± SD (three experiments).
The role of transcriptional and post-transcriptional mechanisms in the expression of WAF1 mRNA

Additional studies were performed to determine the role of transcriptional and post-transcriptional processes in the increase in WAF1 mRNA levels after incubation with 311. In these experiments, SK-N-MC cells were incubated for 5 h at 37°C with the protein synthesis inhibitor cycloheximide (CHX) (5 μg/ml) and the transcription inhibitor Act D (0.4 μM) (Figure 9). These concentrations of CHX and Act D are widely used to inhibit protein synthesis and transcription, and in this study and previous investigations (11), they were shown to markedly inhibit [3H]leucine and [3H]uridine incorporation, respectively (data not shown).

Incubation with 311 markedly increased the WAF1 mRNA level to 19-fold that of the control (Figure 9). Act D at a concentration of 0.4 μM completely abolished WAF1 mRNA in control cells and markedly prevented the 311-mediated increase in WAF1 mRNA levels (Figure 9). It should be noted that this higher concentration of Act D (0.4 μM) acts to inhibit transcription, while lower levels (9 nM) used in the earlier part of the study (see Figures 3–5) result in DNA damage and the induction of WAF1. Our results using Act D in Figure 9 support the nuclear run-on studies (Figure 8) and suggest that the 311-mediated increase in WAF1 mRNA was at least partially mediated through a transcriptional mechanism. Incubation with CHX markedly induced WAF1 mRNA (Figure 9), presumably due to this agent acting to induce transcriptional up-regulation. However, the addition of CHX completely prevented the large increase in WAF1 levels seen in the presence of 311 alone (Figure 9). These results indicate that protein synthesis can influence the levels of WAF1 mRNA and indicate that a post-transcriptional mechanism of regulation may also be involved.

Further studies were performed in SK-N-MC cells to determine the half-life of WAF1 mRNA in the presence of Act D alone or Act D and 311 (Figure 10). In the presence of Act D alone, the half-life of WAF1 mRNA was determined to be ~6 h, and this increased to 10 h in the presence of 311 as well as Act D (Figure 10).

Collectively, the results from Figures 8–10 indicate that Fe chelation increases WAF1 mRNA levels by transcriptional and post-transcriptional mechanisms.

Discussion

A range of recent studies in vitro and a number of clinical trials have indicated that Fe chelators may be useful antitumor agents (1,2). Iron is essential for critical metabolic processes such as energy production and DNA synthesis. Due to their rapid rate of growth, tumor cells have a high Fe requirement such as energy production and DNA synthesis. Due to their rapid rate of growth, tumor cells have a high Fe requirement.

Fig. 7. Reporter construct studies of the WAF1 promoter (1–2280 bp) showing that only Act D but not DFO or 311 can increase the expression of luciferase in cells with wild-type p53. Cells with mutant (SK-N-MC) or wild-type (SH-SY-5Y, MCF-7 and MRC-5) p53 were transiently transfected with the WAF1 reporter construct and incubated for 24 h with DFO (150 μM), 311 (5 and 10 μM) or Act D (9 nM). Luciferase activity was then assessed and normalized to β-galactosidase (β-gal) (see Materials and methods for details). Open bars represent empty pGL2-Basic vector while the closed bars are the same vector containing the WAF1 promoter. Results are means ± SD (three experiments).
As part of our research program to understand the influence of Fe chelation on tumor cell growth, it was important to examine the effect of chelators on molecules that play a role in the expression of genes vital for proliferation. In particular, the G1/S arrest observed after chelation (34,36) suggests a role for p53 and its downstream target WAF1, which regulates S phase entry (12). Previous studies have suggested that p53 can act as a metabolic sensor (38). In fact, when nucleotide pools were disturbed by inhibitors this resulted in a reversible G0/G1 cell cycle arrest associated with induction of p53 and WAF1 (38). Since 311 and DFO inhibit ribonucleotide reductase and decrease [3H]thymidine incorporation (34,42), this could act as a signal to induce p53 transcriptional activity. Certainly, our previous studies examining a variety of chelators demonstrated that high antiproliferative activity was well correlated with the mRNA levels of the downstream p53 targets WAF1 and GADD45 (8,11,35).

The present work shows that incubation of DFO or 311 with normal or neoplastic cells possessing wild-type p53 increased p53 protein levels and p53 DNA-binding activity. Moreover, DFO and 311 were effective at inducing the nuclear accumulation of wild-type p53. These studies were in contrast to those of Sun et al. (19), in which treatment of cells with the chelator 1,10-P was shown to increase p53 transcriptional activity, but have no effect on p53 protein levels. In addition, these observations were different to the results found in vitro using isolated p53 protein, where 1,10-P inactivated p53 DNA-binding activity (19). However, this latter study used very high ligand concentrations (0.625–1.25 mM), which may be necessary to directly remove Zn(II) from p53. Indeed, considering that Zn(II) plays a role in the tertiary structure of this protein, the removal of this ion could disrupt its function. The reason for the difference in the results obtained with 1,10-P compared with DFO or 311 could be related to their different specificities for metal ions. The 1,10-P ligand is a strong divalent metal ion chelator, with high affinity for Fe(II) and Zn(II) (41). In contrast, for DFO and the aroylhydrazone class of chelators, such as 311, the specificity for Fe(III) is extremely high, with much lower affinity for Fe(II) and Zn(II) (9,10). Moreover, 311 or DFO may not be able to gain access to the Zn(II)-binding site of p53 due to steric constraints. It should also be noted that the changes in p53 DNA-binding activity observed could be due to other effects of Fe chelation within the cell. For instance, we cannot exclude the possibility that Fe chelation changes intracellular redox status that may then indirectly alter the DNA-binding activity of p53 and other redox-sensitive transcription factors.

Several studies have examined the effect of DFO on the levels of p53 protein or the transactivation of its target genes.
after exposure to chelators (7,11,15,16,30). However, this is the first work to assess whether there was increased p53 DNA-binding activity that is directly involved in the chelator-mediated up-regulation of WAF1 (11). This was important to assess since p53 activation has been shown to depend on an increase in protein levels and a shift from the latent to the DNA-binding form of the protein (37). We demonstrated that DFO and 311 were less effective than Act D at increasing p53 protein levels and its DNA-binding activity. The increase in p53 DNA-binding activity in mortal MRC-5 fibroblasts after incubation with DFO or 311 was similar to that found for two neoplastic cell types (i.e. MCF-7 and SH-SY-5Y; Fig. 5C). In addition, our immunofluorescence studies clearly showed nuclear targeting of p53 after incubation with DFO or 311 in MRC-5 and MCF-7 cells (Figure 4). These experiments suggest that the response of p53 to Fe chelators was similar in these normal and neoplastic cells and that the protein can be targeted to the nucleus. However, the response of p53 to chelators was less marked than that observed with Act D.

In contrast to our investigation, Ashcroft et al. (30) reported that DFO only partially activated stabilized but latent p53 in MRC-5 fibroblasts and this response was lost in tumor cell lines. While these latter authors did not perform p53 DNA-binding studies to support this suggestion, they did show that a loss of p53 transcriptional activity correlated with its cytosolic accumulation, indicating a defect in pathways allowing nuclear targeting after chelation (30). We have not been able to confirm these latter results in tumor cell types and we did not observe accumulation of cytosolic p53 even when using higher DFO concentrations (i.e. 250 μM; data not shown). In fact, Fe chelators induced similar changes in p53 protein levels, nuclear localization and p53 DNA-binding activity in mortal MRC-5 cells and the two neoplastic cell lines with wild-type p53 (i.e. MCF-7 and SH-SY-5Y).

The precise molecular mechanism(s) responsible for the p53-independent increase in WAF1 and GADD45 expression after exposure to chelators remains unknown. However, our investigation indicates that transcriptional up-regulation is at least partially responsible (Figure 8). It is of interest that recent studies suggest that the p53 homologs, p63 and p73, may play important roles in transactivating p53 target genes when p53 is mutated (14). Indeed, both these molecules bind p53 consensus sequences in the WAF1 and GADD45 promoters (14). It is clear from our reporter construct studies that the regulation appears

Fig. 9. Act D markedly inhibits the 311-mediated increase in WAF1 mRNA expression while cycloheximide partially prevents the 311-mediated increase in WAF1 mRNA levels. The SK-N-MC neuroepithelioma cell line was incubated for 5 h at 37°C with control medium, Act D (0.4 μM), CHX (5 μg/ml), 311 (25 μM), 311 (25 μM) and Act D (0.4 μM) or 311 (25 μM) and CHX (5 μg/ml). After incubation the cells were lysed and northern analyses performed (see Materials and methods for details). Densitometric analyses of the results are shown below the membrane photograph. The results illustrated are a representative experiment from three performed.

Fig. 10. Incubation of cells with the chelator 311 increases WAF1 mRNA half-life. The SK-N-MC neuroepithelioma cell line was incubated for 1–12 h at 37°C with medium containing 311 (25 μM) and Act D (0.4 μM) or Act D (0.4 μM) alone. After this incubation the cells were lysed and northern analyses performed (see Materials and methods for details). Densitometric analyses of the results are shown below the membrane photograph. The results illustrated are a representative experiment from three performed.
to be occurring upstream of the p53, AP2 and Sp1 consensus binding elements that are within the 2280 bp of the WAF1 promoter (Figure 8). Further studies are essential in order to determine the nature of the transcriptional regulation. However, irrespective of the precise molecular mechanism involved, the ability of chelators to potentially inhibit tumor cell growth by a p53-independent pathway is of significance, as p53 is the most frequently mutated gene in human cancer (12). These results may explain why cells with wild-type or mutant p53 are similarly sensitive to the antiproliferative effects of Fe chelators (42). Indeed, it appears that another pathway is vital for the cell cycle arrest observed after Fe chelation. The discovery of this p53-independent pathway could be important for the identification of specific molecular targets that inhibit cancer cell growth by chelators and other drugs.

Apart from transcriptional induction of WAF1 expression, we also obtained evidence for a post-transcriptional mechanism that increased the half-life of WAF1 mRNA (Figure 10). Previous studies have demonstrated that post-transcriptional mechanisms can also be involved in increasing WAF1 mRNA levels (42–45). Of interest to the present study, Esposito et al. (42) showed that during oxidative stress, p21 can be induced via a p53-independent pathway that involves a post-transcriptional mechanism. Obviously, the chelation of Fe from cells could perturb the intracellular redox equilibrium and, hence, a similar mechanism could be responsible for the increased stability of WAF1 mRNA observed in the current investigation.

It is paradoxical that increased p53 protein levels, DNA-binding activity and its targeting to the nucleus did not lead to the observed increase in the mRNA levels of a potential p53 target gene, i.e. WAF1. This is surprising considering that DFO and 311 induce a G1/S arrest and that WAF1 encodes p21(CIP1)/WAF1, the universal inhibitor of cyclin-dependent kinases which prevents cell cycle progression (12). However, it is well known that after exposure to 311 and, to a lesser extent, DFO there is a marked decrease in DNA synthesis due to the inhibition of ribonucleotide reductase (1,34,46), which prevents entrance into S phase. Moreover, exposure of cells to chelators leads to a decrease in the expression of molecules that play key roles in G1 progression, e.g. cyclins D1, D2 and D3 and cdk2 (7). Furthermore, other cell cycle regulators could also be involved in mediating the G1/S arrest after incubation with Fe chelators, e.g. p27Kip1 (47), alleviating the requirement for p21(CIP1)/WAF1.

Recently, we have shown that while WAF1 mRNA is markedly increased after chelation, the protein levels of p21 are decreased (48). Considering this, it is clear that the effect of these ligands on the regulation of WAF1 is complex, with a p53-independent mechanism being involved in the transcriptional and post-transcriptional regulation of the molecule, which results in increased mRNA levels. Paradoxically, Fe chelation then subsequently prevents translation, an effect that can be readily reversed by the addition of Fe (48).

Apart from our studies, others have shown that the transcription of p21 was inhibited after incubating HL-60 cells with DFO and a phorbol ester (49). These results are clearly quite different to those found in the current study and may reflect the specific cell type used and the non-physiological differentiating agent. It is also of interest that the protein levels of p21 can be regulated by proteasomal degradation (50,51). Considering this, Fukuchi et al. (52) demonstrated that p21 accumulation after incubation with an Fe chelator or DNA-damaging agent was dependent on inhibition of the proteasome. However, our previous studies (48) demonstrated that the proteasome played a small role in the chelator-mediated effects on WAF1 expression.

The question remains, what is the function of increased p53 expression and DNA-binding activity observed after incubation with chelators? Recent studies in our laboratory using gene arrays showed that other p53-transactivatable genes were up-regulated after incubation with 311, including mdm2 and GADD45 (N.T.V.Le and D.R.Richardson, unpublished results). Additional studies determining whether these changes in gene expression lead to alterations in protein levels have been initiated using proteomic analysis and are the subject of on-going investigation.

The high Fe chelation activity of 311 in the current study and its ability to markedly induce WAF1 expression is probably at least partially attributed to its high membrane permeability (11). On the other hand, far higher concentrations of DFO were necessary to achieve appropriate Fe chelation efficacy (Figure 2). The low activity of DFO is probably related to the fact that the molecule shows low efficacy at permeating cell membranes (53) and may be internalized by non-specific pinocytosis (54,55).

In summary, we examined the complex mechanisms involved in the chelator-mediated increase in WAF1 expression. The potent chelator 311 and more so the DNA-damaging agent Act D caused an increase in nuclear p53 protein and p53 DNA-binding activity in cells with wild-type or mutant p53. However, reporter construct studies showed that p53 played no role in the 311-mediated increase in WAF1 transcription. Nonetheless, our experiments demonstrated that the elevated WAF1 mRNA expression was at least partially due to increased transcription and also to a post-transcription mechanism that was sensitive to CHX. Our studies indicate that a p53-independent pathway responds to Fe chelation to induce WAF1 transcription.

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References

Iron chelators increase WAF1 expression


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