p53-independent apoptosis mediated by tachpyridine, an anti-cancer iron chelator

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Introduction

Iron is a metal critically important for cell proliferation and survival. It is an essential constituent of haemoglobin and myoglobin, mitochondrial electron transport proteins, hydroxylating enzymes, lipoxygenases and cyclooxygenases, as well as ribonucleotide reductase, the enzyme that catalyzes the rate limiting step in DNA synthesis. Tumor cells frequently exhibit increased uptake and utilization of iron, as evidenced by an increase in transferrin receptors at the cell surface (1,2). This has led to the suggestion that agents that deplete intracellular iron may be useful in cancer therapy (3–5).

Iron deprivation using desferrioxamine (deferoxamine, Desferal), a hexadentate iron chelator and the drug of choice for the treatment of chronic iron overload, has been shown to substantially retard growth of some implanted tumors in animals (6). Conjugation of desferrioxamine to antibodies against the transferrin receptor resulted in a significant reduction in growth of lymphoma both in vitro (7) and in vivo (8). Studies using desferrioxamine alone (9,10), as well as smaller bidentate chelators (11), have further shown that iron chelators cause growth inhibition in leukemia, neuroblastoma and lymphoma cells, and arrest cells in the G1/S phase of the cell cycle. However, desferrioxamine was not designed for anti-tumor therapy. Its activity as an anti-cancer agent is limited (12) and it must be administered by continuous infusion, suggesting the need for development of more effective chelators designed specifically as anti-cancer agents.

\[\text{N,N',N''-tris(2-pyridylmethyl)-cis,cis-1,3,5-triaminocyclohexane}\]

(tachpyridine) is a novel chelator with a unique structure and chemistry that was recently synthesized and described by our group (13–15). Tachpyridine is a hexadentate metal chelator that binds metals through six nitrogen donor ligands, three contributed by pyridyl groups and three contributed by secondary amines. The cytotoxic activity of tachpyridine is likely due to its ability to bind one or more of the metals iron, zinc or copper, since complexes of tachpyridine with these metals (but not others) are non-toxic (14). Because N-alkylated derivatives of tachpyridine that retain an ability to bind copper and zinc but are sterically hindered in their ability to react with iron are no longer toxic, we have postulated that iron deprivation is an important component of the toxicity of tachpyridine (14).

Several reports of the toxic properties of chelators, including their ability to induce apoptosis, have appeared. For example, the iron chelators desferrioxamine, dithizone and hinokitol induced apoptosis in F9 embryonal carcinoma cells (16), desferrioxamine and deferiprone induced internucleosomal DNA cleavage in proliferating T lymphocytes and HL60 promyelocytic leukemia (17) and the chelator O-Trensox induced apoptosis in HepG2 cells (18). Chelator-independent iron deprivation has also been shown to induce apoptosis (19,20). However, apoptotic mechanisms elicited by iron deprivation or iron chelators have not been elucidated.

In the search for the mechanisms of chelator-induced...
apoptosis, the tumor suppressor p53 emerges as a likely candidate (for reviews see refs 21–23). p53 contributes to apoptosis induced by a variety of cellular stresses, including DNA damage, oxidative stress and chemotherapeutic drugs (24). Because over 50% of all human tumors contain functionally mutant p53 (25), the role of p53 in apoptosis has led to concern that many tumors may escape p53-mediated cell death pathways (26). Indeed, several studies have shown that tumor cell lines with mutant p53 are substantially more resistant to a number of commonly used anti-cancer agents, including DNA-damaging agents, anti-metabolites and topoisomerase inhibitors such as cisplatin, 5-fluorouracil and etoposide (27,28). Hence, agents which induce programmed cell death independently of p53 are likely to be of value in the treatment of such tumors, alone or in combination with p53-targeted chemotherapies.

Because tachpyridine may show promise as an anti-cancer agent, we have explored the mechanism by which tachpyridine induces cell death. We report here that tachpyridine induces an apoptotic mode of cell death. We show that although tachpyridine treatment causes the accumulation of p53 protein, it does not result in the transcriptional activation of p53 as measured by induction of p21WAF1. We further demonstrate that tachpyridine can induce apoptosis in cells devoid of p53. Thus, tachpyridine induces apoptosis by a p53-independent pathway. These results suggest that tachpyridine may be of utility in treating the ~50% of human cancers with mutant or absent p53.

Materials and methods

Iron chelators and chemotherapeutics

Tachpyridine was synthesized from cis-1,3,5-triaminocyclohexane as its nitrate salt according to previously published methods (13,15). The identity of tachpyridine was confirmed by 1H, 13C NMR as described (13) and was >99% pure. The compound was prepared as a 1 mM stock in phosphate-buffered saline (PBS), pH 7.4, 0.22 μm filter sterilized and stored at 4°C. Appropriate volumes were added from this stock to cell cultures. Desferrioxamine (95% pure) was purchased from Sigma (St Louis, MO), prepared as a 1 mM stock in complete medium, filter sterilized and added to cultures from this stock. VP16 (etoposide) was purchased from Sigma and reconstituted as a 50 mM stock in dimethyl sulfoxide (DMSO) on the day of the experiment. From this, a working stock of between 1 and 2 mM was prepared by dilution in complete medium for addition to cells. Diluent controls for DMSO contained DMSO present at the highest concentration in the drug screen. For phase contrast microscopy, representative fields of cells were photographed at ×200 magnification using a Nikon Diaphot microscope (Nikon, Melville, NY) and a DC-500 camera (Dage-MTI, Michigan City, IN). In each experiment, at least three fields of cells were photographed per time point for each drug concentration and 96-well plate (Costar, Cambridge, MA). Tachpyridine was added to the cultures at full concentration, a nal concentration, a final concentration, and the cells were observed using fluorescence microscopy under oil immersion with UV illumination (Axiovert II, Zeiss).

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay of fragmented DNA

The degree of DNA fragmentation in chelator-treated cells was quantified using a commercially available TUNEL assay kit (APO-BRDU; Pharmingen, San Diego, CA) according to the manufacturer’s protocol.

Clonogenic assays

Exponentially growing 10(1) or 12(1) cells were allowed to attach overnight before being treated for 72 h with various concentrations of chelator. Chelator-containing medium was replaced with fresh medium and surviving colonies fixed and stained with 0.1% crystal violet after 10–15 days outgrowth.

Western blotting of human p53 and p21WAF1 in MCF7 and ecdysone-inducible H1299 cells

Treated or control cells were harvested by scraping into medium, washed in ice-cold PBS and pellets frozen at −80°C until analysis. Pellets were suspended in 400 μl of ice-cold NP40 lysis buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% NP40) containing a cocktail of protease inhibitors (Complete; Boehringer Mannheim, Indianapolis, IN), including phenylmethyl-sulphonyl fluoride (0.1 mg/ml), and sonicated for 10 s. An aliquot of 20 μg protein from each lysate was separated by 10% SDS-PAGE and analyzed by western blotting using mouse anti-human p53 mAb (Ab-6; Calbiochem, San Diego, CA) or mouse anti-human p21 (Pharmingen) followed by peroxidase-conjugated secondary antibody (goat anti-mouse IgG; Calbiochem) and detection using enhanced chemiluminescence (Amersham, Little Chalfont, UK). Equivalent loading and protein transfer was confirmed by staining membranes with Ponceau S (Sigma).

Growth inhibition of cancer cells by tachpyridine in the NCI anti-cancer drug screen

These studies were conducted at the NCI and the methodology employed has been described in detail elsewhere (28, and references therein).

Results

Tachpyridine induces membrane changes characteristic of apoptosis

In order to determine the mode of cell killing induced by tachpyridine, we first performed time-lapse phase contrast video microscopy, a highly reliable index of apoptosis that enables visualization of successive morphological changes in individual cells (31,33). In these and subsequent studies tachpyridine was used at a 25 μM final concentration, a pharmacologically relevant dose that can be attained in vivo (34). We performed time-lapse video microscopy on all cell lines used in our studies: MCF7 human breast cancer cells, H1299 human non-small cell lung carcinoma cells and 12(1) and 10(1) mouse embryonic fibroblasts containing wild-type
p53-independent apoptosis mediated by tachpyridine

Fig. 1. Cells treated with tachpyridine exhibit membrane changes characteristic of apoptosis. MCF7 (left) or H1299 (right) cells were treated with tachpyridine (25 µM, C-H) or diluent (A and B) in 35 mm tissue culture dishes. After 18 h treatment the dishes were observed under phase contrast and membrane morphology photographed. Characteristics of apoptosis are clearly visible and are indicated by arrows: blebs (C and D), spikes (E and F), blisters (G and H). Magnification ×668; bar 9.5 µm.

p53 or p53 null, respectively. Morphological changes of the cell membrane associated with apoptosis, including the formation of blebs, spikes and blisters (31), were clearly evident in all four cell lines after treatment with 25 µM tachpyridine, as illustrated in Figure I for MCF7 (Figure 1C, E and G) and H1299 (Figure 1D, F and H) cells. Such changes were not observed with untreated (control) cells (Figure 1A and B, respectively). Analysis of the time course of apoptosis demonstrated that for all cell lines examined 100% of the cells in the field of observation underwent apoptotic cell death (data not shown).

Fig. 2. Cells treated with tachpyridine exhibit nuclear changes characteristic of apoptosis. MCF7 (left) or H1299 (right) cells were treated with tachpyridine (25 µM, C-F) or diluent (A and B) in 35 mm tissue culture dishes. After 18 h treatment cells were isolated, fixed onto the dishes and stained with DAPI as described in Materials and methods. Nuclei were observed and photographed under high power. Intra-peripheral chromatin condensation (C and D) and nuclear fragmentation (E and F) are indicated. Magnification ×1060, bar 6 µm.

Tachpyridine induces nuclear changes characteristic of apoptosis

In order to test whether tachpyridine-treated cells exhibited changes in nuclear morphology consistent with apoptotic cell death, cells were stained with DAPI and nuclei examined by fluorescence microscopy as described in Materials and methods. As shown in Figure 2, following treatment with tachpyridine both MCF7 and H1299 cells exhibited nuclear segmentation and intra-peripheral chromatin condensation, hallmarks of apoptosis (31,33).

Tachpyridine induces DNA cleavage as measured by the TUNEL assay

Engagement of apoptotic pathways induces DNA fragmentation through induction of specific DNA endonucleases (35,36). Activation of nucleases can be detected through use of the TUNEL assay, an assay that measures fragmented DNA by flow cytometry following labeling of DNA ends with Br-dUTP (35,37). As seen in Figure 3, although DNA fragmentation was not observed in untreated cells, both 10(1) and 12(1) cells exhibited extensive DNA fragmentation following treatment with tachpyridine. DNA ‘ladders’ characteristic of the inter-
nucleosomal DNA cleavage that occurs during apoptosis were also observed following agarose gel electrophoresis of DNA isolated from cells treated with tachpyridine (data not shown). Tachpyridine induces the accumulation of p53 but not p21WAF1. Many chemotherapeutic agents induce apoptosis by activation of a p53-dependent pathway. In order to determine whether tachpyridine activated a p53-dependent apoptotic pathway, we first asked whether tachpyridine increased levels of p53 protein. MCF7 cells (which contain basal levels of wild-type p53) were treated with 25 µM tachpyridine, cell extracts prepared at various time intervals from 0 to 24 h and p53 levels assessed by western blotting. As a control, cells were treated with etoposide (VP-16), a DNA-damaging drug known to induce p53 (38). As shown in Figure 4, levels of p53 protein were dramatically and rapidly increased following treatment with tachpyridine. Levels of p53 protein induced by tachpyridine were comparable with those induced by VP-16. In order to test whether p53 protein induced by tachpyridine exhibited transcriptional transactivation activity, we then measured levels of p21WAF1, a gene transcriptionally activated by p53 (39). As shown in Figure 4, tachpyridine treatment did not result in detectable levels of p21WAF1. In contrast, activation of p53 by VP16, used as a positive control, led to induction of p21WAF1. These results indicate that tachpyridine treatment leads to accumulation of p53 protein, but that p53 induced by tachpyridine is not competent to transcriptionally induce p21WAF1.

Apoptosis mediated by tachpyridine is p53 independent

Since p53 can activate apoptotic cell death pathways by mechanisms that are both dependent (26,27) and independent (29–31) of its transactivation potential, we asked whether p53 accumulated in response to tachpyridine was required for the cytotoxic effects of tachpyridine. We first compared the sensitivity of p53 wild-type 12(1) cells and p53 null 10(1) mouse embryonic fibroblasts to tachpyridine using a MTT cytotoxicity assay (32). p53 status of these cell lines was confirmed by western blotting (not shown). The sensitivity of 10(1) and 12(1) cells to tachpyridine was very similar, with IC50 values of 4.7 ± 0.27 and 5.2 ± 0.81 µM (n = 5), respectively, despite their differing p53 status. p53-independent killing observed with the MTT assay was confirmed by clonogenic assays (Figure 5).

In order to compare the effects of p53 on tachpyridine sensitivity in isogenic cell lines, we next assessed the response to tachpyridine in p53 null H1299 cells stably transfected with both the edysone receptor and a plIND-based inducible expression vector containing p53 (H1299pINDp53). H1299 cells transfected with edysone receptor alone (pVgRXR) were used as the control. As shown in Figure 6A, p53 protein was undetectable in control cells and in H1299pINDp53 cells in the absence of the edysone analog ponasterone A. Addition of ponasterone A to the medium led to a gradual increase in p53, with maximal levels seen at 48 h. p53 was not detected in control cells transfected with receptor alone under any conditions (Figure 6A). To test the effect of p53 on sensitivity to tachpyridine, H1299pINDp53 cells were pretreated for 24 h with ponasterone A to induce p53. Cells were then exposed to tachpyridine for an additional 72 h in the continuous presence of ponasterone A and viability measured using the MTT assay. H1299 cells expressing edysone receptor alone (pVgRXR) were used to control for effects of ponasterone A. As shown in Figure 6B, the sensitivity of H1299pINDp53 cells remained essentially unchanged in the presence of ponasterone A and was not different from H1299 cells transfected with receptor alone (pVgRXR). These results show that in H1299 cells the cytotoxic effect of tachpyridine is equivalent in the presence and absence of p53.

Under the experimental conditions shown in Figure 6, induction of p53 did not cause substantial growth arrest (Figure 6B). To assess the effects of p53 on tachpyridine-mediated apoptosis at activity levels of p53 sufficient to induce cell cycle arrest, we repeated these experiments using H1299 cells
Cells were grown and treated with different concentrations of tachpyridine (0–25 µM) or diluent for 72 h as described in Materials and methods. The medium was replaced with fresh medium and dishes reincubated until discernible colonies were visible. Colonies from cells treated with two tachpyridine concentrations (8 and 16 µM) as well as those from untreated cells (control) are shown. In this experiment, following treatment with 8 µM tachpyridine there were 122 ± 12 colonies of 12(1) cells (mean ± SD, n = 3) and 103 ± 22 colonies of 10(1) cells. These colony numbers were not statistically different (P = 0.28).

Stably transfected with tetracycline-inducible p53 (HT7 cells) (40). Treating HT7 cells with doxycycline for 24 h resulted in p53 accumulation and growth arrest (data not shown). Induction of p53 in these cells showed a similar lack of effect on sensitivity to tachpyridine: the IC50 of tachpyridine in cells treated with doxycycline was 5.5 µM (95% confidence interval 3.6–8.3), versus 6.1 µM (95% confidence interval 3.9–9.2) in cells that had not been pretreated with doxycycline. Further, as seen in Figures 1–3, p53 null cells treated with tachpyridine exhibited the same hallmarks of apoptosis as p53 wild-type cells, including membrane blebbing, spiking and blistering (Figure 1), as well as nuclear condensation (Figure 2) and DNA fragmentation (Figure 3).

Sensitivity to tachpyridine in the NCI multi-cell line screen is p53 independent

Using a panel of human cancer cell lines, previous studies have shown that the activity of certain drugs as measured by GI50 is correlated with p53 status (28). In order to determine whether tachpyridine would exhibit p53 independence when assayed in multiple cancer cell lines, the GI50 of tachpyridine in cells treated with doxycycline was 5.5 µM (95% confidence interval 3.6–8.3), versus 6.1 µM (95% confidence interval 3.9–9.2) in cells that had not been pretreated with doxycycline. Further, as seen in Figures 1–3, p53 null cells treated with tachpyridine exhibited the same hallmarks of apoptosis as p53 wild-type cells, including membrane blebbing, spiking and blistering (Figure 1), as well as nuclear condensation (Figure 2) and DNA fragmentation (Figure 3).

Discussion

The use of currently available iron chelators as anti-cancer agents has been viewed alternately as a success and failure (12,41,42). The activity of desferrioxamine in neuroblastoma has been viewed as a proof of principle demonstration that iron chelators can target cellular processes critical to cancer cell growth (10,43). On the other hand, thus far neither desferrioxamine nor other available chelators have established themselves as mainstay drugs in neuroblastoma or other cancers. This is not surprising, since these compounds were primarily selected to meet criteria not necessarily pertinent to successful anti-cancer agents, i.e. efficacy in mobilizing body iron stores and lack of toxicity when administered continuously over periods of years in patients with iron overload disorders.

We have begun to consider whether chelators specifically developed with the goal of an anti-cancer application may...
treatment with desferrioxamine, although levels of p53 were lower and appeared with delayed kinetics (data not shown). However, p53 accumulated in response to tachpyridine appeared non-functional in transcriptional transactivation, since p21WAF1 was not observed in tachpyridine-treated cells (Figure 4).

Metal chelating agents have been previously observed to affect p53. 1,10-Phenanthroline and N,N,N',N'-tetrakis(2-pyrimidylimethyl)ethylenediamine were reported to inactivate p53, presumably by coordinating an essential zinc atom (48,49). In contrast, others have found that treatment of intact cells with 1,10-phenanthroline increases p53 transcriptional activation and DNA-binding activity without increasing p53 protein levels (50). Since treatment with tachpyridine results in p53 accumulation, it does not mimic the activity of either of these two metal chelators. Rather, tachpyridine may more closely resemble the iron chelator desferrioxamine, since desferrioxamine similarly induced p53 accumulation without inducing p21WAF1 (51). In contrast, the PII-related chelator 311 has been reported to activate p21WAF1 as well as GADD45 in a p53-independent manner (52). Thus, genes and pathways induced in response to chelator treatment vary in a chelator-specific fashion.

Despite the apparent lack of transcriptional activation activity of p53 induced by tachpyridine, it remained possible that p53 played a role in tachpyridine-mediated apoptosis through its transcriptional repression function (23,53,54). However, using a number of criteria, including cellular and nuclear morphology and TUNEL assays, we found that even p53 null cells could efficiently activate apoptotic pathways when treated with tachpyridine. Similarly, we have observed that p53 null 10(1) and p53 wild-type 12(1) cells exhibit comparable sensitivity to desferrioxamine (data not shown). Thus, the ability to trigger apoptotic pathways in the absence of p53 may be a general feature of iron chelator-mediated cytotoxicity.

Previous characterization of p53 genotype and drug sensitivity of human cancer cell lines has revealed that cells with mutant or absent p53 exhibit less growth inhibition than cells with wild-type p53 to the majority of clinically used anti-cancer agents, including DNA alkylating agents, DNA/RNA anti-metabolites and topoisomerase I and II inhibitors (28). For example, in the cases of bleomycin, 5-fluorouracil and cisplatin the median GI50 ranged from 3- to 10-fold higher in p53 mutant cells than in p53 wild-type cells (28). In contrast to these drugs, screening by the NCI under the same conditions and in the same cell lines revealed no correlation between sensitivity to tachpyridine and p53 status (Figure 7). Over 50% of human tumors contain mutations in p53 (25). The observation that tachpyridine is competent to induce apoptosis in cells with mutant or absent p53 may therefore enhance its utility in anti-cancer therapy. Additionally, since iron depletion defines a biological target different from that addressed by most anti-cancer agents, tachpyridine may exhibit utility not only as a single agent, but as a complement to other anti-cancer drugs directed at alternative cellular targets.

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FIG. 7. Growth inhibition of cancer cells of known p53 status by tachpyridine in the NCI anti-cancer drug screen. The ability of tachpyridine to inhibit the growth of a wide variety of cancer cells of known p53 status is shown. The cell lines are divided into two groups: those which express a mutated form of p53 and those which express wild-type p53. On the ordinate is shown the negative logarithm of the drug concentration required to inhibit growth by 50%. The horizontal bar for each category represents the mean –logGI50.

Meet with greater therapeutic success. Towards that end we have synthesized tachpyridine, a novel iron chelator, and are exploring its utility in the treatment of cancer (14). The anti-neoplastic potential of other chemically unrelated chelators, such as chelators based on pyridoxal isonicotinoyl hydrazone (PIH) (5) and the 8-hydroxyquinoline based chelator O-Trensox, are also being considered in other laboratories (18). Tachpyridine is a hexadentate chelator that preferentially binds Fe(II), although it is also capable of binding Fe(III) by a process of oxidative dehydrogenation (15). Tachpyridine has an n-octanol:water partition coefficient (K_{part}) of 0.4 at pH 7.4, consistent with an ability to penetrate biological membranes (44). Tachpyridine and desferrioxamine are comparable in their ability to repress synthesis of the iron-binding protein ferritin, the synthesis of which is critically dependent upon intracellular iron, suggesting that these two chelators share a similar ability to deplete intracellular iron pools (14,45). Tachpyridine is currently in preclinical development at the NCI as part of the RAID program.

Our initial in vitro studies revealed that tachpyridine exhibited a potent cytotoxic effect (14). The results shown here indicate that tachpyridine exerts its cytotoxic effect by inducing apoptosis. Induction of apoptosis as assessed by DNA fragmentation has also been observed following exposure of cells to other iron chelators, including desferrioxamine and deferiprone (17,20), as well as dithiozone and O-Trensox (16,18). These studies did not address the role of p53 in the apoptotic process.

Many established anti-neoplastic agents, including DNA-damaging drugs such as cisplatin and VP16 (etoposide), as well as γ and UV-irradiation, induce apoptosis by activating the tumor suppressor p53. Western blot analysis of p53 in MCF7 cells treated with tachpyridine showed an early and dramatic increase in the levels of this protein. As reported by others (46,47), we also observed accumulation of p53 following treatment with desferrioxamine, although levels of p53 were
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