

Potent Antitumor Activity of Novel Iron Chelators Derived from Di-2-Pyridylketone Isonicotinoyl Hydrazone Involves Fenton-Derived Free Radical Generation

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ABSTRACT

Purpose: The development of novel and potent iron chelators as clinically useful antitumor agents is an area of active interest. Antiproliferative activity of chelators often relates to iron deprivation or stimulation of iron-dependent free radical damage. Recently, we showed that novel iron chelators of the di-2-pyridylketone isonicotinoyl hydrazone (PKIH) class have potent and selective antineoplastic activity (E. Becker, *et al.*, *Br. J. Pharmacol.*, 138: 819–30, 2003). In this study, we assessed the effects of the PKIH analogues on the redox activity of iron in terms of understanding their antitumor activity.

Experimental Design: We tested the PKIH analogues for their ability to promote iron-mediated ascorbate oxidation, benzoate hydroxylation, and plasmid degradation. Subsequent experiments assessed their ability to bind DNA, inhibit topoisomerase I, and cause DNA damage. To measure intracellular reactive oxygen species, we used the redox-sensitive probe, 2',7'-dichloro-fluorescein-diacetate, to measure intracellular PKIH-dependent redox activity.

Results: The PKIH analogues had relatively little effect on ascorbate oxidation in the presence of Fe(III) but stimulated benzoate hydroxylation and plasmid DNA degradation in the presence of Fe(II) and H₂O₂. These ligands could not inhibit DNA topoisomerase I or cause DNA damage in intact

cells. PKIH markedly increased the intracellular generation of reactive oxygen species, and this was inhibited by catalase. This enzyme also decreased the antiproliferative effect of PKIH, indicating H₂O₂ played a role in its cytotoxic activity.

Conclusions: Our results suggest that the antiproliferative effects of these chelators relates to intracellular iron chelation, followed by the stimulation of iron-mediated free radical generation via the so-formed iron complex.

INTRODUCTION

Some tumors, particularly neuroblastoma and leukemia, are sensitive to iron deprivation (1–5). These rapidly proliferating cell types are known to have an increased requirement for iron (1, 3). This is best reflected by their heightened expression of the transferrin receptor 1 and increased iron uptake that presumably accommodates the need for DNA synthesis and other iron-dependent metabolic processes (3).

The growing body of literature concerning iron chelation as a treatment for cancer illustrates the importance of iron in a variety of crucial metabolic pathways and also the need for a range of chelators to be characterized in this context (1–10). These studies have also attempted to define the mechanisms behind the selective inhibition of tumor cell proliferation by iron chelators. Historically, the clinically used chelator, desferrioxamine, and a variety of α -N-heterocyclic carboxaldehyde thiosemicarbazone chelators have dominated this field (4–7). In large part, this is due to their ability to inhibit ribonucleotide reductase (6–10), an iron-containing enzyme that catalyzes the rate-limiting step of DNA synthesis (11).

Unfortunately, the effectiveness of desferrioxamine is greatly hindered by a range of disadvantages, including a short plasma half-life, poor membrane permeability, and the requirement for long s.c. infusion (12 to 24 hours per day, five to six times per week; ref. 3). In an effort to target ribonucleotide reductase, a range of α -N-heterocyclic carboxaldehyde thiosemicarbazones have been identified that can deprive the R2 subunit of iron and thus destabilize the critical tyrosyl radical that is required for ribonucleotide reductase activity (6, 7). Interestingly, one of the most recent products of α -N-heterocyclic carboxaldehyde thiosemicarbazone development, namely 3-amino-2-pyridyl carboxaldehyde thiosemicarbazone (Triapine; ref. 12), was shown to have selective antiproliferative activity but poor iron chelation efficacy *in vitro* (13). This finding has led to the identification of other modes of antiproliferative activity of these chelators (13). These mechanisms of action include the stimulation of iron-mediated free radical generation and the localization of these radicals to vital biomolecules such as DNA (13).

Aroylhydrazone chelators such as the pyridoxal isonicotinoyl hydrazone analogue, 2-hydroxy-1-naphthaldehyde isonic-

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otinoyl hydrazone, can also inhibit ribonucleotide reductase (9, 13). This is thought to relate to their high chelation efficacy because this class of chelators mobilize cellular iron and prevent iron uptake from transferrin far more effectively than desferrioxamine or Triapine (13, 14). Recently, a series of chelators known as the 2-pyridylcarboxaldehyde isonicotinoyl hydrazone analogues were developed (15). These ligands have high chelation efficacy and low toxicity *in vitro* and have potential for the treatment of iron overload diseases such as β -thalassaemia (15). To increase the understanding of the relationship between structure and antiproliferative activity of chelators, a series of aroylhydrazone chelators known as the di-2-pyridylketone isonicotinoyl hydrazone analogues (Fig. 1) were designed based on 2-pyridylcarboxaldehyde isonicotinoyl hydrazone (16). A number of these ligands show high iron chelation efficacy in terms of increasing iron mobilization from cells and preventing iron uptake from transferrin and also have potent and selective antitumor activity *in vitro* (16). These studies demonstrate that the replacement of the 2-pyridylcarboxaldehyde moiety (2-pyridylcarboxaldehyde isonicotinoyl hydrazone) with a more lipophilic di-2-pyridylketone moiety (di-2-pyridylketone isonicotinoyl hydrazone) results in much greater antiproliferative activity (16).

In the present study, the redox activity of iron in the presence of the di-2-pyridylketone isonicotinoyl hydrazone series of chelators was measured using a variety of assays. These studies were considered crucial as the iron complexes of the di-2-pyridylketone isonicotinoyl hydrazone analogues showed similar antiproliferative activity as the chelators (16). The results of this investigation demonstrated that the antiproliferative activity of these chelators relate, in part, to their ability to enhance the redox activity of cellular iron.

MATERIALS AND METHODS

Chelators. The six di-2-pyridylketone isonicotinoyl hydrazone analogues were synthesized using standard procedures (16). Desferrioxamine was purchased from Novartis (Summit, NJ).

Cell Culture. The human SK-N-MC neuroepithelioma cell line was obtained from the American Type Culture Collection (Manassas, VA). These cells were used so that direct comparison could be made to data presented in previous publications (10, 13, 15–17). The cells were grown as described previously (14, 18).

Ascorbate Oxidation and Benzoate Hydroxylation Assays. These assays were performed as detailed in refs. 17, 19, 20.

Plasmid DNA Degradation Assay. These experiments were performed with standard procedures described previously (17, 21, 22). Briefly, reagents were added to sterile microfuge tubes in the following order: purified sterile water, chelator (1, 10, and 30 $\mu\text{mol/L}$), FeSO_4 (10 $\mu\text{mol/L}$), H_2O_2 (1 mmol/L), and plasmid DNA (pGEM-7Zf(+)) (10 $\mu\text{g/mL}$; Promega Corp., Madison, WI) to a final volume of 100 μL (17, 21).

DNA-binding and Topoisomerase I Assays. The DNA-binding experiments were performed by standard methods (17). Briefly, an appropriate absorbance peak was chosen from spectrophotometric wavelength scans of each chelator and its iron

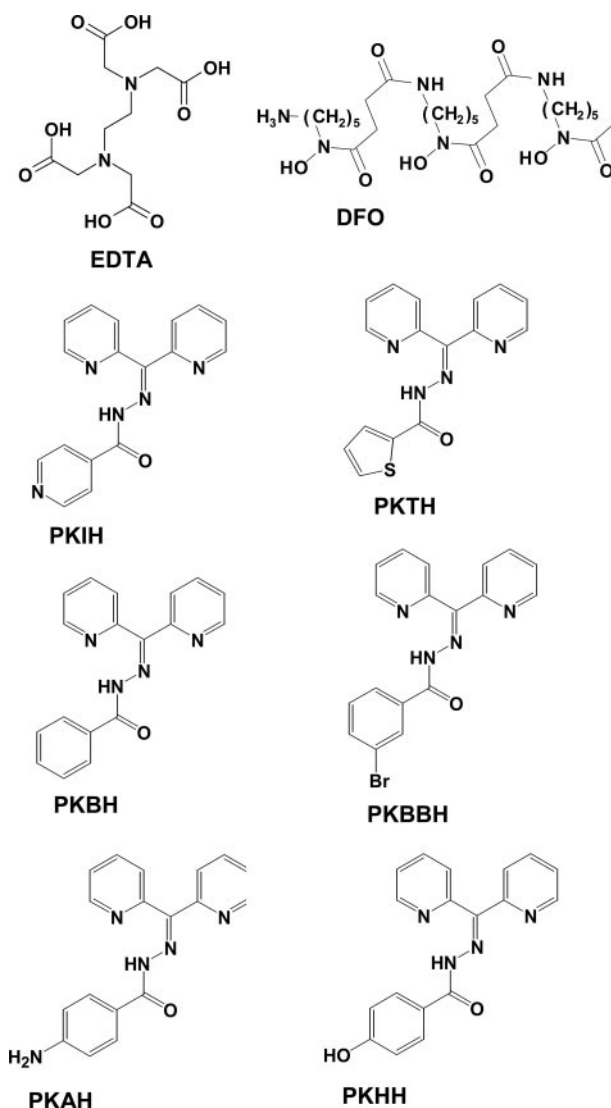


Fig. 1 Structural formulae of EDTA, desferrioxamine (DFO), di-2-pyridylketone isonicotinoyl hydrazone (PKIH), di-2-pyridylketone thiophenecarboxyl hydrazone (PKTH), di-2-pyridylketone benzoyl hydrazone (PKBH), di-2-pyridylketone *m*-bromobenzoyl hydrazone (PKBBH), di-2-pyridylketone *p*-aminobenzoyl hydrazone (PKAH), and di-2-pyridylketone *p*-hydroxybenzoyl hydrazone (PKHH).

complex. Herring sperm DNA Sigma (Chemicals, St. Louis, MO) was added to the ligand, iron complex, or reference solutions, and changes in test compound absorbance were recorded (17, 23). The well-characterized DNA intercalation agent, doxorubicin, was used as a positive control (17, 24, 25).

The topoisomerase I studies were performed as described in Chaston and Richardson (17) to determine the ability of the di-2-pyridylketone isonicotinoyl hydrazone analogues to bind DNA. Intercalation of compounds results in the inhibition of topoisomerase I activity that converts DNA from the supercoiled DNA to open circular DNA form (17, 26). In these experiments, 40 μL of assay buffer [50 mmol/L Tris-HCl, 0.01 mol/L EDTA, 50 mmol/L NaCl, 1 mmol/L DTT, and 20%

glycerol (pH 7.5)] containing 5 $\mu\text{g/mL}$ pBR322 were added to tubes on ice. Doxorubicin (0.1, 1, and 10 $\mu\text{mol/L}$) or the ligands (20, 40, and 60 $\mu\text{mol/L}$) and topoisomerase I (1 unit; Promega Corp.) were added to a total of 50 μL (17).

DNA Precipitation Assay. This method was carried out with standard methodology (17, 27).

Intracellular Reactive Oxygen Species Assay. Intracellular reactive oxygen species generation was measured with the cell-permeable dye, 2',7'-dichloro-fluorescein-diacetate (Sigma; refs. 28, 29). This compound readily diffuses into cells and becomes highly fluorescent when oxidized by superoxide, H_2O_2 , or hydroxyl radicals (28, 29). Cellular fluorescence intensity is directly proportional to the level of intracellular reactive oxygen species (28, 29). SK-N-MC cells (1×10^5 cells per well) were incubated with di-2-pyridylketone isonicotinoyl hydrazone (20, 40, or 60 $\mu\text{mol/L}$) in the presence and absence of ebselen (15 $\mu\text{mol/L}$; Sigma), superoxide dismutase (SOD, 1000 units/mL; Sigma), or catalase (1000 units/mL; Sigma) for 24 hours or 50 $\mu\text{mol/L}$ H_2O_2 (positive control; refs. 28, 29) for 10 minutes at 37°C. The cells were then incubated with 2',7'-dichloro-fluorescein-diacetate (5 $\mu\text{mol/L}$) for 20 minutes at 37°C, washed twice, and detached from the culture plates (28, 29).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Proliferation Assay. Antiproliferative activity of di-2-pyridylketone isonicotinoyl hydrazone was examined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (14, 30).

Statistics. Experimental data were compared using Student's paired *t* test. Results were considered statistically significant when $P < 0.05$. Results are presented as the mean or mean \pm SE of three to four separate experiments.

RESULTS

The Effect of Chelators on the Redox Cycling of Iron in the Presence of Ascorbate. The ability of the chelator to interact intracellularly with the physiologic reductant ascorbate could play a marked effect on reactive oxygen species generation in cells. To determine the effect of chelators on redox cycling of iron in the presence of ascorbate, the rate of ascorbate oxidation was measured in the presence of the chelators and Fe(III) (Fig. 2A). As positive and negative controls, we used EDTA and desferrioxamine, respectively (13, 17, 19). EDTA significantly ($P < 0.00001$) increased ascorbate oxidation to 294 and 302% of the control at an iron-binding equivalent ratio of 1 and 3 but had little effect at an iron-binding equivalent of 0.1 (Fig. 2A). In contrast, desferrioxamine inhibited the reaction to 78 and 9% of the control at iron-binding equivalent ratios of 1 and 3, respectively, whereas it had no significant effect at an iron-binding equivalent of 0.1 (Fig. 2A). The chelators di-2-pyridylketone thiophenecarboxyl hydrazone, di-2-pyridylketone benzoyl hydrazone, and di-2-pyridylketone *p*-aminobenzoyl hydrazone inhibited ascorbate oxidation in a concentration-dependent manner to 26, 46, and 23% of the control, respectively, at an iron-binding equivalent ratio of 3. Interestingly, di-2-pyridylketone *p*-hydroxybenzoyl hydrazone inhibited this reaction to 56% at all tested iron-binding equivalent ratios, whereas di-2-pyridylketone isonicotinoyl hydrazone and di-2-pyridyl-

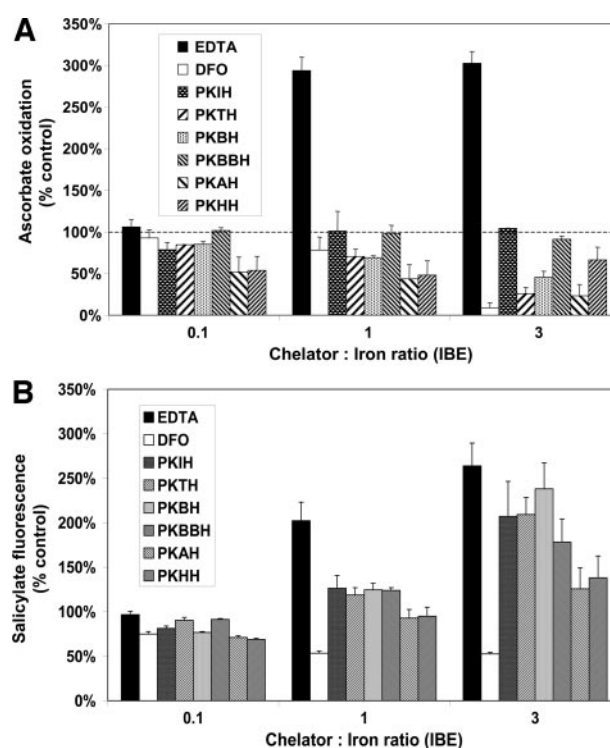


Fig. 2 A. The effect of EDTA, desferrioxamine (DFO), and the di-2-pyridylketone *p*-hydroxybenzoyl hydrazone (PKIH) analogues [PKIH, di-2-pyridylketone thiophenecarboxyl hydrazone (PKTH), di-2-pyridylketone benzoyl hydrazone (PKBH), di-2-pyridylketone *m*-bromobenzoyl hydrazone (PKBBH), di-2-pyridylketone *p*-aminobenzoyl hydrazone (PKAH), and di-2-pyridylketone *p*-hydroxybenzoyl hydrazone (PKHH)] on ascorbate oxidation (A) and the hydroxylation of benzoate in the presence of Fe and hydrogen peroxide (B). A. Ascorbate oxidation studies: chelators at iron-binding equivalent (IBE) ratios of 0.1, 1, and 3 were incubated at room temperature in the presence of iron (10 $\mu\text{mol/L}$) and ascorbate (100 $\mu\text{mol/L}$). The UV-Vis absorbance at 265 nm was recorded after 10 and 40 minutes and the difference between the time points calculated. B. Benzoate hydroxylation studies: chelators at IBE ratios of 0.1, 1, and 3 were incubated for 1 hour at room temperature in the presence of Fe(II) (30 $\mu\text{mol/L}$), hydrogen peroxide (5 mmol/L), and benzoate (1 mmol/L). The fluorescence of hydroxylated benzoate was measured at 308 nm excitation and 410 nm emission. Results are mean \pm SE (four experiments).

etone *m*-bromobenzoyl hydrazone had no significant effect on the rate of ascorbate oxidation under all conditions tested (Fig. 2A).

The Effect of Chelators on the Fenton-dependent Hydroxylation of Benzoate. To assess the ability of chelators to affect the redox cycling of iron in the presence of H_2O_2 , the chelators, iron (added as FeSO_4) and H_2O_2 , were incubated in the presence of benzoate. The degree of benzoate hydroxylation was determined by measuring the presence of the fluorescent product, salicylate (Fig. 2B). Again, EDTA and desferrioxamine acted as positive and negative controls, respectively (13, 17, 19). EDTA stimulated benzoate hydroxylation to 202 and 260% of the control at iron-binding equivalent ratios of 1 and 3, respectively, whereas desferrioxamine inhibited the reaction to 52% of the control at iron-binding equivalent ratios of 1 and 3 (Fig. 2B). In contrast to the ascorbate oxidation results, at an iron-binding

equivalent ratio of 1, di-2-pyridylketone isonicotinoyl hydrazone, di-2-pyridylketone thiophenecarboxyl hydrazone, di-2-pyridylketone benzoyl hydrazone, and di-2-pyridylketone *m*-bromobenzoyl hydrazone significantly ($P < 0.05$) increased the rate of benzoate hydroxylation to 122 to 126% of the control. At an iron-binding equivalent ratio of 3, these latter chelators significantly ($P < 0.01$) increased benzoate hydroxylation to 207, 209, 238, and 178% of the control, respectively (Fig. 2B). In contrast, di-2-pyridylketone *p*-aminobenzoyl hydrazone and di-2-pyridylketone *p*-hydroxybenzoyl hydrazone had no significant activity in this assay when present at iron-binding equivalent ratios of 1 and 3 (Fig. 2B). All of the di-2-pyridylketone isonicotinoyl hydrazone analogues tested caused inhibition of benzoate hydroxylation to between 68 and 91% when present at an iron-binding equivalent of 0.1 (Fig. 2B). It is of interest to note that di-2-pyridylketone *p*-aminobenzoyl hydrazone and di-2-pyridylketone *p*-hydroxybenzoyl hydrazone caused significantly less ($P < 0.05$) redox activity than the other compounds at all iron-binding equivalent ratios (Fig. 2B).

The Effect of the Chelators on Fenton-mediated Degradation of Plasmid DNA. The ability of the chelators to affect Fenton-mediated single- and double-strand breaks in plasmid DNA was assessed by incubating plasmid DNA in the presence of the chelators, Fe(II) and H₂O₂ (10, 13, 17). A single-strand break causes the conversion of supercoiled DNA to the open circular form, whereas a double-stranded break causes conversion of supercoiled DNA to the linear DNA form (10, 13, 17). These three forms run at different rates on agarose gels (Fig. 3, A and B; refs. 10, 13, 17). As a control, plasmid treated with the restriction enzyme *Bam*HI was included, resulting in a single band of linear DNA (Fig. 3, A and B, Lane 2). Untreated plasmid DNA and plasmid treated with H₂O₂ alone appeared on the gels predominately as supercoiled DNA (Fig. 3, A and B, Lanes 3 and 4). When supercoiled plasmid DNA was treated with Fe(II) and H₂O₂, it was completely converted to linear and open circular DNA (Fig. 3, A and B, Lane 5).

Desferrioxamine was not particularly protective of plasmid DNA and was required at an iron-binding equivalent of 3 to markedly prevent Fenton-mediated strand breaks (Fig. 3A, Lane 6, and Fig. 3B, Lanes 7 to 9). Paradoxically, EDTA was highly protective of plasmid DNA when present at iron-binding equivalent ratios of 1 and 3 (Fig. 3A, Lane 7, and Fig. 3B, Lanes 10 to 12). All of the di-2-pyridylketone isonicotinoyl hydrazone analogues markedly stimulated DNA degradation (Fig. 3A, Lanes 9 to 20, and Fig. 3B, Lanes 13 to 18). Even at an iron-binding equivalent of 0.1, very little plasmid DNA was detectable in the presence of the di-2-pyridylketone isonicotinoyl hydrazone analogues (Fig. 3, A and B). Only smears of degraded DNA could be seen in the presence of any of the di-2-pyridylketone isonicotinoyl hydrazone chelators at iron-binding equivalent ratios of 1 and 3 (Fig. 3A, Lanes 9 to 20, and Fig. 3B, Lanes 13 to 18).

Interactions of Chelators and Their Fe(II) Complexes with DNA. The ability of chelators and Fe(II) complexes to interact with DNA was determined through measurement of DNA-dependent hypochromicity of the UV-Vis spectra of the ligand and Fe(II) complex (Fig. 4A; refs. 17, 23, 31). That is, hypochromicity (a decrease in UV-Vis absorbance) or

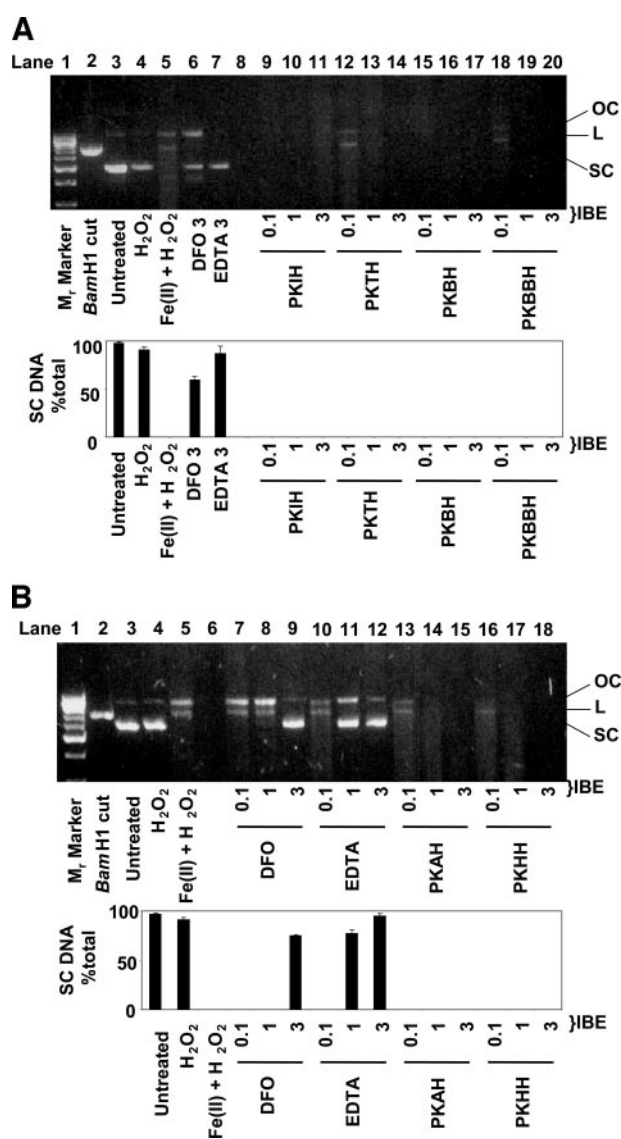


Fig. 3 A and B. The effect of desferrioxamine (DFO), EDTA, and the di-2-pyridylketone *p*-hydroxybenzoyl hydrazone (PKIH) analogues [PKIH, di-2-pyridylketone thiophenecarboxyl hydrazone (PKTH), di-2-pyridylketone benzoyl hydrazone (PKBH), di-2-pyridylketone *m*-bromobenzoyl hydrazone (PKBBH), di-2-pyridylketone *p*-aminobenzoyl hydrazone (PKAH), and di-2-pyridylketone *p*-hydroxybenzoyl hydrazone (PKHH)] on Fenton-mediated plasmid degradation. Chelators at iron-binding equivalent (IBE) ratios of 0.1, 1, and 3 were incubated for 30 minutes in the presence of Fe(II) (10 μ mol/L), hydrogen peroxide (1 mmol/L), and plasmid DNA (10 μ g/mL). Samples were loaded onto an agarose gel and electrophoresed for 1 hour at 90 V (see ref. 17 for details). Densitometric analysis is shown below each gel and illustrates the supercoiled (SC) DNA as a percentage of the total DNA. Results are mean \pm SE (four experiments).

hyperchromicity (an increase in UV-Vis absorbance) upon addition of DNA to the compound indicates an interaction between these molecules (17, 23, 31). As an internal control, the potent intercalating agent and iron-binding ligand, doxorubicin, was used (32) and became subject to 35.8% hypochromicity as a free ligand and 25.0% after preincuba-

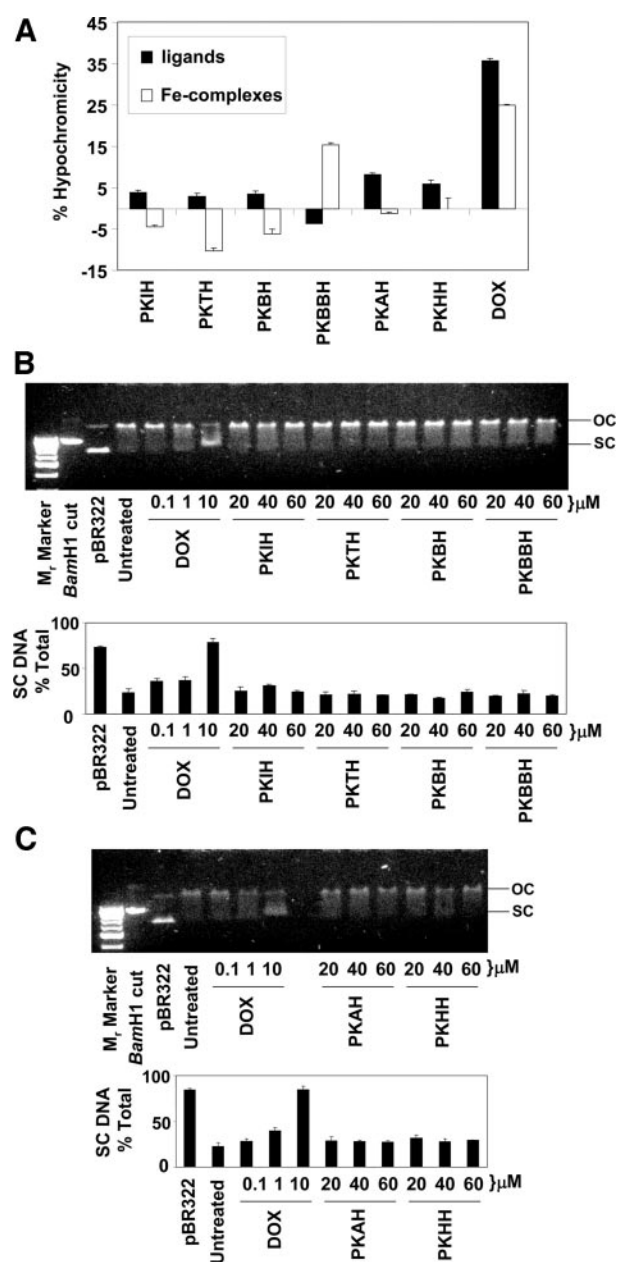


Fig. 4 A. DNA-dependent hypochromicity of doxorubicin (DOX) and the di-2-pyridylketone *p*-hydroxybenzoyl hydrazone (PKIH) analogues [PKIH, di-2-pyridylketone thiophenecarboxyl hydrazone (PKTH), di-2-pyridylketone benzoyl hydrazone (PKBH), di-2-pyridylketone *m*-bromobenzoyl hydrazone (PKBBH), di-2-pyridylketone *p*-aminobenzoyl hydrazone (PKAH), and di-2-pyridylketone *p*-hydroxybenzoyl hydrazone (PKHH)] and their Fe(II) complexes. The effect of DOX, PKIH, PKTH, PKBH, and PKBBH (B) and DOX, PKAH, and PKHH (C) on the activity of DNA topoisomerase I. A. Hypochromicity studies: the ability of the chelators and their Fe(II) complexes to bind DNA was determined through measurement of the DNA-mediated hypochromicity of ligand and Fe(II) complex spectra. An appropriate absorbance peak was chosen from spectrophotometric wavelength scans of each chelator and its iron complex. Concentrations of test compounds were then adjusted so that peak absorbance intensities were <1 (see ref. 17 for details). Data are expressed as the percentage decrease in peak absorbance intensity upon addition of DNA. Results are mean \pm SE (four experiments). B and C. Topoisomerase I studies: DNA topoisomerase I (1 unit), pBR322 plasmid DNA (5 μ g/mL), and the chelators (20 to 60 μ mol/L) were incubated for 1 hour at 37°C.

tion of doxorubicin with a stoichiometric amount of Fe(II) (Fig. 4A).

All of the di-2-pyridylketone isonicotinoyl hydrazone chelators showed very weak DNA-binding activity in the absence of Fe(II), being subject to less than an 8.2% change in peak UV-Vis absorbance intensity upon addition of DNA (Fig. 4A). Interestingly, the iron complexes of these chelators varied widely in their abilities to bind DNA, varying between 0 and 15.4% change in peak absorbance intensity (Fig. 4A). The iron complexes of di-2-pyridylketone thiophenecarboxyl hydrazone and di-2-pyridylketone *m*-bromobenzoyl hydrazone were the most avid binders of DNA as demonstrated by 10.2% hyperchromicity and 15.4% hypochromicity, respectively, in the presence of DNA (Fig. 4A). In contrast, weaker DNA-binding activity was observed for the Fe(II) complexes of di-2-pyridylketone benzoyl hydrazone, di-2-pyridylketone isonicotinoyl hydrazone, di-2-pyridylketone *p*-aminobenzoyl hydrazone, and di-2-pyridylketone *p*-hydroxybenzoyl hydrazone, as indicated by changes in UV-Vis absorbance of 6.1, 3.8, 1.2, and 0%, respectively (Fig. 4A).

Inhibition of DNA Topoisomerase I by Chelators. The ability of iron chelators to inhibit DNA topoisomerase I was determined by incubating the enzyme, plasmid DNA, and the ligand for 60 minutes at 37°C (Fig. 4, B and C). It should be noted that iron complexes of the ligands were not examined because of their marked ability to damage plasmid DNA (see Fig. 3). The intercalating agent doxorubicin was used as a positive control and was found to cause inhibition of enzyme activity at 10 μ mol/L, resulting in the generation of more supercoiled relative to open circular DNA (Fig. 4, B and C). None of the di-2-pyridylketone isonicotinoyl hydrazone analogues could inhibit DNA topoisomerase I significantly at any of the concentrations tested (20, 40, or 60 μ mol/L; Fig. 4, B and C), thus confirming the weakness of DNA binding observed in the hypochromicity experiments (see Fig. 4A).

The Effect of Chelators on the Alkaline Precipitation of Intact Chromosomal DNA from SK-N-MC Neuroepithelioma Cells. The extent of DNA damage caused by chelators was determined by DNA precipitation with the assay of Olive (refs. 17, 27; Fig. 5A). Cells labeled with [3 H]thymidine were lysed under alkaline conditions, and intact genomic DNA was precipitated by centrifugation. Incubation with the positive control, doxorubicin, at 1 and 10 μ mol/L significantly ($P < 0.0001$) decreased DNA precipitation to 52 and 30% of the control indicating direct DNA damage and/or inhibition of cellular DNA repair mechanisms (Fig. 5A). Higher concentrations of doxorubicin could not be used in this experiment because of its marked antiproliferative activity. None of the di-2-pyridylketone isonicotinoyl hydrazone analogues had any significant effect on the proportion of precipitable DNA at 20, 40, or 60 μ mol/L (Fig. 5A).

Samples were loaded onto an agarose gel and electrophoresed for 1 hour (see ref. 17 for details). Densitometric analysis is shown below each gel and illustrates the supercoiled (SC) DNA as a percentage of the total DNA. Note that untreated in this figure denotes topoisomerase I-treated plasmid but without the addition of chelator. Results are a typical experiment from a total of six.

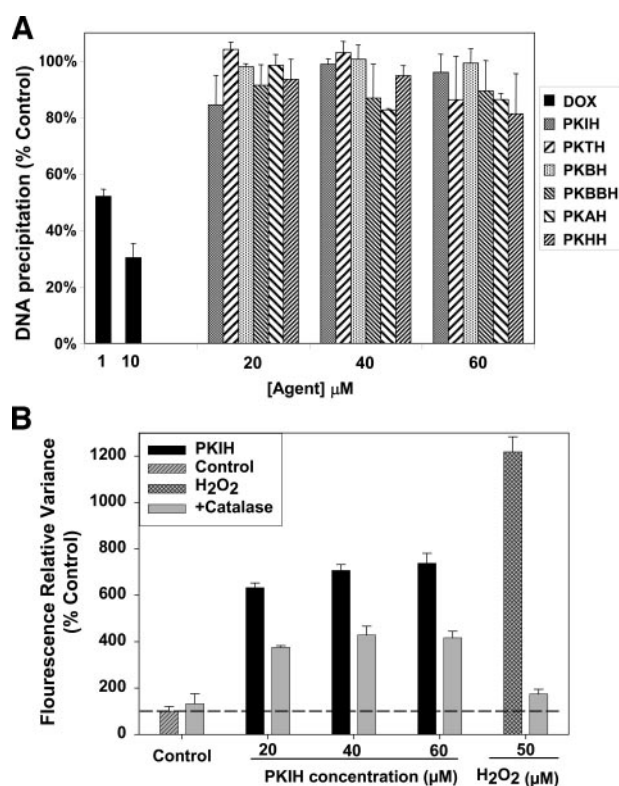


Fig. 5 A. The effect of doxorubicin (DOX) and the di-2-pyridylketone *p*-hydroxybenzoyl hydrazone (PKIH) analogues [PKIH, di-2-pyridylketone thiophenecarboxyl hydrazone (PKTH), di-2-pyridylketone benzoyl hydrazone (PKBH), di-2-pyridylketone *m*-bromobenzoyl hydrazone (PKBBH), di-2-pyridylketone *p*-aminobenzoyl hydrazone (PKAH), and di-2-pyridylketone *p*-hydroxybenzoyl hydrazone (PKHH)] on the proportion of precipitable DNA in SK-N-MC neuroepithelioma cells. B. The effect of PKIH in the presence and absence of catalase on the levels of intracellular reactive oxygen species. A. DNA precipitation studies: SK-N-MC neuroepithelioma cells were labeled with [³H]thymidine (0.5 $\mu\text{Ci}/\text{mL}$) for 24 hours at 37°C, washed, and then treated with DOX (10 $\mu\text{mol}/\text{L}$) or the iron chelators (20, 40, and 60 $\mu\text{mol}/\text{L}$) for 24 hours at 37°C. The cells were then lysed under alkaline conditions and intact genomic DNA was precipitated by centrifugation (see ref. 17 for details). Results are expressed as a percentage of the DNA precipitation found in control cells incubated with medium alone. Results are mean \pm SE (four experiments). B. Intracellular reactive oxygen species studies: the effect of PKIH on the presence of intracellular reactive oxygen species was measured with a flow cytometer with the fluorescent redox probe 2',7'-dichloro-fluorescein-diacetate. The SK-N-MC cells were incubated with PKIH (20, 40, or 60 $\mu\text{mol}/\text{L}$) in the presence or absence of catalase (1000 units/mL) for 24 hours or 50 $\mu\text{mol}/\text{L}$ H₂O₂ (positive control) for 10 minutes at 37°C. The cells were then incubated with 2',7'-dichloro-fluorescein-diacetate (5 $\mu\text{mol}/\text{L}$) for 20 minutes at 37°C, washed twice, and detached from the culture plate. The fluorescence of oxidized 2',7'-dichloro-fluorescein-diacetate in cells was measured with a flow cytometer. Data are expressed as a percentage of the control, *i.e.*, cells incubated with media alone. Results are mean \pm SE (four experiments).

Increase of Intracellular Reactive Oxygen Species Generation after Incubation with Di-2-Pyridylketone Isonicotinoyl Hydrazone. The benzoate hydroxylation and plasmid degradation assays indicate that the di-2-pyridylketone isonicotinoyl hydrazone analogues cause considerable stimulation of

Fenton chemistry in the presence of iron and H₂O₂ (Figs. 2B and 3). Considering this, it was important to examine the generation of free radicals intracellularly after incubation with the chelator. Intracellular reactive oxygen species were measured by flow cytometry with the 2',7'-dichloro-fluorescein-diacetate probe that becomes fluorescent when oxidized by superoxide, H₂O₂, or the hydroxyl radical (refs. 28, 29; Fig. 5B). Experiments were then performed to determine whether the antioxidants superoxide dismutase (1000 units/mL), catalase (1000 units/mL), or ebselen (15 $\mu\text{mol}/\text{L}$) could modulate di-2-pyridylketone isonicotinoyl hydrazone-induced redox activity. These concentrations of antioxidants were used as they have shown to be effective in previous investigations (18, 33, 34).

Incubation of SK-N-MC cells with di-2-pyridylketone isonicotinoyl hydrazone (20, 40, or 60 $\mu\text{mol}/\text{L}$) for 24 hours significantly ($P < 0.001$) increased the levels of the fluorescent, oxidized probe to 632 ± 21 , 707 ± 25 , and $738 \pm 43\%$ of the control, respectively (Fig. 5B). Interestingly, compared with di-2-pyridylketone isonicotinoyl hydrazone alone, incubation of cells with di-2-pyridylketone isonicotinoyl hydrazone (20, 40, or 60 $\mu\text{mol}/\text{L}$) and catalase (1000 units/mL) significantly ($P < 0.001$) decreased fluorescence to 376 ± 8 , 429 ± 38 , and $417 \pm 29\%$ of the control, respectively (Fig. 5B). Incubation of cells with catalase (1000 units/mL) alone for 24 hours did not significantly alter the level of fluorescence ($133 \pm 43\%$ of the control) compared with cells incubated with media alone (the control). These data suggested that incubation of cells with di-2-pyridylketone isonicotinoyl hydrazone resulted in the generation of H₂O₂, and this could be reduced by catalase. For comparison to these results, a 10-minute incubation of cells with the positive control, H₂O₂ (50 $\mu\text{mol}/\text{L}$), increased fluorescence to $1218 \pm 65\%$ of the control (Fig. 5B), whereas incubation of cells with catalase (1000 units/mL) and H₂O₂ (50 $\mu\text{mol}/\text{L}$) markedly decreased fluorescence ($175 \pm 20\%$; Fig. 5B). Interestingly, co-incubation of di-2-pyridylketone isonicotinoyl hydrazone (20, 40, or 60 $\mu\text{mol}/\text{L}$) with ebselen (15 $\mu\text{mol}/\text{L}$) or SOD (1000 units/mL) could not significantly prevent di-2-pyridylketone isonicotinoyl hydrazone-dependent reactive oxygen species formation (data not shown).

The Effects of Ebselen, SOD, and Catalase on the Antiproliferative Activity of Di-2-Pyridylketone Isonicotinoyl Hydrazone. Considering the results above, it can be suggested that the di-2-pyridylketone isonicotinoyl hydrazone analogues, in part, mediate their antiproliferative effects by enhancing iron-dependent reactive oxygen species generation. To confirm this, the antioxidants, ebselen (15 $\mu\text{mol}/\text{L}$), SOD (1000 units/mL), or catalase (1000 units/mL) alone or in combination were tested for their abilities to decrease the antiproliferative activity of di-2-pyridylketone isonicotinoyl hydrazone (Fig. 6). Ebselen was not significantly protective against the antiproliferative effects of di-2-pyridylketone isonicotinoyl hydrazone, whereas SOD slightly enhanced antiproliferative activity in the presence of di-2-pyridylketone isonicotinoyl hydrazone at ligand concentrations greater than 30 $\mu\text{mol}/\text{L}$ (Fig. 6A). In contrast, catalase markedly and significantly ($P < 0.001$) decreased the antiproliferative activity of di-2-pyridylketone isonicotinoyl hydrazone from an IC₅₀ of 0.5 to 7.1 $\mu\text{mol}/\text{L}$ (Fig. 6A).

Co-incubation of cells with di-2-pyridylketone isonicotinoyl hydrazone, ebselen, and SOD had no significant effect

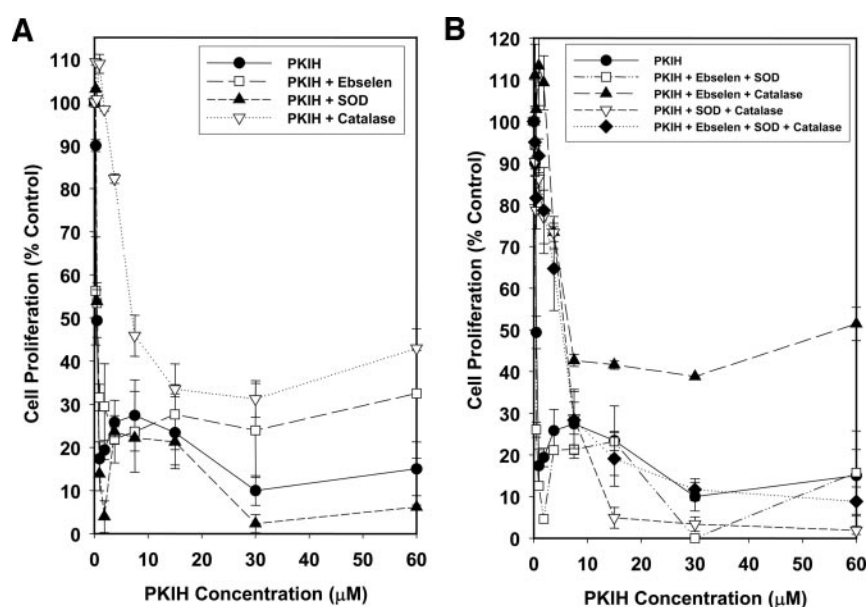


Fig. 6 The effects of antioxidants ebselen, SOD, and catalase (A) and the combinations of these antioxidants (B) on the antiproliferative activity of di-2-pyridylketone *p*-hydroxybenzoyl hydrazone (PKIH). The SK-N-MC neuroepithelioma cells were allowed to grow overnight, and the treatments were then added in 100 µL of complete medium containing diferric transferrin (1.25 µmol/L; iron = 2.5 µmol/L). The cells were then incubated with either (A) PKIH (0.23 to 60 µmol/L); PKIH (0.23 to 60 µmol/L) and ebselen (15 µmol/L); PKIH (0.23 to 60 µmol/L) and SOD (1000 units/mL); and PKIH (0.23 to 60 µmol/L) and catalase (1000 units/mL). B, PKIH (0.23 to 60 µmol/L), ebselen (15 µmol/L) and SOD (1000 units/mL); PKIH (0.23 to 60 µmol/L), ebselen (15 µmol/L), and catalase (1000 units/mL); PKIH (0.23–60 µmol/L), SOD (1000 units/mL), and catalase (1000 units/mL); PKIH (0.23 to 60 µmol/L), ebselen (15 µmol/L), SOD (1000 units/mL), and catalase (1000 units/mL). Control samples contained complete medium and diferric transferrin (1.25 µmol/L). Cells were incubated with treatments for 72 hours at 37°C and cellular proliferation then determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assay (see refs. 14, 30). Data are expressed as cell proliferation (percentage of control). Results and mean ± SE (four experiments).

compared with di-2-pyridylketone isonicotinoyl hydrazone alone (Fig. 6B). In contrast, the combination of di-2-pyridylketone isonicotinoyl hydrazone, ebselen, and catalase had a similar effect as catalase alone, decreasing the antiproliferative activity of di-2-pyridylketone isonicotinoyl hydrazone from an IC_{50} of 0.5 to 6.6 µmol/L. Cotreatment of cells with di-2-pyridylketone isonicotinoyl hydrazone, SOD, and catalase gave an IC_{50} value of 5.7 µmol/L, but at higher concentrations of di-2-pyridylketone isonicotinoyl hydrazone, the antioxidants had no significant effect when compared with di-2-pyridylketone isonicotinoyl hydrazone alone (Fig. 6B). When all three antioxidants were combined with di-2-pyridylketone isonicotinoyl hydrazone, they partially inhibited its antiproliferative activity, increasing the IC_{50} to 5.3 µmol/L, whereas at higher chelator concentrations the presence of antioxidants resulted in no significant difference to di-2-pyridylketone isonicotinoyl hydrazone alone (Fig. 6B).

DISCUSSION

The use of iron chelators as clinical agents against cancer is an area of growing interest (1–7, 9, 10, 12–14, 16, 35, 36). Pivotal to the design of new iron chelators is a focus on structure-activity relationships (13, 14). Previously, the di-2-pyridylketone isonicotinoyl hydrazone analogues were identified as ligands with potent and selective antineoplastic activity that can induce the expression of molecules involved in cell cycle arrest, such as p21^{cip1/waf1} and GADD45 (16). This latter study also

showed that these chelators have high chelation efficacy, indicating a potential mechanism of antitumor activity (16). Indeed, the di-2-pyridylketone isonicotinoyl hydrazone chelators with greatest antiproliferative activity effectively entered cells, bound iron, and induced iron mobilization and prevented iron uptake from transferrin (16). In the present study, we have identified another additional mechanism of cytotoxic activity by showing that once the di-2-pyridylketone isonicotinoyl hydrazone analogues bind iron they promote the production of Fenton-derived radicals.

Interestingly, we showed that the iron complexes of the di-2-pyridylketone isonicotinoyl hydrazone analogues demonstrate the same antiproliferative activity as their free ligands (16). This observation suggested that the iron complex itself could contribute to the antitumor activity observed after incubation of cells with the chelator. Moreover, we demonstrated these compounds formed stable ferrous iron complexes that are potentially redox active (22). Therefore, it was relevant to measure the effects of ligands on the redox cycling of iron (added as FeCl₃) in the presence of the physiologically relevant reducing agent ascorbate. The di-2-pyridylketone isonicotinoyl hydrazone analogues varied in their abilities to inhibit redox cycling of iron in the presence of ascorbate (Fig. 2A), reflecting the effects of each of the aromatic R groups adjacent to the carbonyl oxygen (Fig. 1). However, none of these chelators were capable of markedly stimulating ascorbate oxidation.

To additionally characterize the redox activity of the iron

complexes, a number of other assays were then implemented. The four most cytotoxic di-2-pyridylketone isonicotinoyl hydrazone analogues (namely di-2-pyridylketone isonicotinoyl hydrazone, di-2-pyridylketone thiophenecarboxyl hydrazone, di-2-pyridylketone benzoyl hydrazone and di-2-pyridylketone *m*-bromobenzoyl hydrazone; ref. 16) significantly ($P < 0.05$) increased the rate of benzoate hydroxylation when present at iron-binding equivalent ratios of 1 and 3 (Fig. 2B). This observation provides evidence of a potential mechanism of antiproliferative activity for these chelators and is strengthened by the lack of activity observed for the far less cytotoxic chelators, di-2-pyridylketone *p*-aminobenzoyl hydrazone and di-2-pyridylketone *p*-hydroxybenzoyl hydrazone (ref. 16; Fig. 2B). Again, the differences observed between the di-2-pyridylketone isonicotinoyl hydrazone chelators must relate in part to the electronic influence of the aromatic R group adjacent to the carbonyl oxygen (Fig. 1).

Considering the benzoate hydroxylation data, a more sensitive measure of the ability of the chelator to effect hydroxyl radical production was then used, namely the plasmid degradation assay (10, 13, 17). The greater sensitivity of this latter technique is due to the fact that only one phosphodiester bond in plasmid DNA needs to be hydroxylated in order for it to be converted to the open circular form. However, comparison of the activity of EDTA in the benzoate hydroxylation and plasmid degradation assays demonstrates a fundamental difference between the two methods. EDTA is highly protective of plasmid DNA (Fig. 3), despite its facilitation of redox cycling in both ascorbate oxidation (Fig. 2A) and benzoate hydroxylation assays (Fig. 2B). This can be explained by the ability of the EDTA-iron complex to repel DNA (37), thus preventing proximity of the highly reactive hydroxyl radical to the nucleic acid (37, 38). The importance of this in interpreting our current results is additionally illustrated by the comparative lack of protection offered by desferrioxamine, despite its ability to inhibit Fenton chemistry (19, 21). The di-2-pyridylketone isonicotinoyl hydrazone analogues all caused marked plasmid DNA degradation in the presence of H_2O_2 and iron (added as $FeSO_4$) in an iron-binding equivalent ratio-dependent manner (Fig. 3). This observation indicated that these iron complexes promote Fenton chemistry and cannot prevent the proximity of this reaction to DNA as with EDTA.

Taken together, the results described above strongly suggest that the di-2-pyridylketone isonicotinoyl hydrazone class of chelators promote the production of Fenton-derived radicals, particularly when the ligands are present in excess of iron. This conclusion is consistent with our recent electrochemical studies showing that although the ferrous complexes of the di-2-pyridylketone isonicotinoyl hydrazone analogues are not easily oxidized in aerated solution, oxidation by water yields a highly transient ferric complex that is subsequently protonated or reduced (22). Therefore, in the presence of H_2O_2 , these iron complexes can redox cycle by rapidly returning to the ferrous state after being oxidized (22) but are maintained in the reduced form by ascorbate. Clearly, the properties of the di-2-pyridylketone isonicotinoyl hydrazone analogues are quite different to that of another cytotoxic chelator characterized in our lab, namely 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone (9, 10, 13, 14, 30, 35). This latter ligand binds Fe(III) tightly to

form a complex that is not markedly redox active, and unlike the di-2-pyridylketone isonicotinoyl hydrazone-iron complex (16), the 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone-iron complex does not inhibit cellular proliferation (30). In fact, the high antiproliferative activity of 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone relates predominately from its ability to induce marked intracellular iron depletion (13, 14, 30, 35)

The topoisomerase I inhibition assay was performed to assess the significance of the weak DNA binding observed with the hypochromicity assay (Fig. 4). This latter assay is only sensitive to compounds that can bind DNA very strongly, most commonly through intercalation (17, 26). None of the chelators had any effect on the activity of topoisomerase I, indicating that these ligands do not bind DNA strongly enough to inhibit DNA-binding enzymes. Unlike the planar, fused ring structure of doxorubicin (39), our recent X-ray crystal data shows that neither the free di-2-pyridylketone isonicotinoyl hydrazone ligands nor their iron complexes exhibit coplanarity of aromatic substituents (22). This lack of planarity of the chelators and inability to strongly interact with DNA (Fig. 4A) may play a role in their inability to inhibit topoisomerase I. However, as stated previously, the topoisomerase I assay was not carried out in the presence of iron complexes that may have inhibited the enzyme by degrading DNA. Therefore, it was important to examine the effects of the di-2-pyridylketone isonicotinoyl hydrazone analogues on DNA with intact cells where upon incubation with the ligands iron complexes will form (Fig. 5A). Again, the chelators had little ability to cause DNA damage in the alkaline precipitation assay, neither in confluent (Fig. 5A) nor rapidly proliferating cells (data not shown). Collectively, these results show that the antiproliferative activity of the di-2-pyridylketone isonicotinoyl hydrazone analogues does not relate to a rapid induction of cellular DNA damage under our current incubation conditions.

It is of interest to note that although the di-2-pyridylketone isonicotinoyl hydrazone analogues did cause marked DNA degradation in the isolated plasmid assay (Fig. 3), there was no marked induction of DNA damage in intact SK-N-MC cells with the alkaline precipitation assay (Fig. 5A). These seemingly paradoxical results could be explained by the fact that the plasmid assay uses naked DNA, whereas in mammalian cells, the DNA is probably protected by its compartmentalization in the nucleus and by histone proteins that surround the molecule. However, considering the relationship between lipophilicity and antiproliferative activity shown previously (16) and the redox activity observed in this study, it can be suggested that the di-2-pyridylketone isonicotinoyl hydrazone analogues may associate with and cause oxidation of other important biological molecules, *e.g.*, lipids and/or proteins.

It was critical to examine the relevance of the chemical properties of the di-2-pyridylketone isonicotinoyl hydrazone analogues (22) of their ability to generate radicals intracellularly. To determine this, the cell permeable agent, 2',7'-dichloro-fluorescein-diacetate, was examined as it is oxidized to the highly fluorescent 2',7'-dichloro-fluorescein-diacetate by superoxide, H_2O_2 and hydroxyl radicals, providing a sensitive measure of these species (28, 29). After incubation of cells with di-2-pyridylketone isonicotinoyl hydrazone, the presence of the fluorescent 2',7'-dichloro-fluorescein-diacetate increased dra-

matically (Fig. 5B). This result was markedly inhibited when di-2-pyridylketone isonicotinoyl hydrazone was co-incubated with catalase, demonstrating the role of H₂O₂ in this reaction (Fig. 5B).

To better define the role of redox activity in the antiproliferative activity of di-2-pyridylketone isonicotinoyl hydrazone, experiments were performed in the presence of three different antioxidants. These antioxidants included the cell-permeable glutathione peroxidase mimetic, ebselen (34), the enzyme SOD that removes superoxide, and catalase, which destroys H₂O₂. Neither ebselen nor SOD, alone or in combination, had any significant effect on the antiproliferative activity of di-2-pyridylketone isonicotinoyl hydrazone (Fig. 6). In contrast, catalase was protective to some extent against the antiproliferative effects of di-2-pyridylketone isonicotinoyl hydrazone, suggesting that H₂O₂ was involved in the effects of di-2-pyridylketone isonicotinoyl hydrazone (Fig. 6). Because catalase is a protein, it is unlikely that it could easily gain intracellular access. Hence, the effect of this antioxidant may be mediated through its extracellular action. For instance, catalase may inhibit the detrimental effect of H₂O₂ generated by the redox-active di-2-pyridylketone isonicotinoyl hydrazone iron complex on the cell membrane. Alternatively, because H₂O₂ is diffusible across cell membranes, the extracellular effect of catalase of reducing the media concentration of H₂O₂ may be appreciable enough to reduce the cytotoxic/cytostatic effects of this species. These results using cells in culture support our studies in cell-free systems, demonstrating that di-2-pyridylketone isonicotinoyl hydrazone is redox active (this study and ref. 22) and suggesting the biologically significant participation of di-2-pyridylketone isonicotinoyl hydrazone in Fenton chemistry.

Interestingly, SOD could both reverse the protective effects of catalase and cause some enhancement of the antiproliferative effect of di-2-pyridylketone isonicotinoyl hydrazone at higher concentrations (Fig. 6). This may be explained by an increase in H₂O₂ levels in the presence of SOD. Indeed, SOD reacts with superoxide to generate H₂O₂ that appears to be the cytotoxic species in our studies. The lack of effect of ebselen (Fig. 6) could be due to several possibilities. For instance, ebselen is a relatively hydrophobic compound that may become localized to the wrong cellular location (*e.g.*, in membranes) so that it does not effectively remove H₂O₂. Alternatively, because ebselen is a low molecular weight species that is noncatalytic in nature, there simply may not be sufficient concentrations in the correct microenvironment to scavenge H₂O₂.

Our previous studies have shown that the di-2-pyridylketone isonicotinoyl hydrazone analogues show selective antiproliferative activity against neoplastic cells compared with normal cells (16). From investigations examining the stability constants of iron with the 2-pyridylcarboxaldehyde isonicotinoyl hydrazone ligands (40) that have the same coordination sites as those in the di-2-pyridylketone isonicotinoyl hydrazone group (Fig. 1), we can expect these latter chelators to bind Fe(II) and Fe(III). However, our electrochemical investigations suggest the Fe(II) state is preferred (22). Because iron is probably in equilibrium between the Fe(II) and Fe(III) oxidation states within cells, the ability of the di-2-pyridylketone isonicotinoyl hydrazone chelators to bind both is advantageous in terms of depleting cells of iron.

Considering the structural similarities of the di-2-pyridylketone isonicotinoyl hydrazone analogues to other aroyl-hydrazone chelators, they are unlikely to remove iron directly from the high-affinity transferrin-binding sites (41). The results of this investigation and our previous studies (16) suggest that the antitumor activity of these ligands is derived from their ability to intercept iron from sensitive pools such as those used for DNA synthesis. These iron pools may be more readily bound by the di-2-pyridylketone isonicotinoyl hydrazone analogues in neoplastic cells than their normal counterparts due to the high TFR1 expression and iron uptake rate of the former (3). The subsequent generation of the redox-active di-2-pyridylketone isonicotinoyl hydrazone-iron complex may then act as an additional cytotoxic effector that could then lead to further damage to neoplastic cells. Hence, these chelators act by at least two mechanisms, namely, binding iron from sensitive pools and the subsequent redox activity of the iron complex leading to cytotoxicity. It should be noted that we cannot exclude that the chelators may also bind iron that is present in the extracellular medium at low concentrations before entering cells. However, because the di-2-pyridylketone isonicotinoyl hydrazone chelators can induce a marked net ⁵⁹Fe efflux when added to cells prelabeled with ⁵⁹Fe (16) indicates that they are predominantly in the iron-free state before entering cellular compartments.

In conclusion, the di-2-pyridylketone isonicotinoyl hydrazone analogues have antiproliferative activity that relates to their iron chelation efficacy (16) and consequent inhibition of ribonucleotide reductase and also to the redox chemistry of their iron-complexes. This hypothesis is supported by the observation that the iron complexes of these chelators demonstrate the same antiproliferative activity as their free ligands (16). Additionally, we showed that these chelators form ferrous iron complexes that can be oxidized to a highly unstable ferric complex (22). The consequent redox activity is reflected in our results, which indicate that the iron complexes of the di-2-pyridylketone isonicotinoyl hydrazone series of chelators can enhance Fenton chemistry. However, the resulting oxidative damage is not directed to DNA *in vitro*, at least not under the conditions used in our experiments, and additional studies examining other markers of oxidative damage in cells are warranted.

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REFERENCES

- Buss JL, Torti FM, Torti SV. The role of iron chelation in cancer therapy. *Curr Med Chem* 2003;10:1021–34.
- Donfrancesco A, Deb G, Dominici C, Pileggi D, Castello MA, Helson L. Effects of a single course of deferoxamine in neuroblastoma patients. *Cancer Res* 1990;50:4929–30.
- Lovejoy DB, Richardson DR. Iron chelators as anti-neoplastic agents: current developments and promise of the PIH class of chelators. *Curr Med Chem* 2003;10:1035–49.
- Donfrancesco A, Deb G, De Sio L, Cozza R, Castellano A. Role of deferoxamine in tumor therapy. *Acta Haematol* 1996;95:66–9.

5. Becton DL, Roberts B. Antileukemic effects of deferoxamine on human myeloid leukemia cell lines. *Cancer Res* 1989;49:4809–12.
6. Li J, Zheng LM, King I, Doyle TW, Chen SH. Syntheses and antitumor activities of potent inhibitors of ribonucleotide reductase: 3-amino-4-methylpyridine-2-carboxaldehyde-thiosemicarbazone (3-AMP), 3-aminopyridine-2-carboxaldehyde-thiosemicarbazone (3-AP) and its water-soluble prodrugs. *Curr Med Chem* 2001;8:121–33.
7. Cory JG, Cory AH, Rappa G, et al. Structure-function relationships for a new series of pyridine-2-carboxaldehyde thiosemicarbazones on ribonucleotide reductase activity and tumor cell growth in culture and in vivo. *Adv Enzyme Regul* 1995;35:55–68.
8. Singh S, Khodr H, Taylor MI, Hider JC. Therapeutic iron chelators and their potential side effects. *Biochem Soc Symp* 1995;61:127–37.
9. Green DA, Antholine WE, Wong SJ, Richardson DR, Chitambar CR. 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. *Clin Cancer Res* 2001;7:3574–9.
10. Lovejoy DB, Richardson DR. Novel “hybrid” iron chelators derived from aroylhydrazones and thiosemicarbazones demonstrate selective antiproliferative activity against tumor cells. *Blood* 2002;100:666–76.
11. Thelander L, Reichard P. Reduction of ribonucleotides. *Annu Rev Biochem* 1979;48:133–58.
12. Finch RA, Liu M, Grill SP, et al. Triapine (3-aminopyridine-2-carboxaldehyde-thiosemicarbazone): a potent inhibitor of ribonucleotide reductase activity with broad spectrum antitumor activity. *Biochem Pharmacol* 2000;59:983–91.
13. Chaston TB, Lovejoy DB, Watts RN, Richardson DR. Examination of the antiproliferative activity of iron chelators: multiple cellular targets and the different mechanism of action of triapine compared with desferrioxamine and the potent pyridoxal isonicotinoyl hydrazone analogue 311. *Clin Cancer Res* 2003;9:402–14.
14. Richardson DR, Tran EH, Ponka P. The potential of iron chelators of the pyridoxal isonicotinoyl hydrazone class as effective antiproliferative agents. *Blood* 1995;86:4295–306.
15. Becker E, Richardson DR. Development of novel aroylhydrazone ligands for iron chelation therapy: 2-pyridylcarboxaldehyde isonicotinoyl hydrazone analogs. *J Lab Clin Med* 1999;134:510–21.
16. Becker EM, Lovejoy DB, Greer JM, Watts R, Richardson DR. Identification of the di-pyridyl ketone isonicotinoyl hydrazone (PKIH) analogues as potent iron chelators and anti-tumour agents. *Br J Pharmacol* 2003;138:819–30.
17. Chaston TB, Richardson DR. Interactions of the pyridine-2-carboxaldehyde isonicotinoyl hydrazone class of chelators with iron and DNA: implications for toxicity in the treatment of iron overload disease. *J Biol Inorg Chem* 2003;8:427–38.
18. Richardson DR, Neumannova V, Nagy E, Ponka P. Effects of nitrogen monoxide species on cellular proliferation and transferrin and iron uptake by erythroleukemia (K562) cells. *Blood* 1995;86:3211–9.
19. Dean RT, Nicholson P. The action of nine chelators on iron-dependent radical damage. *Free Radic Res* 1994;20:83–101.
20. Gutteridge JM. Superoxide-dependent formation of hydroxyl radicals from ferric-complexes and hydrogen peroxide: an evaluation of fourteen iron chelators. *Free Radic Res Commun* 1990;9:119–25.
21. Hermes-Lima M, Nagy E, Ponka P, Schulman HM. The iron chelator pyridoxal isonicotinoyl hydrazone (PIH) protects plasmid pUC-18 DNA against *OH-mediated strand breaks. *Free Radic Biol Med* 1998;25:875–80.
22. Bernhardt PV, Caldwell LM, Chaston TB, Chin P, Richardson DR. Cytotoxic iron chelators: characterization of the structure, solution chemistry and redox activity of ligands and iron complexes of the di-2-pyridylketone isonicotinoyl hydrazone (HPKIH) analogues. *J Biol Inorg Chem* 2003;8:866–80.
23. Jin L, Yang P. Synthesis and DNA binding studies of cobalt (III) mixed-polypyridyl complex. *J Inorg Biochem* 1997;68:79–83.
24. Kostoryz EL, Yourtee DM. Oxidative mutagenesis of doxorubicin-Fe(III) complex. *Mutat Res* 2001;490:131–9.
25. Kellogg GE, Scarsdale JN, Fornari FA Jr. Identification and hydrophobic characterization of structural features affecting sequence specificity for doxorubicin intercalation into DNA double-stranded polynucleotides. *Nucleic Acids Res* 1998;26:4721–32.
26. Gewirtz DA. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics Adriamycin and daunorubicin. *Biochem Pharmacol* 1999;57:727–41.
27. Olive PL. DNA precipitation assay: a rapid and simple method for detecting DNA damage in mammalian cells. *Environ Mol Mutagen* 1988;11:487–95.
28. Wang MX, Wei A, Yuan J, Trickett A, Knoops B, Murrell GA. Expression and regulation of peroxiredoxin 5 in human osteoarthritis. *FEBS Lett* 2002;531:359–62.
29. Yuan J, Lovejoy DB, Richardson DR. Novel di-2-pyridyl-derived iron chelators with marked and selective anti-tumor activity: in vitro and in vivo assessment. *Blood* 2004;104:1450–8.
30. Richardson DR, Bernhardt PV. Crystal and molecular structure of 2-hydroxy-1-naphthaldehyde isonicotinoyl hydrazone (NIH) and its iron(III) complex: an iron chelator with anti-tumour activity. *J Biol Inorg Chem* 1999;4:266–73.
31. Trommel JS, Marzilli LG. Synthesis and DNA binding of novel water-soluble cationic methylcobalt porphyrins. *Inorg Chem* 2001;40:4374–83.
32. Kasahara K, Fujiwara Y, Sugimoto Y, et al. Determinants of response to the DNA topoisomerase II inhibitors doxorubicin and etoposide in human lung cancer cell lines. *J Natl Cancer Inst (Bethesda)* 1992;84:113–8.
33. Kwok JC, Richardson DR. Anthracyclines induce accumulation of ferritin iron in normal and neoplastic cells: inhibition of the ferritin Fe mobilization pathway. *Mol Pharmacol* 2003;63:849–61.
34. Konorev EA, Kennedy MC, Kalyanaraman B. Cell-permeable superoxide dismutase and glutathione peroxidase mimetics afford superior protection against doxorubicin-induced cardiotoxicity: the role of reactive oxygen and nitrogen intermediates. *Arch Biochem Biophys* 1999;368:421–8.
35. Darnell G, Richardson DR. The potential of iron chelators of the pyridoxal isonicotinoyl hydrazone class as effective antiproliferative agents III: the effect of the ligands on molecular targets involved in proliferation. *Blood* 1999;94:781–92.
36. Richardson DR. The therapeutic potential of iron chelators. *Exp Opin Investig Drugs* 1999;8:2141–58.
37. Mello Filho AC, Meneghini R. In vivo formation of single-strand breaks in DNA by hydrogen peroxide is mediated by the Haber-Weiss reaction. *Biochim Biophys Acta* 1984;781:56–63.
38. Aruoma OI, Halliwell B, Dizdaroglu M. Iron ion-dependent modification of bases in DNA by the superoxide radical-generating system hypoxanthine/xanthine oxidase. *J Biol Chem* 1989;264:13024–8.
39. Bodley A, Liu LF, Israel M, et al. DNA topoisomerase II-mediated interaction of doxorubicin and daunorubicin congeners with DNA. *Cancer Res* 1989;49:5969–78.
40. Armstrong CM, Bernhardt PV, Chin P, Richardson DR. Structural variations and formation constants of first row transition metal complexes of biologically active aroylhydrazones. *Eur J Inorg Chem* 2003;1145–56.
41. Baker E, Richardson DR, Gross S, Ponka P. Evaluation of the iron chelation potential of hydrazones of pyridoxal, salicylaldehyde and 2-hydroxy-1-naphthylaldehyde using the hepatocyte in culture. *Hepatology* 1992;15:492–501.