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Original Contribution

THE IRON CHELATOR PYRIDOXAL ISONICOTINOYL HYDRAZONE (PIH) PROTECTS PLASMID pUC-18 DNA AGAINST •OH-MEDIATED STRAND BREAKS

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Abstract—Pyridoxal isonicotinoyl hydrazone (PIH) has previously been studied for use in iron chelation therapy in iron-overload diseases. It is an efficient in vitro antioxidant due to its Fe(III) complexing activity (Schulman, H. M., et al. *Redox Report* **1:**373–378; 1995). Pathologies associated with iron-overload include hepatic and other cancers. Since oxidative alterations of DNA can be linked to the development of cancer, we decided to study whether PIH protects DNA against in vitro oxidative stress. We report here that pUC-18 plasmid DNA is damaged by •OH radicals generated from Fe(II) plus H_2O_2 or from Fe(II) plus hypoxanthine/xanthine oxidase. The DNA damage was quantified by determining the diminution of supercoiled DNA forms after oxidative attack using agar gel electrophoresis. Micromolar amounts of PIH (20–30 μM) were able to half-protect DNA from iron (1–7.5 μM)-mediated •OH formation. The antioxidant capacity of PIH was significantly higher than that of some of its analogs and desferrioxamine. PIH and some of its analogues could also inhibit the oxidative degradation of 2-deoxyribose caused by Fenton reagents. Since we observed that PIH enhances the Fe(II) autoxidation rate, measured by the ferrozine technique, PIH may limit •OH formation and consequently DNA damage by decreasing the amount of Fe(II) available to catalyze Fenton reactions. © 1998 Elsevier Science Inc.

Keywords—Free radicals, Pyridoxal isonicotinoyl hydrazone, Desferrioxamine, β-thalassemia, Iron overload, Cancer, Plasmid, DNA Strand break

INTRODUCTION

The iron chelator pyridoxal isonicotinoyl hydrazone (PIH) [1–3] has been used for iron metabolism studies and examined for its potential use in the treatment of secondary iron overload pathologies [1–5] that occur in iron-loading anemias such as β-thalassemias, porphyria cutanea tarda and alcoholic cirrhosis [6]. Experimental and oral administration of PIH induces iron excretion in rats and humans resulting in negative iron balance without serious side effects [4,7–12]. PIH-induced iron excretion in rats occurs mainly through bile [5,12] and is approximately equivalent to that obtained with a comparable dose of desferrioxamine (DFO) given parenterally [7].

Induction of iron excretion can decrease hepatic oxidative stress in iron overload (reviewed in ref. 13). It is well known that iron catalyzes the formation of highly reactive hydroxyl radicals (•OH) that can mediate injury to various biomolecules [14]. Iron-mediated lipid peroxidation, depletion of low-molecular weight antioxidants and single- and double-strand breaks in DNA have been implicated in the pathophysiology of iron overload diseases [13,15–18]. In humans, iron overload correlates with DNA alterations and cancer [13,19,20]. It was reported that the risk of hepatocellular carcinoma is greatly increased in iron overload patients [21–23]. Moreover, i.p. injections of ferric-NTA to experimental animals promotes renal proximal tubular necrosis and a high incidence (60–92%) of renal adenocarcinoma [17,24].

We recently demonstrated that PIH and some of its

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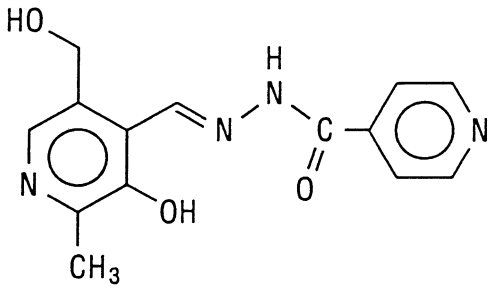
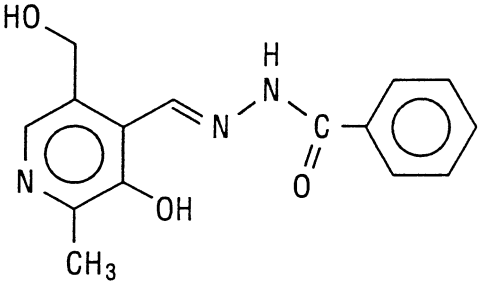
analogs have antioxidant activity against iron-mediated lipid peroxidation of liposomes [25] and can prevent •OH-mediated (formed via Fe(III)EDTA plus ascorbate) release of TBARS from 2-deoxyribose as well as the release of ethylene from 2-keto-4-methiobutyric acid [25]. More recently PIH was shown to inhibit peroxidation and retinal electrophysiological alterations secondary to asphyxia-reoxygenation-induced oxidative stress to newborn animals [26]. Here, we report on the ability of PIH and two of its analogues (pyridoxal benzoyl hydrazone, PBH, and salicylaldehyde isonicotinoyl hydrazone, SIH; Fig. 1) to prevent plasmid pUC-18 DNA strand breaks induced by •OH radicals.



Pyridoxal Isonicotinoyl Hydrazone (PIH)



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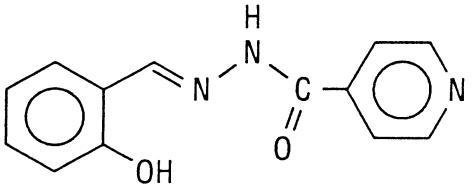

Salicylaldehyde Isonicotinoyl Hydrazone (SIH)

Fig. 1. Structure of iron chelators.

MATERIALS AND METHODS

Chemicals and solutions

PIH, SIH and PBH were synthesized as previously described [25,27]. All solutions were prepared in Milli-Q quality water. Stock solutions (0.75 mM) of the iron chelators were prepared daily in 1 mM Hepes buffer pH 7. Stock solutions of Fe(II) were freshly prepared (1 mM) in water previously bubbled with nitrogen. Dilutions of Fe(II) stock solutions were done in deoxygenated water to prevent autoxidation. *Escherichia coli* cells were transformed with pUC-18 plasmid DNA (2,686 base-pairs) and were grown in LB medium. Plasmid DNA was purified using the QIAprep-spin Plasmid Kit (Quiagen Inc., Chatsworth, USA). Purified samples of plasmid DNA were kept at 20°C in Milli-Q water.

Generation of breaks in DNA by Fenton reactants

Experiments were carried on at room temperature for 5 min, in a final volume of 0.1 mL. In a typical experiment, reactants were added to Eppendorf tubes in the following sequence: 2.4 μL of 420 μg/mL DNA (10 μg/mL final concentration), 13 μL of 100 mM Hepes buffer pH 7.2 (13 mM final concentration), 4 μL of 7.5 mM H_2O_2 (0.3 mM final concentration), 10 μL of 0.75 mM chelator (0.075 mM final concentration) and water to a final volume of 96 μL. Lastly, 4 μL of Fe(II) solution was added to start the Fenton reaction. After 5 min the reaction was stopped by adding 20 μL of 20,000 U/mL catalase followed by 15 μL of loading buffer (0.25% bromophenol blue, 40% w/v sucrose in H_2O). Twenty-five μL of the mixture was then applied to the agarose gel.

Generation of breaks in DNA by hypoxanthine/xanthine oxidase

Experiments were as described above except that H_2O_2 was replaced by 10 μL of 2 mM hypoxanthine. Xanthine oxidase (from Sigma) was added (2.6 μL) a few seconds before the addition of Fe(II). After 10 min the reaction was stopped by catalase as described above. Control experiments demonstrated that PIH does not inhibit xanthine oxidase activity (E. M. Wang and H. M. Schulman, unplublished).

Quantification of strand breaks in treated DNA

Samples of plasmid DNA were electrophoresed in 1% agarose gel. After migration the DNA was stained with

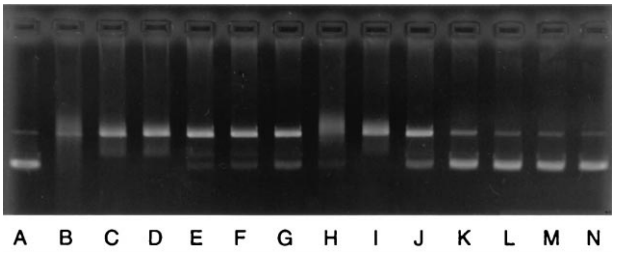

Fig. 2. Picture of a typical agarose gel of pUC-18 DNA showing bands of supercoiled (SC) and open circular (OC) forms. Experimental conditions are as described in Materials and Methods. Lanes on the gel represent: (A) control (no H_2O_2 or Fe(II)); (B–G) samples with 0.3 mM H_2O_2 plus (B) 15 μM Fe(II), (C) 7.5 μM Fe(II), (D) 5 μM Fe(II), (E) 3 μM Fe(II), (F) 2 μM Fe(II), or (G) 1 μM Fe(II); (H–J) samples with 0.03 mM H_2O_2 plus (H) 15 μM Fe(II), (I) 7.5 μM Fe(II), or (J) 2 μM Fe(II); and (K–N) samples with 0.4 mM PIH plus (K) 0.3 mM H_2O_2 + 7.5 μM Fe(II), (L) 0.3 mM H_2O_2 + 2 μM Fe(II), (M) 0.03 mM H_2O_2 + 7.5 μM Fe(II), or (N) 0.03 mM H_2O_2 + 2 μM Fe(II).

Table 1. Protection Against Oxidative Damage to pUC-18 by PIH and Other Iron Chelators

	% DNA as SC[a,b]			
Chelator	2 μM Fe(II)	Protection	7.5 μM Fe(II)	Protection
no chelator	22.1 ± 1.8	—	3.3 ± 1.6	—
PIH	77.7 ± 2.4[c]	(96%)[d]	67.5 ± 2.4	(83%)
SIH	70.9 ± 1.8	(84%)	64.6 ± 1.8	(80%)
PBH	71.0 ± 0.1	(84%)	61.7 ± 2.6	(76%)
DFO	69.4 ± 3.6	(81%)	57.1 ± 0.1	(70%)

[a] Experimental conditions are as described in Materials and Methods. Concentration of H_2O_2 and iron chelators in the reaction media was 0.3 mM and 0.075 mM, respectively.
[b] Controls: 80.3 ± 0.3% DNA as SC.
[c] Values are means ±SD (n = 3).
[d] Number in parenthesis represent the percentage of protection given by the chelators. The % protection was calculated as: % protection = 100× [(control SC − chelator SC/control SC − no chelator SC) − 1].

ethidium bromide and visualized in a UV transiluminator. The DNA bands were quantified by scanning the negatives of the gel pictures with a densitometer. The percent of remaining supercoilded (SC) plasmid DNA was calculated by the formula shown below. A correction factor of 1.4 was applied to account for the relatively lower fluorescence of SC forms compared to the open circular (OC) and linear forms (L) [28,29].

$$\%SC = [1.4 \times SC/(L + OC + (1.4 \times SC))] \times 100$$

Determination of Fe(II) and of damage to 2-deoxyribose

The concentration of Fe(II) was determined by adding 0.5 mL of 5 mM ferrozine and measuring the absorbance at 562 nm in a final volume of 1 mL. Damage to 15 mM 2-deoxyribose caused by H_2O_2 plus Fe(II) (in 15 mM Hepes buffer pH 7.2) was determined by the quantification of TBARS at 532 nm, as previously described [30], except that the 5.6 M HCl acid used in the analytical part of the assay was replaced by 4% phosphoric acid.

RESULTS AND DISCUSSION

Characterization of the damage to plasmid pUC-18

Control preparations of pUC-18 contained 80 ± 3.9% supercoiled (SC) DNA (Fig. 2, lane A), levels of which are not affected by incubation with 0.3 mM H_2O_2 for 5–30 min. However, addition of increasing concentrations of Fe(II) causes, after 5 min, breakage of pUC-18 (Fig. 2, lanes B–G). With 1 μM Fe(II) there was breakage of approximately half the SC DNA while its complete elimination occured with 15 μM Fe(II), in the presence of 0.3 mM H_2O_2 (Fig. 2, lanes B–G).

The breakage of pUC-18 is also dependent on the levels of H_2O_2 at a fixed Fe(II) concentration. More damage occurred with 0.3 mM H_2O_2 than with 0.03 mM H_2O_2 (Fig. 2, lanes H–J). With 2 μM Fe(II), SC DNA was 39.8% and 20.6% of the original DNA in the presence of 0.03 mM and 0.3 mM H_2O_2, respectively. When Fe(II) was raised to 7.5 μM, the SC DNA forms were 23.4% and 4.0% with 0.03 mM and 0.3 mM H_2O_2, respectively.

Protection of DNA damage by PIH and other chelators

PIH at a concentration of 0.4 mM protected pUC-18 from damage when oxyradicals were generated by 0.03 mM H_2O_2 plus either 2 or 7.5 μM Fe(II), and by 0.3 mM H_2O_2 plus either 2 or 7.5 μM Fe(II) (Fig. 2, lanes K–N). Even under the conditions that caused much DNA breakage (0.3 mM H_2O_2 plus 7.5 μM Fe(II)), PIH almost completely protected the DNA (see Table 1).

Titration studies showed that 23 ± 6 μM PIH gave half-maximal protection against DNA breakage caused by 0.3 mM H_2O_2 plus 2 μM Fe(II) (Fig. 3). Half-maximal protection was at 34 ± 4 μM PIH when the iron concentration was 7.5 μM. The results show that PIH is more effective in preventing DNA damage when oxyradicals are generated by low iron concentrations indicating that the antioxidant action of PIH against DNA damage is related to its iron binding activity, inhibiting Fe(II) participation in the Fenton reaction. Although PIH may also have •OH scavenging activity (possibly by hydroxylation of one of its aromatic rings), this may be relevant only at higher levels as observed for other scavengers [31] and DFO at 1 mM [32].

A comparizon of the protective effect of DFO, PIH, SIH and PBH against the DNA damage induced by Fenton reagents is shown in Table 1. Although all che-

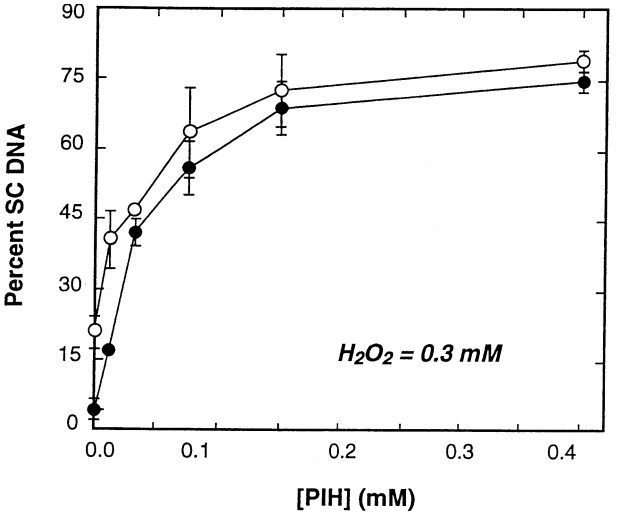

Fig. 3. Effect of PIH concentration on the oxidative damage to pUC-18 induced by 0.3 mM H_2O_2 plus 2 μM Fe(II) (open symbols) or 7.5 μM Fe(II) (closed symbols). Values are means ± SD (n = 3).

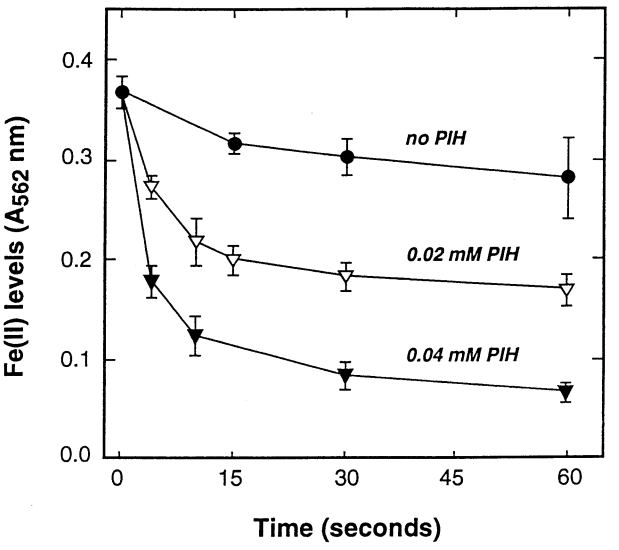

Fig. 4. Time course of 20 μM Fe(II) autoxidation in 2 mM Hepes buffer (pH 7.2) in the absence or in the presence of PIH. Values are means ± SD (n = 3).

lators had similar efficiencies in protecting against DNA damage, PIH was the most effective.

DNA damage by hypoxanthine/xanthine oxidase versus PIH

Plasmid pUC-18 is also damaged by oxyradicals generated from Fe(II) (1 μM) and hypoxanthine/xanthine oxidase. Table 2 shows that PIH, in a concentration dependent manner, protected against oxidative damage to the plasmid DNA in this system also. The calculated half-maximal protection was achieved at 40–50 μM PIH. The efficacy of PIH was slightly better than that of DFO. Again, the protection effect of PIH can be explained by its ability to chelate iron and prevent the Fenton reaction.

Table 2. Effect of PIH and DFO on Damage to pUC-18 Induced by Xanthine Oxidase/Hypoxanthine Plus Fe(II)

Experimental condition[a]	% DNA as SC	Protection
Control	88.3 ± 0.3[b]	—
Fe(II) + hp/xo[c]	68.9 ± 1.7	—
+ 0.030 mM PIH	76.2 ± 1.9	(37.6%)[d]
+ 0.075 mM PIH	79.9 ± 0.7	(56.7%)
+ 0.400 mM PIH	83.1 ± 1.8	(73.2%)
+ 0.075 mM DFO	77.8 ± 1.9	(45.9%)

[a] Experimental conditions are as described in Materials and Methods. Briefly, concentrations of Fe(II), hypoxanthine (hp) and xanthine oxidase (xo) were 1 μM, 0.2 mM and 25 mU/mL, respectively. Incubation time was 10 min.

[b] Values are means ± SD (n = 3–4).

[c] % SC DNA after 10 min treatment with 1 μM Fe(II) alone was 86.8% (n = 2).

[d] Numbers in parenthesis represent the percentage of protection given by the chelators. The % protection was calculated as described in the legend to Table 1.

PIH prevents the formation of $^{\bullet}$OH radicals

We demonstrated that PIH (0.4 mM) inhibits oxidative damage to 2-deoxyribose caused by Fe(II) plus 0.3 mM H_2O_2 (76% protection in media containing 10 μM Fe(II) plus H_2O_2 and 73% protection in media containing 20 μM Fe(II) plus H_2O_2; data not shown). Nearly 50% protection was obtained with 0.07 mM PIH. PBH, SIH and DFO were similarly effective. Degradation caused by H_2O_2 (0.3 mM) alone, that is not mediated by $^{\bullet}$OH radicals, was not inhibited by 0.4 mM PIH (data not shown). Preliminary EPR studies using DMPO as a spin trap also indicate that PIH can inhibit the generation of $^{\bullet}$OH radicals via the Fenton reaction with an effectiveness similar to DFO [33].

We have previously shown that PIH and some of its analogues inhibit $^{\bullet}$OH generation from an Fe(III)EDTA/ascorbate/O_2 system [25]. In this case, the antioxidant action of PIH was attributed to its capacity to prevent Fe(III) reduction by ascorbate. Since Fe(III) forms a complex with two PIH molecules and the 6 coordination sites of the metal become occupied, Fe(III) may become inaccessible to reduction by ascorbate [25]. The next set of experiments deal with the mechanism by which PIH inhibits $^{\bullet}$OH formation from Fe(II) plus H_2O_2.

Effect of PIH on the autoxidation of Fe(II)

Figure 4 shows that PIH (20 or 40 μM) accelerated the autoxidation of Fe(II) in Hepes buffer (2 mM). This

may be due to the much higher affinity of PIH for Fe(III) than for Fe(II) (Webb and Vitolo [7] observed a formation constant (log β_{11}) of 12.5 for Fe(II)PIH_2 and 24.8 for Fe(III)PIH_2 in neutral pH), that would change the equilibrium of the autoxidation reaction and enhance the rate of Fe(II) formation. A transient green complex of PIH with Fe(II) is formed with an absorbance peak at 426 nm (data not shown) that disappears in seconds when a peak at 476 nm, characteristic of the PIH_2-ferric iron complex [1,2], appears. Together these results suggest that the antioxidant action of PIH is due to its ability to diminish the concentration of Fe(II) available for Fenton reactions producing •OH radicals. DFO, which also has a higher affinity for Fe(III) than Fe(II) [9,12], also accelerates Fe(II) autoxidation with a rate similar to that of PIH (data not shown).

CONCLUSIONS

Free radical damage to DNA has been implicated in the development of renal and hepatic cancer in iron overload [13,21–24] and, in general, there is thought to be a link between free radical damage to DNA and cancer [15,19,20]. Therefore, studying iron-mediated in vitro damage to DNA is relevant for understanding the biochemical basis of the proposed iron-mediated carcinogenesis [19,20].

In the present paper we demonstrate that the iron chelators, PIH, SIH and PBH, inhibit •OH-mediated in vitro damage to pUC-18 plasmid DNA. In the presence of PIH, Fe(II) from solution may bind to the chelator and thus accelerate its autoxidation and prevent its participation in the Fenton reaction and the formation of •OH. A similar mechanism has been proposed to explain how EDTA inhibits free radical reactions initiated with Fe(II) [34].

In hepatic iron overload PIH might be effective in preventing •OH formation catalyzed by "free iron," since intracellular levels of "free iron" are elevated in iron overload [13]. High ferritin levels in iron overload are also a threat to DNA integrity because iron can be mobilized (as Fe(II)) from ferritin by reducing agents, such as ascorbate or superoxide, and participate in Fenton reactions [35]. PIH may also be able to remove iron bound to DNA and prevent in situ damage mediated by •OH, as proposed elsewhere for the mechanism of the antioxidant action (against DNA fragmentation) of the iron chelator Quin-2 [36]. Studies on the effect of PIH on iron binding to DNA are under way.

Since low micromolar concentrations of PIH prevent oxidative damage to DNA it may be suitable for pharmacological experiments. Furthermore, PIH traverses biological membranes efficiently and chelates intracellular iron [7,37] resulting in relevant antioxidant activity [25, 26]. Thus, it may be superior to DFO which does not cross membranes easily [7]. PIH is far more active than DFO in mobilizing ^{59}Fe from prelabelled rabbit reticulocytes and human melanoma cells [1,12].

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ABBREVIATIONS

DFO—desferrioxamine
PBH—pyridoxal benzoyl hydrazone
PIH—pyridoxal isonicotinoyl hydrazone
SIH—salicylaldehyde isonicotinoyl hydrazone
SC—supercoiled
TBARS—thiobarbituric acid reactive substances