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Original Contribution

NEW ISONICOTINOYLHYDRAZONES WITH SSA PROTECT AGAINST OXIDATIVE-HEPATIC INJURY OF ISONIAZID

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ABSTRACT

To determine whether recently synthesized isonicotinoylhydrazone analogs of the antituberculosis drug Isoniazid (INH) could prevent isoniazid free radical induced hepatotoxicity and thereby confer hepatoprotection. The level of lipid peroxidation products and the activities of antioxidant defense enzymes, superoxide dismutase and catalase, in liver homogenates of mice were investigated. The results showed that liver homogenates from mice treated with INH had higher levels of lipid peroxidation products compared to Controls ($p<0.05$). This was consequential to the decreased activities of the antioxidant defense enzymes superoxide dismutase and catalase ($p<0.05$). When mice were treated with combinations INH + SH7 or INH + SH8, lipid peroxidation products (MDA liver) were decreased and SOD and CAT activities were restored to levels close to the Control. Bearing in mind the results from previously demonstrated tuberculostatic and superoxide scavenger activity (SSA) of the isonicotinoylhydrazones, SH7 and SH8, and the results from the present study a new chemotherapeutic regimen, comprising isoniazid combination with SH7 or SH8 to produce decreased hepatotoxicity, is being proposed.

Key words: Isoniazid; Isonicotinoylhydrazones**;** Hepatoprotection; Lipid peroxides, Catalase, Superoxide dismutase

1. INTRODUCTION

Reactive oxygen species (ROS), such as $\cdot O_2$, H_2O_2, and $\cdot OH$, are highly reactive species generated by the biochemical redox reactions that occur as part of normal cell metabolism and by exposure to environmental factors such as UV light, cigarette smoke, environmental pollutants and gamma radiation. Some toxic compounds, including anticancer drugs, anesthetics, analgesics, etc, can result in the production of free radicals (1,2) and could be a reason for some toxic side effects such as hepatotoxicity, cardiotoxicity, pulmonary fibrosis, etc (3-10). Prime targets of ROS are the polyunsaturated fatty acids (PUFA) in the membrane lipids. This attack causes lipid peroxidation and further decomposition of peroxidized lipids yields a wide variety of end-products including malondialdehyde (MDA) that is widely used in practice as an indicator of free radical damages. To circumvent the damages caused by the ROS, multiple defense systems, collectively called antioxidants superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione (GSH), etc, are present in living organisms (11-13). However, the protective efficiency against lipid peroxidation would depend on the balance between oxidant species and the availability of antioxidant defense systems (14-17).

Tuberculosis is still a leading cause of death among those infections with a single etiology and one-third of the world population is latently infected with *Mycobacterium tuberculosis*. First-line drug, with superior efficacy used for treating infections caused by *Mycobacterium tuberculosis*, is isoniazid (isonicotinic acid hydrazide, INH) (17, 18). Problems associated with drug resistance and

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potential adverse reactions, such as hepatotoxicity, indicate the need for new effective anti-tuberculosis drugs and for alternative therapy regimens. Several drug combinations have been studied in order to increase the therapeutic efficacy and to reduce the toxicity of isoniazid. Recent studies have suggested that superoxide is involved in INH activation and reactive oxygen species, such as NO, O_2^-, H_2O_2, and ·OH, arise during this activation (3,19-21). The role of these ROS in the oxidative-stress and isoniazid-induced hepatic injury has been postulated (22). Isonicotinoylhydrazones are compounds structurally related to isoniazid and have antibacterial, especially antimycobacterial, activities. We have synthesized a new class of isonicotinoylhydrazones, analogs of the antituberculosis drug isoniazid (INH), and a few members showed pharmacological advantages over isoniazid; they had lower toxicity and higher antimycobacterial activity (23, 24). When the superoxide scavenging activity (SSA) of two of these new isonicotinoylhydrasones was investigated, N-isonicotinoyl-N´-(3,5-dichloro-2-hydroxybenzaldehyde)hydrazone (SH7) and N-isonicotinoyl-N´-(3-etoxy-2-hydroxybenzaldehyde) hydrazone (SH8) possessed SSA comparable to that of the reference antioxidant ascorbic acid, whereas the clinically used drug INH did not show any SSA. We then hypothesized that the antioxidant action of these isonicotinoylhydrazones may be responsible for their superior beneficial effects, which are: higher tuberculostatic activity and lower acute toxicity (24).

Therefore, the aim of the present study was to determine whether N-isonicotinoyl-N´-(3,5-dichloro-2-hydroxybenzaldehyde) hydrazone (SH7) and N-isonicotinoyl-N´-(3-etoxy-2-hydroxybenzaldehyde) hydrazone (SH8) prevent isoniazid free radical-induced hepatotoxicity and display hepatoprotective effects. For this purpose we investigated the levels of lipid peroxidation products and the activities of antioxidant defense enzymes superoxide dismutase and catalase from liver homogenates of mice treated with INH in combinations with either SH7 or SH8.

2. MATERIALS AND METHODS

2.1. Compounds tested

Isoniazid (*Rimifon*) was obtained from Bristol-Myers Squibb Co. (Connecticut, USA). *Buttermilk xanthine oxidase* and *trolox* were obtained from Fluka (Germany). TMPO was purchased from Aldrich (Milwaukee, U.S.A.). All isonicotinoylhydrazones were synthesized according to Varbanova and Georgieva (22). The test compounds were dissolved *ex tempore* as follows: first step in DMSO (to 3 ml) and second step in double distilled water (to 10 ml) and incubated at 37^0C.

2.2. Animal studies and treatment schedules

This study was carried out on white healthy mice (18-22 g body weight) divided into six groups of 6 animals per group. One of these groups served as the Control. The animals were housed in plastic cages, fed a normal laboratory diet and water *ad libitum*.

The mice were treated with solutions of INH, SH7, SH8 and vitamin A in different combinations in accordance with the routine methods described in the literature (25) but with slight modifications.

Four hours after administration of the drugs mice were sacrificed by cervical decapitation. Livers were removed and kept on ice until homogenization on the same day. The samples were first washed with deionized water to separate blood and then homogenized. After centrifugation for 20 min at 2,000g, the supernatant was removed for protein determination (26), and then diluted with PBS (1:10) (pH 7.4). The homogenates were extracted with ethanol/chloroform to eliminate lipids, which would have interfered with the measurement of SOD and CAT activity.

2.3. Estimation of products of lipid peroxidation

Basal levels of lipid peroxidation as indicated by thiobarbituric acid-reactive substances (TBARS) were determined using the thiobarbituiric acid (TBA) method, which measures the malondialdehyde (MDA) reactive products (27). In the TBARS assay 1 ml of the supernatant, 1 ml of normal saline and 1 ml of 25 % trichloroacetic acid (TCA) were mixed and centrifuged at 2000g for 20 minutes. One ml of protein free supernatant was taken, mixed with 0.25 ml of 1 % thiobarbituric acid (TBA) and boiled 1h at 95ºC. After cooling the absorbance of the resulting pink color was determined spectrophotometrically at 532 nm.

2.4. Measurement of antioxidant enzyme activity

SOD activity was determined by the xanthine/xanthine-oxidase/nitroblue tetrazolium (NBT) method according to Sun *et al* (28), but with minor modification. Xanthine/xanthine-oxidase-produced $\cdot O_2^-$ reduces NBT to formazan, which can be assessed spectrophotometrically at 560 nm. SOD competes with NBT for the dismutation of $\cdot O_2^-$ and inhibits its reduction. The level of this reduction is used as a measure of SOD activity.The total SOD activity is expressed in units/mg of protein, where one unit is equivalent to the SOD activity that causes 50% inhibition of the reaction rate without SOD.

The assay of CAT activity was done according to the method of Beers and Sizer (29). Briefly, hydrogen peroxide (30 mM) was used as a substrate and the decrease in H_2O_2 concentration at 22ºC in a phosphate buffer (50 mM, pH 7.0) was followed spectroscopically at 240 nm for 1 min. The activity of the enzyme was expressed in units per mg of protein and 1 unit equals the amount of an enzyme that degrades 1M H_2O_2 per minute.

2.5. Statistical analysis

The results were reported as mean ± S.D. Statistical analysis was done using the Student's t-test and multiple regression analysis. $p<0.05$ was considered statistically significant.

3. RESULTS

The course of the enzymatic activities is presented in **Table 1**. There were differences in the enzyme activities among the different treatment groups.

Table 1 and **Figure 1** show the results from lipid peroxidation products from liver homogenates of mice treated with isoniazid at dose 151mg/kg i.p.= $LD_{50.}$ MDA level was significantly increased compared to the Controls (mean 2.578 μM/L *vs* 2.024μM/L, respectively, p=0.001). Isoniazid showed reduced levels of MDA when combined with either of the isonicotinoylhydrazones SH7 or SH8, a result that was close to the Control, as could be seen in the combination INH + SH8 (mean 2.291μM/L, p=0.004). When mice were treated with isoniazid and Vitamin A (i.m.), the levels of MDA were reduced but not significantly (mean 2.419 μM/L, p>0.05). The combination INH with SH7 showed lower level of MDA compared to INH administrated alone (mean 2.059 μM/L, p=0.03 and 2.152 μM/L, p=0.02 for concentrations 30 mg/kg p.o. and 15 mg/kg p.o., respectively).

Table 1. MDA, SOD and CAT activities from liver homogenates of mice after administering INH in combinations with SH7, SH8 and vitamin A.

Groups	MDA (μM/l)	SOD (U/mgPr)	CAT (U/mgPr)
Control	2.024 ± 0.164	2.273 ± 0.317	47.070 ± 16.490
INH 151 mg/kg i.p.= LD_{50}	*2.578 ± 0.349	***1.583 ± 0.562	***30.176 ± 7.300
INH 151 mg/kg i.p. +15mg/kg p.o. SH7	#2.152 ± 0.203	***1.636 ± 0.648	38.812 ± 9.970
INH 151 mg/kg i.p. +30mg/kg p.o. SH7	#2.059 ± 0.377	2.166 ± 0.341	39.964 ± 15.450
INH 151 mg/kg i.p. +30mg/kg p.o. SH8	**2.291 ± 0.025	1.681 ± 0.746	39.585 ± 13.500
INH 151 mg/kg i.p. + vit. A i.m. ther. dose	**2.419 ± 0.235	*1.210 ± 0.061	36.904 ±1.560

**p≤0.001 vs Controls; **p<0.005 vs Controls; ***p<0.05 vs Controls; #p<0.05 vs INH.*
Results are presented as mean ± SD (6 mice in a group).

When SOD activity was studied in liver homogenates of mice treated with INH at dose 151mg/kg i.p, statistically significant decrease was observed compared to that of the Controls (1.583U/mg Pr, *vs* 2.273U/mg Pr, p=0.03) (**Figure 2**). SOD activity was increased when INH was administered in combination with SH7 or SH8. There was no

significant difference in SOD activities between groups treated with the combinations 151mg/kg i.p INH + 30 mg/kg p.o. SH7 or 30 mg/kg p.o. SH8 and Controls, ($p>0.05$). The most effective combination with the lowest reduction in the levels of SOD compared to the Controls was the combination 151 mg/kg i.p INH + 30 mg/kg p.o. SH7 (2.166 U/mg Pr). Statistically significant reduction in the level of SOD was established in mice treated with the combination INH and vitamin A, compared to the Control (1.210 U/mg Pr, $p<0.001$).

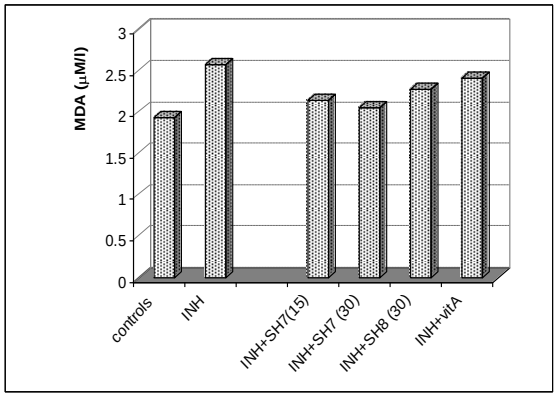

Figure 1. MDA from liver homogenates of mice after treatment with INH alone and in combinations with SH7, SH8 and vitamin A.

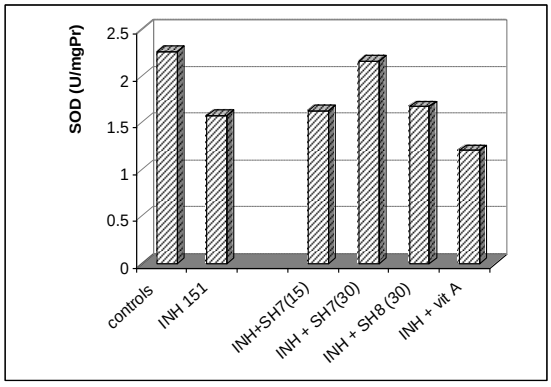

Figure 2. SOD activities from liver homogenates after mice were treated with INH alone and in combinations with SH7, SH8 and vitamin A.

The results from CAT activity in liver homogenates are shown in **Table 1** and **Figure 3**. Mice treated with INH, 151 mg/kg i.p, showed a statistically decreased level of CAT compared to the Controls (30.176 U/mg Pr, *vs* 47.07 UCAT/mgPr, $p=0.03$). Mice treated with the combinations of 151 mg/kg i.p INH and 30 mg/kg p.o. SH7 or 15 mg/kg p.o. SH7 or 30 mg/kg p.o. SH8, showed CAT activity that was only slightly higher compared to CAT activity of mice treated with INH alone (39.964 U /mgPr, 39.585 U/mgPr and 38.812 U/mgPr, respectively). There were no significant differences in CAT between groups treated with these combinations and the Control group ($p>0.05$). The most effective combinations that produced levels of CAT close to the Controls were: 151 mg/kg i.p INH + 30 mg/kg p.o. SH7 (39.964 U/mgPr, $p>0.05$) and INH + 30 mg/kg p.o. SH8 (39.585 U/mg Pr, $p>0.05$). Liver homogenates from mice treated with the combination INH and Vitamin A showed significantly reduced levels of CAT, compared to the Controls (36.904 U/mg Pr).

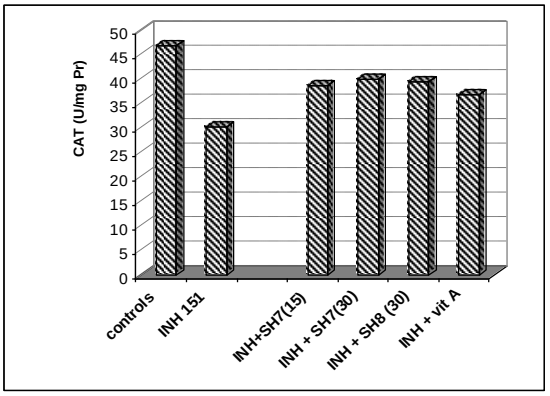

Figure 3. CAT activities from mice liver homogenates after mice were treated with INH alone and in combinations with SH7, SH8 and vitamin A.

Comparison of the levels of MDA, SOD and CAT showed a slight negative correlation (MDA *vs* SOD, $p=0.608$ and MDA vs CAT, $p=0.58$).

4. DISCUSSION

One of the major obstacles in the use of isoniazid as antituberculosis drug is its toxic side effects, notably its strong hepatotoxicity. Some earlier studies have pointed to cytolytic liver injury in rat studies, as a result of possible free radical generation, when isoniazid was used. These cytotoxic materials had been shown to come from lipid peroxidation and the suppression of the antioxidant system (6). Other studies relating suppression of the antioxidant system in antituberculosis drugs studies with rats have also been reported (30). Isoniazid is known to induce the cytochrome P450 system, resulting in increased metabolism, formation of toxic metabolites, including ROS, depletion of glutathione stores and subsequent hepatocellular damage. Our results confirmed that liver homogenates of mice treated with INH had high plasma formation of lipid

peroxidation products and it was accompanied by a decrease in the activity of the antioxidant defense enzymes: superoxide dismutase and catalase.

The observed decrease of SOD and CAT activities in mice treated with INH might be due to both the additional depletion of antioxidant enzyme system by newly produced ROS during the isoniazid metabolism (3) and activation of the processes leading to the peroxidation of lipids, nucleic acids, proteins and other macromolecules (31). In addition, mice treated with INH might have a weakened enzyme production. The decreased activities of SOD and CAT, the primary antioxidant enzymes, observed in isoniazid-treated mice may be due to the interaction of the accumulated free radicals with the associated metal ions or with the active amino acids of these enzymes (32, 33). During hepatotoxicity these enzymes are structurally and functionally impaired by free radical resulting in liver damage. Some studies provide information about possible mechanisms by which low antioxidant defense can increase the risk of various associated diseases and promote the progression of these diseases. Treatment with antioxidants offers protection against isoniazid-induced hepatotoxicity by reducing lipid peroxidation and restoring the antioxidant defence system (6).

In view of the notable superoxide scavenging activity (SSA) of the isonicotinoylhydrazones (24) synthesized by our laboratory (23), it was reasonable to hypothesize that the combination of isoniazid with either (SH7) or (SH8) could decrease the toxic side effects by scavenging the O_2-.

Our results showed that after administration of isoniazid in combination with either SH7 or SH8 the levels of MDA were decreased and the activities of the antioxidant enzymes SOD and CAT were restored. The most effective combination was: 151 mg/kg i.p INH + 30 mg/kg p.o. SH7. The restoration of the activities of antioxidant enzymes could be due to the ability of SH7 and SH8 to scavenge reactive superoxide radicals produced as consequence of isoniazid metabolism within the lipid peroxidation region of the membrane. Similar results have been reported for other hepatoprotective agents (34).

In conclusion, our results confirm that there is an increased oxidative stress and decreased antioxidant defense factors in mice treated with INH. However, previous results have demonstrated the tuberculostatic activity and SSA of the isonicotinoylhydrazones SH7 and SH8 but our results have demonstrated their hepatoprotective effect in mice. It could be suggested that the dosage regimen, comprising a combination of isoniazid at lower doses with SH7 or SH8, would decrease the isoniazid-induced hepatotoxicity and would be a beneficial approach to tuberculosis treatment.

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