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STUDY ON THE INFLUENCE OF ISONIAZID ALONE OR COMBINED WITH NEWSYNTHETIZED ISONIAZID STRUCTURAL ANALOGUES UPON CATALASE ACTIVITY

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Summary

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The in vivo influence of isonicotinic acid hydrazide (isoniazid, INH), a medicine used in the chemotherapy of tuberculosis and that of isoniazid combined with its structural analogues, synthetized by us: N-isonicotinoyl-N´-(3,5-dichloro-2-hydroxybenzal)hydrazone (SH7) and N-isonicotinoyl-N´-(3-etoxy-2-hydroxybenzal)hydrazone (SH8), both with a proven antioxidant and tuberculostatic activity, on the activity of the antioxidant enzyme catalase, was tested under conditions of acute isoniazid toxicity. The data showed a statistically significant decrease in catalase levels in murine liver homogenates under conditions of acute INH toxicity compared to control untreated mice (30.176 ± 7.30 CAT U/mg Pr vs 47.07 ± 16.49 CAT U/mg Pr, p=0.03). Following the treatment of experimental mice with INH combined with isonicotinoylhydrazones SH7 and SH8, liver homogenate catalase activities were similar to those in controls. There was not a statistically significant difference in catalase activities for the following combinations: INH i.p. at 151 mg/kg + SH7 p.o. at 30 mg/kg (39.964 ± 15.45 CAT U/mg Pr) and INH i.p. at 151 mg/kg + SH8 p.o. at 30 mg/kg (39.585 ± 13.53 CAT U/mg Pr), compared to activities in controls (47.07 ± 16.49 CAT U/mg Pr).

Key words: antioxidants, catalase, isoniazid, isonicotinoylhydrazones, reactive oxygen species

INTRODUCTION

Isonicotinic acid hydrazide (INH) has a powerful tuberculostatic activity and is used in tuberculosis therapy under the name of isoniazid (Rimifon, INH). The preparation is also used for isoniazid prophylaxis of HIV-positive patients that are at high risk for tuberculosis morbidity (Gordin *et al.*, 1997; Nolan, 2003). According to literature data the bacteriostatic activity of the preparation is increased under the influence of reactive oxygen species (ROS): superoxide radical ($^{\cdot}O_2$-), nitrogen oxide (NO), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$), formed during its metabolism (Albano and Tomasi, 1987; de Zwart *et al.*, 1999; Mates *et al.*, 1999). Despite its strong antibacterial efficacy, the application of

isoniazid is sometimes limited because of the side toxic effects and the rapid development of resistance of tuberculosis bacteria. The hepatotoxic effect of isoniazid is well known (Byrd and Nelson, 1972; Farrer *et al.*, 1977; Sodhi *et al.*, 1997; 1998). One of the possible mechanisms of the hepatotoxic action of isoniazid is said to be related to the production of free radicals during its intercellular metabolism (Johansson *et al.*, 1995; Wang *et al.*, 1998; Attri *et al.*, 2001). Via the production of ROS, isoniazid exerts a general toxic effect on proteins, DNA and other macromolecules (Rosner and Storz, 1994; Zhang *et al.*, 1996). The hepatotoxicity of isoniazid is related to oxidative stress, occurring as a result of INH treatment (Sodhi *et al.*, 1997; 1998).

The deactivation of ROS depends on the activity of antioxidant system of defense that includes the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (Gpx). The role of this enzymatic system is to protect the cell from ROS (Esterbrauer *et al.*, 1992; Johansson & Schultz, 1994; Siems *et al.*, 2000).

An effective means for avoiding the abnormal production of free radicals is antioxidant prophylaxis and therapy. The use of appropriate antioxidants leads to decrease of lipid peroxidation and enhanced antioxidant protection (lower oxidative stress) and is therefore defined as a modern field of contemporary chemotherapy for infectious disease control, including tuberculosis (Meral *et al.*, 2001; Yoshida *et al.*, 2003).

The limited number of available effective tubericulostatic drugs as well as their side effects (including hepatotoxicity) are a prerequisite for the synthesis of new isoniazid analogues or perfection of the known schedules of its application. That is why the research of new antituberculosis drugs and the effective chemotherapy is always actual.

In previous studies of ours, we showed that isonicotynoilhydrazones, synthetized by us (Varbanova and Georgieva, 1993), possessed a similar or higher tuberculostatic activity than isoniazid against the standard *Mycobacterium tuberculosis* $H_{37}R_V$*-London* strain, accompanied by a lower toxicity and antioxidant properties (Georgieva and Gadjeva, 2002). We observed a decreased concentration of one of the end products of lipid peroxidation – malone dialdehyde (MDA) as a marker of radical-induced damage (Georgieva *et al.*, 2002) and normalization of SOD activity (Georgieva *et al.*, 2003) in liver homogenates, obtained from mice, treated with isoniazid combined with some new synthetized isonicotyoilhydrazones vs mice, treated only with isoniazid.

The aim of the present study was to investigate the *in vivo* effects of isoniazid alone and that of isoniazid combined with antioxidants possessing a tuberculostatic activity, including N-isonicotinoyl-N´-(3,5-dichloro-2-hydroxybenzal)hydrazone (SH7) and N-isonicotinoyl-N´-(3-etoxy-2-hydroxybenzal)hydrazone (SH8), upon one of oxidative stress parameters – the antioxidant enzyme CAT under conditions of acute toxicity in albino mice.

MATERIALS AND METHODS

Experimental animals

The study was performed with 36 albino mice from a conventional ICR line, divided into groups of 6 animals (equal number from both genders). The mice were fed with standard pelleted food and were given water *ad libitum.* The regimen of housing included 12 h light (luminis-

cent system)/12 h dark, air humidity of 60–66 % and ambient temperature of 18-20 °C. Each of the groups was housed separately in standard plastic cages with metal covers according to the Bulgarian State Standard.

Substances

The used substances included isonicotinic acid hydrazide (Rimifon, Bristol-Myers Squibb Co, Connecticut, USA), retinol palmitate (Roche, Basel, Switzerland) and isonicotinoyl hydrazones – N-isonicotinoyl-N′-(3,5-dichloro-2-hydroxybenzal)hydrazone (SH7) and N-isonicotinoyl-N′-(3-etoxy-2-hydroxybenzal)hydrazone (SH8), synthetized by us (Varbanova and Georgieva, 1993).

Treatment

Experimental mice were treated as followed: Group I: control (untreated); group II – treated with INH i.p. at 151 mg/kg (= LD_{50}); group III – with INH i.p. at 151 mg/kg + SH7 p.o. at 15 mg/kg; group IV – with INH i.p. at 151 mg/kg + SH7 p.o. at 30 mg/kg; group V – with INH i.p. at 151 mg/kg + SH8 p.o. at 30 mg/kg and group VI – with INH i.p. at 151 mg/kg + retinol palmitate i.m. at 4000 IU/kg.

The studied isonicotinoylhydrazones were dissolved *ex tempore* in dimethylsulfoxide and bidistilled water, warmed to 37 °C as followed: first, the compound was dissolved with 1 mL dimethylsulfoxide and then, bidistilled water was added to 10 mL working solution. The isoniazid was dissolved with bidistilled water.

After a 18 h fasting, each group was treated as listed via a metal gastric tube (oral application). The isoniazid solution was injected i.p. with a 20-gauge injection needle in the left inguinal region.

Determination of CAT activity

Enzyme activities were determined in liver homogenates of mice, treated with forementioned substances. Liver samples were obtained from all experimental mice, euthanized 4 hours post treatment. Samples were stored at –4 °C until homogenization, that occurred within 1 h of obtaining. Liver homogenates were prepared with ice-cold PBS buffer containing 1 mM EDTA-2Na. The ratio of biological tissue and buffer was experimentally determined as 1:10. The CAT activity was determined in supernatant, obtained after a 15 min centrifugation of liver homogenates at 4000–5000 rpm. The CAT activities were measured according to the method of Beers and Sizer (Beers and Sizer, 1952), modified by us: 30 mM hydrogen peroxide was used as a substrate. To each sample, 1.9 mL phosphate buffer (pH) and 1 mL 30 mM H_2O_2 were added. The decrease in H_2O_2 concentration at 25 °C and pH 7 was spectrophotometrically determined at 240 nm. The extinction was read exactly 1 min after H_2O_2 addition.

CAT activities presented as units per mg protein (U/mg Pr) were calculated according to the equation:

$$\text{CAT [U/mg Pr]} = \frac{(E_{blank} - E_{av}) \times \text{dilution}}{0.066 \times \text{Pr}}$$

where E_{blank} is the extinction of blank sample at 240 nm; E_{av} is the average of 2 extinctions of the studied solution); 0.066 – a coefficient showing the extinction of pure catalase; Pr – the concentration of total protein in the sample, g/L.

Determination of total protein in tissue homogenate

Total protein concentrations in liver homogenates of experimental mice were

determined with commercial kit (Human, Germany) in supernatants.

Statistical analysis

The data were presented as means ± standard deviations and analysed by the Student's t-test. The level of $p< 0.05$ was accepted as statistically significant.

RESULTS

The results for CAT activities in liver homogenates of treated mice are presented in Table 1. After treatment with INH i.p. at 151 mg/kg, equal to LD_{50}, a statistically significant decrease in CAT activities occurred compared to controls (30.176 ± 7.3 CAT U/mg Pr vs 47.07 ± 16.49 CAT U/mg Pr, p=0.03). There were not significant differences between CAT acti-vities of groups, treated with INH i.p. at 151 mg/kg combined with oral administration of: SH7 at 30 mg/kg (39.964 ± 15.45 CAT U/mg Pr); SH7 at 15 mg/kg (38.812 ± 9.97 CAT U/mg Pr); SH8 at 30 mg/kg (39.585 ± 13.50 CAT U/mg Pr) and CAT activity of untreated controls. The levels of the antioxidant enzyme were within normal ranges after the treatment with the combinations INH i.p. at 151 mg/kg + SH7 p.o at 30 mg/kg and INH i.p. at 151 mg/kg + SH8 p.o. at 30 mg/kg. The CAT activities in liver homogenates from mice, treated with INH i.p. at 151 mg/kg + retinol palmitate i.m. at 4000 IU/kg were statistically insignificantly lower than those in untreated mice (36.904 ± 1.56 CAT U/mg Pr vs 47.07 ± 16.49 CAT U/mg Pr).

DISCUSSION

The enzyme CAT protects the cells from drug-induced oxygen consumption via degradation of hydrogen peroxide or via

Table 1. CAT activities in liver homogenates from mice under conditions of acute isoniazid toxicity, treated with various combinations of isoniazid (INH) and N-isonicotinoyl-N′-(3,5-dichloro-2-hydroxybenzal)hydrazone (SH7), N-isonicotinoyl-N′-(3-etoxy-2-hydroxybenzal)hydrazone (SH8) and retinol palmitate (vitamin A). Data are presented as mean ± SD (n=6)

Group	Treatment	CAT (U/mg Pr)	MDA (μM/L) [1]
I	Untreated controls	47.070 ± 16.490	2.024 ± 0.164
II	INH i.p. at 151 mg/kg (= LD_{50})	30.176 ± 7.300***	2.578 ± 0.349*
III	INH i.p. at 151 mg/kg + SH7 p.o. at 15 mg/kg	38.812 ± 9.970	2.152 ± 0.203$^{\#}$
IV	INH i.p. at 151 mg/kg + SH7 p.o. at 30 mg/kg	39.964 ± 15.450	2.059 ± 0.377$^{\#}$
V	INH i.p. at 151 mg/kg + SH8 p.o. at 30 mg/kg	39.585 ± 13.530	2.291 ± 0.025**
VI	INH i.p. at 151 mg/kg + retinol palmitate i.m. at 4000 IU/kg	36.904 ± 1.560	2.419 ± 0.235**

[1] The data for MDA are according to Georgieva *et al.* (2002).* p≤0.001 vs controls; ** p<0.005 vs controls; ***p<0.05 vs controls; $^{\#}$p<0.05 vs INH-treated group.

direct drug interaction (Speranza *et al.*, 1993). The decreased CAT activity could be due to the enhanced generation of ROS, during isoniazid metabolism (Albano and Tomasi, 1987; de Zwart *et al.*,1999; Mates *et al.*, 1999), that are deactivated by CAT as well as to the activation of the peroxide oxidation of lipids, proteins, nucleic acids and other macromolecules (Rosner and Storz, 1994; Zhang *et al.*, 1996). It is known that the antioxidant enzyme CAT is especially important for cell adaptation to oxidative stress. We suppose that the impaired CAT levels after treatment with INH i.p. at 151 mg/kg (=LD_{50}) is due to the shift of the antioxidant-prooxidant systemic balance towards prooxidants and that it is related to the production of oxidative stress after the treatment of experimental mice with isoniazid. The increased levels of MDA, another marker of radical damage, reported in previous studies of ours (Georgieva *et al.*, 2002) and the decreased SOD activities (Georgieva *et al.*, 2003) in murine liver homogenates under conditions of acute isoniazid toxicity confirmed those data as well.

When isoniazid was used simultaneously with 15 or 30 mg/kg SH7 or with 30 mg/kg SH8, liver homogenate CAT levels did not differ significantly from those in controls (Table 1). Therefore, the decreases in CAT values were antagonized when INH was used in combination with the isonicotinoylhydrazones SH7 and SH8, that were shown to have a tuberculostatic (Varbanova and Georgieva, 1993) and antioxidant activity (Georgieva and Gadjeva, 2002). Most possibly, the antioxidant systemic defense was improved due to antiradical properties of SH7 and SH8. Our results are in accordance with some recent data (Attri *et al.*, 2001) showing that INH-induced oxidative damage (including hepatotoxicity) could be reduced via the use of antioxidants providing supplementary antioxidant mechanisms of defense. Similar results are also reported from other investigators, that applied various antioxidants aiming to provide an additional cellular antioxidant protection against isoniazid oxidative toxicity (Suja *et al.*, 1997; Reiter *et al.*, 1997, 2002; Attri *et al.*, 2001).

The effect of restored CAT activity was less evident when INH was combined with retinol palmitate and thus it could be assumed that its hepatoprotective effect was lower than that of SH7 and SH8. This is seen from the higher concentrations of MDA (Georgieva *et al.*, 2002) and the lower CAT activities (Table 1) in mice, treated with this combination.

The normalization of CAT activity under conditions of acute isoniazid toxicity after the application of isoniazid combined with SH7 and SH8, was probably related to their hepatoprotective effect. We suppose that isonicotinoylhydrazones protect the cells from oxidative stress via inhibition of the hepatotoxic effect of isoniazid due to their antioxidant properties.

CONCLUSIONS

According to our data it could be concluded that the combined administration of isoniazid and the isonicotinoylhydrazones SH7 and SH8, synthetized by us, reduced the oxidative stress under conditions of acute isoniazid toxicity (normalized the activities of the anti-oxidant enzyme catalase) and therefore, manifested a hepatoprotective effect.

The hepatotoxicity of isoniazid could probably be decreased via its application with compounds possessing a tuberculostatic and antioxidant activity at a time,

such as SH7 and SH8. There were, therefore, prerequisites to decrease the dose of isoniazid in cases of combined application.

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