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Hepatoprotective effect of isonicotinoylhydrazone SH7 against chronic isoniazid toxicity

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This study was carried out to investigate whether 3,5-dichloro-salicylaldehyde isonicotinoyl hydrazone (SH7), an analogue of the antituberculosis drug isoniazid (INH), recently synthesized in our laboratory, could prevent isoniazid-induced liver damage. Forty-two white healthy mice were treated, once daily for 30 days, with solutions of INH, SH7 and combinations of them at doses of 7.5 mg/kg p.o. and 15 mg/kg p.o. At the end of this period, livers were harvested for histopathological analysis. The levels of lipid peroxidation products (MDA) and the activities of antioxidant defense enzymes, superoxide dismutase (SOD) and catalase (CAT), were also measured in the liver homogenates. Treatments with combinations of INH and SH7 decreased the levels of MDA, normalized the previously depressed levels of SOD and CAT and prevented isoniazid-induced liver damages. A previously demonstrated tuberculostatic and superoxide scavenger activity (SSA) of the isonicotinoylhydrazone SH7 and results from the present study thus set out a proposal for a new isoniazid/SH7 combination therapy designed to provide hepatoprotection.

1. Introduction

Tuberculosis is still a leading cause of death among those infections with a single etiology and one-third of the world's population is latently infected with *Mycobacterium tuberculosis* (Snider and La Montagne 1994). First-line drug with superior efficacy, used in treating *Mycobacterium tuberculosis* infection, is isoniazid (isonicotinic acid hydrazide, INH). Problems associated with drug resistance (Chauhan and Mande 2001; Ramaswamy et al. 2003) and potential adverse reactions such as hepatotoxicity (Scharer and Smith 1969; Byrd and Nelson, 1972; Black et al. 1975; Farrer et al. 1977; Sokolova et al. 1989; Vidal Pla et al. 1991; Sodhi et al. 1998) indicate the need for new effective anti-tuberculosis drugs and for alternative therapy regimens. Several drug combinations have been studied to increase the therapeutic efficacy and reduce the toxicity of the anti-microbacterial agent isoniazid.

The ability of isonicotinoylhydrazones to specifically inhibit the growth of *Mycobacterium tuberculosis*, the high selectivity index and their ability to enhance the activity of standard antituberculosis drugs *in vitro* indicate that they may serve as promising lead compounds in future development of drugs for the treatment of *M. tuberculosis* infections (De Logu et al. 2002). Though progress in tuberculosis chemotherapy is predicated on development of new drugs, much still depends on fully unraveling the mechanism of action of existing ones like isoniazid and those to be introduced into the pharmacologic armamentarium.

Recent studies have suggested that superoxide is involved in INH activation. Reactive oxygen species (ROS) such as NO, $\cdot O_2^-$, H_2O_2, $\cdot OH$ arise during these activations (Chauhan and Mande, 2001; Ramaswamy et al. 2003) and probably account for INH toxicity.

INH in combined therapy with melatonin, a known radical scavenger, leads to a three-fold increase in tuberculostatic activity and an appreciable decrease in toxic side effects. (Wiid et al. 1999; Reiter et al. 2002). These findings suggest that tuberculosis chemotherapy could be improved by applying molecules having the same action as melatonin. Our previous studies have shown that some isonicotinoyl

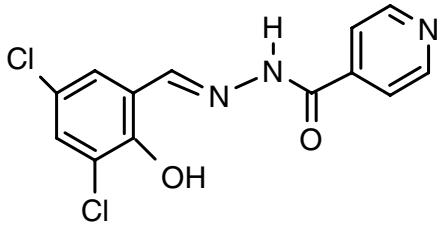

Table: MDA, SOD and CAT in mouse liver homogenates after 30 days treatment with INH, SH7 and their combinations

Groups	MDA (μM/l)	SOD (U/mgPr)	CAT (U/mgPr)
Control (Non treated)	1.734 ± 0.194	4.292 ± 0.46	17.358 ± 6.49
7.5 mg/kg p.o. INH	***1.935 ± 0.146 $p = 0.05$	*2.524 ± 0.43 $p = 0.0000$	*9.568 ± 4.54 $p = 0.03$
15 mg/kg p.o. INH	*2.195 ± 0.105 $p = 0.0004$	*2.517 ± 0.18 $p = 0.0000$	*8.348 ± 1.73 $p = 0.008$
7.5 mg/kg p.o. SH7	***1.704 ± 0.219 $p = 0.005$	**3.640 ± 0.59 $p = 0.004$	**15.685 ± 4.73 $p = 0.04$
15 mg/kg p.o. SH7	***1.725 ± 0.283 $p = 0.003$	***4.160 ± 0.28 $p = 0.0000$	**16.115 ± 2.73 $p = 0.01$
7.5 mg/kg p.o. INH + 7.5 mg/kg p.o. SH7	***1.749 ± 0.259 $p = 0.003$	**3.157 ± 0.22 $p = 0.0000$	14.565 ± 3.35
7.5 mg/kg p.o. INH + 15 mg/kg p.o. SH7	***1.743 ± 0.101 $p = 0.00002$	***3.422 ± 0.27 $p = 0.0000$	15.473 ± 1.53

* – vs. control; ** – vs. INH 7.5 mg/kg; *** – vs. INH 15 mg/kg

hydrazones, synthesized in our laboratory, had tuberculostatic activity (Varbanova and Georgieva 1993). They also had antioxidant activity, as measured by their level of SSA (Georgieva and Gadjeva 2002). All these information calls for intense studies on these newly synthesized compounds. Therefore, the aim of the present study was to determine whether 3,5-dichloro-salicylaldehyde isonicotinoylhydrazone (SH7) would display hepatoprotective effects. In this study we investigated isoniazid-induced liver damages and the level of lipid peroxidation products. We studied also the activities of antioxidant defense enzymes, superoxide dismutase and catalase, in livers of mice treated with INH in combinations with SH7.

2. Investigations and results

2.1. Biochemical findings

The results of the levels of lipid peroxidation and the activities of antioxidant enzymes SOD and CAT are presented in the Table. The levels of MDA were significantly increased in mouse liver homogenates after 30 days of treatment with INH at doses of 7.5 mg/kg p.o. and 15 mg/kg p.o., compared to the control group (non treated mice) (mean 1.935 μM/l and 2.195 μM/l, vs 1.734 μM/l, $p < 0.05$). There was no difference in MDA concentration compared to the controls after treatment with SH7 at doses 7.5 mg/kg p.o and 15 mg/kg p.o. (mean 1.718 μM/l and 1.769 μM/l, $p > 0.05$).

The combination INH (7.5 mg/kg p.o) with SH7 (7.5 mg/kg p.o) and INH (7.5 mg/kg p.o.) with SH7 (15 mg/kg p.o), showed lower levels of MDA compared to INH administrated alone (mean 1.749 μM/L, $p = 0.003$ and 1.743 μ/L, $p = 0.00002$).

The activities of the antioxidant enzymes SOD and CAT decreased significantly in mouse liver homogenates after 30 days of treatment with INH at doses 7.5 mg/kg p.o. and 15 mg/kg p.o., compared to the Controls (for SOD mean 2.524 U/mgPr and 2.17 U/mgPr, vs 4.292 U/mgPr, $p < 0.0001$ and for CAT mean 9.568 U/mgPr and 8.348 U/mgPr, vs 17.358 U/mgPr, $p < 0.05$). The levels of the antioxidant enzymes increased significantly following treatment with combinations of INH (7.5 mg/kg p.o) and SH7 (7.5 mg/kg p.o) and INH (7.5 mg/kg p.o.) with SH7 (15 mg/kg p.o), compared to INH administrated alone, as shown in this order: for SOD (3.157 U/mgPr and 3.422 U/mgPr, $pp < 0.0001$) and for CAT (14.565 U/mgPr and 15.473 U/mgPr, $p < 0.05$).

2.2. Histopathological findings

The livers from all control mice showed normal microstructure under light microscopy. Livers from the third experimental group (7.5 mg/kg p.o. INH) presented moderate total hyperemia of all functional and nutritive vessels, but the parenchyma was intact. Few hepatocytes showed weakly condensed nuclear chromatin. Livers from the fourth group (15 mg/kg p.o. INH) showed, on the other hand, well defined total stasis hyperemia of the vessels in all liver lobules; this was accompanied by destructive changes in the hepatocytes. Most of them had pycnotic nuclei; their cytoplasms contained inclusions characteristic of granular dystrophy. There were solitary hepatocytes with signs of fatty dystrophy. There were rare signs of micro necrosis (Fig. 1).

The histological condition of the livers in the fifth group (7.5 mg/kg p.o. SH7) (Fig. 2) was defined by weak hyperemia of the vessels, different degrees of cariopycnosis in few parts of the hepatocytes and isolated cases of micro lymphocyte infiltrates in some lobules. The sixth group, (15 mg/kg p.o. SH7), presented almost the same histological picture except that there were more frequent perivascular infiltrates associated with the small and big vessels (Fig. 3). The histological condition of the livers in the seventh group (7.5 mg/kg p.o. INH + 7.5 mg/kg SH7) was identical to that of the fifth group (Fig. 2). Livers from the eighth group, (7.5 mg/kg p.o. INH + 15 mg/kg SH7), presented the most pronounced vascular hyperemia among

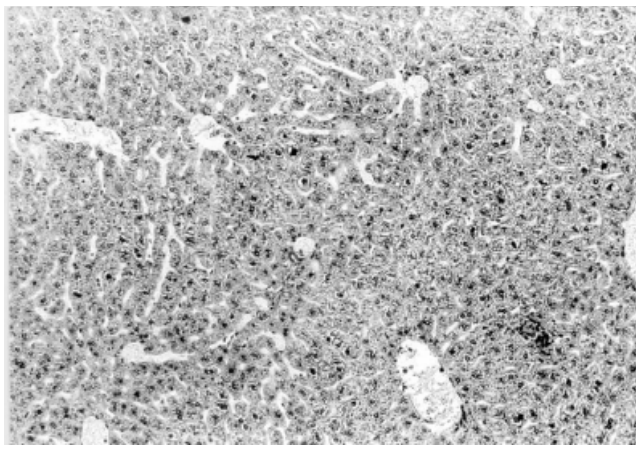

Fig. 1: A few contiguous liver lobules with structural changes characteristic of toxic liver dystrophy of mice treated with INH 15 mg/kg p.o.; H E × 100

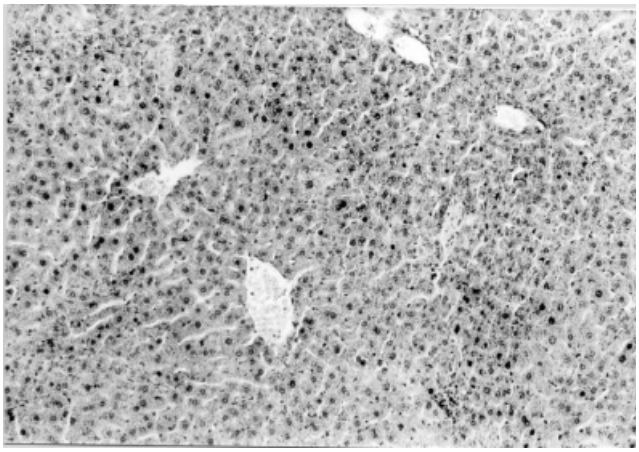

Fig. 2: Weak vessel hyperemia. Some hepatocytes are with pycnotic nuclei in livers of mice treated with 7.5 mg/kg SH7 p.o. In the background are local microlymphocytic infiltrations; H E × 100

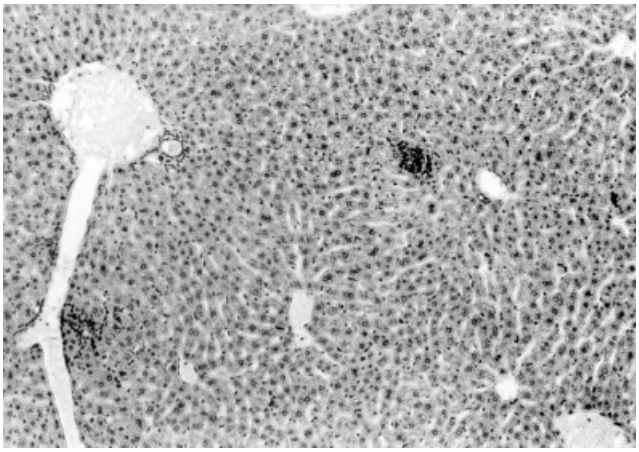

Fig. 3: Local and diffuse microlymphocytic perivascular infiltrations in liver parenchyma of mice treated with 15 mg/kg SH7 p.o.; H E × 100

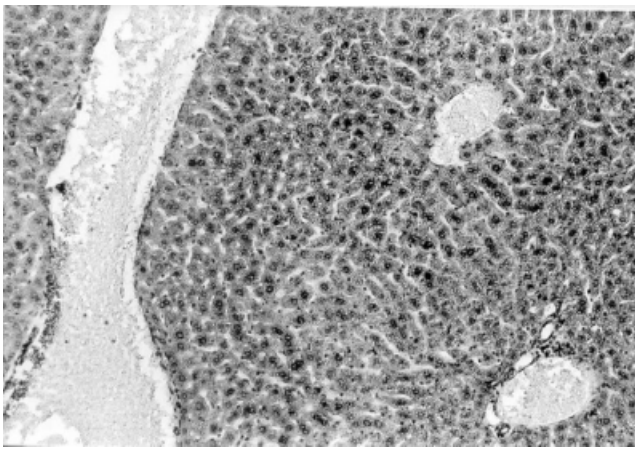

Fig. 4: An area with well expressed hyperemia of all types of vessels in liver parenchyma of mice treated with 7.5 mg/kg INH p.o. + 15 mg/kg SH7 p.o. Perivascular cellular infiltrates are lacking; H E × 100

the groups. There were no perivascular cellular infiltrates in the samples of this group. But for those near the vessel walls the integrity of all hepatocytes could have been intact. The cytoplasm contained small amounts of granular inclusions (Fig. 4).

3. Discussion

In our study we found that 30 days oral treatment of mice with INH led to an increase in lipid peroxidation levels and a decrease in liver SOD and CAT activities, a condition that signaled oxidative stress (Buss et al. 2002). This condition, ostensibly deriving from isoniazid metabolism, could have arisen from the consequential increase in reactive oxygen species generation (Albano and Tomasi 1987), as well as the activation of the processes of lipid peroxidation, peroxidations of proteins, nucleic acids and other macromolecules. We found that, though free radical production increased, antioxidant levels decreased in mice treated with INH. Our data suggest that the reduced antioxidant production was as a result of the increased reactive oxygen series metabolites that caused a decrease in the activity of the antioxidant defense system.

In our study we found also that treatment with the combinations of INH and SH7 decreased these elevated free radicals, and reversed the INH condition by bringing back the depressed levels of the antioxidants to normal. This normalization of the levels of the antioxidant enzymes, following INH and SH7 combined application, could be linked to the observed SH7 prevention of INH-induced oxidative damage of cell structures. Our results are consistent with earlier observations of Skakun and Slivka Yun (1992), Oswelier (1996), Suja et al. (1997), Wiid et al. (1999), Attri et al. (2001), and Reiter et al. (2002).

Another aim of this study was to investigate whether SH7 could prevent INH-induced liver damage. Our histological findings in pursuance of this aim showed various grades of structural changes in livers obtained from mice treated with different amounts of this agent. For example, we found hepatocellular degenerative changes in mice treated with INH alone, that is, in our fourth group (15 mg/kg p.o. INH). This confirmed the existence of toxic liver dystrophy. The liver parenchyma from other groups showed various degrees of these changes with the least changes occurring in the fifth (7.5 mg/kg p.o. SH7) and seventh (7.5mg/kg INH + 7.5 mg/kg SH7) groups. These data show that SH7, when used alone or in combination with therapeutic doses of INH, could provide the least hepatotoxic effect similar to what the experimental doses used in the fifth and seventh experimental groups of this investigation did. Thus, the oxidative decomposition of the liver occurring because of the increased lipid peroxidation could be the reason for the hepatocellular degeneration (Turkdogan et al. 2001).

These results have elucidated the role of ROS in the untoward effects of isoniazid and, particularly, its hepatotoxicity. The fall in MDA levels, following application of the above-mentioned combinations, indicated that SH7 could probably provide some hepatoprotection. In view of this there could be some merit and safer therapeutic endpoint in proffering a combination of INH and its tuberculostatic and hepatoprotective analogues in which the former would be present in a sub-maximal dose (Varbanova and Georgieva 1993; Georgieva and Gadjeva 2002). This belief is in agreement with the hypothesis of Wiid et al. (1999) that any compound that could synergize or complement isoniazid without amplifying the latter's toxicity, would be efficient in tuberculosis chemotherapy.

In conclusion, our data show that isonicotinoyl hydrazone, SH7, a compound synthesized in our laboratory, could reduce the oxidative stress resulting from isoniazid chronic toxicity during our 30-day investigation. It lowered lipid peroxidation (characterized by decreased MDA levels) and re-

versed the depressed antioxidant enzyme defense system by bringing the levels of SOD and CAT activity back to normal. There is therefore every reason to suggest that SH7 could provide hepatoprotective action in the presence of chronic isoniazid toxicity. It does this by inactivating ROS produced during INH activation.

4. Experimental

4.1. Animal studies

This study was carried out on 42 white healthy mice, weighing between 18 and 22 g. They were divided into groups of 6 animals per group in which they were matched for gender. The animals were housed in plastic cages, fed a normal laboratory diet and water *ad libitum*.

4.2. Treatment

Solutions of the test compounds, INH and SH7, were made as follows: they were dissolved *ex tempore* firstly in 1 to 3 ml DMSO, then made up to 10 ml in double distilled water and incubated at 37 °C.
All mice received daily for 30 days solutions of INH (7.5 mg/kg p.o. and 15 mg/kg p.o.), SH7 (7.5 mg/kg p.o. and 15 mg/kg p.o.) and combinations of them in the following order: INH (7.5 mg/kg p.o.) + SH7 (7.5 mg/kg p.o.) and INH (7.5 mg/kg p.o.) + SH7 (15 mg/kg p.o.) in line with methods described in the literature (Geran et al. 1972).

4.3. Spectrophotometric studies

The liver from each treated mouse was homogenized. The homogenate was diluted 1 : 10 with PBS buffer (pH = 7.4). It was then centrifuged for 10 min at 2000 rpm. The supernatant was deproteinated with 25% solution of trichloroacetic acid (TCA), then centrifuged again for 20 min at 2000 rpm. This preparation was used for the spectrophotometric investigations.

4.3.1. Products of lipid peroxidation (MDA)

Basal levels of lipid peroxidation, as indicated by thiobarbituric acid-reactive substances (TBARS), were determined using the thiobarbituric acid (TBA) method, which measures the malondialdehyde (MDA) reactive products (Plaser et al. 1966). In the TBARS assay 1 ml of the supernatant, 1 ml of normal saline and 1 ml of 25% trichloroacetic acid were mixed and centrifuged for 20 min at 2000 rpm. One ml of protein free supernatant was taken, mixed with 0.25 ml of 1% thiobarbituric acid (TBA) and boiled for 1 h at 95 °C. After cooling the absorbance of the resulting pink color was read at 532 nm.

4.3.2. Measurement of antioxidant enzyme activities

SOD activity was determined by the xanthine/xanthine-oxidase/nitroblue tetrazolium (NBT) method according to Sun et al. (1988) 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 using Beers and Sizer method (Beers and Sizer 1952). Briefly, hydrogen peroxide (30 mM) was used as a substrate and the decrease in H_2O_2 concentration at 22 °C 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 one unit equals the amount of an enzyme that degrades 1 M H_2O_2 per minute.

4.4. Histological analysis

At the end of the experiment mice were killed after narcosis. The livers were harvested and tissue fragments obtained, fixed in 10% neutralized formaldehyde, embedded in paraffin, and then stained with haematoxylin and eosin. Canadian balm was dropped on the stained slices and then were covered with glass, thus providing permanent histological preparations (Lilie 1965). Histological specimens were examined under the light microscope ("Ergaval" model, Karlzeiss Jena, Germany).

4.5. Statistical analysis

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

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