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Isonicotinoylhydrazone Analogs of Isoniazid: Relationship between Superoxide Scavenging and Tuberculostatic Activities

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Abstract—Superoxide scavenging activity (SSA) of recently synthesized isonicotinoylhydrazones, analogs of the clinically used anti-tuberculosis drug isoniazid (INH), was investigated using xanthine/xanthine oxidase system to generate the superoxide anion. The isonicotinoylhydrazones exhibited well expressed SSA, whereas INH did not show any SSA. All of the isonicotinoylhydrazones had a tuberculostatic activity when tested with the standard strain of *Mycobacterium tuberculosis* $H_{37}R_v$ and some of them had a higher tuberculostatic activity than INH. A lower acute toxicity was also observed compared to INH. Moreover, a correlation was observed between LD_{50} and SSA for the isonicotinoylhydrazones studied. An explanation is suggested for the higher tuberculostatic activity and lower acute toxicity of some of the isonicotinoylhydrazones as compared to that of INH. A new route to less toxic derivatives of INH with potential tuberculostatic activity is proposed.

Key words: isonicotinoylhydrazones, superoxide scavenging activity, anti-tuberculosis, isoniazid

Reactive oxygen species (ROS), such as $O_2^{\cdot-}$, H_2O_2, and $OH^{\cdot}$, are highly reactive species generated by biochemical redox reactions as a part of the normal cell metabolism. Exposure to environmental factors, such as UV light, cigarette smoke, environmental pollutants, and gamma radiation, accelerates the generation of ROS [1, 2]. Some exogenous compounds including anticancer drugs, anesthetics, and analgesics can also result in increased production of free radicals [3].

Isoniazid (isonicotinic acid hydrazide, INH) has been a front-line anti-tubercular agent for decades, but only recently has an understanding of its action against *Mycobacterium tuberculosis* emerged [4-6]. It was shown that superoxide stimulates INH activity in mycobacteria [7]. Activation of INH may also affect DNA, proteins, and other macromolecules through formation of ROS [8]. Thus, oxidative activation of INH can both have a specific effect on mycolic acid synthesis and be generally toxic for proteins and nucleic acids. The limited number of effective anti-tuberculosis drugs available and the problems associated with drug resistance and potential adverse reactions such as hepatotoxicity became a prerequisite for synthesis of more effective analogs of INH.

We have synthesized isonicotinoylhydrazones, analogs of INH with different substitutes in the benzene ring at the N′ position [9].

The present study was undertaken to determine the superoxide scavenging activity (SSA) of newly synthesized isonicotinoilhydrazone analogs of isoniazid with respect to their tuberculostatic activity and acute toxicity. Further, we hypothesized that antioxidant action of the isonicotinoylhydrazones may be responsible for the beneficial effects of these compounds.

MATERIALS AND METHODS

Chemicals. The structures of the isonicotinoylhydrazones used for SSA measurements are shown in the table. Isoniazid (Rimifon) was obtained from Bristol-Myers Squibb Co. (USA), buttermilk xanthine oxidase and trolox from Fluka (Germany), 2,2,6,6-tetramethyl-4-oxopiperidine-1-oxyl (TMPO) from Aldrich (USA). All isonicotinoylhydrazones were synthesized according to Varbanova and Georgieva [9]. The test compounds were dissolved in dimethylsulfoxide (DMSO)/phosphate buffered saline (PBS; 10 mM phosphate, 120 mM NaCl, 2.7 mM KCl, pH 7.4) (1 : 10 v/v).

Abbreviations: ROS) reactive oxygen species; INH) isoniazid; DMSO) dimethylsulfoxide; SSA) superoxide scavenging activity; TMPO) 2,2,6,6-tetramethyl-4-oxopiperidine-1-oxyl; NBT) nitroblue tetrazolium.

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Assay of superoxide scavenging activity. SSA measurements were performed by the methods of Gadzheva et al. [10] or Sun and Oberley [11] with minor modifications. Briefly, the xanthine/xanthine oxidase system was used to generate the superoxide anion. This anion reduces nitroblue tetrazolium (NBT) to formazan, which is monitored at 560 nm. The final concentrations of xanthine, xanthine oxidase, NBT, and EDTA in the assay were 50 μM, 10 U/ml, 0.125 mM, and 0.85 mM, respectively. The test compounds (25 μM) were mixed in DMSO/PBS (pH 7.4) (1 : 10 v/v). Compounds with SSA (superoxide dismutase (SOD)-like activity) in the sample remove the superoxide anion and inhibit the reduction of NBT. The level of this reduction is used as a measure of SSA. Results are expressed as SOD units (U_{SOD}). One unit of SOD activity is defined as the amount resulting in 50% inhibition of the reduction of NBT to formazan. SSA method is valid only when the following two criteria are met: scavengers do not react with NBT, and they do not interfere with superoxide generating system. To check the first criterion, we also measured SSA using two different concentrations of NBT (0.125 and 0.2 mM). To exclude the possibility that the isonicotinoylhydrazones interfere with superoxide generating system, xanthine oxidase activity in the presence of either isonicotinoylhydrazones or TMPO was also measured from uric acid formation. Xanthine (0.1 mM), EDTA (0.85 mM), and the compounds tested (0.1 mM) were mixed in 0.1 M sodium phosphate buffer (pH 7.4), and the reaction was started by adding xanthine oxidase (0.1 U/ml). The generation of uric acid was monitored at 290 nm ($\varepsilon_{290} = 9.6\ mM^{-1}\cdot cm^{-1}$).

Tuberculostatic activity. The tuberculostatic activity was determined on the standard strain of *M. tuberculosis* typus humanus $H_{37}R_v$ – London. The culture medium of Loewenschtain–Yensen, accepted by the World Health Organization as the standard medium for research on mycobacterium, was used.

Isonicotinoylhydrazones were dissolved in dimethylformamide and the dilutions were made with sterile distilled water. The dissolved isonicotinoylhydrazones were added at various concentrations to the culture medium while it was liquid. The coagulation of the medium in test tubes was done at 85-90°C for 1 h. The suspensions of *M. tuberculosis* were prepared from an actively growing 3-4-week culture, which was added into test tubes with a sterile physiological solution. The results were evaluated after 28 days.

Acute toxicity. The acute toxicity of the isonicotinoylhydrazones was evaluated in inbred white mice line ICR with 20-25 g body weight. Various doses of the compounds studied were administrated i.p. in a suspension stabilized with 1% carboxymethyl-cellulose. The control group obtained only the same volume of saline. The mean lethal dose LD_{50} was the dose required to achieve 50% death rate of the treated mice.

Statistical analysis. The results are reported as means ± SD. Statistical analysis was performed with Student's *t*-test and multiple regression analysis. $p < 0.05$ was considered statistically significant.

RESULTS

Superoxide scavenging activity assay. Superoxide scavenging activity (SSA) of isonicotinoylhydrazones studied in comparison with the well known superoxide scavengers ascorbic acid (1.32 ± 0.14 U_{SOD}) and TMPO (1.36 ± 0.03 U_{SOD}) are shown in the table. Our results show that all of these compounds possess SSA. For compounds SH7 and SH8 it was comparable with that of ascorbic acid and TMPO (table). The clinically used analog INH actually exhibited no significant SSA (0.006 U_{SOD}).

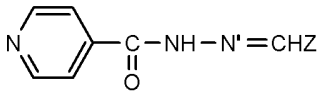
Adequacy of superoxide scavenging activity assay. In order to exclude the possibility that the isonicotinoylhydrazones may directly react with NBT, experiments with two different NBT concentrations were performed. Similar slopes of the inactivation curves measured with SH7 at two concentrations of NBT (figure) indicated that SH7 did not react with NBT. Similar data were obtained for all the isonicotinoylhydrazones studied. We determined also whether the compounds directly interfere with the superoxide generating system. Compounds SH4, SH8, and TMPO did not show any inhibitory effect on the enzymic activity of xanthine oxidase as estimated from uric acid formation rate.

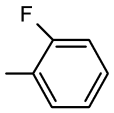
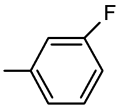
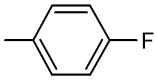
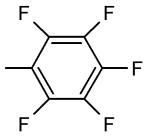
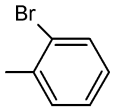
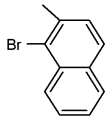
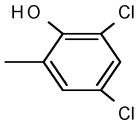
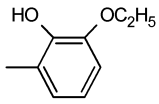
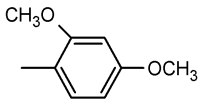
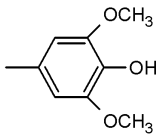
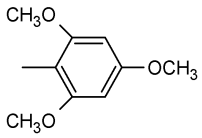
Tuberculostatic screening. The results from the tuberculostatic screening of the isonicotinoylhydrazones showed that compounds SH5, SH6, and SH11 have a tuberculostatic activity comparable to that of INH when tested with the standard strain of *Mycobacterium tuberculosis* typus humanus $H_{37}R_v$ – London (table). Moreover compounds SH1, SH3, SH4, SH8, SH9, and SH10 showed higher tuberculostatic activity than INH. We did not observe statistically significant correlation between SSA and minimum inhibitory concentrations (MIC) ($R = 0.219$, $p = 0.52$). The observed weak tendency was negative ($k = -2.6$).

Acute toxicity. The acute toxicity was tested only for the isonicotinoylhydrazones with high tuberculostatic activity. Three of them—SH2, SH4, and SH8—were less toxic than INH as judged by the LD_{50} values (table). Interestingly, a definitive positive correlation existed between SSA and LD_{50} ($R = 0.712$, $p = 0.0495$).

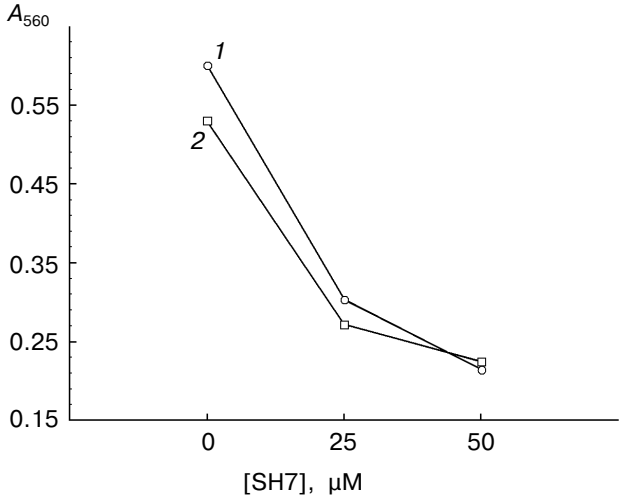
Structure–activity relationships. The results from the SSA assay prompted us to search for a relationship between the chemical structure and the superoxide scavenging activity of the new compounds. The highest SSA was measured for compounds SH7 and SH8, which possess a hydroxyl group in the *ortho*-position of the benzene ring. Addition of a methoxy group(s) to the benzene ring did not have any effect on SSA. Among the halogen-substituted isonicotinoylhydrazones, the highest SSA was measured for SH5 with Br in the *ortho*-position of the benzene ring followed by SH4 with five fluorine atoms. The number of the fluorine atoms in the benzene ring was not critical.

The results of the tuberculostatic screening revealed thus the following relationship between the chemical

Minimum inhibitory concentrations (MIC) of the new isonicotinoylhydrazones for the *Mycobacterium tuberculosis* strain $H_{37}R_v$ – London 

Compound	Z	MIC, μM	LD_{50}, mg/kg	SSA*, U_{SOD}
SH1		0.041		0.39 ± 0.24
SH2		0.206	430	0.57 ± 0.20
SH3		0.082		0.57 ± 0.20
SH4		0.013	1280	0.59 ± 0.25
SH5		0.164		0.69 ± 0.30
SH6		0.141		0.031 ± 0.016
SH7		0.070		1.07 ± 0.50
SH8		0.070	1012	0.89 ± 0.34
SH9		0.070		0.32 ± 0.08
SH10		0.065		0.45 ± 0.15
SH11		0.159		0.63 ± 0.24
INH		0.146	151	0.062 ± 0.045

* Mean ± SD (n = 6).



Inhibition of the reduction of NBT (0.125 (*1*) and 0.20 mM (*2*)) to formazan in the xanthine/xanthine oxidase system by SH7. A_{560} values represent the means of three measurements. SD values are within 6%

structure and the antibacterial activity of the new compounds: the position of the methoxy groups in the benzene ring does not have any effect on the tuberculostatic activity; the presence of more than two methoxy groups in the benzene ring leads to a sharp decrease of the tuberculostatic activity; in the group of the isonicotinoylhydrazones with halogen substituents, the highest tuberculostatic activity is achieved when a fluorine atom is present in the *ortho*-position of the benzene ring (SH1); the presence of several fluorine atoms in the benzene ring leads to a considerable increase in the tuberculostatic activity (SH4).

DISCUSSION

Recent studies have suggested that superoxide is involved in INH activation, and reactive oxygen species arise during this activation [8]. Wild et al. [12] reported that melatonin can cause at least a threefold increase in the efficacy of isoniazid and is thought to do so via its prooxidative properties. The authors supposed that the addition of melatonin to INH may initiate INH activation through formation of $O_2^{\cdot-}$. The addition of melatonin to isoniazid has additive or even synergistic effects in *M. tuberculosis*. Therefore, agents which potentiate the effect of INH (without adverse side effects) or allow the use of a lower INH concentration may be extremely important. It was also found that all the isoniazid-resistant *M. tuberculosis* strains were deficient in the activity of mycobacterial catalase-peroxidase [13]. KatG, the gene encoding the catalase-peroxidase of *M. tuberculosis* is required for activation of isoniazid and defects in KatG lead to INH resistance [14]. This suggests that tuberculosis chemotherapy can be improved by molecules with SOD- or catalase-like activity, to mitigate the added burden imposed by $O_2^{\cdot-}$ or organic peroxides on *M. tuberculosis* strains.

Based on both above mentioned facts it was of interest to investigate the behavior towards $O_2^{\cdot-}$ of formerly synthesized isonicotinoylhydrazones**,** analogs of the anti-tuberculosis drug INH. Since, some of the isonicotinoylhydrazones possessed SSA comparable with that of the reference antioxidant ascorbic acid, we hypothesized that the antioxidant action of these isonicotinoylhydrazones may be responsible for their beneficial effects such as higher tuberculostatic activity and lower acute toxicity as compared to that of INH. The compound SH8 exhibited both higher tuberculostatic activity and lower toxicity than INH. This compound showed also a well expressed SSA. Therefore, we assume that a combination of a lower concentration of INH with the compound SH8 could decrease the toxic side effects of the tuberculostatics by scavenging $O_2^{\cdot-}$.

Thus, introducing of OH-substituted benzene moieties in the structure of INH leads to a higher superoxide scavenging activity, higher tuberculostatic activity and lower acute toxicity compared to those of INH. We also consider that the combination of INH in low doses and a isonicotinoylhydrazone such as SH8 would be a promising anti-tuberculosis therapy approach without adverse side effects. Further detailed analyses are warranted to clarify the effect of the combinations of isonicotinoylhydrazone with INH on the tuberculostatic activity and toxicity.

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