NEW ISONIAZID DERIVATIVES FOR ANTIMICROBIAL SPECTRUM EXTENSION AND HEPATOTOXICITY REDUCTION OF PARENT COMPOUND: IN VITRO AND IN VIVO ASSAYS

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Abstract

One of the most common adverse effects, in the case of medicines used voluntarily or not, of over dosage, or even of drugs administered in therapeutic doses, is hepatic injury, which is why, in the pharmaceutical research, the experimental compounds are assessed before and during clinical trials, so that only compounds that prove to be safe for commercial use will be approved. In our case, compounds obtained prior to this study were evaluated from a biological point of view, following in vitro tests, establishing the antimicrobial potential, on bacterial strains and fungal strains. In addition, in vivo tests were performed on mice, for completing the pharmacotoxicological profile: evaluation of the hepatic markers (GPT, GOT, alkaline phosphatase, total serum cholesterol and serum albumin). The results demonstrate that different structural modulations of isoniazid can favourably influence the antimicrobial activity and may lead to an improvement of liver markers, after oral administration.

Keywords: isoniazid derivatives, hepatotoxicity, antimicrobial potential, liver injury

Introduction

In the case of the administration of medicines administered voluntarily or not, in overdose, or even in therapeutic doses, the process of hepatocyte injury occurs. In preclinical toxicology studies, hepatotoxicity is a major obstacle to the development of new drugs, the liver being the most exposed organ to the adverse reactions induced by their use. In addition, compared to other tissues, the liver is the most exposed to high concentrations of a drug, when administered orally [19]. In the drug industry, using a series of toxicological tests, experimental compounds are evaluated before and during clinical trials, to ensure that only compounds that will prove to be safe for commercial use, will be approved. In this context, liver damage is closely related to the consumption of a large number of drugs, preclinical toxicological studies having a particularly important role in detecting molecules with high toxicological potential. At the same time, this is the most common reason for disposing of drugs already approved for therapeutic use [11]. Thus, over 900 drugs were considered responsible for
the production of liver injury, which is why many of them were withdrawn from use [12], thus causing significant financial losses. In addition to the chemical substances used as medicines, it is worth noting that other compounds, including ethanol, herbal remedies and food supplements can also induce liver damage, sometimes serious [17]. In the case of isoniazid, studied in this paper, after its biotransformation in the liver, the production of nitrogen-centred free radicals takes place, which generates reactive oxygen species that stimulates lipid peroxidation, ultimately causing cell death and liver necrosis [1, 13].

On the other hand, improving the pharmacotoxicological profile of isoniazid by introducing chemical modifications into its main structure in order to increase the biological response to Mycobacterium tuberculosis, to reduce liver toxicity or to avoid the resistance phenomena, continues to be an increasingly intriguing scientific challenge [4, 8]. In addition, the present work is outlined as a continuation of previous research [5], where the new derivatives obtained by synthesis (HIN-a, HIN-b and HIN-c) (Figure 1) demonstrated remarkable antimicrobial activity against M. tuberculosis strains and significantly reduced toxicity.

The present study was designed to evaluate the in vitro antimicrobial properties on different bacterial and fungal strains. In addition, the pharmacotoxicological study was initiated in order to highlight also the effect of per os administration of the mentioned compounds, on the biochemical parameters. Finally, a statistical analysis of these parameters completed this work, in order to understand the correlation of liver markers in laboratory animals treated with isoniazid and its derivatives.

**Materials and Methods**

*Evaluation of the antimicrobial potential of new isoniazid derivatives*

Isoniazid (HIN) and its new derivatives (HIN-a, HIN-b, HIN-c) were tested for evaluation of antimicrobial activity against the bacterial and pathogenic fungal strains: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* ATCC 90028, ATCC 14053. The culture media used were Mueller-Hinton agar, for the study of antibacterial activity and Sabouraud agar, for the study of the antifungal activity. The culture media were divided into sterile Petri dishes in a volume of 25 mL/plate. Isoniazid and its new derivatives were dissolved in DMSO, at a concentration of 25 µg/mL. The antibacterial and antifungal activity of the new compounds was assessed in vitro, by using the agar disk-diffusion method, according to the CLSI specifications [20]. The bacterial and fungal suspensions were uniformly inoculated on the surface of the culture media distributed in Petri dishes. Sterile cylinders, made of stainless steel, with a height of 10 mm and an inside diameter of 5 mm, were deposited on the surface of the sown plates [6, 9]. The samples to be analysed, obtained by dissolving the substances in DMSO at a concentration of 25 µg/mL, were deposited inside the sterile cylinders in volume of 100 µL, the final tested concentration being of 2.5 µg. Commercial discs containing Ciprofloxacin (5 µg/disc), Fluconazole (25 µg/disc) and Voriconazole (1 µg/disc) were used as positive controls. DMSO was used as a negative control. The plates were incubated for 24 hours at 37°C for evaluation of antibacterial activity and at 24°C for 48 hours for evaluation of antifungal activity. After incubation, the diameters of the inhibition zones of the microbial growth were measured and compared in mm, including the diameter of the disc.

*Evaluation of biochemical parameters*

The evaluation of liver markers was carried out on Swiss white male mice, in accordance with the current guidelines on the ethics of laboratory animal testing [3] and with the approval of the UMF Research Ethics Commission “Grigore T. Popa” Iași, Romania. The animals were divided into 6 groups (7 animals per group) - groups 1 to 4 received suspensions of HIN
and its derivatives (HIN-a, HIN-b and HIN-c); group 5 received the vehicle sodium carboxymethylcellulose (1% CMC-Na solution) and group 6 has been preserved as an untreated control. On the basis of the results obtained in the acute toxicity test [5], the evaluation of biochemical parameters was monitored after administering the new compounds at doses of 1/10 from LD50, by oral gavage. Thus, the substances were dispersed in 1% CMC-Na solution and administered in a single dose, for 30 days. Serum aminotransferases (GPT and GOT), alkaline phosphatase, total cholesterol and serum albumin were determined on the ABX Pentra 400 automated biochemical analyser, Horiba manufacturing company. Substrate concentrations and enzyme activities are determined on serum, by spectrophotometric measurements after colour reactions or reactions based on UV detection. The kits are from Diamedix and the reagents are in boxes which ensure their increased stability and extended linearity of results.

Statistical methods for evaluating biochemical parameters

In calculating the significant difference between two or more groups, depending on the distribution of the series of values, at the significance threshold of 95%, for the quantitative variables was applied the Student test - parametric test comparing the average values recorded in 2 groups with normal distributions and test F (ANOVA) used when 2 or more average values from groups with normal distributions are compared. Multiple linear regression aims to highlight the relationship between a dependent variable (explained, endogenous, resultant) and a series of independent variables (explanatory, factorial, exogenous, predictive).

Results and Discussion

Evaluation of the antimicrobial potential of new isoniazid derivatives

The antibacterial activity of isoniazid and its derivatives against Gram-positive species (Staphylococcus aureus ATCC 25923) is illustrated in Figure 2.

Figure 2. Antibacterial activity against S. aureus ATCC 25923

Figure 2 shows an antibacterial activity superior to isoniazid for HIN-a and HIN-b derivatives. For the HIN-c derivative, its activity is comparable to that of isoniazid. Analysing the influence of the structural modifications, carried out on the isoniazid nucleus, on the antimicrobial activity, it can be observed that the introduction of aromatic benzaldehydes, on this structure, through an azomethine bond, had a favourable influence especially for the HIN-a and HIN-b derivatives. Condensation of the isoniazid with the two aromatic benzaldehydes increased the antimicrobial activity, by recording the diameters of the inhibition zones larger than the parent compound. Thus, they increased from 13 to 15 mm, while for HIN-c there was a slight decrease up to 12 mm.

The antibacterial activity of the new compounds against Gram negative species (Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853) is illustrated in Figure 3.

Figure 3. Antibacterial activity against E. coli ATCC 25922 (a) and P. aeruginosa ATCC 27853 (b) 1: HIN; 2: HIN-a; 3: HIN-b; 4: HIN-c; 5: DMSO

In these cases, the results highlight the absence of antibacterial activity, both for isoniazid and for its derivatives, the different structural modifications having no influence on the activity against the tested Gram negative bacteria.

Antifungal activity (Candida albicans ATCC 90028 (c), Candida albicans ATCC 14053 (d)) of the tested substances is shown in Figure 4.

Figure 4. Antifungal activity of the new compounds 1: HIN; 2: HIN-a; 3: HIN-b; 4: HIN-c; 5: DMSO

Antifungal activity evaluation of isoniazid derivatives, evaluated as 25 µg/mL solutions, revealed that all tested compounds exhibit inhibitory activity at this concentration in the case of Candida albicans ATCC.
In this case, the inhibition diameter values between 12 and 15 mm were registered, while for *Candida albicans* ATCC 14053 the values were between 13 and 16 mm.

The values of the registered diameters of the inhibition zones are shown in Table I.

<table>
<thead>
<tr>
<th>Sample</th>
<th>S. aureus ATCC 25923</th>
<th>E. coli ATCC 25922</th>
<th>P. aeruginosa ATCC 27853</th>
<th>C. albicans ATCC 90028</th>
<th>C. albicans ATCC 14053</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIN</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>HIN-a</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>HIN-b</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>HIN-c</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ciprofloxacin 5 µg/disk</td>
<td>26</td>
<td>30</td>
<td>25</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Fluconazole 25 µg/disk</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>28</td>
<td>29</td>
</tr>
<tr>
<td>Voriconazole 1 µg/disk</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>25</td>
<td>24</td>
</tr>
</tbody>
</table>

NT = not tested

Regarding the antimicrobial action, chemical modifications made to the functional amino group of isoniazid, changed the biological profile of the obtained HIN derivatives. The obtained results show, for isoniazid and its derivatives an extended antimicrobial spectrum, unlimited for the *M. tuberculosis* strains (as shown in previous papers [5, 6]), including Gram positive and fungal species. Isoniazid and its synthesized derivatives exhibited no action against the Gram negative bacteria tested. The results also demonstrate that different structural modulations of isoniazid can favourably influence the antimicrobial activity.

**Evaluation of the biochemical parameters**

In the case of substance administration in a suspension of 1% CMC-Na (0.1 mL/kg body weight), after analysing the obtained results, the highest average values of GPT were found in the group of animals that received isoniazid (106.20 U/L – group 1), significantly higher values compared to those recorded in the other studied groups, which received the corresponding derivatives: HIN-a (91.09 U/L – group 2), HIN-b (86.75 U/L – group 3) and HIN-c (84.87 U/L – group 4). The lowest mean GPT values were found in the untreated group (23.70 U/L – group 6) and the 1% CMC-Na group (0.1 mL/kg body weight) (27.01 U/L – group 5) (Figure 5).

The mean value close to the group mean value suggests that the GPT value series was homogeneous, that is to say significance tests can be applied for the continuous variables. By comparing the GPT values of all the studied groups (Figure 5), one may notice a slight decrease in these values, in the case of the groups that received HIN derivatives, as compared to the group that received HIN.

**Figure 5.**

GPT (U/L) mean values after the administration of the tested compounds in 1% CMC-Na

Regarding the GOT values (Figure 6), in the case of the administration of the suspended substances of 1% CMC-Na (0.1 mL/kg body weight), after analysing the obtained results the highest average values were found in the group of animals which received isoniazid (166.58 U/L – group 1), higher values compared to those recorded in the other studied groups, which received the corresponding derivatives: HIN-a (154.89 U/L – group 2), HIN-b (152.18 U/L – group 3) and HIN-c (151.21 U/L – group 4). The lowest mean GOT values were found in the untreated control group (74.76 U/L – group 6) and the 1% CMC-Na group (0.1 mL/kg body weight) (73.87 U/L – group 5).
The mean value close to the group mean value suggests that the GOT value series was homogeneous. As in the case of the GPT value determination, in the case of the GOT enzyme, too, after comparing all the values, one may notice a slight decrease in the values corresponding to the HIN derivatives.

The highest mean values of alkaline phosphatase were found in the HIN group (189.74 U/L – group 1), significantly higher values than those recorded in the 1% CMC-Na groups (96.69 U/L; p = 0.001) and the untreated control group (97.21 U/L; p = 0.001), where the lowest mean values of alkaline phosphatase were recorded (Figure 7).

The highest mean values of total serum cholesterol were found in the HIN group (57.64 mg/dL – group 1), higher than those recorded in the 1% CMC-Na groups (51.24 mg/dL; p = 0.001) and the untreated control group (51.66 mg/dL; p = 0.001), where the lowest average cholesterol values were recorded (Figure 8). For the groups that received the other isoniazid derivatives, the total serum cholesterol was between 54.07 and 54.84 mg/dL.

The mean value close to the group mean value suggests that the total serum cholesterol value range was homogeneous, so significance tests can be applied for continuous variables. After comparing the cholesterol values of all studied groups (Figure 8), a significant decrease may be noticed in the groups that received HIN derivatives substances as compared to the groups that received HIN in an oral suspension.
The highest mean serum albumin values were found in the 1% CMC-Na group (4.61 g/dL – group 5), but also in the group of animals receiving HIN (4.18 g/dL – group 1). The lowest serum albumin values were recorded in the group of animals receiving HIN-a (3.23 g/dL – group 2), values close to those recorded in the animals from the untreated group (3.15 g/dL – group 6) (Figure 9).

**Correlation of liver markers in laboratory animals treated with isoniazid and its derivatives**

In animals treated with HIN and its derivatives, GPT registered: direct, high intensity correlations with GOT ($r = 0.903; p = 0.001$), alkaline phosphatase ($r = 0.893; p = 0.001$) and cholesterol ($r = 0.812; p = 0.001$) and an indirect correlation, moderate in intensity, with albumin ($r = -0.660; p = 0.001$) (Figure 10).

In animals treated with HIN and its derivatives, GOT recorded: direct, high intensity correlations with alkaline phosphatase ($r = 0.913; p = 0.001$) and cholesterol ($r = 0.758; p = 0.001$) and indirect correlation, moderate in intensity, with albumin ($r = -0.668; p = 0.001$) (Figure 11).

In animals treated with HIN and its derivatives, alkaline phosphatase was significantly correlated with cholesterol ($r = 0.708; p = 0.001$) and by indirect correlation with albumin ($r = -0.695; p = 0.001$) (Figure 12).
In animals treated with HIN and its derivatives, the correlation between cholesterol and albumin was indirect, moderate in intensity, statistically significant \( (r = -0.439; p = 0.015) \) (Figure 13).

Aminotransferases are the most specific index of hepatic impairment, rather reflecting the integrity of the hepatocyte or bile epithelial cell and less its function. The activity of liver enzymes can increase up to ten times in different pathologic conditions. Although GPT and GOT are found in high concentrations in hepatocytes, only GPT describes the normal functioning of the liver, being predominantly localized at this level, while GOT is present in a variety of tissues and organs: liver, myocardium, skeletal muscle, brain, kidneys and pancreas [10]. Thus, GPT is the most commonly used hepatic cytolysis indicator, able to detect even minor liver lesions, as the enzyme is
released from hepatocytes as a result of increased cell membrane permeability or cell necrosis. On the other hand, mitochondrial GOT release from hepatocytes indicates severe hepatocyte impairment, whereas cytoplasmic GOT and GPT releases do not have such significance. When the membrane of a liver cell is damaged, a variety of cytosolic enzymes are released into the blood, so their estimation in serum is a useful quantitative marker of the size and type of hepatocellular impairment.

The degree of hepatic impairment, in the present study, was evidenced by the significant increase of approximately four times the activity of the liver enzymes.

Most reports of hepatotoxicity induced by antiTB medication have shown an increase in alanine (GPT) or aspartate transaminase (GOT) three times over the normal limit, with symptoms such as: abdominal pain, nausea, vomiting, unexplained fatigue or jaundice, symptoms which can be attributed to liver injury [2]. However, asymptomatic increases in aminotransferases are frequent and do not always justify the withdrawal of drugs. However, especially in the cases with increased transaminase activity, more than three times the upper limit of normal (in the presence of hepatitis and/or jaundice symptoms) or five times the upper limit (in the absence of symptoms), treatment should be discontinued [14].

The fulminant hepatic impairment, induced by antiTB drugs, appears to have a degradable result, compared with that of acute viral hepatitis, with a mortality rate between 0.042 - 0.07 per 100 persons [17]. In this case, as markers for the onset of liver injury, serum concentrations of GPT and GOT increased significantly in animals receiving isoniazid, indicating hepatotoxicity. Hepatotoxicity induced by isoniazid is explained by the oxidative destruction of hepatocytes, which resulted in the release of enzymes into the vascular compartments. Elimination of aminotransferases from isoniazid intoxicated cells was high, while the impact was diminished by the administration of the appropriate derivatives (HIN-a, HIN-b and HIN-c), which suggests the ameliorating effect of blocking the free amino group of isoniazid on GOT and GPT activity, in mice.

As can be seen from the presented results, the chronic administration of isoniazid and its derivatives, lowers the concentrations of serum albumin and increases the concentrations of total cholesterol.

In this regard, the liver is a key regulator of plasma cholesterol levels by intervening in the elimination of cholesterol from lipoproteins in the bloodstream. Under these conditions, the proper functioning of this organ is absolutely essential to overcome hypercholesterolemia and related co-morbidities [10].

The presence of relatively high intracellular (free) cholesterol levels can induce oxidative stress, which directly leads to high toxicity and cell death. In our case, the use of isoniazid led to an increase in the total cholesterol level, while the use of the three derivatives of isoniazid has made a significant impact in reducing these values.

In addition, when there is an acute inflammatory process or in some chronic inflammatory conditions, serum albumin levels are reduced, its loss from vascular to extravascular space being due to increased vascular permeability [7]. In our case also, liver damage is indicated, in addition, by the reduction of albuminaemia values, in the groups of animals that received the tested substances, while no significant differences were recorded between the parent compound and the three derivatives.

Conclusions

Hepatotoxicity induced by tuberculostatic therapy is an important adverse effect that interferes with the effective administration of the medication. Liver cells are involved in a variety of metabolic activities and their transport function is disrupted in particular, after liver damage, as a result of the deterioration of the plasma membrane, ultimately causing the loss of functional integrity of the cell membranes of the liver. This study shows that the derivatives of isoniazid (the new isonicotinoylhydrazones) have an antimicrobial spectrum that is not limited to the M. tuberculosis species, also including Gram positive and fungal species. In addition, the three derivatives have shown improvement of liver markers, after oral administration. In conclusion, the new derivatives of isoniazid have potential application as antimicrobial agents in the treatment of tuberculosis, where both a favourable antimicrobial action and a lower incidence of adverse reactions are required, especially in the context of the high toxicity of current tuberculostatic medication.

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Conflict of interest

The authors declare no conflict of interest.

References


