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ABSTRACT OF Ph. D. THESIS

SYNTHESIS OF NEW ISONIAZID DERIVATIVES WITH POTENTIAL IMPLICATIONS IN THE INDIVIDUALIZED THERAPY OF TUBERCULOSIS

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Tuberculosis is an infectious disease caused by *Mycobacterium tuberculosis*, an aerobic pathogenic bacterium. The disease progression is dependent on the host immune response that either can immediately eliminate the aerobic pathogen and/or induce a dormant state or fail, resulting in the development of an active disease. In 1993 the World Health Organization declared tuberculosis a global emergency.[3]

At present, the treatment of tuberculosis is based on two groups of drugs: the first-line drugs with a greater efficacy and acceptable toxicity (isoniazid, rifampin, streptomycin, ethambutol, and pyrazinamide) and the second-line ones characterized by a lower efficacy or a greater toxicity (i.e. kanamycin, ethionamide, thiacetazone, rifabutin).

Isoniazid (INH), the hydrazine of isonicotinic acid, is one of the most active drugs used against active metabolizing and multiplying *Mycobacterium tuberculosis* bacteria,[5] but is less active against the dormant ones.[7] Isoniazid is a prodrug that crosses the *mycobacterial* cell wall by passive diffusion. Once inside the cells it is activated by KatG (mycobacterial catalase-oxidase).[9] The active forms of isoniazid exhibit various effects on the synthesis of cell wall mycolic acids, nucleic acids replication and transcription, and bacterial respiratory metabolism. The mechanism of action of this prodrug is very complex and still incompletely elucidated.[13]

The activity of isoniazid and its toxicity is influenced by its metabolism in the human body. The main degradation pathway of this drug is acetylation by *N*-acetyltransferase (NAT), the resulted metabolite, N^1-acetyl-N^2-isonicotinylhydrazine (acetylisoniazid), being metabolized further in mono-acetylhydrazine and the non-toxic di-acetylhydrazine.[23] The metabolism of isoniazid is influenced by genetic polymorphisms of genes that codify NATs. The presence of different polymorphisms determines different degrees of activity for NATs and an individual acetylation profile. The human population can be therefore divided into slow, intermediate and fast acetylators.[15] The fast acetylation of isoniazid decreases the concentration of the active drug and affects the success of therapy, especially in patients with weekly single doses. At the opposite side, in slow acetylators the risk of side effects due to the toxicity of high concentrations of isoniazid metabolites could occur and determine hepatotoxic effects.[17]

There are some reports stating that the modification of isoniazid molecule by the addition of a functional group to the hydrazine entity could block the acetylation of the drug by NATs, therefore influencing its activity and toxicity.[20]

The **aim** of this thesis was the synthesis and characterization of new isoniazid derivatives in order to improve the tuberculosis therapy for slow acetylation phenotype subjects.

Objectives:

1. The assessment of genotype / phenotype / pharmacokinetics correlation regarding the genes involved in the metabolism of anti-tuberculosis drugs.
2. Synthesis and evaluation of biologic activities of new isoniazid derivatives with potential anti-mycobacterial activity.

The thesis is divided in two parts: the theoretical part, which contains data from literature regarding the research topics approached, and the original part, which presents the results of research performed in the PhD theme.

The theoretical part consists of three chapters. In the first chapter are presented data on tuberculosis, the etiologic agent, *Mycobacterium tuberculosis*, and its particularities,

tuberculosis transmission and pathogenesis, and current treatment schemes, used drugs and resistance. The second chapter is dedicated to isoniazid, the drug most commonly used in tuberculosis treatment, its mechanism of action and resistance, isoniazid metabolism and its implications in the development of adverse effects that influence anti-tuberculosis treatment and determinant factors. Also in this chapter are described isoniazid derivatives reported in literature, methods of obtaining and their biological activity. The third chapter is devoted to drug metabolism, being briefly described the main reactions of the two metabolism phases of xenobiotics, factors that influence drug metabolism and the role of metabolism in drug-induced toxicity. There are also provided methods used for drug metabolism study and toxicity.

In the second part of the work are presented the original results obtained in the thesis. This part is composed of seven chapters, the last chapter including used materials and methods.

Chapter IV. NAT Polymorphisms and Acetylator Status of Patients with Tuberculosis

The first part of this chapter presents the analysis of genotype / phenotype / pharmacokinetics correlations regarding the genes involved in the metabolism of anti-tubercular drugs and the determination of NAT genes polymorphisms that are responsible for the adverse effects induced by isoniazid in the population of Romania. The second part of this chapter describes the selection of cell lines that can be used to assess the biotransformation and elimination of new synthesized substances in patients with an acetylator profile responsible for the isoniazid adverse effects.

NAT genetic polymorphisms have a significant effect on the half-life of isoniazid, acetylation rate influencing the concentration of free drug in plasma and its half-life in circulation, but also the early bactericidal effect of isoniazid against *M. tuberculosis*. Also, isoniazid can cause adverse effects such as liver disorders and peripheral neurotoxicity. Thus, to achieve the first objective, we assessed NAT gene polymorphisms responsible for isoniazid-induced side effects in 37 patients diagnosed with pulmonary tuberculosis, with or without side effects.

The evaluation of *NAT1* gene polymorphisms revealed the presence of two polymorphisms located in the promoter region, $C^{-344}\rightarrow T$ and $C^{-40}\rightarrow T$, non-synonymous polymorphisms $G^{445}\rightarrow A$ ($Val^{149}\rightarrow Ile$), $G^{560}\rightarrow A$ ($Arg^{187}\rightarrow Gln$) and $G^{640}\rightarrow T$ ($Ser^{214}\rightarrow Ala$) and synonymous polymorphism $G^{459}\rightarrow A$ ($Thr^{153}\rightarrow Thr$). $C^{-344}\rightarrow T$, $C^{-40}\rightarrow T$, $G^{445}\rightarrow A$, $G^{640}\rightarrow T$ and $G^{459}\rightarrow A$ polymorphisms were associated with *NAT1*11* allele, and $G^{560}\rightarrow A$ polymorphism with *NAT1*14* allele. Thus, 16.22% of studied subjects presented a *NAT1*11/*4* heterozygous genotype and 2.7% - *NAT1*14/*4* heterozygous genotype, other patients (81.08%) being *NAT1*4* homozygous. In the case of two patients were detected mutations in the positions $C^{309}\rightarrow T$ (heterozygous) and $C^{383}\rightarrow T$ (homozygous) of *NAT1* gene.

In the case of *NAT2* gene, the analysis revealed the presence of $C^{282}\rightarrow T$ ($Ile^{94}\rightarrow Ile$) in 51.35% of patients, $T^{341}\rightarrow C$ ($Ile^{114}\rightarrow Thr$) and $C^{481}\rightarrow T$ ($Leu^{161}\rightarrow Leu$) in 72.97% of cases and of $G^{590}\rightarrow A$ ($Arg^{197}\rightarrow Gln$) polymorphism in 45.95%. Thus, 27.03% of the study group presented *NAT2*5* homozygous genotype and 10.81% - *NAT2*6* homozygous genotype, only 5.4% being homozygous for *NAT2*4*. The other patients had *NAT2*5/*4* (27.03%), *NAT2*6/*4* (10.81%) and *NAT2*5/*6* (18.92%) heterozygous genotypes.

According to the literature data, *NAT2*4* genotype, considered wild type, is associated with a rapid acetylation phenotype. The presence of *NAT2* polymorphisms (heterozygous/ homozygous) changes this phenotype to intermediate / slow.

Of the 37 subjects with tuberculosis introduced in the study, 24 had hepatotoxic reactions after taking anti-tubercular medication. Genetic analysis found gene changes compared to the *NAT2* reference employing 21 patients in slow acetylator genotype, while two patients had intermediate acetylator genotype and one presenting a rapid acetylator genotype. Regarding *NAT1* gene, only 18.92% of patients showed homozygous genotype s, being recorded also two cases of NAT1 gene mutations at positions 309 and 383, these polymorphisms not being correlated with adverse effects induced by the administered anti tuberculosis treatment.

Although most of the patients with adverse hepatotoxic effects presented a slow acetylator genotype, in some cases hepatotoxicity seems to be due to other factors. The analysis of genetic alterations in other genes involved in the metabolism of anti-tubercular drugs could reveal the possible causes of rate changes of isoniazid metabolism in the three patients with hepatotoxic effects, but who did not present a slow acetylator genotype. The hepatotoxicity induced by isoniazid may be enhanced also by associated therapies.[104] On the other hand, the phenotype is the result of interactions between environmental factors and genetic structure, thus it is not always consistent with the genotype.[523]

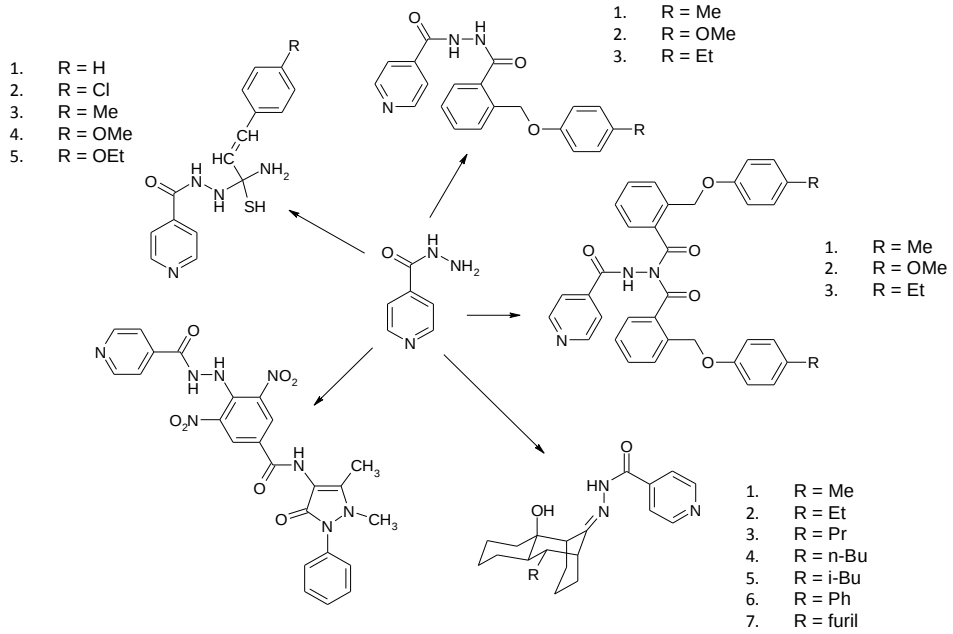
In order to select a study model for evaluation of the biotransformation and elimination of new synthesized substances for slow acetylator profiles responsible for isoniazid adverse effects, we determined the polymorphisms of the *NAT1* and *NAT2* genes in three cell lines derived from intestinal tissue, Caco-2, HCT-8 and HT-29, and a newly initiated cell culture, 1002_TACI.

The four analysed cell lines presented wild-type genotypes for *NAT1* gene. In the case of the *NAT2* gene, in HT-29 and 1002_TACI cell lines were detected two homozygous polymorphisms, $T^{341}\rightarrow C$ and $C^{481}\rightarrow T$, characteristic for *NAT2*5* alleles, while in the Caco-2 and HCT-8 cell lines were detected $C^{282}\rightarrow T$ and $G^{590}\rightarrow A$ homozygous polymorphisms, characteristic for *NAT2*6* alleles. The four analysed cell lines presented slow acetylator genotype, two of them containing *NAT2*5* allele, while the other two - *NAT2*6* allele. The same alleles were detected in Romanian patients with tuberculosis presenting hepatotoxic side effects from drugs administration. Since the analysed cell lines present two different allelic variants, *NAT2*5* and *NAT2*6*, only two cell lines were selected to study the potential metabolic pathways of new isoniazid derivatives. The initialized cell line, 1002_TACI, still not a stable line, shows a heterogeneous cell population with a high population doubling time. Also, Caco-2 cell line, although it is often used in metabolism studies, has a population doubling time of about 62 hours, in contrast to the HCT-8 and HT-29 cell lines, which are characterized by population doubling time of 18 hours, respectively, for 19 hours. Thus, it was considered that the use of HCT-8, *NAT2*6* homozygous, and HT-29, *NAT2*5* homozygous, cell lines reduces the number of variables in further experiments.

Chapter V. Synthesis of New Compounds with Potential Antituberculosis Activity and Their Structural Characterization

Most of isoniazid derivatives reported in the literature exhibit anti-mycobacterial activity, including against isoniazid resistant strains, after their hydrolysis and release of the

active principle in bacterial microenvironment.[557,558] Usually these compounds being evaluated only in terms of their anti-mycobacterial or antimicrobial activity, without assessment of their impact on eukaryotic cells, one of the objectives of this thesis is the synthesis of new compounds, isoniazid derivatives, with potential anti-mycobacterial activity, and the evaluation of their biologic activities. To achieve this objective 19 new isoniazid derivatives were synthesized (Scheme 35) and were evaluated in terms of antibacterial activity (*M. tuberculosis* and other non-tuberculosis strains), possible metabolic pathways, cytotoxicity (by evaluating the capacity to induce apoptosis), and the influence on the cell cycle.



Scheme 35. Newly synthesized isoniazid derivatives

N'-[1-amino-1-mercapto-3-(*p*-substituted-phenyl)-allyl]-hydrazides of isonicotinic acid (**INH-TCA**) were synthesized by a simple coupling reaction between isoniazid and α,β-unsaturated thioamides (obtained previously by Pappalardo method[560]). The chemical structure of the newly synthesized compounds was confirmed by NMR spectral analysis and by elemental analysis. The ^{1}H-NMR spectra of **INH-TCA 1-5** showed singlets at 10.06 – 10.10 ppm and 9.52 – 9.56 ppm, which represent the two hydrazide protons (NH-NH) that are deuterable. There are also singlets at δ = 9.23 – 9.30 ppm and at δ = 4.52 – 4.57 ppm that represents thiol group (SH) and amino group (NH2) protons.

Isoniazid derivatives of 2-(4-substituted-phenoxymethyl)-benzoic acids (**INH-AC**) were obtained by interaction between isoniazid and crude acid at room temperature in dichloromethane. The obtained products were separated and purified on chromatographic columns. The chemical structure of the newly synthesized compounds was confirmed by ^{1}H-, ^{13}C-NMR and IR and by elemental analysis. The ^{1}H-NMR spectra of compounds **INH-AC 1** – **INH-AC 3** showed two singlets at 10.11 – 10.96 ppm and 9.72 – 10.55 ppm, which represent the two hydrazide protons (NH-NH, deuterable), while the ^{1}H-NMR spectra of compounds **INH AC 4** – **INH-AC 6** showed only one singlet at 8.53 – 11.97 ppm which correspond to NH–N proton. This indicates that compounds **INH-AC 1** – **INH-AC 3** are N-

substituted hydrazides of 2-(4-substituted-phenoxymethyl)-benzoic acids, while compounds **INH AC 4** – **INH-AC 6** are *N,N*-disubstituted hydrazides of 2-(4-substituted-phenoxymethyl)-benzoic acids. Compound **INH-AC 6** was subjected to MS analysis and a signal of molecular ion [M+H] was observed at 614.1, corresponding to the presumed molecular weight and structure of this compound.

N-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-3,5-dinitro-4-[*N*-(pyridine-4-carbonyl)hydrazino]-benzamide (**INH-AP**) was obtained by a simple coupling reaction between isoniazid and 4-chloro-*N*-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-3,5-dinitro-benzamide. The chemical structure of this compound was confirmed by ^{1}H-, ^{13}C-NMR and IR and by elemental analysis

The isonicotinic acid (2-hydroxy-8-substituted-tricyclo[7.3.1.0$^{2.7}$]tridec-13-ylidene)-hydrazides were obtained by condensation of 2-hydroxy-8-substituted-tricyclo[7.3.1.0$^{2.7}$]tridecan-13-ones (obtained by Michael reaction[591]) with isoniazid in methanol. The chemical structure of the newly isoniazid derivatives was confirmed by NMR and IR spectral analysis. The ^{1}H-NMR spectra of all compounds showed singlets at 10.47 – 10.25 ppm, corresponding to hydrazide proton NH, and at 4.09 – 4.45 ppm, corresponding to hydroxyl group OH.

Chapter VI. Antibacterial Activity Study

The *in vitro* anti-mycobacterial activity of newly synthesized derivatives of isoniazid was tested against a clinical strain of *M. tuberculosis* according to the method reported by Franzblau et al.[574]

From *N'*-[1-amino-1-mercapto-3-(*p*-substituted-phenyl)-allyl]-hydrazides of isonicotinic acid (**INH-TCA**) the best anti-mycobacterial activity had been proved by compound **INH-TCA 1** (the minimum inhibitory concentration (MIC) is 0.391 μg/mL), followed by compounds **INH-TCA 3** and **INH-TCA 5** (CMI = 0.0781 μg/mL). In this set of compounds, the worst activity had been shown by **INH-TCA 2** (MIC = 6.25 mg / mL). The mycobacterial cell wall fluid gradient has an opposite orientation compared to other gram-negative bacteria, the inner layer being characterized by a low fluidity due to the packaging and the structure of mycolic acids, and the outer layer - by higher fluidity determined by the presence of glycolipids,[40] which provides low permeability to the mycobacterial cell wall. Thus, since the chlorine atom increases the molecule lipophilicity, inducing a higher partition in the lipophilic phase of the cell membrane, the low anti-mycobacterial activity of **INH-TCA 2** can be partly explained by its lipophilicity.

Regarding isoniazid derivatives of 2-(4-substituted-phenoxymethyl)-benzoic acids (**INH-AC**), none of the new synthesized compounds proved to have anti-tubercular activity, with the exception of the compound **INH-AC 6** that inhibited the growth of *M. tuberculosis* at a concentration of 6.25 μg/mL. *N*-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-3,5-dinitro-4-[*N*-(pyridine-4-carbonyl)hydrazino]-benzamide (**INH-AP**) also proved a moderate anti-mycobacterial activity (MIC = 6,25 μg/mL), which can be explained by the large radical in the *para* position of the benzene ring and the presence of two nitro groups.

The isonicotinic acid (2-hydroxy-8-substituted-tricyclo[7.3.1.0$^{2.7}$]tridec-13-ylidene)-hydrazides (**INH-TCT**) proved the best anti-mycobacterial activity from newly synthesized isoniazid derivatives. The best anti-mycobacterial activity was proved by compounds **INH-TCT 2**, **INH-TCT 3**, **INH-TCT 4** and **INH-TCT 5** (MIC = 0.195 μg/mL), followed by

compound **INH-TCT 7** (MIC = 0.391 μg/mL) and, the weakest ones, compounds **INH-TCT 1** and **INH-TCT 6** (MIC = 0.781 μg/mL). The majority of isoniazid derivatives are activated before reaching the intracellular environment of *M. tuberculosis* and it is presumed that isoniazid derivatives activation involve the formation of electrophilic intermediate species (hydrazyl radical or ion) that are transformed in acyl radicals that act as active isoniazid forms.[557,584] The hydrazones double bond increase the anti-tuberculosis due to its easy hydrolysis and release of isoniazid in the cell, which may explain the higher anti-mycobacterial activity of **INH-TCT** in comparison with the other isoniazid derivatives evaluated in our study.

Thus, the synthesized novel compounds show a good anti-Mycobacterium tuberculosis, as compared to the other derivatives of isoniazid reported in the literature, the majority of inhibiting the development of mycobacteria in concentrations of less than 6.25 μg/mL, value postulated by the Global Program for the Discovery of New Anti-Tuberculosis Drugs as the upper threshold for the evaluation of new anti-*M. tuberculosis* agents. [256]

In order to establish if our new synthesized compounds have antibacterial activity against other microbial species and to determine the spectrum of their activity, the novel derivatives were tested on other, non-tuberculosis, Gram-positive and Gram-negative, bacterial strains isolated from clinical specimens.

From thioamide derivatives, the most active compound proved to be **INH-TCA 2**, substituted with a chloride atom in *para*-position (active against all tested bacterial strains), followed by **INH-TCA 1** and **INH-TCA 4**. All new synthesized derivatives from this group proved to be more active on the tested strains than isoniazid and the thioamides they derived from, except compound **INH-TCA 5**.

Compound **INH-AC 6** proved to be the most efficient from all 2-(4-substituted-phenoxymethyl)-benzoic acids derivatives, inhibiting the growth of the majority of tested strains. The antibacterial activity decreased in the direction **INH-AC 6** > **INH-AC 3** > **INH-AC 4** > **INH-AC 5 > INH-AC 2** > **INH-AC 1**. The *N*,*N*-disubstituted hydrazides of 2-(4-substituted-phenoxymethyl)-benzoic acids **INH-AC 4** – **INH-AC 6** exhibited a better antibacterial activity than their corresponding *N*-substituted hydrazides **INH-AC 1** – **INH-AC 3**. *N*-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-3,5-dinitro-4-[*N*-(pyridine-4-carbonyl)hydrazino]-benzamide (**INH-AP**) did not proved any antibacterial activity on tested strains.

From (2-hydroxy-8-substituted-tricyclo[7.3.1.$0^{2.7}$]tridec-13-anoic derivatives, the best antibacterial activity was proved by compound **INH-TCT 6**, followed by compounds **INH-TCT 3**, **INH-TCT 4** and **INH-TCT 5**. The less active compounds were **INH-TCT 1** and **INH-TCT 7**.

In conclusion, compound **INH-AC 6** showed the best antibacterial activity from novel derivatives of isoniazid synthesized in our study.

Chapter VII. The Study of Metabolic Mechanisms of New Isoniazid Derivatives

The analysis of the influence of a compound on the gene expressions of enzymes involved in drug metabolism allows the evaluation of its primary metabolic pathways. Thus, in order to identify how newly synthesized substance may be degraded and eliminated from the body, we evaluated the expression of some genes involved in the metabolism of pharmacological substances in two study models selected, HCT-8 and HT-29.

NAT1 and *NAT2* genes expression was influenced differently by the novel compounds in the cell lines HT-29 and HCT-8. **INH-TCA 1**, **INH-TCA 2** and **INH-TCA 3** have increased the gene expression levels of *NAT1* and *NAT2* in both cell lines, while the **INH-TCA 4** increased NATs gene expression in HT-29 cells, and **INH-TCA 5** increased the *NAT2* gene expression level in HCT-8 cells. *NAT1* gene expression was increased by the majority of 2-phenoxymethylbenzoic derivatives in HCT-8 cell line (with the exception of **INH-AC 1**) and by compounds **INH-AC 2**, **INH-AC 3** and **INH-AC 6** in HT-29 cell line. *NAT2* expression was enhanced by all compounds, except **INH-AC 2** in HCT-8 cells and **INH-AC 1** in both cell lines used. Generally, the *NAT2* gene expression was more influenced by tested compounds in HCT-8 cell line, which is *NAT2*6* homozygote, than in HT-29 cell line, which is *NAT2*5* homozygote, with the exception of compound **INH-AC 6**. Regarding antipyrin derivative (**INH-AP**), it significantly increased NAT2 expression level in HCT-8 line against HT-29. Derivatives **INH-TCT** increased *NAT1* and *NAT2* gene expression level in HT-29 cell line, *NAT2* being minimally affected by the compound **INH-TCT 4**. In HCT-8 cell line, *NAT1* gene expression was increased by **INH-TCT 2**, **INH-TCT 3**, **INH-TCT 4** and **INH-TCT 6**, while *NAT2* gene expression was increased by **INH-TCT 4**, **INH-TCT 5** and **INH-TCT 6**.

Thus **INH-TCA 1**, **INH-TCA 2**, **INH-TCA 3**, **INH-AC 3**, **INH-AC 4**, **INH-AC 5**, **INH-AC 6**, **INH-AP**, **INH-TCT 5** and **INH-TCT 6** could be used to improve isoniazid metabolism in slow acetylators, especially derivatives **INH-TCA 3**, **INH-AC 5** and **INH-AP** that stronger induced *NAT2* gene expression. Among the compounds that showed good anti-mycobacterial activity, compounds **INH-TCT 4**, **INH-TCT 5** and **INH-TCA 1** induced a slight increase of *NAT1* and *NAT2* gene expression, suggesting a better rate of metabolization and thus, a lower risk for side effects that could be induced by the accumulation of the compound in high concentrations within the host. Similar behaviour was observed for compounds **INH-TCA 3** and **INH-TCT 6**, which presented also good inhibitory activity against *M. tuberculosis*. These results suggest that these compounds could be better tolerated than isoniazid.

In order to determine other possible pathways of metabolism for the new synthesized compounds we evaluated the expression of other genes involved in drug metabolism.

Cytochrome P450 enzymes are responsible for the metabolism of two-thirds of drugs,[608] playing an important role in regulating both the intensity and duration of drug action, and in detoxification, or their activation. In human body, isoniazid inhibits several CYP450 enzymes (1A, 2A, 2C, 2E and 3A), increasing plasmatic concentrations of other potentially hepatotoxic drugs. Isoniazid reversibly inhibit the activity of CYP2C19 and CYP3A4 at clinically relevant concentrations (isoniazid peak serum concentration placing around 30-50 μM) and mechanical inactivates CYP1A2, CYP2A6, CYP2C19 and CYP3A4 in human microsomes (metabolites formed by these enzymes bind directly and irreversibly inhibits the enzyme, blocking the catalytic site).[624] Thus, isoniazid interferes with the metabolism of many drugs such as phenytoin, warfarin, carbamazepine, primidone, tolbutamide, benzodiazepines, etc., inhibiting the activity of CYP1A1,[180] CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2E1 and CYP3A4 isoenzymes. [630] The analysis of isoniazid effect on CYP isoforms expression in HT-29 and HCT-8 cell lines showed a decrease of *CYP1A1* and *CYP2C19* gene expression in both used cell lines and an increase of CYP3A4 expression in the HCT-8 cell line.

Regarding the action of the compounds evaluated in our study, *CYP1A1* gene expression was influenced more than *CYP2C19* or *CYP3A4* gene expression. The expression of *CYP1A1* isoform was increased by the majority of new synthesized compounds in both cell lines, *CYP1A1* being involved in the metabolism of aromatic molecules, excepting **INH-TCA 5**, which determined a decrease of *CYP1A1* gene expression in both cell lines, and **INH -TCA 1**, which induced a decrease of this gene expression only in HT-29 cells.

CYP2C19 isoform is also implicated in the metabolism of aromatic compounds. This isoform was influenced differently in the two cell lines. **INH-TCA** derivatives have increased *CYP2C19*gene expression levels in both analysed cell lines, except compound **INH-TCA 5**, which determined a slight decrease of *CYP2C19* expression in HT-29 cells. However, the expression of this gene was more induced in the HCT-8 cells, rather in HT-29 cells. **INH-AC 1** increased *CYP2C19* gene expression level in HT-29 cell line, and **INH-AP** and **INH-TCT 1** decreased this gene expression in HCT-8 cell line. The other isoniazid derivatives increased *CYP2C19* gene expression in the HCT-8 cells, and decreased it in HT-29 cells. Different profiles of CYP2C19 expression in the two cell lines used could account for the existence of a polymorphic locus.

Although untreated HCT-8 cells do not express *CYP3A4* isoform, compounds **INH-TCA 2**, **INH-TCA 4**, **INH-TCA 5**, **INH-AC 6**, **INH-TCT 1**, **INH-TCT 6**, **INH-TCT 7**, including isoniazid, have caused an increase of the expression level of this gene. In HT-29 cells, which express this isoform, *CYP3A4* expression levels were increased by **INH-TCA 3**, **INH-TCA 4**, **INH-TCA 5**, **INH-AC 1** and **INH-AC 6**, and decreased by **INH-AC 2** and **INH-AC 5**, **INH-TCT** derivatives not influencing this gene expression level.

The increase of CYP isoforms gene expression by new compounds indicates a possible involvement of this isoforms in their metabolism. Cytochrome P450 enzymes are responsible for most reactions that occur in phase I of drug metabolism and aim the introduction of new functional groups in the drug structure to facilitate its elimination.[633] Thus, one of the possible ways of new isoniazid derivatives metabolism involves oxidation or reduction by cytochrome P450 enzymes.

Among the enzymes involved in phase II of drug metabolism, the expression of glutathione S-transferase 3 (GSTA3) was analysed, glutathione S-transferases (GST) being responsible for drug conjugation with glutathione, one of the major pathways of drug metabolism, resulting metabolite being characterized by an increased hydrophility and fast elimination from organism.[371] **INH-TCA** induced *GSTA3* gene expression in both cell lines, except **INH-TCA 4** and **INH-TCA 5** in HT-29 cell line. Of other studied compounds, *GSTA3* expression was increased by **INH-AC 5** and **INH-TCT 3** in HT-29 and by **INH-AC 6**, **INH-TCT 4** and **INH-TCT 6** in HCT-8 cells. The effect of thioamide derivatives on the expression of GST can be explained by the fact that these enzymes catalyse isomerization reactions,[643] **INH-TCA** derivatives being obtained and analysed as E / Z mixtures. GST enzymes are involved also in conjugation with glutathione, facilitating drug elimination from the body, including cinamic acid derivatives.[651] On the other hand, GST enzymes are involved in other non-enzymatic processes, such as cell proliferation and apoptosis.[652]

Chapter VIII. The Study of Cytotoxic Activity of Newly Synthesized Compounds

The cytotoxicity of new isoniazid derivatives was analysed by evaluating the capacity to induce apoptosis, the discrimination between intact and apoptotic cells being performed by flow cytometry using FITC-labelled annexin-V and propidium iodide.

Among **INH-TCA** derivatives, the most toxic compound proved to be **INH-TCA 2**, inducing necrosis in 23,7% of cells and apoptosis in 20,93% at the tested concentration of 25 μg/mL, followed by **INH-TCA 5** (necrosis – 28,3%; apoptosis – 24,41%) and **INH-TCA 3** (necrosis – 28,5%; apoptosis – 17,16%), both at the tested concentration of 50 μg/mL. Less toxic from this group proved to be **INH-TCA 4**. The cytotoxicity of **INH-AC** was evaluated by flow cytometry at 24, 48 and 72 hours. The most toxic compound proved to be the **INH-AC 4**, followed by **INH-AC 6** and **INH-AC 5**. The treatment of HT-29 cells with **INH-AC 4** had no toxic effect after 24 hours, but after 48 and 72 hours it induced necrosis in 28.6% and, respectively, 54% of the eukaryotic cells, only 70.6% cells being viable after 48 hours and 43% after 72 hours. The cell treatment with 100 μg/mL **INH-AP** for 24 hours induced necrosis in 88.0% and apoptosis in 10,04% of treated cells. Among **INH-TCT** derivatives, **INH-TCT 6** is highly toxic, inducing apoptosis in 35% of the cells , followed by **INH-TCT 4** (apoptosis in 12.41% cells) and **INH-TCT 2** (necrosis in 13.6% cells).

The cytotoxicity of the newly synthesized compounds was also studied at molecular level by analyzing the gene expression levels of caspase 3, 7, 8 and 9, implicated in apoptosis initiation and propagation, and of Bax, Bcl-2 and Mcl-1, involved in apoptosis regulation. The activation of the effector caspase 3 can be induced by the extrinsic pathway via tumor necrosis factor (TNF) family receptors (e.g., Fas), FADD (Fas-activated death domain protein) and caspase-8, or by the intrinsic pathway via the mitochondrial release of cytochrome c and Apaf-1–mediated processing of caspase-9.

In our study the influence of isoniazid on the expression levels of caspases genes is minor which correlates with its low toxicity. On other side, the increased expression level of caspase 9 induced by **INH-TCA** could be associated with activation of intrinsic apoptotic pathway. **INH-TCA 2** toxicity can be explained by the increased caspase 3 and 7 gene expression, and also by caspase 9 gene expression level.

Among **INH-AC** compounds, **INH-AC 4** increased the expression level of caspase 9 that could be associated with activation of intrinsic apoptotic pathway. Although the phenotypic changes induced by the treatment with 50 μg/mL of compound **4** during 24 hours are minor, the increased expression of mRNA caspase 3 and 7 predicts the apoptotic phenomena occurring at 48 and 72 hours.

Compound **INH-AP** increased caspase 3 gene expression level after cell treatment with 50 μg/mL, a fact that may partly explain the cytotoxic effect of this compound observed by flow cytometry. However, cell treatment with 100 μg/ml **INH-AP** induced necrosis in 88% of cells, this process being mediated through signalling pathways other than those analysed.

INH-TCT derivatives increased caspase 3 and 9 gene expression, with the exception of **INH-TCT 3** that determined a decrease of caspase 3 expression levels. This can means that all compounds induced apoptosis through mitochondrial stress pathways. **INH-TCT 6**, the most toxic compound from this group of new isoniazid derivatives, increased the expression of activating caspase 8 and 9 and also of effector caspase 3 and 7. The increased expression level of caspase-8 induced by **INH-TCT 6** indicates a possibility of apoptosis activation via

extrinsic pathway. On the other hand, the low cytotoxicity of compound **INH-TCT 3** can be explained by low expression of effector caspase 3.

The mechanism of action of the Bax proteins involves their ability to form pores or channels in the mitochondrial membrane. Mitochondria are involved in the biochemical processes of apoptosis and are able to release cytochrome c during apoptosis. In the cytoplasm, cytochrome c, together with Apaf-1, forms apoptosome that activates caspase-9, the latter resulting in the onset of caspase cascade. In contrast to Bax, Bcl-2 protein acts on mitochondrial membranes by blocking the formation of pores. Mcl-1 blocks apoptosis by preventing mitochondrial dysfunction determined by Bax and Bak activation.

Regarding the influence of isoniazid on regulatory proteins of apoptosis, this drug caused a decrease in gene expression of *Bax* and *Mcl-1* and a slight increase of the anti-apoptotic *Bcl-2* gene expression level, but the relationship between *Bax* and *Bcl-2*, involved in mitochondrial membranes permeabilization, indicates failure to initiate an apoptotic process.

All the thioamide derivatives have decreased the *Bax* and *Bcl-2* gene expression levels, and the ratio of Bax / Bcl-2 indicates an induction of apoptosis. Higher toxicity of the compound **INH-TCA 5** in comparison with **INH-TCA 3** can be explained by the Mcl-1 gene expression level, involved in preventing mitochondrial dysfunction. The high level of Mcl-1expression, with the ratio of Bax / Bcl-2, may explain also the low cytotoxicity of the compound **INH-TCA 4**, although it induces an increased expression of caspase 9.

Carboxylic acid derivatives also have decreased the expression level of *Bax* and *Bcl-2* genes, and their ratio shows the induction of the apoptotic program. The high *Mcl-1* gene expression level induced by **INH-AC 4** indicates an activation of anti-apoptotic mechanism in the cell and can explain the lack of an apoptotic effects after 24 hours. However, this compound causes an increase in the expression levels of analysed caspases, and the Bax gene expression level is higher than the Bcl-2 gene expression level, indicating the initiation of the apoptotic process. **INH-AP** has little influence on the expression level of proteins involved in the regulation of the apoptotic process.

The influence of **INH-TCT** on *Bax* and *Mcl-1* gene expression is different, all compounds in this series leading to a decrease in *Bcl-2* gene expression. However, they induced a greater decrease of *Bcl-2* gene expression levels than the case of *Bax* gene, which indicates induction of apoptosis by the intrinsic pathway.

The cytotoxicity of the new synthesized compounds was also evaluated through analysis of the amount of cytochrome c in the cytosolic and mitochondrial fractions. The release of cytochrome c from the mitochondria can be performed by Bid or Bax. In cytosol, cytochrome c binds to Apaf-1 and caspase 9 forming an active apoptosome. Cytochrome c release may increase caspase cascade during apoptosis.

All compounds exhibited higher concentrations of cytochrome c in the mitochondrial fraction, as compared to the cytosolic one, with the exception of **INH-TCA 2** and **INH-TCA 3**. **INH-TCA 2** induced the release of much higher amounts of cytochrome c in the cytosolic fraction compared to the mitochondria one, indicating the increased toxicity of this compound, which is in concordance with flow cytometry analysis (cell death of more than 60% of the cells at a concentration of 50 μg/mL for 24 hours). These results correlate, also, with the expression levels of caspase 9 and 3 observed after treatment of the cells with 25 μg/mL **INH-TCA 2** for 24 hours.

The toxicity of newly synthesized isoniazid derivatives can be partially explained by the activity of potential metabolites. Thus, **INH-TCA 2** it contains chlorine atom in the *para* position of the benzene ring. The activity of chlorinated compounds is determined by the reactivity of the electrophilic carbon center adjacent to chlorine atom. This facilitates the replacement of the chlorine atom with nucleophilic biomolecules, reaction resulting in irreversible attachment of the chlorinated compounds to proteins or DNA bases, causing mutations appearance or disturbance of the protein functions.

Also, the chlorine atom increases the lipophilicity of the molecule which causes a greater partition of the compound in the lipophilic phase of the cell membrane or the lipophilic domain of a protein, and a higher concentration of chlorine compound in cells.[582] On the other hand, the properties of chlorine atom, such as electronegativity, the presence of 3 pairs of electrons not involved in chemical bonds and the geometric size of the atom determines steric and / or electronic effects that induce local electronic attraction or repulsion or steric interference with amino acid residues surrounding the chlorine atom in the binding pocket of the protein. This can cause a close interaction or relaxation of linkages between amino acids near chlorine atom or other areas of the active molecule. Any of these conditions could affect the function of the targeted protein and can lead to an increased or decreased biological activity. In other cases, however, the presence of the chlorine atom cannot affect the biological properties of the molecule to which it is attached by electrostatic forces.[582]

IN the case of **INH-AP**, its toxicity can be determined by intermediate radicals that are formed as a result of reducing the nitro group to the corresponding aniline metabolites. These radicals can damage DNA molecules by breaking the double-stranded chain. Additional DNA damage can be caused by the formation of covalent bonds with some active metabolites, such as *N*-hydroxylamine and *O*-esterified derivative. The detoxification route of formed metabolites involve *N*-acetylation of the aniline nitrogen to the corresponding amide.[654] Since the cell line used to evaluate the cytotoxic effect, HCT-8, has a slow acetylator genotype, the process of detoxification by this means takes place more slowly, resulting in accumulation of toxic metabolites in cells.

The compounds containing benzene substituents present variable toxicities. The greater toxicity is shown by compounds containing a methyl group in the *para* position of the benzene ring, **INH-TCA 3** and **INH-AC 4**. The tolyl is oxidized to the corresponding alcohol and carboxylic acid and to *o*-cresyl.[669] The presence of a hydroxyl group in the *ortho* position of the benzene ring plays an important role in the cytotoxicity and antitumor activity of the compounds and is a good ligand for metals. The mechanism of action of such derivatives can be based on the formation of complex compounds which appear to inactivate cell enzymes.[761] The derivative containing an ethyl group in the *para* position of the benzene ring (**INH-AC 5**) is less toxic. The metabolism of this compound involves also the oxidation of the alkyl group and / or the introduction of hydroxyl groups in *ortho* position, but the formation rate of these compounds can be lower. The compounds containing *p*-methoxy/ethyl phenyl moieties (**INH-TCA 4**, **INH-TCA 5** and **INH-AC 6**) are less toxic, biotransformation of these compounds involving *O*-dealkylation and formation of *p*-hydroxyphenyl group. These results are consistent with studies on other isoniazid derivatives that were evaluated in order to determine their cytotoxicity.[761,762] However, **INH-AC** derivatives are less toxic than **INH-TCA**. This can be explained by the possible *O*-dealkylation of **INH-AC**, phenoxy groups being removed,

while in the case of **INH-TCA** derivatives, thiol groups can covalently modify proteins by interaction with the cysteine-disulfide links.[654]

In the case of **INH-TCT 6**, the most toxic of **INH-TCT** derivatives, it contains a benzene ring which may be enzymatically oxidized to intermediary arene oxides that are further biotransformed in stable phenolic metabolites. Formed arene oxides are considered to be responsible for the formation of covalent bonds with cellular macromolecules and, respectively, for the toxicity induced by this type of compounds.[654]

Although several analysed compounds induced apoptosis, only **INH-TCA 2** caused a substantial increase in cytochrome c in the cytosolic fraction, and a decrease in the mitochondrial one, which indicate high toxicity of this compound and the irreversibility of the induced apoptotic process.

Chapter IX. The Influence of New Isoniazid Derivatives on Cell Cycle

The cell cycle represents the sum of events that govern cell transition from a state of rest to proliferation and ensure fidelity genetic transcript. Cell cycle analysis by flow cytometry involves the quantification of the amount of DNA contained in the nucleus and the cell cycle phase distribution of the cells based on it.

The treatment of cells with **INH-TCA 3** determined an increased number of cells that are in the G0 / G1 phase (from 46.43% to 72.35% in the control) and reduction of the number of cells in the S phase (from 39.43% in control to 13.1%). The influence of other **INH-TCA** derivatives and of **INH-AP** on the cell cycle is minor. *N*, *N'*-disubstituted hydrazides **INH-AC 4 - INH-AC 6** increased G0/G1 phase and decreased the S and G2/M, whereas **INH-AC 1 - INH-AC 3** slightly increased the G2 / M phase. **INH-TCT 3**, **INH-TCT 4** and **INH-TCT 6** determined an increase of G0/G1 phase and a decrease of S and G2/M phases.

As a result, **INH-AC 4**, **INH-AC 5**, **INH-AC 6**, **INH-TCT 3** and **INH-TCT 6** led to a block of cells in the G0 / G1 cell cycle phase which may be associated with a potential antumor activity.

At the molecular level, the cell cycle is controlled by a series of linked intra- and extracellular signalling pathways based on the activity of cyclin-dependent kinases (Cdk).[782] The study at the molecular level of the effect of the new synthesized compounds on the progression of the cell cycle included the analysis of expression levels of the genes: cyclin A (involved in progression through the S and G2 phases), cyclin B (mediates the passage through G2 and M phases), CDK1 (interacts with cyclin B1 and form "mitosis promoting factor") and Cdc20 (combined with the anaphase promoting complex/ cyclosome (APC/C) is involved in metaphase-anaphase transition).

INH-TCA reduced the expression levels of cyclin A, cyclin B, CDK1 and Cdc20 involved in S and G2 / M phase regulation. Antipyrine derivative, **INH-AP**, showed a weak influence on the expression levels of genes involved in cell cycle regulation, which is correlated with its effect on cell cycle observed by flow cytometry.

INH-AC 1 and **INH-AC 2** slightly reduced cyclin A, CDK1 and Cdc20 gene expression and induced the expression of cyclin B gene. **INH-AC 3** proved a weak influence on expression of genes implicated in cell cycle by slightly increasing the expression of cyclin A, cyclin B and CDK1 genes and slightly reducing the expression of Cdc20. **INH-AC 4 – INH-AC 6** reduced the expression levels of cyclin A, cyclin B, CDK1 and Cdc20 genes

indicating an inhibition of cell cycle progression, which comes into agreement with the decrease of S and G2 phases demonstrated by flow cytometry.

INH-TCT 3, **INH-TCT 4** and **INH-TCT 6** induce a decrease in expression levels of analyzed genes. Most affected by **INH-TCT 3** is the expression of cyclin A that is in accordance with the decreased S phase observed in flow cytometry. In the case of **INH-TCT 6**, the most decreased is the expression of cyclin A and CDK1 genes, which is in accordance with the decrease of G2/M phase observed by flow cytometry. Thus, **INH-TCT 3** and **INH-TCT 6** caused cell cycle arrest in the G1 phase.

As a result, **INH-AC 4**, **INH-AC 5**, **INH-AC 6**, **INH-TCT 3** and **INH-TCT 6** have decreased the expression level of analysed cyclins, cyclin A and cyclin B, and of genes Cdk1 and Cdc20, which correlates with cell cycle blocking in the G0 / G1 observed by flow cytometry.

Most of the mutations that cause tumors are in genes encoding proteins involved in the G1 phase of the cell cycle.[771] In our case, some of the newly synthesized derivatives of isoniazid block the cell cycle progression, inducing cell accumulation in G0 / G1 phase. Also, some of the examined compounds have cytotoxic effect by inducing apoptosis. The toxicity of these compounds can be partially explained by the formation of toxic metabolites, but which can also be removed from the body, avoiding their accumulation. However, to elucidate the antitumor potential of these compounds requires further studies regarding their mechanism of action.

In order to evaluate the influence of new compounds on celular energy level, d the expression of genes encoding the alpha subunit of AMPK, *PRKAA1* and *PRKAA2*, were examined. AMPK protects cells from stress which causes depletion of ATP by disconnecting energy-activated biosynthetic pathways, activates energy production pathways and inhibits consuming processes in response to the decreasing level of intracellularly ATP, regulates polarity of the cell through remodeling the actin cytoskeleton and, possibly, by indirect activation of miosis. This protein acts through direct phosphorylation of metabolizing enzymes and, for a longer lasting effect, through phosphorylation of transcription regulators.

All **INH-TCA** derivatives have increased the gene expression level of *PRKAA2*, and slight decreased gene expression level of *PRKAA1*. The *PRKAA1*/*PRKAA2* ratio indicates an increase of expression level of alpha subunit of AMPK induced by **INH-TCA** (except **INH-TCA 5**). In the case of **INH-AC**, gene expression level of *PRKAA2* was significant increased by **INH-AC 4**, **INH-AC 5** and **INH-AC 6**. The *PRKAA1*/*PRKAA2* ratios for these compounds indicate also an increased expression of the alpha subunit of AMPK. **INH-AP** did not affect significantly the gene expression *PRKAA1* and *PRKAA2*, which correlates with poor influence of this compound on cell cycle progression observed by flow cytometry. In the case of **INH-TCT**, *PRKAA2* gene expression level was increased following treatment of cells with **INH-TCT 2**, **INH-TCT 3** and **INH-TCT 6**. The *PRKAA1*/*PRKAA2* ratios indicate an increase of AMPK alpha subunit expression induced by these compounds.

The increased expression of AMPK alpha subunit, induced by **INH-AC 4**, **INH-AC 5**, **INH-AC 6**, **INH-TCT 3** and **INH-TCT 6**, can be linked to cell cycle arrest in the G0 / G1 phase, and that is in the point of metabolic control, the increase in *PRKAA1* and *PRKAA2* gene expression levels indicating activation of AMPK that determine inhibition of mTOR and cell cycle arrest.[842] On the other hand, the modulation of apoptosis by p53 by AMPK[846] may explain the increase of gene expression induced by **INH-TCA 1**, **INH-TCA 2**, **INH-TCA 3**,

INH-TCA 4, **INH-AC 4**, **INH-AC 5**, **INH-AC6** and **INH-TCT 6**. Also, the increased gene expression induced by **INH-TCA** can be determined by the increase of the expression level of *GSTA3*, a condition in which the formation of the GST/AMPK enzyme leads to the *in vitro* activation of AMPK.[844]

CONCLUSIONS

Objective 1. The assessment of genotype / phenotype / pharmacokinetics correlation regarding the genes involved in the metabolism of anti-tuberculosis drugs

1. The determination of *NAT1* and *NAT2* genotype in subjects with tuberculosis, with or without side effects, revealed:
   - 16,22% have a *NAT1*11/*4* heterozygous genotype and 2,7% –*NAT1*14/*4* heterozygous genotype, other patients (81,08%) being *NAT1*4* homozygous;
   - there is no correlation between NAT1 genetic polymorphisms and hepatotoxic effects induced by tuberculosis treatment;
   - 27.03% have *NAT2*5* homozygous genotype, 10.81% – *NAT2*6* homozygous genotype, and only 5.4% being *NAT2*4* homozygous. The other patients are heterozygous for *NAT2*5/*4* (27.03%), *NAT2*6/*4* (10.81%) and *NAT2*5/*6* (18.92%);
   - slow acetylator genotype determined by *NAT2* genetic polymorphisms is responsible for hepatotoxic adverse effects induced of anti-tuberculosis treatment.

Hepatotoxicity may be determined also by other factors, i.e. the presence of polymorphisms in other genes involved in the metabolism of other anti-tuberculosis drugs or concurrent therapy.

Objective 2. Synthesis and evaluation of biologic activities of new isoniazid derivatives with potential anti-mycobacterial activity

1. Were synthesized 19 novel derivatives of isoniazid 5 *N'*-[1-amino-1-mercapto-3- (*p*-substituted-phenyl)-allyl]-hydrazides of isonicotinic acid, 6 hydrazides of 2-(4-substituted-phenoxymethyl)-benzoic acids, *N*-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-3,5-dinitro-4-[*N*-(pyridine-4-carbonyl)hydrazino]-benzamide and 7 isonicotinic acid (2-hydroxy-8-substituted-tricyclo[7.3.1.$0^{2.7}$]tridec-13-ylidene)-hydrazides, their structures being confirmed by chemical analysis. With respect to biological studies, results have suggested that isoniazid novel derivatives show various biological activities depending on their structure

2. The study of the biological activity of *N'*-[1-amino-1-mercapto-3- (*p*-substituted-phenyl)-allyl]-hydrazides of isonicotinic acid (**INH-TCA**) highlighted:
   - The most active compound in terms of the anti-*Mycobacterium tuberculosis* activity was *N'*-[1-amino-1-mercapto-3-phenyl-allyl]-hydrazide of isonicotinic acid (**INH-TCA 1**). This compound has increased *NAT1* and *NAT2* gene

expression, involved in isoniazid metabolism, demonstrating that can be easily metabolized and therefore better tolerated by the human host;

- The best antibacterial activity was presented by *N'*-[1-amino-1-mercapto-3- (*p*-chloro-phenyl)-allyl]-hydrazides of isonicotinic acid (**INH-TCA 2**);
- Thioamide derivatives have increased gene expression levels of *CYP1A1*, *CYP2C19*, *CYP3A4* and *GSTA3* analysed in both cell lines indicating their possible involvement in the metabolism of new compounds;
- **INH-TCA** induce apoptosis by intrinsic pathway, the most toxic compound being *N'*-(1-amino-1-mercapto-3-(4-chlorophenyl)-allyl)-hydrazide of isonicotinic acid (**INH-TCA 2**), and the less toxic - *N'*-(1-amino-1-mercapto-3-(4-methoxyphenyl) allyl)-hydrazide of isonicotinic acid (**INH-TCA 4**).

3. The study of the biological activity of hydrazides of 2-(4-substituted-phenoxymethyl)-benzoic acids (**INH-AC**) highlighted:

- The best antibacterial activity (including anti-*Mycobacterium tuberculosis*) has been presented by *N*-[2-(4-ethyl-phenoxymethyl)-benzoyl]-*N'*-(pyridine-4-carbonyl)-hydrazide of 2-(4-ethyl-phenoxymethyl)-benzoic acid (**INH-AC 6**);
- All compounds have increased gene expression levels of *NAT2*, *CYP1A1* and *CYP2C19* in HCT-8 cell line indicating their possible involvement in the metabolism of new compounds;
- *N*-[2-(4-methyl-phenoxymethyl)-benzoyl]-*N'*-(pyridine-4-carbonyl)-hydrazide of 2-(4-methyl-phenoxymethyl)-benzoic acid (**INH-AC 4**) is the most toxic compound from this group and induces apoptosis in 54% cell in 72 hours, an effect that can be predicted by the increased expression of mRNA caspase 3 and 7 after a 24 hours treatment;
- *N*-[2-(4-methyl-phenoxymethyl)-benzoyl]-*N'*-(pyridine-4-carbonyl)-hydrazide of 2-(4-methyl-phenoxymethyl)-benzoic acid (**INH-AC 4**), *N*-[2-(4-methoxy-phenoxymethyl)-benzoyl]-*N'*-(pyridine-4-carbonyl)-hydrazide of 2-(4-methoxy-phenoxymethyl)-benzoic acid (**INH-AC 5**) and *N*-[2-(4-ethyl-phenoxymethyl)-benzoyl]-*N'*-(pyridine-4-carbonyl)-hydrazide of 2-(4-ethyl-phenoxymethyl)-benzoic acid (**INH-AC 6**) block the cell cycle in the G1 phase, which may indicate their potential use as a cytostatic.

4. The study of the biological activity of *N*-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-3,5-dinitro-4-[*N*-(pyridine-4-carbonyl)hydrazino]-benzamide (**INH-AP**) highlighted:

- This compound has a moderate anti-mycobacterial activity and shows no antibacterial activity against other tested non-tuberculosis strains;
- **INH-AP** increases gene expression level of *NAT1*, *NAT2* and *CYP1A1* and decrease the gene expression of *CYP2C19* isoform expression in both cell lines;
- **INH-AP** is more toxic than its precursors without significantly affecting cell cycle.

5. The study of the biological activity of isonicotinic acid (2-hydroxy-8-substituted-tricyclo[7.3.1.$0^{2.7}$]tridec-13-ylidene)-hydrazides (**INH-TCT**) highlighted:

- The best anti-mycobacterial activity has been presented by compounds containing ethyl (**INH-TCT 2**), propyl (**INH-TCT 3**), butyl (**INH-TCT 4**) and isobutyl (**INH-TCT 5**) moieties in 8 position of tricyclo[7.3.1.$0^{2.7}$]tridec-13-ylidene group;
- Isonicotinic acid 2-(2-hydroxy-8-phenyl-tricyclo-[7.3.1.$0^{2.7}$]tridec-13-ylidene)-hydrazide (**INH-TCT 6**) is the most efficient antibacterial compound, but has poor activity against *M. tuberculosis* used strain;
- This compounds induce *CYP1A1* gene expression in HT-29 and HCT-8 cell lines, and *CYP2C19* gene in HCT-8 cell line;
- The most toxic compound from this group is isonicotinic acid 2-(2-hydroxy-8-phenyl-tricyclo-[7.3.1.$0^{2.7}$]tridec-13-ylidene)-hydrazide (**INH-TCT 6**) that induces apoptosis by extrinsic pathway and causes blocking of the cell cycle in the G1 phase;
- The less toxic the compound is isonicotinic acid 2-(2-hydroxy-8- propyl-tricyclo-[7.3.1.$0^{2.7}$]tridec-13-ylidene)-hydrazide (**INH-TCT 3**), which has a good antibacterial activity both against the *M . tuberculosis* and non-tuberculosis strains against, but also affects cell cycle progression in eukaryotic cells by blocking the G1 phase.

6. Thus, of the 19 new synthesized isoniazid derivatives:

- The best anti-M. tuberculosis has been shown by (isonicotinic acid (2-hydroxy-8-substituted-tricyclo[7.3.1.$0^{2.7}$]tridec-13-ylidene)-hydrazides (**INH-TCT**)
- The best antibacterial activity has been presented by *N*-[2-(4-ethyl-phenoxymethyl)-benzoyl]-*N'*-(pyridine-4-carbonyl)-hydrazide of 2-(4-ethyl-phenoxymethyl)-benzoic acid (**INH-AC 6**), which inhibited the growth of the majority of non-tuberculosis strains tested;
- Most of newly synthesized isoniazid derivatives determined an increase of NAT genes expression levels in the two cell lines used, HT-29 and HCT-8, but also of other genes involved in metabolism, suggesting that the novel compounds may be metabolised by routes other than isoniazid, which indicates the possibility of their use in combination with isoniazid in order to improve isoniazid metabolizing rate in slow acetylators, avoiding adverse effects.
- *N*-[2-(4-methyl-phenoxymethyl)-benzoyl]-*N'*-(pyridine-4-carbonyl)-hydrazide of 2-(4-methyl-phenoxymethyl)-benzoic acid (**INH-AC 4**), *N*-[2-(4-methoxy-phenoxymethyl)-benzoyl]-*N'*-(pyridine-4-carbonyl)-hydrazide of 2-(4-methoxy-phenoxymethyl)-benzoic acid (**INH-AC 5**), *N*-[2-(4-ethyl-phenoxymethyl)-benzoyl]-*N'*-(pyridine-4-carbonyl)-hydrazide of 2-(4-ethyl-phenoxymethyl)-benzoic acid (**INH-AC 6**), isonicotinic acid 2-(2-hydroxy-8- propyl-tricyclo-[7.3.1.$0^{2.7}$]tridec-13-ylidene)-hydrazide (**INH-TCT 3**) and isonicotinic acid 2-(2-hydroxy-8-phenyl-tricyclo-[7.3.1.$0^{2.7}$]tridec-13-ylidene)-hydrazide (**INH-TCT 6**) block the cell cycle in the G1 phase and have anti-tumor potential.
- Even though some of the compounds with antibacterial or anti-tumor activity show increased toxicity, it can be explained partly by the formation of toxic metabolites, but which can also be removed from the body, avoiding their accumulation.

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