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Original article

Efficient synthesis of new (*R*)-2-amino-1-butanol derived ureas, thioureas and acylthioureas and *in vitro* evaluation of their antimycobacterial activity

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ABSTRACT

The synthesis of 22 structurally diverse urea, thiourea and acylthiourea derivatives containing the (*R*)-2-amino-1-butanol motif has been performed. The evaluation of their *in vitro* activity against *Mycobacterium tuberculosis* (H_{37}Rv and strain 43) showed promising results in the case of the acylthiourea derivatives (MIC range 0.36–7.46 μM for H_{37}Rv strain).

1. Introduction

Tuberculosis (TB) is one of the most devastating diseases primarily due to several decades of neglect, HIV infection, immigration and globalization [1]. Approximately one-third of the world's population has been infected with the causative organism *Mycobacterium tuberculosis* (MTB), eight million become sick with TB and globally it accounts for approximately two million deaths per year. The spreading of collaborative TB/HIV infections [2] and the reemergence of TB accompanied by an increasing number of drug resistant and multidrug-resistant (MDR) strains MTB (i.e. resistant to at least rifampin [RIF] and isoniazid [INH]) has been noted since the mid-1980s [3–5]. Thus, management of tuberculosis is complicated, which has become a serious health problem worldwide.

The frontline drugs INH, RIF, pyrazinamide (PZA) and ethambutol (EMB) are currently recommended by the World Health Organization (WHO) for the treatment of TB [6]. The problems with current TB treatment are complex and include: a prolonged standard course regimen of six months, which often result in patient noncompliance; emergency of extremely drug-resistant tuberculosis (XDR-TB) strains; lack of effective drugs against the latent state. One approach to decrease treatment time is improvement of potency of currently used anti-tuberculosis drugs [7], mainly through discovery of more effective combinations with newer, more potent and less toxic active compounds [8,9]. There is a clear trend toward gradually increasing partition of new active compounds, including derivatives of known anti TB drugs [10] and natural products [11]. Except of few new chemical entities [12,13] no other anti MDR-TB drugs with proved novel mechanism of action are available in clinical use since last 40 years, but many classes of new potent compounds [13–15] are currently in different steps of their anti TB evaluation.

EMB is a simple (*S*)-2-amino-1-butanol derived 1,2-diamine, clinically used as primarily bacteriostatic anti-tuberculosis agent (Scheme 1) with not fully known mechanism of action. It targets the arabinosyl transferases responsible for arabinogalactan biosynthesis, a key component of the unique mycobacterial cell-wall [16–18]. Despite modest antimycobacterial activity and due to its synergy with other drugs and lower toxicity, EMB is used in combination with more potent frontline antimycobacterial agents. The configuration of

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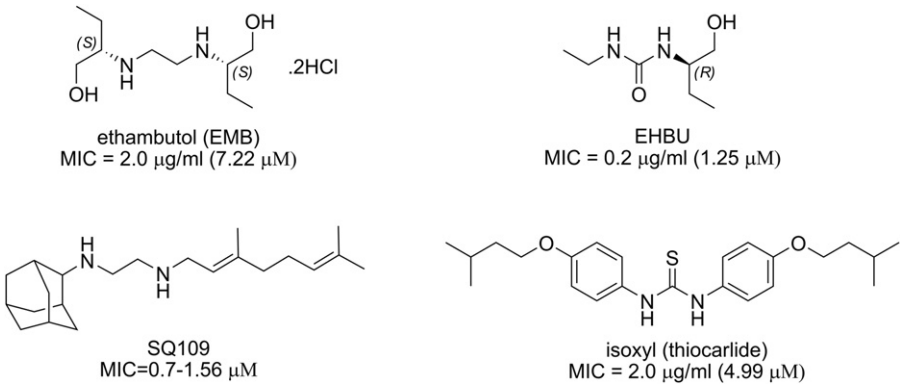


Scheme 1. Representative examples of compounds possessing antimycobacterial activity.

the molecule is decisively important for the activity, since EMB is approximately 200–500 fold more potent than its (*R*,*R*) enantiomer [19].

In recent years diverse derivatives of (*S*)-2-amino-1-butanol and 1,2-ethylenediamine have been synthesized and evaluated in respect of their antimycobacterial activities and mechanisms of action [14,20–23]. One of the most active (up to 35 fold more active *in vitro*) analogues of EMB of late years is 1,2-diamine SQ109 [14,15,21,24] (Scheme 1). This compound possesses low cytotoxicity, excellent pharmacokinetic properties and selectivity. It demonstrates synergistic interactions with some "classic" anti TB drugs, as well. SQ109 and its analogues are currently under the procedures of their clinical evaluation [24,25].

In our recent study [26] the significant role of the chirality has been demonstrated on the example of a series of (*R*)-2-amino-1-butanol derivatives, some being up to 11 times more active than the *S*,*S*-configured EMB. One of the urea derivatives obtained, namely (*R*)-1-ethyl-3-(1-hydroxybutan-2-yl)urea (EHBU, Scheme 1), showed very promising activity and low toxicity together with good water solubility.

A variety of structurally related thioureas (*N*-aryl-*N*′-alkyl and *N*,*N*′-diaryl substituted) have been extensively evaluated against different strains of MTB [27–32] showing valuable activities, sometimes greatly exceeding EMB. One representative example is isoxyl (thiocarlide; 4,4-diisoamyloxydiphenylthiourea; Scheme 1), efficiently clinically used drug since 1960 [33,34]. Recently, the subclass of acylthioureas have been also an object of interest showing promising anti-TB activity, as summarized by Ananthan et al. [35,36].

Taking into account the above presented results, we were encouraged to perform the synthesis of new series of ureas, thioureas and acylthioureas incorporating the (*R*)-2-amino-1-butanol motif and to evaluate their *in vitro* antimycobacterial activity.

2. Results and discussion

2.1. Chemistry

We have optimized efficient pathways to obtain small libraries of chiral ureas, thioureas and acylthioureas. All compounds described have been isolated in pure form and have been characterized by means of NMR, spectroscopy, mass spectrometry and elemental analysis. Detailed description of the experimental procedures and the data obtained are available in Supplementary data.

2.1.1. Synthesis of ureas *15*–*27*, *30*–*31* and thioureas *34*–*35*

The synthesis of compounds **15**–**27** was performed by mixing **1** and the corresponding isocyanates **2**–**14**, respectively, in THF or dichloromethane (DCM) as a solvent (Scheme 2). They were obtained in very high yields and excellent purity. The preparation of **21**, **22** [37], and **24** [38,39] has been described previously, however as racemic mixtures and without evaluation of their bioactivity.

The urea derivative **30** was synthesized from **1** and **28** (Scheme 3) using a published solvent-free procedure [40]. Compound **31** was obtained by reacting **1** with **29** under standard acylation conditions (0 °C and Et_3N in dry DCM). The thioureas **34** and **35** were obtained in high yields in the same manner as ureas **15**–**27**, by mixing **1** and the isothiocyanates **32** and **33**, respectively (Scheme 3). The preparation of **34** has been mentioned earlier [41].

2.1.2. Synthesis of acylthioureas *41*–*45*

The synthesis of compounds **41**–**45** (Scheme 4) was performed by recently published procedure [42] by using one-pot reaction of acylchlorides **36**–**40** with NH_4SCN in the presence of catalytic amounts of PEG-400, followed by addition of **1** to the reaction mixture. The yields were moderate, however the application of this method was easy to perform and convenient in respect of the purification of the desired products (simple filtration through a pad of silica provided excellent pure products).

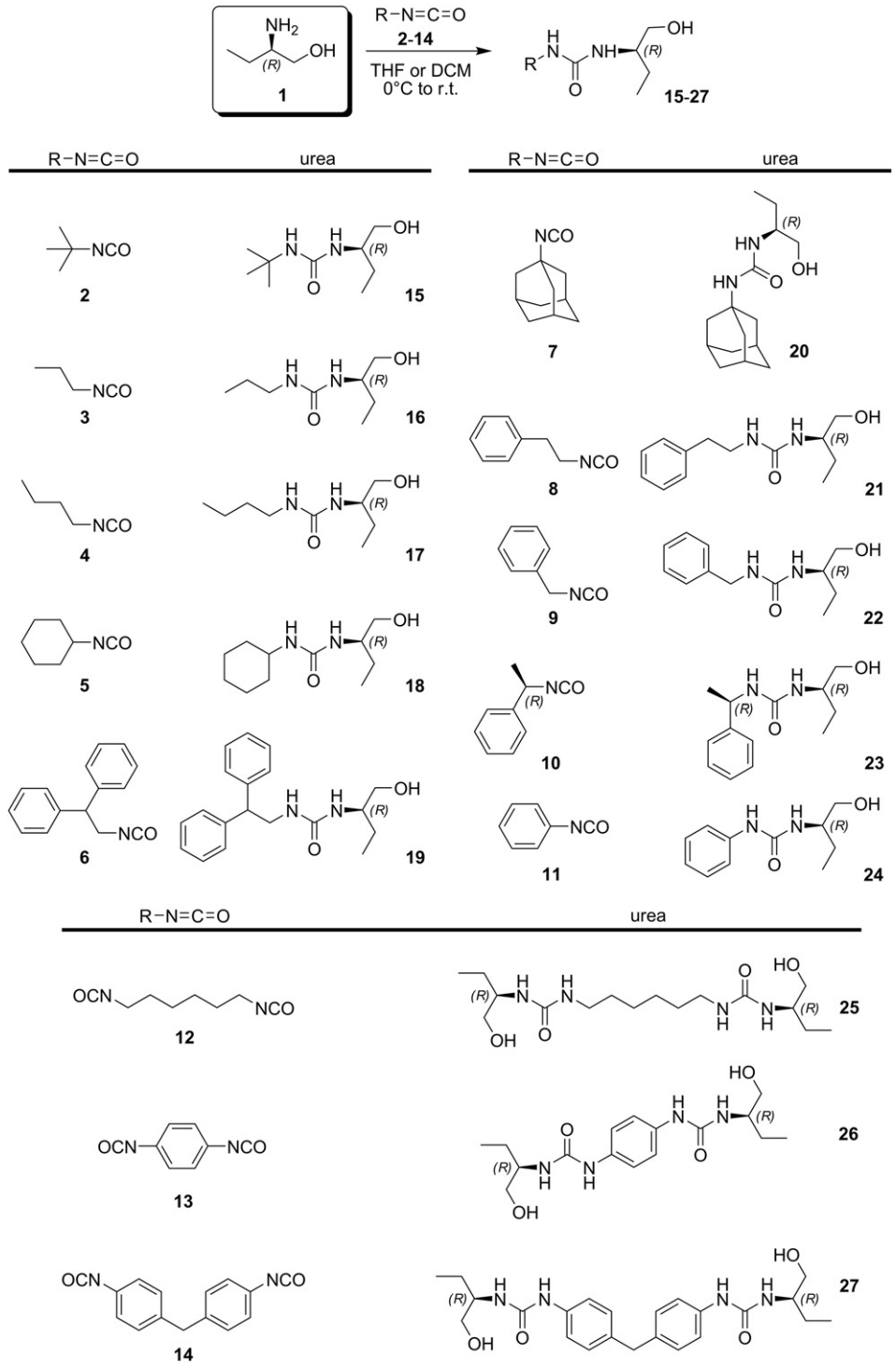
2.2. Biology

There are no data regarding the antimycobacterial and cytotoxic activity of the 22 newly synthesized compounds.

2.2.1. In vitro antimycobacterial activity

The synthesized compounds were evaluated for their *in vitro* activity against *M. tuberculosis* $H_{37}Rv$ and MDR strain 43 (Table 1; results are recalculated in μM) using the method of Canetti (see Section 4.2). All the compounds synthesized are in agreement with the formal Lipinski's rule of five. The first 17 derivatives of (*R*)-2-amino-1-butanol – ureas **15**–**27**, **30**–**31** and thioureas **34**–**35** (Schemes 2 and 3) were inactive against *M. tuberculosis* $H_{37}Rv$ even at concentrations of 5 μg/ml (100% growth of the bacteria was observed). The only observed exception was compound **19**, showing activity close to EMB. It is interesting to point out, that even a small structural change in the molecule EHBU [26] (Scheme 1) induce lack of activity. For example, its inactive near homologues possess propyl (**16**), *n*-butyl (**17**) and *t*-butyl (**15**) groups. Replacement of the carbonyl group with thiocarbonyl (thioureas **34**–**35**) leads to the same consequences. Similar negative trend was observed for many derivatives of EMB [7,19].

Other series of five new acylthioureas **41**–**45** (Scheme 4) was designed to contain important pharmacophore groups (discovered in our previous study [26]), attached to acylthioureas containing (*R*)-2-amino-1-butanol moiety. Compounds **41** and **43**–**45** showed



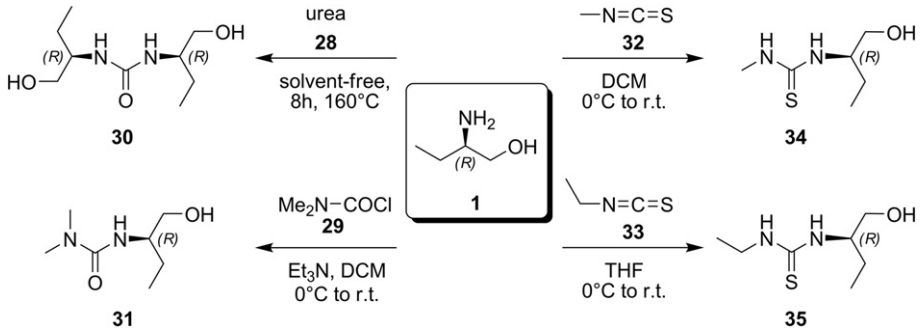
Scheme 2. Synthesis of compounds **15**–**27**.

activity against *M. tuberculosis* $H_{37}Rv$ comparable to EMB. Cinnamic derivative **42** is the most active compound in this study with MIC 0.36 μM. Besides, relatively low cytotoxicity and excellent selectivity index (SI) (119.2) of **42** suggest that this compound is a good lead structure for further investigations. Encouraging from above results, we tested **41**–**45** against MDR strain 43. In this case compounds **41**–**43** lose their activity (100% growth of bacteria at concentrations 5 μM). On the other hand, **44** preserved its activity and **45** was only 2 fold less active.

2.2.2. Cytotoxic activity of the complexes

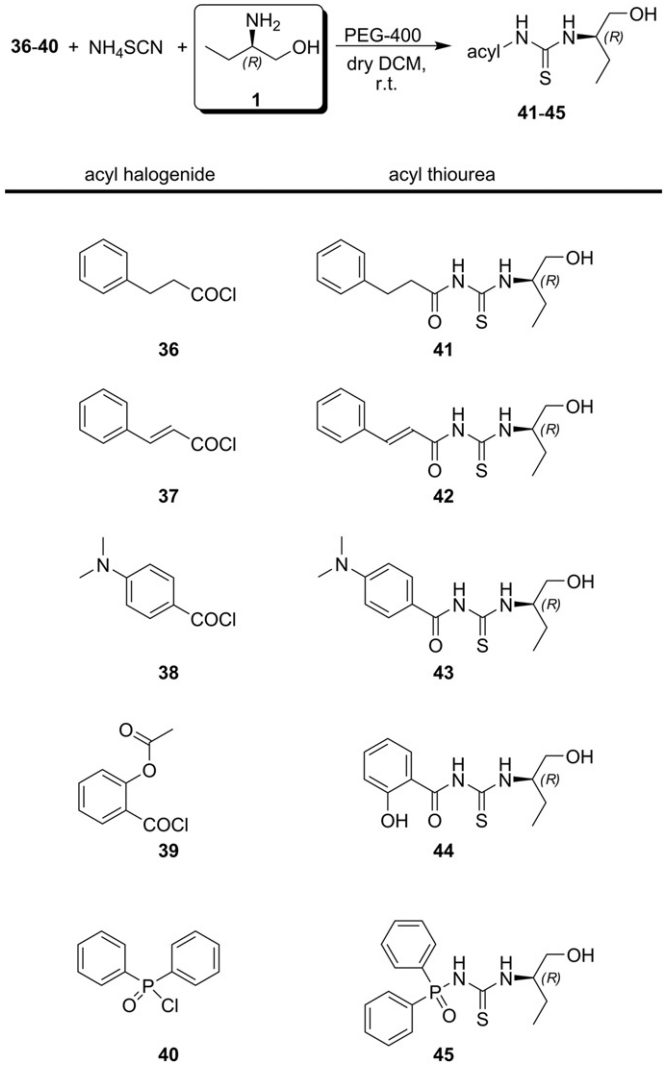
The findings that the acylthioureas **41**–**45** exerted good antimycobacterial activity gave us a good reason for further more detailed evaluation of the cytotoxic effect of those compounds. The cytotoxic activity of the tested compounds was investigated against human embryonic kidney cell line (HEK293) using the MTT dye reduction assay.

The corresponding IC_{50} values of the tested compounds were calculated using nonlinear regression analysis and summarized in



Scheme 3. Synthesis of compounds **30**–**31** and **34**–**35**.

Table 1. As it could be seen the compounds demonstrated a wide range of cytotoxicity with IC_{50} between 12.8 and 179.6. Perceptible cytotoxicity to the cells was exhibited by the substances **41**–**42** while compound **43** showed strong cytotoxic effect. It is noteworthy that the most potent antimycobacterial sample within this series, e.g. **42**, showed excellent SI (119.2). Therefore it turned out that compound **42** is of particular interest due to its highly favorable features – low cytotoxicity and significant antimycobacterial activity which makes it a good candidate for an efficient therapeutic agent.



Scheme 4. Synthesis of acylthioureas **41**–**45**.

3. Conclusion

An efficient synthesis of chiral urea, thiourea and acylthiourea derivatives containing the (*R*)-2-amino-1-butanol moiety has been demonstrated. After purification and structure characterization, the antimycobacterial activity of the compounds was evaluated *in vitro* against two MTB strains ($H_{37}Rv$ and strain 43). The most active compounds among the evaluated series were the acylthiourea derivatives **41**–**45**.

4. Experimental

4.1. Chemistry

For thin layer chromatography (TLC) aluminum sheets precoated with silica gel 60 F_{254} (Merck) were used. Flash column chromatography was carried out using silica gel 60 (0.040–0.063 mm, 230–400 mesh ASTM, Merck). Commercially available solvents for reactions, TLC and column chromatography were used after distillation (and were dried when needed) – hexane, heptane, diethyl ether (Et_2O), dichloromethane (DCM), methyl *tert*-butyl ether (MTBE), tetrahydrofurane (THF), methanol (MeOH), ethanol (EtOH), ethylacetate (EtOAc). Melting temperatures were determined in capillary tubes on an Electrothermal MEL-TEMP 1102D-230 VAC apparatus without corrections. The NMR spectra were recorded on a Bruker Avance DRX-250 (250.13 for ^1H and 62.90 MHz for ^{13}C) and on a Bruker Avance II+ 600 (600.13 for ^1H and 150.92 MHz and for ^{13}C NMR) spectrometers. In case of $CDCl_3$ TMS was used as internal standard for chemical shifts (δ, ppm) and ^1H spectra were calibrated to the signal of TMS ($\delta = 0.0000$). For other deuterated solvents ^1H spectra were calibrated to the residual solvent peaks (DMSO-d_6 $\delta = 2.50$). ^{13}C spectra were calibrated in all cases to the residual solvent peaks ($CDCl_3$ $\delta = 77.00$, DMSO-d_6 $\delta = 39.52$). ^{31}P NMR spectra were recorded with full proton decoupling and using 85% H_3PO_4 as external standard. The calibration of the ^{31}P NMR spectra was performed through changing of the spectrum reference frequency (specific for the used NMR probe). The following additional NMR techniques were used for all compounds: DEPT 135, COSY, HSQC and HMBC. ^1H and ^{13}C NMR data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), integration, identification, and coupling constants (in Hz). For numbering of the atoms see Supplementary data. Mass spectra (MS) were recorded on a Thermo Scientific High Resolution Magnetic Sector MS DFS by chemical ionization (CI) or negative-ion electrospray ionization (-ESI), and are reported as fragmentation in

Table 1
In vitro screening data for antimycobacterial activity and cytotoxicity of synthesized compounds **15**–**27**, **30**–**31**, **34**–**35** and **41**–**45**.

Entry	Compound	Antimycobacterial activity toward reference strain of *Mycobacterium tuberculosis* $H_{37}Rv$, MIC (μM)	Antimycobacterial activity toward MDR strain 43 of *Mycobacterium tuberculosis* MIC (μM)[a]	*In vitro* cytotoxicity toward human embryonal kidney cell line 293T, IC_{50} (μM)[a]	Selectivity index, SI[b]	Log *P*[c]
1	**15**	>26.56	NT	NT	NT	0.46 ± 0.36
2	**16**	>28.71	NT	NT	NT	0.30 ± 0.35
3	**17**	>26.56	NT	NT	NT	0.83 ± 0.35
4	**18**	>23.33	NT	NT	NT	1.30 ± 0.35
5	**19**	4.80	NT	NT	NT	3.04 ± 0.38
6	**20**	>18.77	NT	NT	NT	2.12 ± 0.39
7	**21**	>21.16	NT	NT	NT	1.43 ± 0.36
8	**22**	>22.49	NT	NT	NT	1.01 ± 0.37
9	**23**	>21.16	NT	NT	NT	1.36 ± 0.38
10	**24**	>24.01	NT	NT	NT	1.51 ± 0.40
11	**25**	>14.43	NT	NT	NT	−0.16 ± 0.49
12	**26**	>14.76	NT	NT	NT	0.41 ± 0.56
13	**27**	>11.67	NT	NT	NT	2.80 ± 0.56
14	**30**	>24.48	NT	NT	NT	−0.51 ± 0.39
15	**31**	>31.21	NT	NT	NT	0.05 ± 0.39
16	**34**	>30.82	NT	NT	NT	−0.09 ± 0.27
17	**35**	>28.36	NT	NT	NT	0.44 ± 0.27
18	**41**	7.13	>17.83	66.2	9.3	1.75 ± 0.60
19	**42**	0.36	>17.96	42.9	119.2	2.29 ± 0.61
20	**43**	3.39	>16.92	12.8	3.8	1.73 ± 0.61
21	**44**	7.46	7.46	104.4	14.0	1.58 ± 0.62
22	**45**	5.74	11.49	179.6	31.3	1.86 ± 0.62
23	EMB·2HCl[d]	7.22	NT	NT	NT	0.06[e]

[a] NT – not tested – for low active compounds against $H_{37}Rv$ strain; cytotoxicity and SI were tested/calculated only for selected active compounds.
[b] Selectivity index: SI = IC_{50}/MIC ($H_{37}Rv$).
[c] Log *P*, octanol–water partitioning coefficient, was calculated using ACDLabs/ChemSketch 12 (www.acdlabs.com).
[d] EMB·2HCl – ethambutol dihydrochloride (reference compound).
[e] Log *P* of EMB·2HCl is known in the literature: N.R. Budha, R.E. Lee and B. Meibohm, Curr. Med. Chem. 15 (2008) 809.

m/*z* with relative intensities (%). Optical rotation $[\alpha]_D^{20}$ measurements were obtained using a Perkin–Elmer 241 polarimeter. Elemental analyses were performed by the Microanalytical Laboratory for Elemental Analysis of the Institute of Organic Chemistry, Bulgarian Academy of Sciences. All starting chemicals were commercially available (from Sigma–Aldrich, Merck, Fluka, Acros, Alfa Aesar). Dimethyl sulfoxide (DMSO) for testing of bioactivities was commercial (spectroscopic grade) and was used without distillation. (*R*)-2-Amino-1-butanol (**1**) was obtained from Alfa-Aesar, >98 ee (proved as described elsewhere [26]).

4.2. Methodology for evaluation of antimycobacterial activity

The antimycobacterial activity was determined through the proportional method of Canetti towards reference strain *M. tuberculosis* $H_{37}Rv$ and multi-drug resistant strain 43 (resistant to Rifampin and Isoniazid), recovered from Bulgarian adult HIV-negative pulmonary TB patient, who was permanent resident of the country. This method, recommended by the WHO, is the most commonly used one worldwide for exploration of sensitivity/resistance of tuberculosis strains towards chemotherapeutics [43–47]. It allows precise determination of the proportion of resistant mutants to a certain drug.

A sterile suspension/solution of each tested compound was added to Löwenstein–Jensen egg based medium before its coagulation (30 min at 85 °C). Each compound was tested at four concentrations – 5 μg/ml, 2 μg/ml, 0.2 μg/ml and 0.1 μg/ml (in DMSO). For some compounds, additional tests at concentrations 0.05, 1, 2 and 3 μg/ml were performed. Tubes with Löwenstein-Jensen medium (5 ml) containing tested compounds and those without them (controls) were inoculated with a suspension of *M. tuberculosis* $H_{37}Rv$ (10^5 cells/ml) and incubated for 45 days at 37 °C. The ratio between the number of colonies of *M. tuberculosis* grown in medium containing compounds and the number of colonies in control medium were calculated and expressed as percentage of inhibition. The MIC is defined as the minimum concentration of compound required to inhibit bacterial growth completely (0% growth). The MIC values are calculated and given as μM.

4.3. Methodology for evaluation of cytotoxicity

The cell viability was assessed using the standard MTT-dye reduction assay as described by Mosmann [48] with some modifications. The method is based on the biotransformation of the yellow tetrazolium salt MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a violet formazan via the mitochondrial succinate dehydrogenase in the viable cells. Briefly: the exponentially growing cells (HEK human embryonic kidney fibroblasts) were seeded in 96-well flat-bottomed micro-plates (100 μL/well) at a density of 1×10^5 cells per ml and after 24 h incubation at 37 °C they were exposed to various concentrations of the tested compounds for 72 h. At least 8 wells were used for each concentration. After the incubation with the test compounds 10 μL MTT solution (5 mg/mL in PBS) aliquots were added to each well. The microplates were further incubated for 4 h at 37 °C and the formazan crystals formed were dissolved through addition of 100 μL DMSO into each well. The absorbance was measured on an ELISA plate reader (Bio-Tek Instruments) with a test wavelength of 550 nm and a reference wavelength of 630 nm to obtain sample signal (OD570–OD630). The cell survival fractions were calculated as percentage of the untreated control. In addition, IC_{50} values were derived from the concentration response curves.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.02.034.

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