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Synthesis of novel 3-cyclohexylpropanoic acid-derived nitrogen heterocyclic compounds and their evaluation for tuberculostatic activity

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ABSTRACT

A series of novel 3-cyclohexylpropanoic acid derivatives and 3-cyclohexylpropanoic acid-derived nitrogen heterocyclic compounds (**1–8**) have been synthesized and evaluated for tuberculostatic activity. Compounds **1a**, **1c**, **1e** and **1f** bearing benzimidazole or benzimidazole-like systems showed the most potent tuberculostatic activity against *Mycobacterium tuberculosis* strains with MIC values ranging from 1.5 to 12.5 μg/mL. More importantly **1a** (6-chloro-2-(2-cyclohexylethyl)-4-nitro-1*H*-benzo[*d*]imidazole) and **1f** (2-(2-cyclohexylethyl)-1*H*-imidazo[4,5-*b*]phenazine) appeared selective for *M. tuberculosis* as compared with eukaryotic cells (human fibroblasts), and other antimicrobial strains. These compounds may thus represent a novel, selective class of antitubercular agents. Additionally compound **1a** stimulated type I collagen output by fibroblasts, in vitro.

1. Introduction

Tuberculosis caused by *Mycobacterium tuberculosis* takes the leading place in the reports of incidence and mortality among populations of developed countries.[1] The infection can affect the respiratory system, central nervous system, lymphatic system, genitourinary system, bones, and even skin.[2] In 1990, there were over 6 million new cases, and in 2007 this figure rose to 9.2 million.[3] Furthermore, new multidrug-resistant tuberculosis strains (MDR-TB) are appearing at an alarming speed. The cure of multidrug-resistant tuberculosis requires a longer treatment period, and the success rate is only 52% in newly diagnosed patients, and 29% among previously treated patients.[4]

Tuberculosis is one of the opportunistic infections in AIDS patients. For this reason, it represents a serious threat for this group of individuals. Immune deficiency increases vulnerability incidence to tuberculosis up to 50%.

At the same time there is only a small number of effective antituberculous chemotherapeutics. Among them isoniazid (INH) and pyrazinamide (PZA) belong to the most commonly administrated drugs (Fig. 1). Unfortunately, the most effective chemotherapeutics, as well as antibiotics such as rifampicin (RMP), rapidly induce multidrug resistance (MDR) and cause serious side effects, like hepatotoxicity, neurotoxicity, acute pancreatitis and hypersensitivity reactions.[5,6] Therefore, the search for new antituberculous drugs active against resistant strains of *M. tuberculosis* should be one of the priority tasks of medicinal chemistry.

Most of the administrated drugs belong to the group of nitrogen heterocyclic compounds. That is why potential antituberculous drugs are searched for in this chemical group. Many pyridine and pyrazine derivatives obtained in the course of these studies exhibited high antituberculous activity, for example 4-(5-pentadecyl-1,3,4-oxadiazol-2-yl)pyridine containing 1,3,4-oxadiazole ring fused with pyridine system.[7] Other examples of potent compounds are pyrazinamidine-, pyridinamidine-, pyrazinecarbohydrazide- and pyridinecarbohydrazide-derived hydrazinecarbodithioic acid esters and amides described earlier by us.[8,9] After numerous syntheses of pyridine and pyrazine derivatives, our interest turned in the direction of other nitrogen heterocyclic systems including benzimidazole.

The compounds of the benzimidazole structure described previously, just like their biological effects, are characterized by great variety. An example might be 2-benzylbenzimidazole, for which the SciFinder Database provides over three hundred reports concerning its biological activity. The same 2-benzylbenzimidazole and its derivatives have effects on the cardiovascular system[10]

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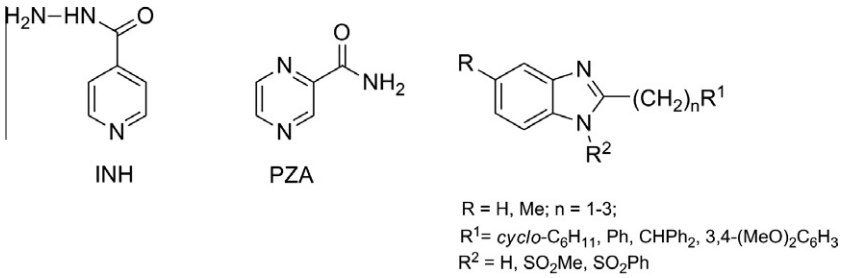


Figure 1. Structures of some tuberculostatic drugs and benzimidazoles of tuberculostatic activity.

and cholesterol level.[11] They exhibit anti-inflammatory and analgesic,[12] antineoplastic,[13] anticonvulsant, antidiabetic[14] and also anthelmintic activity.[15] In addition, benzimidazoles are gonadotropin-releasing hormone (GnRH) receptor antagonists.[16] Their antiviral[17] and antimicrobial[18] activities have been also demonstrated.

However, there are only few reports on the antituberculous activity of benzimidazoles.[19] Significant tuberculostatic activity of 2-phenylalkyl- and 2-cyclohexylalkylbenzimidazoles (Fig. 1) has been presented by us.[20] The designated minimum concentration inhibiting the growth of *M. tuberculosis* strains (MIC) in vitro was at the level appropriate for applied chemotherapeutics.[20,21] We previously found that tested compounds were more active against resistant than sensitive strains and the presence of cyclohexylethyl substituent at position C-2 of the benzimidazole system was important for their activity.

These findings prompted us to extend our studies on the development of novel tuberculostatic agents, here we disclose the synthesis of novel 3-cyclohexylpropanoic acid derivatives.

2. Results and discussion

2.1. Chemistry

We synthesized structures in which the cyclohexylethyl group is connected to the benzimidazole system or systems of benzimidazole type structure, as well as other heterocyclic rings. Derivatives of 3-cyclohexylpropanoic acid hydrazide were also obtained: mono- and diester of hydrazinecarbodithioic acid and appropriate hydrazinecarbothioamides. The synthesized compounds have been screened for their tuberculostatic, antibacterial and cytotoxic activities. Finally, target structures were planned in order to investigate the preliminary structure–activity relationship, with the emphasis on the crucial role of the benzimidazole system.

The synthetic route of the studies was outlined in Scheme 1. Syntheses of the target 3-cyclohexylpropanoic acid derivatives **1–8** were achieved in 36–98% yields by procedures described below. The starting compound for a series of syntheses was the commercially available 3-cyclohexylpropanoic acid. 6-Chloro-2-cyclohexylethyl-4-nitrobenzimidazole **1a** was obtained by the heating of 3-cyclohexylpropanoic acid with 5-chloro-3-nitrobenzene-1,2-diamine according to the method described by Algul et al.[22] and based on the use of polyphosphoric acid (PPA) as a solvent with strong acidic properties (method A).

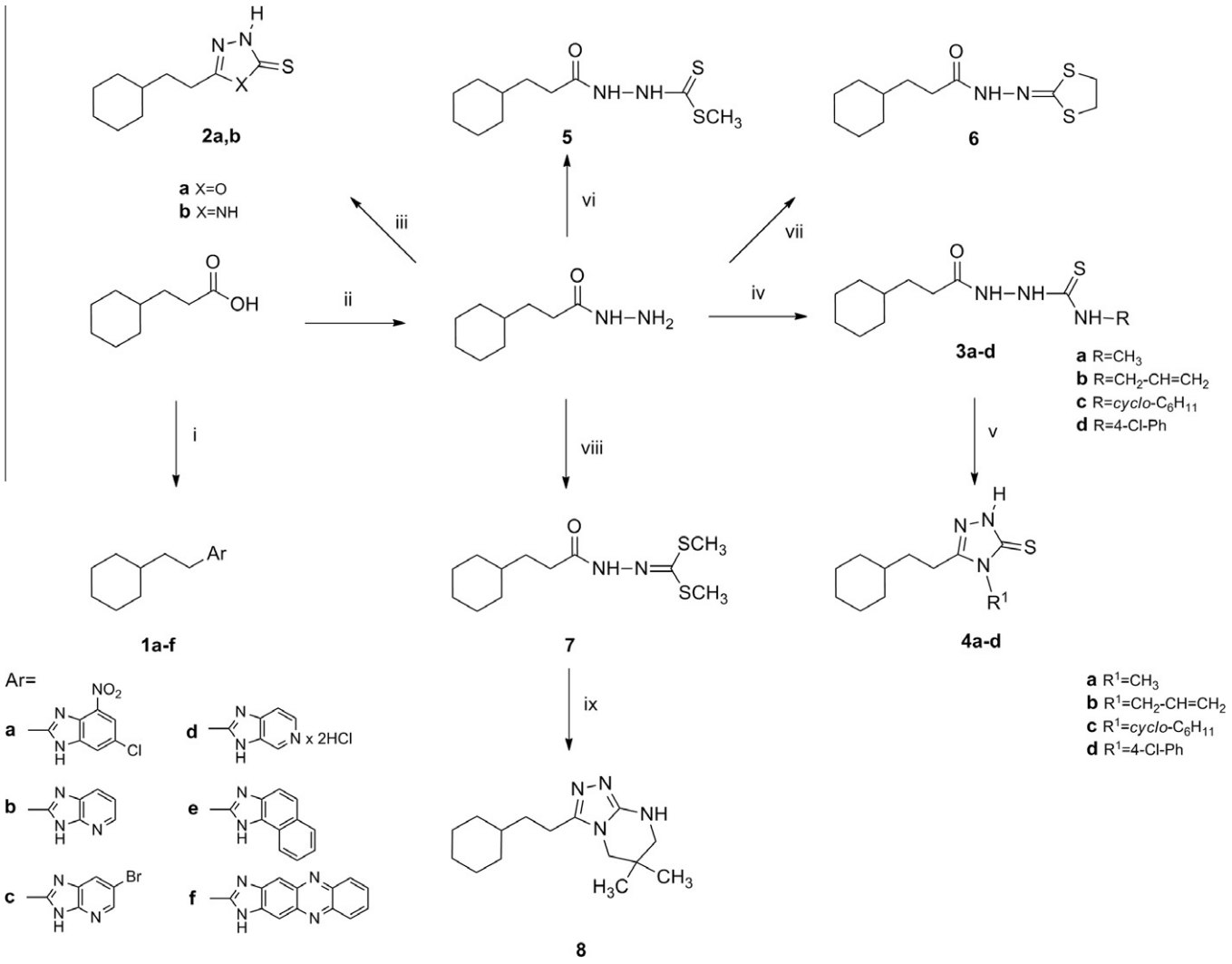
Compounds **2–8** were synthesized from 3-cyclohexylpropanoic acid hydrazide, which was obtained from starting acid via methyl ester in the reaction with hydrazine hydrate. Then, upon treatment of carbon disulfide in an alkaline water–ethanol solution, hydrazide gave 1,3,4-oxadiazole-2-thione derivative **2a**. The same substrate in the reaction with ammonium thiocyanate without solvent underwent cyclization to 1,2,4-triazole-5-thione **2b**. The reaction of hydrazide with appropriate isothiocyanates (methyl, allyl, cyclohexyl, 4-chlorophenyl) yielded compounds **3a–d** of thiosemicarbazide structure. Those derivatives cyclized to 4-substituted 1,2,4-triazole-5-thiones **4a–d** in a 10% solution of lye. Hydrazinecarbodithioic acid esters **5–7** were synthesized in the reaction of 3-cyclohexylpropanoic acid hydrazide with carbon disulfide and suitable halides (methyl iodide, 1,2-dibromoethane) in a methanol solution of triethylamine (TEA). In the reaction with 1,2-dibromoethane a two-fold molar excess of triethylamine was used to obtain 1,3-dithiolane **6**. In the case of methyl diester **7**, a two-fold molar excess of both triethylamine and methyl iodide was needed. That methyl diester was used to obtain 5,6,7,8-tetrahydro-1,2,4-triazolopyrimidine **8** in the reaction with 1,3-diamino-2,2-dimethylpropane. The formation of this kind of compounds has been already reported by our research team.[23]

Promising results of biological studies encouraged us to undertake the synthesis of other benzimidazole type compounds **1b–f**. Imidazopyridines **1b** and **1d** were obtained by the heating of 3-cyclohexylpropanoic acid and appropriate diamine in a diglyme (di(2-dimethoxyethyl) ether) solution (method B). Due to the liquid form of 2-(2-cyclohexylethyl)-3*H*-imidazo[4,5-*c*]pyridine the compound **1d** was synthesized as dihydrochloride. Syntheses of compounds **1c**, **1e** and **1f** were carried out according to the method described above for benzimidazole **1a**.

All the newly synthesized compounds were characterized by IR and ^{1}H NMR spectra as well as the elemental analysis listed in the experimental section. The spectral analyses were in accordance with the assigned structures. The spectral data of compound **6** indicated two multiplets for SCH_2 groups at 3.45 and 3.64 ppm in the ^{1}H NMR spectrum, which suggested the magnetic inequivalence of both groups. A similar phenomenon was observed in the case of the compound **7** with two SCH_3 groups observed in the ^{1}H NMR spectrum as two singlets at 2.46 and 2.51 ppm. Magnetic inequivalence seen in the ^{1}H NMR spectrum of compound **6** was confirmed by the ^{13}C NMR spectrum performed for that derivative. Carbon atoms of SCH_2 groups gave no signal together, but did apart at 37.8 and 39.4 ppm.

2.2. Biological activity

All of the obtained 3-cyclohexylpropanoic acid derivatives **1–8** were evaluated for their in vitro tuberculostatic activity against the *M. tuberculosis* H_{37}Rv strain and two 'wild' strains isolated from tuberculosis patients: one (Spec. 210) resistant to *p*-aminosalicylic acid (PAS), isonicotinic acid hydrazide (INH), etambutol (ETB) and rifampicin (RMP) and the another (Spec. 192) fully sensitive to the administrated tuberculostatics. The MIC values were determined as the minimum concentration inhibiting the growth of tested tuberculous strains in relation to the probe with no tested compound. INH, PZA and RMP were used as reference drugs. Compounds were also tested for their antibacterial activity against *Propionibacterium acnes* (ATCC11827) and *Brevibacterium linens* (ATCC9174). The most active compounds of the benzimidazole structure **1a–f** which exhibited the highest tuberculostatic activity



Scheme 1. Synthesis of novel 3-cyclohexylpropanoic acid derivatives **1**–**8**. Reagents and conditions: (i) method A (compounds **1a**, **1c**, **1e**, **1f**): diamine, PPA, 180–200 °C; $NaHCO_3/H_2O$; method B (compounds **1b**, **1d**): diamine, dyglime, reflux; (ii) hydrazine hydrate, MeOH, reflux; (iii) for **2a**: KOH/H_2O, CS_2, EtOH, reflux; for **2b**: NH_4SCN, 170–180 °C; $NaOH/H_2O$, reflux; (iv) R^4NCS, MeOH, reflux; (v) $NaOH/H_2O$, reflux, conc. HCl; (vi) TEA, CS_2, MeI, EtOH, room trmperature; (vii) TEA (2 equiv), CS_2, 1,2-dibromoethane, EtOH, room temperature; (viii) TEA (2 equiv), CS_2, MeI (2 equiv), EtOH, room temperature; (ix) 1,3-diamino-2,2-dimethylpropane (2 equiv), reflux.

were then tested for effect on the proliferation of neonatal human dermal fibroblasts (ATCC PCS-201–010). MAP (magnesium ascorbyl phosphate) and bFGF (basic fibroblast growth factor) were used as the positive control. Non-cytotoxic compounds **1a**, **1d**, **1f** were additionally tested for effect on type I collagen output in human dermal fibroblast populations by ELISA. Type I collagen is the predominant form of collagen in the body and its decrease is associated with the ageing of many organs, such as skin and tendons. The ELISA test used in the studies detects stimulation of soluble type I collagen by human dermal fibroblast populations, due either to the stimulation of fibroblast proliferation, stimulation of collagen production or the inhibition of metaloproteinases, which digest collagen.

The bioactive data were summarized in Tables 1–3.

2.2.1. Tuberculostatic activity

The results of tuberculostatic activity indicated that most of the synthesized compounds exhibited moderate to low activity against *M. tuberculosis* strains in vitro. As seen in Table 1, compounds **2b–8** exhibited comparable tuberculostatic activity against sensitive (Spec. 192) and resistant (Spec. 210) strains, as well as the standard $H_{37}Rv$ strain. MIC values ranging from 50 to100 μg/mL indicated low tuberculostatic activity against all strain types. Three of the obtained compounds **1b**, **1d** and **2a** exhibited moderate tuberculostatic activity with MIC values in range 12.5–50 μg/mL. Their activities against sensitive and resistant strain were also comparable.

The tuberculostatic activity of benzimidazole **1** and compounds of the benzimidazole type structure **1c**, **1e** and **1f** was much better than the other 3-cyclohexylpropanoic acid derivatives. Among this 'benzimidazole' series, the compound **1a** containing the benzene ring in benzimidazole system and two substituents—a chlorine atom in the C-6 position and a nitro group in the C-4 position—exhibited the weakest tuberculostatic activity with MIC values of 12.5 μg/mL against all tested strains. Compound **1c** containing a pyridine ring instead of benzene in the benzimidazole system and a bromine atom in the C-6 position showed good tuberculostatic activity with MIC values of 6.2 μg/mL. Noticeably, compounds **1e** and **1f** with larger condensed systems instead of benzimidazole exhibited excellent tuberculostatic activity with MIC values at 1.5–3.1 μg/mL. In the **1e** case, we also observed no difference in activity against sensitive and resistant strains.

All of the tested compounds showed tuberculostatic activity lower than INH and RMP used as reference drugs with MIC values at 0.5–1.1 μg/mL and 1.2–2.5 μg/mL respectively. However, they

Table 1
In vitro tuberculostatic and antibacterial activities of compounds **1–8**[a,b,c]

Compds	MIC [μg/mL]				
	M. tuberculosis			*P. acnes*	*B. linens*
	$H_{37}Rv$	Spec. 192	Spec. 210		
1a	12.5	12.5	12.5	>100	>100
1b	25	50	50	>100	>100
1c	6.5	6.2	6.2	>100	>100
1d	25	25	25	>100	>100
1e	1.5	1.5	1.5	>100	>100
1f	3.1	1.5	3.1	>100	>100
2a	25	25	12.5	>100	>100
2b	50	50	25	>100	>100
3a	50	100	50	>100	>100
3b	50	100	50	>100	>100
3c	50	100	50	>100	>100
3d	50	50	50	>100	>100
4a	50	100	50	>100	>100
4b	50	50	50	>100	>100
4c	50	100	50	>100	>100
4d	50	100	50	>100	>100
5	50	50	50	>100	>100
6	50	100	50	>100	>100
7	50	50	50	>100	>100
8	50	100	50	>100	>100
INH	0.5	0.5	1.1	—	—
PZA	25	25	>400	—	—
RMP	1.2	1.2	2.5	—	—

[a] Minimum inhibitory concentrations for bacterial strains were determined by two-fold serial dilution method for microdilution plates and for mycobacterial strains by two-fold classical test-tube method of successive dilution.
[b] **INH** isoniazid; **PZA** pyrazinamide; **RMP** rifampicin.
[c] *M. tuberculosis* $H_{37}Rv$, Spec. 192, Spec. 210, *P. acnes* (ATCC 11827), *B. linens* (ATCC 9174).

Table 2
Effect of compounds **1a-f** on the proliferation of human fibroblasts at compounds concentration 10 and 100 μg/mL.[a,b,c]

Compds	Inhibition (%)	
	10 μg/mL	100 μg/mL
1a	0	18
1b	0	41
1c	17	100
1d	0	0
1e	16	74
1f	0	11
MAP 100 μg/mL	138	
bFGF 15 ng/mL	160	

[a] Effect expressed as % of non-treated control.
[b] MAP and bFGF were used as the positive controls.
[c] Neonatal human dermal fibroblasts (ATCC PCS-201-010).

Table 3
Stimulation of type I collagen output by compound **1a**.[a]

Compound	Collagen concentration (ng/mL]	Stimulation (%)
Control	11.3	100
1a	18.8	166
MAP	141.9	1255
bFGF	23.1	204

[a] Stimulation of type I collagen output as compared with positive controls (bFGF and MAP).

exhibited higher activity than PZA with MIC values at 25 to >400 μg/mL, commonly used as second line chemotherapeutic. Importantly, as opposed to clinically used drugs, these compounds did not have a decreased activity towards resistant strain 210.

The obtained results indicate that tuberculostatic activity of 3-cyclohexylpropanoic acid derivatives was associated with the presence of benzimidazole or other benzimidazole type system in their molecules. The structures containing other nitrogen heterocyclic rings (1,2,4-triazole-5-thione, 1,3,4-oxadiazole-2-thione) or hydrazinecarbothioyl group fused with cyclohexylethyl moiety did not exhibit enhanced antituberculous activity in vitro.

2.2.2. Antibacterial activity against anaerobic strains

Interestingly, all of the synthesized compounds exhibited poor antibacterial activity against the anaerobic bacteria *P. acnes* and *B. linens*, (Table 1), which indicates that their mechanism of action may involve interference with aerobic functions of *M. tuberculosis*. Indeed, this microorganism unusually has a high oxygen requirement for growth.

2.2.3. Cytotoxic activity

The results of cytotoxicity tests have indicated that compounds **1a–f** represent a whole range of cytotoxic activity between 10 μg/mL and 100 μg/mL, from no effect (**1d**), to mildly cytostatic (**1a**, **1f**), to 100% kill (**1c**) (Table 2). It appears that compounds **1a** and **1f** have the best therapeutic potential, because of their high tuberculostatic activity and no (or low) cytotoxicity to tested eukaryotic cells.

2.2.4. Effect of 1a on type I collagen output

Besides having substantial anti-tuberculotic activity and no cytotoxic effect on fibroblasts, the compound **1a** stimulated type I collagen output in a statistically-significant manner at 100 μg/mL (Table 3). This stimulation was not due to the increase of cell number, as measured by the MTT assay. Furthermore, it appears to be selective to type I collagen, as measured by the sulforhodamine B total insoluble protein stain. No other tested compounds and concentrations stimulated type I collagen output. Benzimidazole **1a** seems to be the most interesting among three tested compounds and further investigations of its mechanism of collagen I stimulation by itself and its derivatives is greatly warranted.

3. Conclusions

In conclusion, a series of novel 3-cyclohexylpropanoic acid derivatives were successfully synthesized. All these new compounds were confirmed by IR and ^{1}H NMR spectra as well as elemental analysis. Their tuberculostatic activity was evaluated against *M. tuberculosis* $H_{37}Rv$, Spec. 192 and Spec. 210 strains, using the two-fold serial dilution MIC method. The results showed that most of the synthesized derivatives exhibited moderate tuberculostatic activity; however compounds **1a**, **1c**, **1e** and **1f** bearing benzimidazole or benzimidazole-like systems exhibited potent tuberculostatic activity with MIC values ranging from 1.5 to 12.5 μg/mL. More importantly, active compounds **1a** and **1f** exhibited no cytotoxic effect on the proliferation of neonatal human dermal fibroblasts in vitro. Compound **1a** also stimulated type I collagen output. These findings demonstrated that 2-cyclohexylethylbenzimidazoles are of biological significance, with the potential to become a new member of tuberculostatic agents.

4. Experimental

4.1. Chemistry

All materials and solvents were of analytical reagent grade. Thin-layer chromatography was performed on Merck silica gel $60F_{254}$ plates and visualized with UV. The results of elemental analyses (% C, H, N) for all of obtained compounds were in agreement with calculated values within ±0.3% range. ^{1}H NMR spectra and ^{13}C NMR spectra in $CDCl_3$ or DMSO-d_6 were recorded on Varian Unity Plus (500 MHz) and Varian Gemini (200 MHz) instruments.

IR Spectra (KBr) were determined as KBr pellets of the solids on a Satellite FT-IR spectrophotometer. Melting points were determined on a BOETIUS apparatus and were uncorrected.

4.1.1. Synthesis of 3-cyclohexylpropanoic acid hydrazide

A stirred solution of 3-cyclohexylpropanoic acid (10 mL, 64 mmol) and 3 mL of thionyl chloride (137 mmol) in 30 mL of methanol was refluxed for 3 h. Then methanol was evaporated, residue was neutralized with a saturated solution of sodium hydrogen carbonate and extracted with dichloromethane (2 × 40 mL). After drying, the solvent was evaporated, and 40 mL of methanol and 10 mL of 98% hydrazine hydrate were added to the oily residue. The mixture was refluxed for 3 h. Then methanol was evaporated, and the residue was treated twice with 30 mL of benzene, which in each case was evaporated. The oily residue crystallized when cooled on ice. The step of recrystallization from methanol yielded 10 g (90%) of hydrazide, which was obtained as colourless crystals melting at 92 °C, in agreement with literature data.[24]

4.1.2. General procedure for the synthesis of arenoimidazoles (1a–f)

Method A: 3-Cyclohexylpropanoic acid (15 mmol), appropriate diamine (10 mmol) and PPA (5 mL) were heated and stirred at 180–200 °C for 5 h, then the mixture was allowed to cool to ambient temperature and poured into cold water (50 mL). The mixture was neutralized with a saturated solution of sodium hydrogen carbonate and the resulting precipitate was filtered off, washed several times with water and recrystallized from a methanol/water mixture (1:1) with the addition of activated carbon.

Method B: 3-Cyclohexylpropanoic acid (2 mL, 12 mmol) was added to a stirred mixture of appropriate diaminopyridine (1.09 g, 10 mmol) and 2 mL of diglyme. The mixture was refluxed for 3 h. After cooling, 20 g of ice was added. The precipitate was filtered and purified. The hot methanol solution of crude product with the addition of activated carbon was filtered and cooled. Then the product was precipitated by the water addition, filtered off, dried and recrystallized again.

4.1.2.1. 6-Chloro-2-(2-cyclohexylethyl)-4-nitro-1*H*-benzo[*d*]imidazole (1a). Compound **1a** was obtained by method A as pale yellow crystals in 84% yield (2.6 g), mp 190–192 °C. IR (KBr): 3095, 3015, 2925, 2849 (*ν* C–H), 1517 (*ν* NO_2), 1462 (*ν* C=C), 1402, 1339 (*ν* NO_2), 1279 (*δ* C–H), 898 (*γ* C–H), 671 (ν C–Cl) cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): *δ* 0.99 (t, 2H, CH_2, *J* = 12 Hz), 1.11–1.41 (m, 4H, $2CH_2$), 1.64–1.85 (m, 7H, 6H $3CH_2$ and 1H CH), 3.01 (t, 2H, CH_2, *J* = 7.5 Hz), 7.99 (m, 1H, Ph), 8.10 (m, 1H, Ph), 10.35 (br s, 1H, NH) ppm; ^{13}C NMR (200 MHz, $CDCl_3$): *δ* 26.2, 26.9, 27.3, 33.5, 35.7, 37.9, 119.1, 121.8, 126.8, 127.6, 132.9, 147.3, 160.0 ppm. Anal. Calcd for $C_{15}H_{18}ClN_3O_2$ (307.78): C, 58.54; H, 5.89; N, 13.65. Found: C, 58.48; H, 5.88; N, 13.64.

4.1.2.2. 2-(2-Cyclohexylethyl)-3*H*-imidazo[4,5-*b*]pyridine (1b). Compound **1b** was obtained by method B as colourless crystals (after crystallization from toluene) in 94% yield (3 g), mp 139–140 °C. IR (KBr) 3250 (*ν* N–H), 3033, 2922, 2851 (*ν* C–H), 1643, 1569 (*δ* N–H), 1447 (*ν* C=C), 1398 (*δ* C–H), 889, 763 (*γ* C–H), 591 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): *δ* 0.92 (t, 2H, CH_2, *J* = 12 Hz), 1.15–1.32 (m, 4H, $2CH_2$), 1.55–1.74 (m, 7H, 6H $3CH_2$ and 1H CH), 2.33 (t, 2H, CH_2, *J* = 7.7 Hz), 6.54–6.60 (m, 1H, pyridine), 6.89–6.93 (m, 1H, pyridine), 7.35–7.38 (m, 1H, pyridine), 11.20 (br s, 1H, NH) ppm. Anal. Calcd for $C_{14}H_{19}N_3$ (229.32): C, 73.33; H, 8.35; N, 18.32. Found: C, 73.45; H, 8.33; N, 18.35.

4.1.2.3. 6-Bromo-2-(2-cyclohexylethyl)-3*H*-imidazol[4,5-*b*]pyridine (1c). Compound **1c** was obtained by method A as white solid in 82% yield (2.5 g), mp 229–231 °C. IR (KBr) 3089, 2921, 2850 (*ν* C–H), 1621, 1535 (*δ* N–H), 1425, 1394 (*ν* C=C), 1266 (*δ* C–H), 930 (*γ* C–H), 685 cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6): *δ* 0.92, (t, 2H, CH_2, *J* = 11 Hz), 1.11–1.21 (m, 4H, $2CH_2$), 1.51–1.75 (m, 7H, 6H $3CH_2$ and 1H CH), 2.83 (t, 2H, CH_2, *J* = 7.5 Hz), 8.13 (d, 1H, pyridine, *J* = 1.8 Hz), 8.31 (d, 1H, pyridine, *J* = 1.8 Hz), 11.08 (br s, 1H, NH) ppm. Anal. Calcd for $C_{14}H_{18}BrN_3$ (308.22): C, 54.56; H, 5.89; N, 13.63. Found: C, 54.62; H, 5.89; N, 13.63.

4.1.2.4. 2-(2-Cyclohexylethyl)-3*H*-imidazo[4,5-*c*]pyridine dihydrochloride (1d). In the case of compound **1d** obtained by method B, the water solution was extracted with dichloromethane (3 × 8 mL). Organic fractions were collected, dried with $MgSO_4$ and evaporated. The crude product was diluted with 10 mL of methanol and treated with 10 mL of dry diethyl ether saturated with hydrochloride. The precipitate was filtered, dried and recrystalized. Compound **1d** in dihydrochloride form was obtained as white solid (after crystallization from the methanol/ethyl ether mixture 1:1) in 36% yield (1 g), mp 148–150 °C. IR (KBr): 3024, 2920, 2849 (*ν* C–H), 2572 (*ν* $^+$N–H), 1639, 1497, 1473 (*ν* C=C), 1405 (*δ* C–H), 1226, 801 (*γ* C–H), 626 cm^{-1}; ^1H NMR for free amine (500 MHz, DMSO-d_6): *δ* 1.02–1.08 (m, 2H CH_2) 1.19–1.44 (m, 4H, $2CH_2$), 1.69–1.90 (m, 7H, 6H $3CH_2$ and 1H CH), 3.24 (t, 2H, CH_2, *J* = 8.0 Hz), 8.27 (d, 1H, pyridine, *J* = 6.3 Hz), 8.72 (d, 1H, pyridine, *J* = 6.3 Hz), 9.40 (s, 1H, pyridine), 11.18 (br s, 1H, NH) ppm. Anal. Calcd for $C_{14}H_{19}N_3 \times 2HCl$ (302.24): C, 55.63; H, 7.00; N, 13.90. Found: C, 55.68; H, 7.01; N, 13.87.

4.1.2.5. 2-(2-Cyclohexylethyl)-1*H*-naphto[2,3-*d*]imidazole (1e). This compound was obtained by method A as beige solid in 87% yield (2.42 g), mp 157–158 °C. IR (KBr) 2923, 2851 (*ν* C–H), 1564 (*δ* N–H), 1449, 1384 (*ν* C=C), 1087, 1004, 809, 754 (*γ* C–H), 523 cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6): *δ* 0.96 (t, 2H, CH_2, *J* = 12 Hz), 1.13–1.44 (m, 4H, $2CH_2$), 1.62–1.80 (m, 7H, 6H CH_2 and 1H CH), 2.93 (t, 2H, CH_2, *J* = 7.8 Hz), 7.43 (t, 1H, ArH, *J* = 7.2 Hz), 7.57 (t, 1H, ArH, *J* = 7.1 Hz), 7.96 (d, 1H, ArH, *J* = 8.1 Hz), 8.36 (d, 1H, ArH, *J* = 7.9 Hz), 10.46 (br s, 1H, NH) ppm. Anal. Calcd for $C_{19}H_{22}N_2$ (278.39): C, 81.97; H, 7.97; N, 10.06. Found: C, 82.11; H, 7.99; N, 10.04.

4.1.2.6. 2-(2-Cyclohexylethyl)-1*H*-imidazo[4,5-*b*]phenazine (1f). Compound **1f** was obtained by method A as yellow solid in 90% yield (2.96 g), mp >270 °C (decomp.). IR (KBr) 3085, 2921, 2849 (*ν* C–H), 1563, 1535 (*δ* N–H), 1425 (*ν* C=C), 1310, 1220, 752 (*γ* C–H) cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): 0.87 (t, 2H, CH_2, *J* = 12 Hz), 1.01–1.38 (m, 4H, $2CH_2$), 1.58–1.70 (m, 5H, 4H $2CH_2$ and 1H CH), 1.76–1.87 (m, 2H, CH_2), 3.02 (t, 2H, CH_2, *J* = 7.8 Hz), 7.71–7.76 (m, 2H, ArH), 8.15–8.20 (m, 2H, ArH), 8.34 (s, 2H, ArH), 10.52 (br s, 1H, NH) ppm; ^{13}C NMR (200 MHz, $CDCl_3$): *δ* 26.5, 26.8, 28.0, 33.4, 35.5, 37.9, 129.5, 130.1, 140.9, 142.8, 165.1 ppm. Anal. Calcd for $C_{21}H_{22}N_4$ (330.43): C, 76.33; H, 6.71; N, 16.96. Found: C, 76.42; H, 6.72; N, 16.95.

4.1.3. Synthesis of 5-(2-cyclohexylethyl)-1,3,4-oxadiazole-2(3*H*)-thione (2a)

3-Cyclohexylpropanoic acid hydrazide (0.85 g, 5 mmol) was dissolved in a solution of 1 g (17 mmol) of KOH in 3 mL of water and 20 mL of ethanol. Then 1 mL (17 mmol) of carbon disulfide was added and the mixture was refluxed for 4 h. After ethanol evaporation, 10 mL of water was added and the solution was acidified with concentrated hydrochloric acid. The precipitate was filtered off, dried and recrystallized from cyclohexane/petroleum ether mixture (1:1) giving colourless needles in 70% yield (0.74 g), mp 81–82 °C. IR (KBr): 3100 (*ν* N–H), 2925, 2850 (*ν* C–H), 1616, 1527 (*δ* N–H), 1451 (*δ* C–H), 1191 (*γ* C–O), 975, 956 (*γ* C–H), 758, 667 (*γ* N–H) cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): *δ* 0.94 (t, 2H, CH_2, *J* = 11 Hz), 1.03–1.39 (m, 4H, $2CH_2$), 1.56–1.75 (m, 7H,

6 H $3CH_2$ and 1H CH), 2.72 (t, 2H, CH_2, J = 7.7 Hz), 11.60 (br s, 1H, NH) ppm. Anal. Calcd for $C_{10}H_{16}N_2OS$ (212.31): C, 56.57; H, 7.60; N, 13.19. Found: C, 56.62; H, 7.61; N, 13.21.

4.1.4. Synthesis of 3-(2-cyclohexylethyl)-1*H*-1,2,4-triazole-5(4*H*)-thione (2b)

3-Cyclohexylpropanoic acid hydrazide (1.7 g, 10 mmol) and ammonium thiocyanate (1.9 g, 25 mmol) were heated in a metal bath at 170–180 °C for 0.5 h. After cooling to ambient temperature, 25 mL of 10% NaOH aqueous solution was added and the mixture was refluxed for 2 h. Then the mixture was cooled and acidified with acetic acid. The precipitate was filtered off and recrystallized from ethanol giving 1.6 g (75%) of colourless prisms, mp 233–234 °C. IR (KBr): 3115 (ν N–H), 2922, 2850 (ν C–H), 1604 (δ N–H), 1480 (δ C–H), 1026 (ν N–N), 986 (γ C–H), 798, 669 (γ N–H) cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6): δ 0.88 (t, 2H, CH_2, J = 11 Hz), 1.05–1.27 (4H, $2CH_2$), 1.50–1.74 (m, 7H, $3CH_2$ and 1H CH), 2.47–2.55 (m, 2H, CH_2), 13.11–13.25 (br s, 2H, 2NH) ppm. Anal. Calcd for $C_{10}H_{17}N_3S$ (211.33): C, 56.83; H, 8.11; N, 19.88. Found: C, 56.92; H, 8.13; N, 19.88.

4.1.5. General procedure for the synthesis of hydrazinecarbothioamides (3a–d)

3-Cyclohexylpropanoic acid hydrazide (5 mmol) was refluxed in 5 mL of methanol with addition of stechiometric quantity (5 mmol) of appropriate isothiocyanate (methyl, allyl, cyclohexyl, 4-chlorophenyl). Then the mixture was cooled, precipitate was filtered off and recrystallized from suitable solvent.

4.1.5.1. 2-(3-Cyclohexylpropanoyl)-*N*-methylhydrazinecarbothioamide (3a). Compound **3a** (0.92 g) was obtained as white solid in 77% yield, mp 173–174 °C after crystallization from methanol. IR (KBr): 3285, 3138 (ν N–H), 2926, 2850 (ν C–H), 1695 (ν C=O), 1564 (δ N–H), 1447 (δ C–H), 1273, 1220 (ν C–N), 1043 (ν N–N), 574 (γ N–H) cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6): δ 0.87 (t, 2H, CH_2, J = 11 Hz), 1.15–1.26 (m, 4H, $2CH_2$), 1.35–1.46 (m, 2H, CH_2), 1.58–1.74 (m, 5H, $2CH_2$ and 1H CH), 2.16 (t, 2H, CH_2), 2.84 (s, 3H, NCH_3), 7.80 (s, 1H, NH), 9.11 (s, 1H, NH), 9.58 (s, 1H, NH) ppm; ^{13}C NMR (200 MHz, DMSO-d_6): δ 26.0, 26.4, 31.1, 32.2, 32.9, 37.0, 172.5, 182.5 ppm. Anal. Calcd for $C_{11}H_{21}N_3OS$ (243.37): C, 54.29; H, 8.70; N, 17.27. Found: C, 54.13; H, 8.69; N, 17.28.

4.1.5.2. *N*-Allyl-2-(3-cyclohexylpropanoyl)hydrazinecarbothioamide (3b). Compound **3b** (1.2 g) was obtained as colourless crystals in 89% yield, mp 132–134 °C after crystallization from methanol/water mixture (1:1). IR (KBr): 3275, 3182 (ν N–H), 2922, 2851 (ν C–H), 1689 (ν C=O), 1544 (δ N–H), 1196 (ν C–N), 969 (γ C–H), 621 (γ N–H) cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): δ 0.91 (t, 2H, CH_2, J = 11 Hz), 1.09–1.32 (m, 4H, $2CH_2$), 1.48–1.72 (m, 7H, $3CH_2$ and 1H CH), 2.32 (t, 2H, CH_2, J = 7.4 Hz), 4.22 (d, 2H, CH_2, J = 5 Hz), 5.15–5.28 (m, 2H, CH_2), 5.79–5.98 (m, 1H, CH), 8.15 (br s, 1H, NH), 9.00 (br s, 1H, NH), 9.60 (br s, 1H, NH) ppm. Anal. Calcd for $C_{13}H_{23}N_3OS$ (269.41): C, 57.96; H, 8.61; N, 15.60. Found: C, 57.85; H, 8.60; N, 15.58.

4.1.5.3. *N*-Cyclohexyl-2-(3-cyclohexylpropanoyl)hydrazinecarbothioamide (3c). Compound **3c** (1.1 g) was obtained as white solid in 71% yield, mp 158–160 °C after crystallization from dioxane/water mixture (1:1). IR (KBr): 3295, 3189 (ν N–H), 2924, 2852 (ν C–H), 1685 (ν C=O), 1556 (δ N–H), 1450 (δ C–H), 1212 (ν C–N), 985 (γ C–H), 586 (γ N–H) cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): δ 0.92 (t, 2H, CH_2, J = 11 Hz), 1.15–1.80 (m, 19H, 18H $9CH_2$ and 1H CH), 1.99–2.05 (m, 2H, CH_2), 2.32 (t, 2H, CH_2, J = 7.6 Hz), 3.98–4.28 (m, 1H, CH), 7.00 (br s, 1H, NH), 8.1 (br s, 1H, NH), 9.20 (br s, 1H, NH) ppm. Anal. Calcd for $C_{16}H_{29}N_3OS$ (311.49): C, 61.69; H, 9.38; N, 13.49. Found: C, 61.73; H, 9.40; N, 13.50.

4.1.5.4. *N*-(4-Chlorophenyl)-2-(3-cyclohexylpropanoyl)hydrazinecarbothioamide (3d). Compound **3d** (1.5 g) was obtained as colourless needles in 88% yield, mp 160–161 °C after crystallization from dioxane. IR (KBr): 3363, 3243 (ν N–H), 2923, 2852 (ν C–H), 1681 (ν C=O), 1524, 1491 (ν C=C), 1088, 1015, 830 (γ C–H), 725 (C–Cl) cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): δ 0.83 (t, 2H, CH_2, J = 11 Hz), 1.14–1.30 (m, 4H, $2CH_2$), 1.49–1.69 (m, 7H, 6H $3CH_2$ and 1H CH), 2.37 (t, 2H, CH_2, J = 7.4 Hz), 7.29 (d, 2H, Ph, J = 8.8 Hz), 7.50 (d, 2H, Ph, J = 8.8 Hz), 9.17 (br s, 1H, NH), 10.15 (br s, 1H, NH), 10.50 (br s, 1H, NH) ppm. Anal. Calcd for $C_{16}H_{22}ClN_3OS$ (339.88): C, 56.54; H, 6.52; N, 12.36. Found: C, 56.48; H, 6.53; N, 12.37.

4.1.6. General procedure for the synthesis of 1,2,4-triazole-3-thiones (4a–d)

Appropriate hydrazinecarbothioamide **3a–d** (2 mmol) was refluxed in 20 mL of 10% NaOH aqueous solution for 2 h. The hot mixture was filtered, cooled and acidified with concentrated hydrochloric acid to the precipitation of 1,2,4-triazole-3-thione. The crude product was filtered and recrystalized from ethanol/water mixture (1:1).

4.1.7.1. 3-(2-Cyclohexylethyl)-4-methyl-1H-1,2,4-triazole-5(4*H*)-thione (4a). Compound **4a** (0.4 g) was obtained as colourless prisms in 89% yield, mp 171–172 °C. IR (KBr): 3113, 3055 (ν N–H), 2922, 2849 (ν C–H), 1576 (δ N–H), 1499, 1450 (δ C–H), 1343, 1083, 968 (γ C–H), 758 (γ N–H) cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): δ 0.96 (t, 2H, CH_2, J = 11 Hz), 1.12–1.42 (m, 4H, $2CH_2$), 1.55–1.76 (m, 7H, 6H $3CH_2$ and 1H CH), 2.65 (t, 2H, CH_2, J = 8.1 Hz), 3.53 (s, 3H, NCH_3), 11.80 (br s, 1H, NH) ppm. Anal. Calcd for $C_{11}H_{19}N_3S$ (225.35): C, 58.63; H, 8.50; N, 18.65. Found: C, 58.69; H, 8.48; N, 18.67

4.1.7.2. 4-Allyl-3-(2-cyclohexylethyl)-1*H*-1,2,4-triazole-5(4*H*)-thione (4b). Compound **4b** (0.49 g) was obtained as white solid in 98% yield, mp 120–171 °C. IR (KBr): 3098, 3052 (ν N–H), 2923, 2849 (ν C–H), 1573 (δ N–H), 1501, 1445 (δ C–H), 1366, 1273 (ν C–N), 917 (γ C–H), 787 (γ N–H) cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): δ 0.88 (t, 2H, CH_2, J = 11 Hz), 1.15–1.37 (m, 4H, $2CH_2$), 1.56–1.74 (m, 7H, 6H $3CH_2$ and 1H CH), 2.62 (t, 2H, CH_2, J = 7.8 Hz), 4.65 (d, 2H, CH_2, J = 5.3 Hz), 5.20 (dd, 2H, CH_2, J_1 = 17 Hz, J_2 = 11 Hz), 5.79–5.98 (m, 1H, CH), 11.70 (br s, 1H, NH) ppm. Anal. Calcd for $C_{13}H_{21}N_3S$ (251.39): C, 62.11; H, 8.42; N, 16.72. Found: C, 62.18; H, 8.42; N, 16.70.

4.1.7.3. 4-Cyclohexyl-3-(2-cyclohexyl)-1*H*-1,2,4-triazole-5(4*H*)-thione (4c). Compound **4c** (0.47 g) was obtained as colourless needles in 84% yield, mp 157–158 °C. IR (KBr): 3125, 3046 (ν N–H), 2923, 2851 (ν C–H), 1562 (δ N–H), 1502, 1448 (δ C–H), 1290 (ν C–N), 983 (γ C–H), 739 (γ N–H) cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): δ 0.97 (t, 2H, CH_2, J = 11 Hz), 1.10–1.40 (m, 21H, 20H $10CH_2$ and 1H CH), 2.72 (t, 2H, CH_2, J = 7.8 Hz), 4.35–4.65 (m, 1H, CH), 11.80 (br s, 1H, NH) ppm. Anal. Calcd for $C_{16}H_{27}N_3S$ (293.47): C, 65.48; H, 9.27; N, 14.32. Found: C, 65.52; H, 9.29; N, 14.33.

4.1.7.4. 4-(4-Chlorophenyl)-3-(2-cyclohexylethyl)-1*H*-1,2,4-triazole-5(4*H*)-thione (4d). Compound **4d** (0.51 g) was obtained as white solid in 80% yield, mp 210–212 °C. IR (KBr): 3089 (ν N–H), 2925, 2848 (ν C–H), 1570 (δ N–H), 1495, 1487 (ν C=C), 1331, 1092, 1008, 840 (γ C–H), 728 (ν C–Cl) cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 0.82 (t, 2H, CH_2, J = 12 Hz), 1.07–1.29 (m, 4H, $2CH_2$), 1.47–1.51 (m, 2H, CH_2), 1.59–1.68 (m, 5H, 4H $2CH_2$ and 1H CH), 2.50 (t, 2H, CH_2, J = 7.8 Hz), 7.32 (d, 2H, Ph, J = 7.8 Hz), 7.57 (d, 2H, Ph, J = 8.3 Hz),

11.80 (br s, 1H, NH) ppm. Anal. Calcd for $C_{16}H_{20}ClN_3S$ (321.87): C, 59.70; H, 6.26; N, 13.06. Found: C, 59.54; H, 6.25; N, 13.04.

4.1.8. Synthesis of methyl 2-(3-cyclohexylpropanoyl) hydrazinecarbodithioate (5)

3-Cyclohexylpropanoic acid hydrazide (1.7 g, 10 mmol) was dissolved in 10 mL of ethanol. Then triethylamine (1.4 mL, 10 mmol), carbon disulfide (0.60 mL, 10 mmol) and methyl iodide (0.62 mL, 10 mmol) were added. The mixture was stirred at room temperature for 0.5 h. Then the mixture was cooled and precipitate was filtered off, dried and recrystallized from toluene giving 1.5 g (58%) of colourless crystals, mp 122–124 °C. IR (KBr) 3171, 3052 (ν N–H), 2970, 2919, 2849 (ν C–H), 1688 (ν C═O), 1546 (δ N–H), 1220 (ν C–N), 1056, 618 (γ N–H) cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): δ 0.87–1.82 (m, 12H $6CH_2$), 2.35 (t, 2H, CH_2, J = 7.5 Hz), 2.63 (s, 3H, SCH_3), 3.15–3.24 (m, 1H, CH), 9.60 (br s, 1H, NH), 10.70 (br s, 1H, NH) ppm. Anal. Calcd for $C_{11}H_{20}N_2OS_2$ (260.42): C, 50.73; H, 7.74; N, 10.76. Found: C, 50.67; H, 7.76; N, 10.76.

4.1.9. Synthesis of 3-cyclohexyl-*N*′-(1,3-dithiolan-2-ylidene)propanehydrazide (6)

3-Cyclohexylpropanoic acid hydrazide (1.7 g, 10 mmol) was dissolved in 10 mL of ethanol. Then triethylamine (2.8 mL, 20 mmol) and carbon disulfide (0.60 mL, 10 mmol) were added and the mixture was stirred at room temperature for 0.5 h. After that time 1,2-dibromoethane (0.86 mL, 10 mmol) was added and the mixture was stirred for another 0.5 h. Then 20 g of ice was added and the oily product precipitated, which crystallized when frozen for few hours. The crude solid was filtered off, dried and recrystallized from cyclohexane/petroleum ether mixture (1:1) giving 2.5 g (93%) of bright solid, mp 91–92 °C. IR (KBr) 3188, 3017 (ν N–H), 2922, 2850 (ν C–H), 1679 (ν C═OC═O), 1638, 1579, 1534 (δ N–H), 1448 (δ C–H), 1280 (ν C–N), 1121, 1033, 852 (γ C–H) cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): δ 0.83 (t, 2H, CH_2, J = 10 Hz), 1.12–1.32 (m, 4H, $2CH_2$), 1.65–1.75 (m, 7H, 6H $3CH_2$ and 1H CH), 2.55 (t, 2H, SCH_2, J = 7.7 Hz), 3.42–3.52 (m, 2H, SCH_2), 3.61–3.69 (m, 2H, SCH_2), 7.89 (br s, 1H, NH) ppm; ^{13}C NMR (200 MHz, $CDCl_3$): δ 26.8, 27.1, 30.7, 32.3, 33.6, 35.8, 37.8, 39.4, 154.4, 175.7 ppm. Anal. Calcd for $C_{12}H_{20}N_2OS_2$ (272.43): C, 52.90; H, 7.40; N, 10.28. Found: C, 52.81; H, 7.39; N, 10.27.

4.1.10. Synthesis of dimethyl 3-cyclohexylpropanoylcarbonohydrazonodithioate (7)

3-Cyclohexylpropanoic acid hydrazide (0.8 g, 5 mmol) was dissolved in 10 mL of methanol. Then triethylamine (1.4 mL, 10 mmol), carbon disulfide (0.30 mL, 5 mmol) and methyl iodide (0.62 mL, 10 mmol) were added. The mixture was stirred at room temperature for 2 h and 40 g of ice was added. The crude product precipitate was filtered off, dried and recrystallized from petroleum ether giving 1.2 g (89%) of white solid, mp 45–46 °C. IR (KBr) 3166, 3073 (ν N–H), 2992, 2915, 2849 (ν C–H), 1664 (ν C═O), 1450 (δ C–H), 1375, 1102, 942 (γ C–H), 587 (γ N–H) cm-1; ^1H NMR (500 MHz, $CDCl_3$): δ 0.89–0.97 (m, 2H, CH_2), 1.12–1.32 (m, 4H, $2CH_2$), 1.57–1.76 (m, 7H, 6H $3CH_2$ and 1H CH), 2.45 (s, 3H, SCH_3), 2.51 (s, 3H, SCH_3), 2.64 (t, 2H, CH_2, J = 8.3 Hz), 8.91 (br s, 1H, NH) ppm. Anal. Calcd for $C_{12}H_{22}N_2OS_2$ (274.45): C, 52.52; H, 8.08; N, 10.21. Found: C, 52.46; H, 8.06; N, 10.22.

4.1.11. Synthesis of 3-(2-cyclohexylethyl)-6,6-dimethyl-5,6,7,8-tetrahydro-[1,2,4] triazolo[4,3-*a*]pyrimidine (8)

Compound **7** (1.7 g, 6.4 mmol) was refluxed with 1,3-diamino-2,2-dimethylpropane (1.5 mL, 12.7 mmol) for 1.5 h. Then 20 g of ice was added and product precipitated. The crude product was filtered off, dried and recrystallized from dioxane giving 0.6 g (36%) of white solid, mp 156–158 °C. IR (KBr) 3241, 3168, 3108 (ν N–H), 2923, 2849 (ν C–H), 1630, 1546 (δ N–H), 1447 (δ C–H), 1299 (ν C–N), 1121, 1105 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): δ 0.95–0.97 (m, 2H, CH_2), 1.11 (s, 6H, $2CH_3$), 1.16–1.28 (m, 2H, CH_2), 1.54–1.79 (m, 7H, 6H $3CH_3$ and 1H CH), 2.57 (t, 2H, CH_2, J = 8.3 Hz), 3.08 (s, 2H, CH_2), 3.43 (s, 2H, CH_2), 3.71 (s, 2H, CH_2), 5.65 (br s, 1H, NH) ppm; ^{13}C NMR (200 MHz, $CDCl_3$): 22.9, 24.7, 26.7, 33.5, 34.9, 37.7, 51.4, 52.6, 150.2, 153.1 ppm. Anal. Calcd for $C_{15}H_{26}N_4$ (262.39): C, 68.66; H, 9.99; N, 21.35. Found: C, 68.78; H, 9.97; N, 21.33.

4.2. Biological assays

4.2.1. Tuberculostatic assay

The synthesized compounds were examined in vitro for their tuberculostatic activity against the *M. tuberculosis* $H_{37}Rv$ strain and two 'wild' strains isolated from tuberculosis patients: one (Spec. 210) resistant to *p*-aminosalicylic acid (PAS), isonicotinic acid hydrazide (INH), etambutol (ETB) and rifampicine (RMP) and the another (Spec. 192) fully sensitive to the administrated tuberculostatics. Investigations were performed by a classical test-tube method of successive dilution in Youmans' modification of the Proskauer and Beck liquid medium containing 10% of bovine serum.[25,26] Bacterial suspensions were prepared from 14 days old cultures of slowly growing strains and from 48 h old cultures of saprophytic strains.[27,28] Solutions of compounds in ethylene glycol were tested. Stock solutions contained 10 mg of compounds in 1 millilitre. Dilutions (in geometric progression) were prepared in Youmans' medium. The medium containing no investigated substances and containing isoniazid (INH), pyrazinamide (PZA) or rifampicin (RMP) as reference drugs were used for comparison. Incubation was performed at a temperature of 37 °C. The MIC values were determined as minimum concentration inhibiting the growth of tested tuberculous strains in relation to the probe with no tested compound. The influence of the compound on the growth of bacteria at a certain concentration, 3.1, 6.2, 12.5, 25, 50 and 100 μg/mL, were evaluated.

4.2.2. Anaerobic bacteria assay

Compounds **1–8** immediately before use were dissolved in 100% DMSO at 5 mg/mL and further to 1 mg/mL in 10% DMSO. Compounds were tested at serial dilutions in bacterial broth starting at 100 μg/mL (final concentration). *P. acnes* (ATCC11827) was grown in thioglycolate nutrient broth (Hardy Diagnostics K29) for 72 h at 33 °C, then inoculated at the density equivalent to 0.5 McFarland standard and incubated with the compounds for another 72 h in an anaerobic environment. *B. linens* (ATCC9174) culture was started from an agar plate, grown in nutrient broth (Hardy Diagnostics K243) for 24 h at 30 °C, then inoculated at the density equivalent to 1 MsFarland standard and incubated with tested compounds for another 24 h. At the end of the incubation MIC was assessed optically as the lowest concentration of a compound, which caused no bacteria growth.[29] This optical assessment was further confirmed by measuring the absorbance at 655 nm with the BioRad 3550-UV microplate reader. MIC was defined as at least 50% inhibition of the control's (vehicle) OD (optical density).

4.2.3. Cytotoxicity and type I collagen quantification assay

Compounds were dissolved in DMSO at 20 mg/mL and tested at 100 μg/ml and 10 μg/mL. Magnesium ascorbyl phosphate (MAP, 100 μg/mL and basic fibroblast growth factor (bFGF, 15 ng/mL) were used as positive controls. Neonatal human dermal fibroblasts (ATCC PCS-201–010) were plated in high glucose DMEM with 5% cosmic calf serum (Hyclone, Salt Lake City, UT). Cells were seeded at a density of 3×10^3 cells/well in 96 well plates and were exposed to compounds tested for 72 h. The fibroblast number and total insoluble proteins were quantified using

the MTT and sulforhodamine B methods as described earlier.[30,31] Briefly, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, St. Louis, MO) was added to cell cultures at the end of the 72 h incubation period and incubation was pursued for additional 2.5 h. The culture media were then discarded and the intracellular MTT reduction product formazan was solubilised in isopropanol. Alternatively, at the end of the experiment cells were fixed in trichloroacetic acid and stained with protein-binding dye sulforhodamine B (Sigma). The colorimetric signals proportional to cell numbers were measured with the BioRad microplate spectrophotometer 3550-UV at 550 nm (MTT) or 570 nm (sulforhodamine B).

At the end of the incubation with the tested compounds, cell culture conditioned media were harvested and assayed for type I collagen concentration by a sandwich ELISA using affinity-purified antibodies, followed by streptavidin/avidin-HRP conjugate and ABTS, according to standard enzyme-linked immunosorbent assay (ELISA) protocol.[32,33] The colorimetric signal proportional to the collagen content was quantified with the BioRad microplate spectrophotometer 3550-UV at 405 nm. Type I collagen concentrations were calculated from standard curve made with serial dilutions of purified human collagen I. All ELISA reagents were from Southern Biotechnology Associates, Birmingham, AL.

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