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Original article

Synthesis, cytotoxicity and effects of some 1,2,4-triazole and 1,3,4-thiadiazole derivatives on immunocompetent cells

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Abstract

Novel derivatives of 4,5-substituted-1,2,4-triazole-thiones and 2,5-substituted-1,3,4-thiadiazoles were synthesized and evaluated for their cytotoxicity. The biological study indicated that compounds 4-ethyl-5-(4,5,6,7-tetrahydro-1-benzothien-2-yl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione **13**, *N*-ethyl-5-(4,5,6,7-tetrahydro-1-benzothien-2-yl)-1,3,4-thiadiazol-2-amine **16**, 4-amino-5-(4,5,6,7-tetrahydro-1-benzothien-2-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione **20** and 4-amino-5-(5-phenylthien-2-yl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione **21** possessed high cytotoxicity in vitro against thymocytes. The corresponding IC_{50} values were 0.46 μM, 5.2×10^{-6} μM, 0.012 μM and 1.0×10^{-6} μM. Most toxic against lymphocytes was compound **21**, IC_{50} − 0.012 μM. The tested compounds showed a general stimulation effect on B-cells' response.

Keywords: 1,2,4-Triazoles; 1,3,4-Thiadiazoles; Cytotoxicity; PFC; LIF

1. Introduction

A large number of compounds containing 1,2,4-triazole system have been investigated as therapeutically interesting drug candidates because of their properties both as selective COX-2 inhibitors [1] and as anti-acetylcholinesterase [2], antimicrobial and antimycotic agents [3–5].

The efficacy of anastrozole and letrozole as aromatase inhibitors and their use as non-steroidal drugs for the treatment of estrogen-dependent cancer as well as the anticancer properties of ribavirine led to the investigation of many 1,2,4-triazole derivatives in laboratorial conditions for their anti-tumor activity [6–8].

Among the 1,2,4-triazole derivatives, the mercapto- and the thione-substituted 1,2,4-triazole ring systems have been studied and so far a variety of anti-tumor properties have been reported for a large number of these compounds [9–12].

Several derivatives of 1,3,4-thiadiazole were prepared starting from the precursors, used for the synthesis of 1,2,4-triazoles and large diversity of effects such as anti-inflammatory, antituberculosis, anticonvulsant, and antibacterial were proved [13–16]. A number of *N*-substituted 2-amino-1,3,4-thiadiazoles were synthesized and evaluated for their antiproliferative activities [17–20].

The similarity in the structure of the 2-amino-1,3,4-thiadiazole and the mercapto- and thion-substituted 1,2,4-triazole ring systems assumes similar biological properties. Series of heterocyclic mercaptans incorporating 1,3,4-thiadiazole- and 1,2,4-triazole rings have been prepared and investigated for antiproliferative activity against human cancer cell lines and tumor-associated carbonic anhydrase isoenzymes I, II, and IX [21,22].

Many types of chemotherapeutic agents have been shown to be effective against cancer and tumor cells, but many of these agents also destroy the normal cells. Despite advances in the field of cancer treatment the discovery of cytotoxic agents that have specificity for cancer and tumor cells remains pendant. Unfortunately, none have been found and instead of

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that, substances whose target is especially rapid dividing cells have been used. Alternatively the substances that were cytotoxic to tumor cells while exerting mild effects on normal cells would be desirable.

Therefore, the search and synthesis of new 3-mercapto-5-substituted-1,2,4-triazole and 2,5-disubstituted-1,3,4-tiadiazole derivatives are determined by the above mentioned facts.

Previously we have reported the synthesis and the cytotoxicity of some 1,2,4-triazoles against Graffi tumor cells [23]. Having in view the high cytotoxicity of these compounds, we undertook synthesis of some derivatives of 3-mercapto-1,2,4-triazoles and 2-amino-1,3,4-thiadiazoles and in vitro investigation of their cytotoxicity against thymocytes and lymphocytes as well as an estimation of their influence on T- and B-cells' response.

The choice of these structures was in accordance with the fact that the thiophene heterocycle takes part in the structure of substances possessing anti-tumor activity, therefore, it was of pharmacological interest to incorporate a thiophene or a tetrahydrobenzothiophene cycle in the structure of 3-mercapto-1,2,4-triazoles and 2-amino-1,3,4-thiadiazoles [24–26].

The structures of 5-aryl-1,2,4-triazoles and 1,3,4-triazoles give the opportunity to realize changes in two directions: introduction of some substituents either in the 1,2,4-triazole and 1,3,4-thiadiazole cycle or in the aromatic ring. That alteration in the structure of the main model compounds may enhance the interaction of these molecules with the biological targets.

2. Chemistry

The synthesis of the 1,2,4-triazole and 1,3,4-thiadiazole derivatives is illustrated and outlined in Fig. 1.

3-Chloro-acrylaldehydes were synthesized according to the procedure described in Ref. [27]. Because of their instability the aldehydes have to be kept only for few hours in refrigerator and must be used as soon as possible.

The interaction of ethyl thioglycolate with 3-chloro-acrylaldehydes **1–3** in pyridine medium in the presence of triethylamine led to ethyl thiophene-2-carboxylates **4–6** [28]. The hydrazides **7–9**, obtained by refluxing of ethyl carboxylates **4–6** with hydrazine hydrate in ethanol medium, were used as precursors for the synthesis of *N*-ethyl hydrazinecarbothioamides **10–12** as well as for the preparation of potassium arylcarbonylhydrazinecarbodithionate **18**, **19**.

The reaction of hydrazides **7–9** with ethyl isothiocyanate resulted in *N*-ethyl hydrazinecarbothioamides **10–12**. Thiosemicarbazides undergo different cyclization reactions to give five member heterocycles. The product of cyclization depends on the reagent used. This cyclization leads to the formation of 1,3,4-oxadiazole, 1,3,4-thiadiazole and 1,2,4-triazole derivatives. When the *N*-ethyl hydrazinecarbothioamides **10–12** were refluxed with solution of sodium hydroxide for 6–12 h, 4-ethyl-5-aryl-1,2,4-triazole-3-thiones **13–15** were synthesized. In sulfuric acid medium the hydrazinecarbothioamides **10–12** formed *N*-ethyl-5-substituted-1,3,4-thiadiazol-2-amines **16**, **17**.

In ethanol solution of potassium hydroxide carbohydrazides **3a–c** interacted with carbon disulfide to potassium hydrazinecarbodithionates **18**, **19**, which were used as precursors for the synthesis of 4-amino-1,2,4-triazoles **20**, **21**. The refluxing of **18**, **19** and hydrazine hydrate in molar ratio 1:2 led to the preparation of 4-amino-1,2,4-triazol-yl-3-thiones.

The chemical structures of the compounds were established by elemental analyses, IR-, ^{1}H NMR and ^{13}C NMR spectra and the results are presented in Section 6. The elemental analyses indicated by the symbols of the elements were within ±0.4% of theoretical values.

3. Pharmacology

3.1. Cytotoxicity

Compounds **10**, **11**, **13**, **16**, **20**, and **21** were evaluated for their cytotoxicity using thymocytes and lymphocytes, derived from sexually mature hamsters, bearing the solid form of the Graffi myeloid tumor.

Migration, rosette forming and plaque forming tests were accomplished on spleen cells, thymocytes and lymphocytes using compounds **10**, **11**, **13**, **16**, **20**, and **21** and the effect on T- and B-cells' response was estimated.

4. Results and discussion

The derivatives of 1,2,4-triazole and 1,3,4-thiadiazoles containing ethyl or amino group at 4th position of 1,2,4-triazole, respectively, ethylamino-group at second position of 1,3,4-thiadiazole cycle as well as 4,5,6,7-tetrahydro-1-benzothiophene-2- ,5-phenylthiophene-2- and 5-(4-methylphenyl)thiophene-2-substituents at the 5th position of 1,2,4-triazole, respectively, 1,3,4-thiadiazole cycles were obtained in order to estimate their cytotoxicity and influence on T- and B-cells' response. The yields of 4-amino-1,2,4-triazoles were in the range 78–96% and that of 4-ethylamino-1,2,4-triazoles accordingly 78–98%, while the yield of *N*-ethyl-5-(4,5,6,7-tetrahydro-1-benzothienyl)1,3,4-thiadiazol-2-amine was only 43% and that of *N*-ethyl-5-(5-phenylthien-2-yl)-1,3,4-thiadiazol-2-amine – 80%.

4.1. Cytotoxicity

The exclusive trypan blue test for the estimation of cytotoxicity in vitro was performed with compounds **10**, **11**, **13**, and **16** as well as **20**, **21** according to the method given in Ref. [29] on thymocytes and blood lymphocytes, derived from sexually mature hamsters. The compounds were dissolved in DMSO at the concentration of 0.5 mg/ml. The investigation was carried out by dilution of the stock solution in ratio 1:10, 1:100, 1:1000 and 1:100,000. To 0.1 μl cell solution was added 0.1 μl of the corresponding series of compounds. The preparations were incubated for 24 h at 37 °C under humidified atmosphere in the presence of 5% carbon dioxide. Trypan blue, 0.1 μl of a 0.2% solution was added to each preparation. The cells' survival was evaluated by means of electronic microscope and the percentage of cytotoxicity was

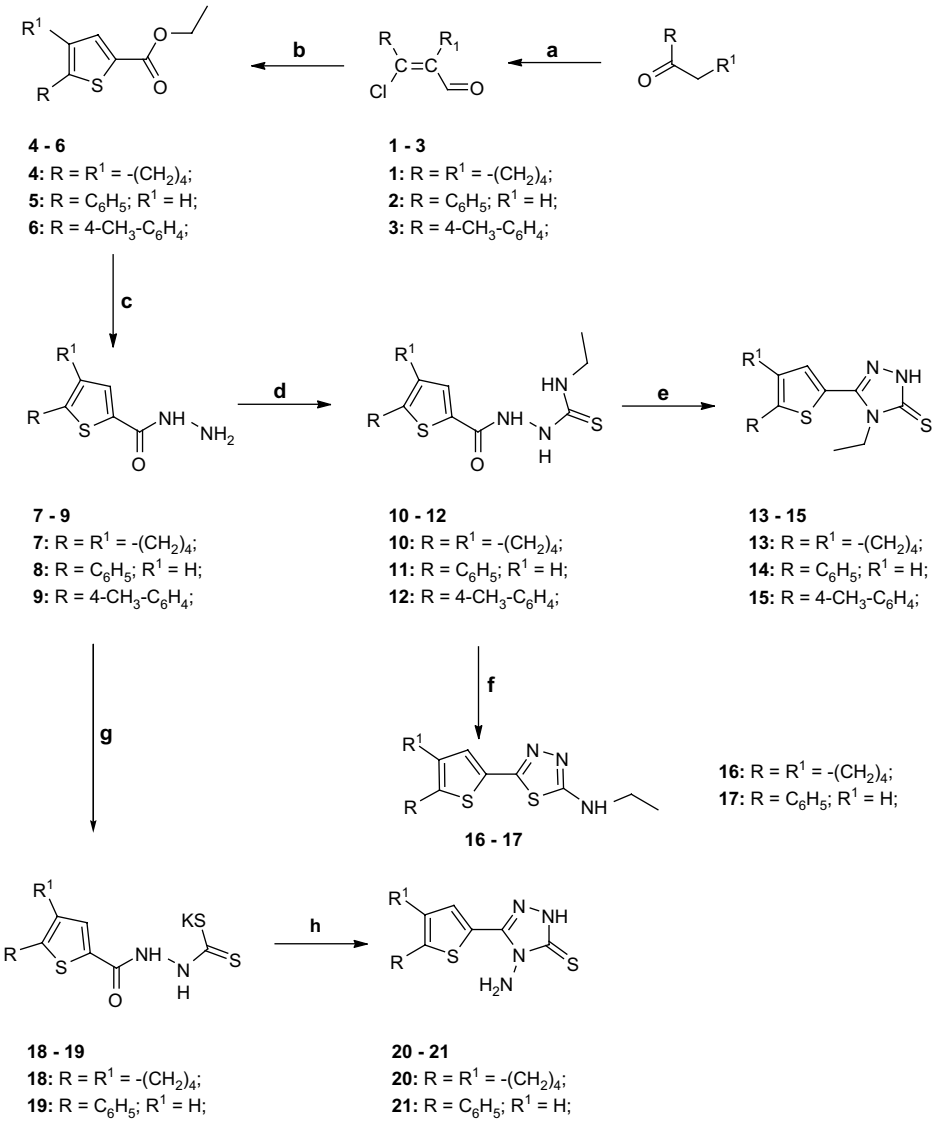

Fig. 1. Synthesis of 1,2,4-triazole and 1,3,4-thiadiazole derivatives. Regents and conditions: (a) DMF, $POCl_3$, trichloroethylene; (b) thioglycolate, triethylamine, pyridine, 10–15 °C; (c) hydrazine hydrate, ethanol, reflux; (d) ethyl isothiocyanate, ethanol, reflux; (e) 10% NaOH, reflux; (f) concentrated sulfuric acid, 0 °C; (g) NaOH, CS_2, absolute ethanol; (h) hydrazine hydrate, reflux.

calculated. The obtained results were plotted and IC_{50} was estimated. The data are given in Table 1. The proved compounds exhibited relative high cytotoxicity against thymocytes (IC_{50} values were in the range 0.1–2.43 μM) but lower cytotoxicity against blood lymphocytes (IC_{50} – 1.64–2.67 μM).

Because after treatment in vivo the experimental animals can develop a response reaction, it was interesting to estimate the cytotoxicity of the studied compounds on cells, derived from preliminary treated hamsters. That is why a second group of hamsters, bearing the solid form of the Graffi myeloid

Table 1
In vitro cytotoxicity

Compound	$IC_{50} \pm SE$ (μM)		After treatment $IC_{50} \pm SE$ (μM)	
	Type of cells		Type of cells	
	Thymocytes	Blood lymphocytes	Thymocytes	Blood lymphocytes
10	0.11 ± 8.23	NA	0.011 ± 14.89	0.014 ± 15.81
11	2.10 ± 9.43	1.64 ± 5.68	3.5 ± 14.37	0.32 ± 13.77
13	0.12 ± 12.69	1.81 ± 10.87	0.46 ± 17.19	0.29 ± 17.93
16	0.26 ± 11.51	0.32 ± 9.69	5.2×10^{-6} ± 13.17	0.41 ± 13.77
20	2.20 ± 7.47	2.67 ± 9.65	0.012 ± 14.13	0.14 ± 12.68
21	0.24 ± 16.20	NA	1.0×10^{-6} ± 14.75	0.012 ± 16.53

NA – cytotoxicity not determined.

tumor was treated in vivo with the substances **10**, **11**, **13**, **16**, **20**, and **21** with a dose at concentration of 0.5 mg/ml, i.p. The in vitro effect of the compounds on thymocytes and lymphocytes, extracted from the hamsters five days after treatment was determined. The results for cytotoxicity of compounds **10**, **11**, **13**, **16**, **20**, and **21** in vitro after treatment of the experimental hamsters are presented in Table 1. Each value represents a mean value of three parallel samples in three independent experiments. As it can be seen that the cytotoxic effects of the compounds on thymocytes increased and the IC_{50} values of compounds **13**, **16**, **20** and **21** varied between 0.46 μM and 1.0×10^{-6} μM. The highest activity against blood lymphocytes exhibited compound **21**, IC_{50} – 0.012 μM.

It was important for us to establish if the studied compounds can cause T- and B-cells' response. Migration, rosette forming and plaque forming tests were accomplished on spleen cells, thymocytes and lymphocytes extracted from treated hamsters as described in Refs. [30–32]. As control cells were used cells from not intact animals. The data present a mean value of three individual experiments and are given in Table 2. Rosette formation was not observed for all of the tested compounds. The number of the plaque forming cells for compound **21** in the test with thymocytes was counted as haemolytic zone and compared to those of the healthy controls, was the highest – 19,200. Regarding the control cells all studied compounds, excluding compound **10**, exhibited higher value number of the plaque forming cells in screening with blood lymphocytes. In comparison to the control group compound **21** showed the highest value number of PFC– 19,920, followed by compound **16** – 14,288, but these compounds were not active in the LIF test regarding to spleen cells and blood lymphocytes, while compound **20** showed the highest LIF against blood lymphocytes 1.654.

Table 2
Effects of the compounds in vitro on the immunocompetent cells

Compound	Cells	Migration (mm^2)	LIF	PFC
10	Spleen cells	NA		Full lys.
	Thymocytes	146	0.08	2040
	Blood lymphocytes	NA	NA	768
11	Spleen cells	822	NA	2010
	Thymocytes	202	0.104	7800
	Blood lymphocytes	NA	NA	5200
13	Spleen cells	1823	0.00	3700
	Thymocytes	997	0.518	5287
	Blood lymphocytes	NA	0.001	4992
16	Spleen cells	1788	NA	3928
	Thymocytes	1118	0.581	15,300
	Blood lymphocytes	NA	NA	14,288
20	Spleen cells	1286	NA	3920
	Thymocytes	1024	0.532	7250
	Blood lymphocytes	1800	1.654	5869
21	Spleen cells	2332	NA	4700
	Thymocytes	1836	0.954	19,200
	Blood lymphocytes	NA	NA	19,920
Control cells	Spleen cells	NA	NA	5400
	Thymocytes	1924	NA	6033
	Blood lymphocytes	1088	NA	680

NA – not response determined.

The above-mentioned facts indicated that the compounds have a general stimulating effect on the B-cells' response, but additional experiments are required to elucidate their efficacy. When there is a stimulation of the immunological response it may be supposed that the resistance of the organism to the tumor cells will increase.

Statistical significant differences in the level of cells in both control and experimental groups were determined ($p \leq 0.05$).

5. Conclusion

New derivatives of 3,4,5-substituted-1,2,4-triazole and 2,5-substituted-1,3,4-thiadiazoles were synthesized using as precursors 4,5,6,7-tetrahydro-1-benzothiophene-2, as well as 5-phenylthiophene-2, and 5-(4-methylphenyl)thiophene-2-carbohydrazide.

The initial biological screening in vitro showed that the studied compounds possessed relative high cytotoxicity against thymocytes and low cytotoxicity against blood lymphocytes, derived from sexually mature hamsters.

After treatment of the experimental animals with a dose of the tested compounds at and following estimation of the cytotoxicity in vitro, the results showed increase in the cytotoxicity of compounds **13**, **16**, **20** and **21** against thymocytes. IC_{50} values were in the range $0.46–1.0 \times 10^{-6}$ μM. With respect to blood lymphocytes the most cytotoxic was compound **21**, IC_{50} was 0.012 μM.

The PFC, LIF and the migration tests' study indicated that the compounds revealed a general stimulating effect on the B-cells' response. Compound **20** exhibited a general stimulation regarding blood lymphocytes, LIF – 1.654, while compound **21** showed the highest value number of PFC, which surpasses 29 times than that of the control cells.

The above results also confirmed the hypothesis that the introduction of a 5-phenylthiophene-2- and tetrahydrobenzothiophene-2-substituent at 5th position in the structure of 3-mercapto-1,2,4-triazoles and 2-amino-1,3,4-thiadiazoles are auspicious to the interaction of these molecules with the biological targets.

6. Experimental part

Melting points (mp) were determined on an Electrothermal AZ 9000 3MK4 apparatus and were uncorrected. The thin layer chromatography (TLC, R_f values) was performed on Al_2O_3 60 plates F_{254} or silica gel plates (Merck, 0.2 mm thick) using mobile phase benzene/ethanol – 2:0.5, respectively, benzene/ethanol – 4:2, and visualization was effected with ultraviolet light. IR spectra were recorded on a Specord 71 IR spectrophotometer as potassium bromide discs. All NMR spectra were recorded on a Bruker Avance DRX 250 spectrometer (Bruker, Faelanden, Switzerland) operating at 250.13 MHz for ^1H and 62.89 MHz for ^{13}C, using a dual 5 mm $^1H/^{13}C$ probehead. Chemical shifts were expressed relative to tetramethylsilane (TMS) and were reported as δ (ppm). The measurements were carried out at ambient

temperature (300 K). The microanalyses for C, H, N and S were performed on Perkin–Elmer elemental analyzer.

6.1. Chemistry

6.1.1. General procedure for compounds **1–3**

Phosphoric chloride 0.044 mol was dropped to 0.054 mol DMF dissolved in 10 ml dry trichloroethylene by stirring at 10 °C. In absence of humidity a solution of 0.041 mol cyclohexanone in 10 ml trichloroethylene was added dropwise at ambient temperature for 30 min. The reaction mixture was stirred at 60 °C for 3 h. After cooling the solution was poured out in 50 ml water containing 14 g sodium acetate dihydrate. The organic layer was separated and the water layer was extracted with trichloroethylene. The combined layers were dried with sodium sulfate and the solvent was evaporated in vacuum. The aldehydes were distillated under reduced pressure (5 mm/Hg). Because of their instability they have to be kept in refrigerator only for a few hours.

6.1.2. General procedure for compounds **4–6**

To a cooled solution of 0.025 mol of 2-chloro-acrylaldehyde **1–3** and 0.03 mol ethyl thioglycolate in pyridine 5.1 ml triethylamine was added at 10 °C drop by drop by stirring. The temperature must be maintained between 10 °C and 15 °C. The solution colored darker and triethylamine hydrochloride was formed. The mixture was stirred for 1 h more. After cooling with ice 48% potassium hydroxide (5.12 ml) was added by stirring for 15 min and the reaction mixture was diluted with 150 ml water. The organic layer was separated and the water layer was extracted with chloroform. The combined organic layers were dried with Na_2SO_4. The solvent was removed in vacuum evaporator and the compound was distilled under reduced pressure.

*6.1.2.1. Ethyl 4,5,6,7-tetrahydro-1-benzothiophene-2-carboxylate **4**.* Yield – 60%; mp – 30–31 °C, re-crystallized with ethanol; NMR ($CDCl_3$): 1.22 (t, 3H, CH_3); 1.74 (t, 2H, $2CH_2$); 2.42 (t, 2H, CH_2); 2.61 (t, 2H, CH_2); 4.18 (q, 2H, CH_2); 7.1 (s, 1H, 1H-Th); Analysis: calc.: C, 62.83; H, 6.71; O, 15.22; S, 15.25; Found: C, 62.73; H, 6.61; O, 15.15; S, 15.15.

*6.1.2.2. Ethyl 5-phenylthiophene-2-carboxylate **5**.* Yield – 40%; mp – 29 °C, re-crystallized with ethanol; NMR ($CDCl_3$): 1.24 (t, 3H, CH_3); 4.22 (q, 2H, CH_2); 6.98 (m, 1H, Th); 7.22 (m, 1H, Bz); 7.44 (m, 2H, Bz); 7.54 (m, 2H, Bz); 7.63 (m, 1H, Th); Analysis: Calc.: C, 67.21; H, 5.21; O, 13.77; S, 13.80 Found: C, 67.11; H, 5.31; O, 13.57; S, 13.68.

*6.1.2.3. Ethyl 5-(4-methylphenyl)thiophene-2-carboxylate **6**.* Yield: 56%; mp – 85–88 °C, re-crystallized with ethanol; $R_f = 0.63$ mobile phase benzene/ethanol – 3:1, Al_2O_3 plates; NMR ($CDCl_3$): 1.42 (t, 3H, CH_3); 2.30 (s, 3h, CH_3); 4.24 (q, 2H, CH_2); 6.88 (m, 1H, Th); 7.16 (m, 2H, Bz); 7.34 (m, 2H, Bz, $^3J = 8.02$ Hz); 7.58 (m, 1H, Th); Analysis: Calc.: C, 68.26; H, 5.73; O, 12.99; S, 13.02; Found: C, 68.16; H, 5.83; O, 12.79; S, 13.18.

6.1.3. General procedure for compounds **7–9**

A solution of 0.01 mol **4–6** and 0.02 mol hydrazine hydrate in 30 ml ethanol was refluxed for 6 h. The hydrazide was crystallized after cooling. TLC: mobile phase benzene–ethanol – 2:0.4, Al_2O_3 plates.

*6.1.3.1. 4,5,6,7-Tetrahydro-1-benzothiophene-2-carbohydrazide **7**.* Yield – 85%; mp – 146–148 °C; $R_f = 0.54$; ^{1}H NMR (C_5D_5N): 1.76 (t, 4H, $2CH_2$); 2.35 (t, 2H, CH_2); 2.45 (t, 2H, CH_2); 5.10 (bs, 2H, NH_2, exchangeable with D_2O); 6.90 (s, 1H, Th); 11.4 (bs, 1H, NH, exchangeable with D_2O); Analysis: Calc.: C, 55.08; H, 6.15; N, 14.27; S, 16.34; Found: C, 55.18; H, 6.05; N, 14.40; S, 16.18.

*6.1.3.2. 5-Phenylthiophene-2-carbohydrazide **8**.* Yield – 75%; mp – 164–166 °C; $R_f = 0.63$; ^{1}H NMR (C_5D_5N): 5.20 (bs, 2H, NH_2, exchangeable with D_2O); 6.93 (d, 1H, Th); 7.25 (m 3H, Bz); 7.5 (m 2H, Bz); 7.78 (d 1H, Th); 10.74 (s, 1H, NH, exchangeable with D_2O); Analysis: Calc.: C, 60.53; H, 4.62; N, 12.83; S, 14.69; Found: 60.48; H, 4.66; N, 12.77; S, 14.78.

*6.1.3.3. 5-(4-Methylphenyl)thiophene-2-carbohydrazide **9**.* Yield: 92%; mp – 154–157 °C; $R_f = 0.45$; ^{1}H NMR (C_5D_5N): 2.31 (s, 3H, CH_3); 5.32 (bs, 2H, NH_2, exchangeable with D_2O); 6.94 (m, 3H, 2H-Bz, 1H-Th); 7.41 (m, 2H, Bz); 7.71 (m, 1H, Th); 10.90 (s, 1H, NH, exchangeable with D_2O); Analysis: Calc.: C, 62.04; H, 5.21, N, 12.06; S, 13.80; Found: C, 62.10; H, 5.16, N, 12.16; S, 13.88.

6.1.4. General procedure for compounds **10–12**

Hydrazides **7–9**, 0.023 mol, were suspended in 25 ml ethanol and 0.028 mol of ethyl isothiocyanate was added. The mixture was refluxed for 3 h. The product was isolated after cooling. TLC: mobile phase benzene/ethanol – 4:2.

*6.1.4.1. N-Ethyl-2-(4,5,6,7-tetrahydro-1-benzothien-2-ylcarbonyl)hydrazinecarbothioamide **10**.* Yield – 64%; mp – 189–190 °C; $R_f = 0.57$; ^{1}H NMR (DMSO): 1.02 (t, 3H, CH_3); 1.74 (m, 4H, $2CH_2$); 2.53 (m, CH_2); 2.72 (t, 2H, CH_2); 3.44 (q, 2H, $-CH_2-N$); 7.48 (s, 1H, Th); 8.04 (bs, 1H, NH, exchangeable with D_2O); 9.20 (s, 1H, NH, exchangeable with D_2O); 10.14 (s, 1H, NH exchangeable with D_2O); Analysis: Calc.: C, 50.85; H, 6.05; N, 14.83; S, 22.63; Found: C, 50.75; H, 6.01; N, 14.76; S, 22.58.

*6.1.4.2. N-Ethyl-2-[(5-phenylthien-2-yl)carbonyl]hydrazinecarbothioamide **11**.* Yield – 85%; mp – 205–207 °C; $R_f = 0.53$; ^{1}H NMR (DMSO): 1.12 (t, 3H, CH_3); 3.49 (q, 2H, CH_2); 6.97 (m, 1H, Th); 7.18 (m, 3H, Bz), 7.38 (m 1H, Bz); 8.04 (bs, 1H, NH, exchangeable with D_2O); 9.20 (s, 1H, NH exchangeable with D_2O); 10.14 (s, 1H, NH, exchangeable with

D_2O); Analysis: Calc.: C, 55.06; H, 4.95; N, 13.76; S, 21.00; Found: C, 55.00; H, 4.91; N, 13.81; S, 21.10.

6.1.4.3. N-Ethyl-2-{[5-(4-methylphenyl)thien-2-yl]carbonyl}-hydrazinecarbothioamide ***12***. Yield – 47%; mp – 210–213 °C; $R_f = 0.61$; ^{1}H NMR (C_5D_5N): 1.18 (t, 3H, CH_3); 2.31 (s, 3H, CH_3); 3.50 (q, 2H, CH_2); 7.11 (m, 3H, 2H-Bz, 1H-Th); 7.37 (m, 2H-Bz); 7.54 (d, 1H, Th); 7.78 (bs, 1H, NH, exchangeable with D_2O); 9.1 (s, 2H, NH, exchangeable with D_2O); Analysis: Calc.: C, 56.40; H, 5.36; N, 13.15; S, 20.08; Found: C, 56.34; H, 5.42; N, 13.25; S, 20.18.

6.1.5. General procedure for compounds ***13–15***

To 0.01 mol of compounds **10–12** was added 2 ml 10% NaOH and the mixture was refluxed for 5–12 h. After cooling the solution was acidified with concentrated hydrochloric acid and the obtained precipitate was filtered and re-crystallized with ethanol. TCL was performed on silica gel plates using mobile phase benzene/ethanol – 2:0.5.

6.1.5.1. 4-Ethyl-5-(4,5,6,7-tetrahydro-1-benzothien-2-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione ***13***. Reaction time: 5 h. Yield – 97%; mp – 183–185 °C; $R_f = 0.63$; ^{1}H NMR (DMSO): 1.23 (t, 3H, CH_3); 1.71 (m, 4H, $2CH_2$); 2.54 (m, 2H, CH_2); 2.71 (m, 2H, CH_2); 4.18 (q, 2H, CH_2); 7.37 (s, 1H, Th); 13,88 (s, 1H, NH, exchangeable with D_2O); Analysis: Calc.: C, 54.31; H, 5.70; N, 15.83; S, 24.16; Found: C, 54.15; H, 5.45; N, 15.66; S, 24.00.

6.1.5.2. 4-Ethyl-5-(5-phenylthien-2-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione ***14***. Yield – 87%; mp – 195–197 °C; $R_f = 0.63$; ^{1}H NMR (DMSO): 1.32 (t, 3H, CH_2–CH_3); 4.28 (q, 2H, CH_2–CH_3); 7.06 (d, 1H, Th); 7.2 (m, 2H, Ar), 7.48 (m,1H-Th, 3H, Ar); Analysis: Calc.: C, 58,51; H, 4.56; N, 14.62; S, 22.31; Found: C, 58.37; H, 4.67; N, 14.41; S, 22.47.

6.1.5.3. 4-Ethyl-5-[5-(4-methylphenyl)thien-2-yl]-2,4-dihydro-3H-1,2,4-triazole-3-thione ***15***. Yield – 98%; mp – 226–228 °C; $R_f = 0.63$; ^{1}H NMR (C_5D_5N): 1.34 (t, 3H, CH_3); 2.30 (s, 3H, CH_3); 4.38 (q, 2H, CH_2); 7.10 (m, 1H, Th); 7.58 (m, 5H, 1H-Th, 4H, Ar); 9.41(bs, 1H, NH, exchangeable with D_2O); Analysis: Calc.: C, 59.77; H, 5.02; N, 13.94; S, 21.28; Found: C, 59.49; H, 5.15; N, 14.08; S, 21.37.

6.1.6. General procedure for compounds ***16, 17***

To 1 ml cooled concentrated sulfuric acid was added 0.01 mol carbohydrazides **10**, **11** for 90 min in portions by cooling (0 °C) and stirring. The solution was allowed to stay for 2 h at ambient temperature by stirring. The yellowish orange colored solution was poured into ice by stirring. The obtained precipitate was filtered, washed with water and re-crystallized with ethanol.

6.1.6.1. N-Ethyl-5-(4,5,6,7-tetrahydro-1-benzothien-2-yl)-1,3,4-thiadiazol-2-amine ***16***. Yield – 43%; mp – 137–139 °C; $R_f = 0.56$; ^{1}H NMR (C_5D_5N): 1.2 (t, 3H, CH_2–CH_3); 1.52 (m, 4H, $2CH_2$); 2.50 (m, 2H, CH_2); 2.74 (m, 2H, CH_2); 3.82 (q, 2H, CH_2–CH_3); 7.71 (s, 1H, Th); 9.16 (bs, 1H, NH, exchangeable with D_2O); Analysis: Calc.: C, 54.31; H, 5.70; N, 15.83; S, 24.16; Found: C, 54.20; H, 5.68; N, 15.64; S, 24.31.

6.1.6.2. N-Ethyl-5-(5-phenylthien-2-yl)-1,3,4-thiadiazol-2-amine ***17***. Yield – 80%; mp – 145–147 °C; $R_f = 0.48$; ^{1}H NMR (C_5D_5N): 1.2 (t, 3H, CH_2–CH_3); 3.9 (q, 2H, CH_2–CH_3); 7.2 (m, 3H, 2H-Bz, 1H-Th); 7.4 (m, 2H,Bz); (d, 1H, Th); Analysis: Calc.: C, 58.51; H, 4.56; N, 14.62; S, 22.31; Found: C, 58.42; H, 4.46; N, 14.71; S, 22.42.

6.1.7. General procedure for the synthesis of compounds ***17–19***

To a solution of 0.023 mol carbohydrazides **7**, **8** and 0.027 mol of potassium hydroxide in 20 ml absolute ethanol was added 0.034 mol of carbon disulfide. The solution became orange colored and was stirred for 2–10 h. The obtained precipitate was filtered. TLC: mobile phase: benzene/ethanol – 5:2, silica gel plates (Merck, 0.2 mm thick).

6.1.7.1. Potassium 2-(4,5,6,7-tetrahydro-1-benzothien-2-ylcarbonyl)hydrazinecarbodithionate ***18***. Yield – 66%; mp – 268–270 °C; $R_f = 0.59$

6.1.7.2. Potassium 2-[(5-phenylthien-2-yl)carbonyl]hydrazinecarbodithionate ***19***. Yield – 66%; mp – 253–255 °C; $R_f = 0.57$.

6.1.8. General procedure for compounds ***20***, ***21***

To a solution of 0.012 mol of potassium hydrazinecarbodithionate **18**, **19** in 3 ml of water was added 0.024 mol hydrazine hydrate and the mixture was heated on steam bath for 1 h. After cooling the solution was quenched with 10 ml water and acidified with acetic acid. The obtained solid was filtered and re-crystallized with water.

6.1.8.1. 4-Amino-5-(4,5,6,7-tetrahydro-1-benzothien-2-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione ***20***. Yield: 78%; mp – 181–183 °C; $R_f = 0.4$, mobile phase: benzene/ethanol – 3:0.5, silica gel plates (Merck, 0.2 mm thick); ^{1}H NMR (DMSO-d_6): 1.75 (m, 4H, $2CH_2$); 2.37 (m, 2H, CH_2); 2.73 (m, 4H, $2CH_2$); 7.57 (s, 1H, Th); 13.78 (s, 2H, NH_2, exchangeable with D_2O); 14.54 (bs, 1H, NH, exchangeable with D_2O); Analysis: Calc.: C, 47.59; H, 4.79; N, 22.20; S, 25.41; Found: C, 47.71; H, 4.92; N, 22.09; S, 25.23.

6.1.8.2. 4-Amino-5-(5-phenylthien-2-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione ***21***. Yield: 96%; mp – 185–187 °C; $R_f = 0.66$, mobile phase: benzene/ethanol – 3:0.5, silica gel plates (Merck, 0.2 mm thick); ^{1}H NMR (C_5D_5N): 6.5 (s, 2H, NH_2, exchangeable with D_2O); 7.15 (m, 1H, Th); 7.3 (m, 5H, Bz); 7.5 (d, 1H, Th); 8.1 (s, 1H, NH, exchangeable with D_2O); Analysis: Calc.: C, 52.53; H, 3.67; N, 20.42; S, 23.37; Found: C, 52.61; H 4.55; N, 20.54; S, 23.48.

6.2. Biological evaluation

6.2.1. Cell preparation

Cells from thymus and spleen were obtained by grinding the tissue in cold PBS, pH 7.2 in Potter homogenizator and

filtering the cells through a 60 mesh and after 10 min centrifugation at 1800 rpm the thin white over layer was aspirated and used in the experiments. Peripheral blood lymphocytes were obtained from heparinized blood centrifuged at 1100 rpm for 15 min and the white ring was taken out after that laid on LYMPHOPREP (''Nyccomed'', Norway) and centrifuged for further 30 min at 20 °C and 2800 rpm. All the lymphocytes were finally washed 2 times in cold PBS. The cell vitality was measured with 0.2% trypan blue and was found to be 85–96%. The number of the immunocompetent cells (RFC and PFC) was calculated among 10^6 lymphocytes.

6.2.2. Cytotoxicity assay

The compounds were dissolved in DMSO at concentration of 5 mg/ml. The investigation was carried out by dilution of the stock solution as described above. To 0.1 μl cell solution was added 0.1 μl of the corresponding series of compounds concentration. The preparations were incubated for 24 h at 37 °C under humidified atmosphere in the presence of 5% CO_2. After incubation of the preparations to 0.1 μl of each of them 0.1 μl of 0.2% solution of Trypan blue was added and the cells' vitality was estimated.

6.2.3. Rosette forming test

The rosette forming cells among the lymphocytes in the peripheral blood, spleen and thymus were counted following the procedure of Biozzi et al. [30]. A lymphocyte surrounded by more than four sheep erythrocytes was considered as a rosette.

6.2.4. Plaque forming test

The plaque forming cells (PFCs = haemolysin producing B-cells) were detected using the method of Cunningham and Szenberg [31] which is briefly as follows: the experimental cells were mixed with complement and sheep red blood cells (SRBC) in definite concentrations and 50 μl chambers were filled with them. After 24-h keeping at 37 °C and 5% CO_2 the PFCs were counted as a haemolytic zone and compared to these of the healthy controls.

6.2.5. Leucocyte migration inhibition

The evaluation was carried out by the procedure of Anders and Natvig [32] which allows following the activation or inhibition of migration of the experimental leucocytes. The test was undertaken in 50 mm plastic Petri dishes with monolayer of agarose, containing different components. 3-mm holes in the agarose were filled with the examined cell suspension and after 16 h preserving at 37 °C and 5% CO_2 the migration area of the cells was calculated. The migration index (leucocyte migration factor − LIF) was the ratio between the area migration of the experimental cells and the area of migration of the control ones.

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