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EXAMINATIONS ON THE CYTOTOXICITY OF SOME 2,5-DISUBSTITUTES-1,2,4-TRIAZOLES AND 1,3,4-THIADIAZOLES AND THEIR PRECURSORS TOWARDS GUINEA HEN CELLS

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ABSTRACT

The biological activity of several new derivatives of 1,2,4-triazoles and 1,3,4-thiadiazoles as well as of their precursors - thiosemicarbasides to normal immunocompetent cells and to cells from virus-induced tumor-bearing guinea hens was investigated by trypan blue exclusion test, morpholigical observations and propidium iodine labeling. The incubation procedure of the examined cells with the substances was done at two different temperatures and the vitality of the cells was established. From all tested compounds, only these which were less or not cytotoxic to normal cells and more toxic to the cells from infected guinea hens can be assessed as suitable for further steps in chemotherapy examinations.

Keywords: 1,2,4-triazoles, 1,3,4-thiadiazoles, thiosemicarbazide, cytotoxicity, Pts56-virus, lymphocytes.

INTRODUCTION

The 1,2,4-thriazole heterocycle is an important scaffold in the structure of several drug candidates. The application of anastrozole and letrozole as aromatase inhibitors 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 [1-5]. Among the 1,2,4-triazole derivatives, the mercapto- and the thione-substituted 1,2,4-triazole ring systems were reported to possess a variety of anti-tumor properties [6-9].

The 1,3,4- thiadiazoles may be regarded as isosters of 5-mercapto-substituted 1,2,4-triazoles and thus may be expected to have similar biological activity. Many 1,3,4-thiadiazoles were synthesized and investigated for their anticancer properties [10-12]. Since the thiophene ring system participates in the structure of many compounds possessing antineoplastic properties, it was of pharmacological interest to incorporate a thiophene or a tetrahydrobenzothiophene ring in the structure of 3-mercapto-1,2,4-triazoles and 2-amino-1,3,4-thiadiazoles [13-15].

We already reported the cytotoxicity and the effect of 1,2,4-triazole and 1,3,4-thiadiazole derivatives on thymocytes and lymphocytes, derived from sexually mature hamsters, bearing the solid form of the Graffi myeloid tumor [16]. In this paper we report the cytotoxic activity *in vitro* against tumor cells and immunocompetent cells (spleen lymphocytes) derived from tumor bearing (virus positive) and from healthy animals, because these lymphocytes take part in the antitumor response and are expected to be changed during the disease. The osteopetrosis virus strain Pts56 induces mainly pancreatic tumors (very malignant and invasive) in the guinea fowls and it is proved that these tumors have very common features with the same human tumors.

EXPERIMENTAL
MATERIALS AND METHODS

The tested compounds (Fig 1) were synthesized as we described in [16].

Melting points (mp) were determined on an Electrothermal AZ 9000 3MK4 apparatus without correction. The thin layer chromatography (TLC, Rf 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).

General procedure for compounds 1-3: To a suspension of appropriate hydrazide (0.023 mol) in 25 ml ethanol, 0.028 mol of the corresponding isothiocyanate was added. The mixture was refluxed for 3 hours. The product was isolated after cooling. TLC: mobile phase benzene/ethanol 4:2.

General procedure for the synthesis of compounds 4-5: To a solution of 0.023 mol carbohydrazides 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 - 6 hours. The obtained precipitate was filtered. TLC: mobile phase: benzene/ethanol 5:2, silica gel plates (Merck, 0.2 mm thick).

General procedure for compounds 6-8: To 0.01 mol of compounds **1-3** was added 2 ml 10 % NaOH and the mixture was refluxed for 5 – 12 hours. 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.

General procedure for compounds 9-10: To 1 ml cooled concentrated sulfuric acid was added 0.01 mol thiosemicarbazides **1-3** for 90 minutes in portions by cooling (0 °C) and stirring. The solution was allowed to stay 2 hours at ambient temperature by stirring. The yellow-orange colored solution was poured into ice by stirring. The obtained precipitate was filtered, washed with water and re-crystallized with ethanol. The thiadiazole **13** was obtained analogous using of potassium hydrazinecarbodithionate **4.**

General procedure for compounds 11-12: To a solution of 0.012 mol of potassium hydrazinecarbodithionate **4-5** in 3 ml of water was added 0.024 mol hydrazine hydrate and the mixture was heated on steam bath for 1 hour. 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.

Synthesis of compound **14**: To a solution of 0.004 mol (0.16 g) of NaOH in 10 ml ethanol and 0.005 mol of

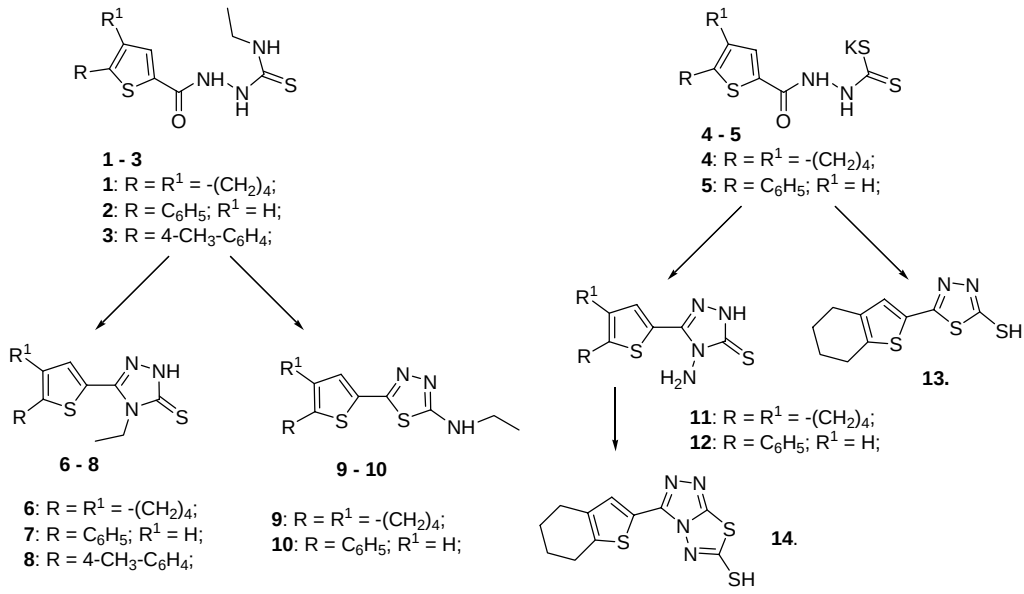

Fig. 1. The synthesized and studied thiosemicarbazides, 1,2,4-triazoles and 1,3,4-thiadiazoles.

4-amino-5-mercapto-3-(4,5,6,7-tetrahydrobenzothienyl-2)-1,2,4-triazole was added (0.026 mol) carbon disulphide. The solution was refluxed 18 hour. After cooling the obtained solid was filtered and re-crystallized from ethyl acetate/ benzene. Mp - 215-217, yield 63 %.

Pharmacology

Animals: In the experiments there have been used 2-days old guinea-hens, who were treated *in vivo* with 0.2-0,5 ml Pts56-virus [3]-containing blood plasma and after showing disease clinic (1-2 to 5 weeks) they were sacrificed and in case they had macroscopic alterations, their spleen lymphocytes were used in the further experiments.

Lymphocytes: Lymphocytes from healthy or tumor-bearing quinea hens were taken by grinding the spleens in a loose Potter homogenizer, separating them through 80-mesh sieve and washing 3 times in PBS.

In vitro cytotoxicity: The toxic impact of each compound was assigned *in vitro* conditions after incubation of 1 ml cell suspension (10^6cells/ml) of these lymphocytes (separately) with 0.1 ml 10 % solution of the corresponding compound in DMSO. After 24 h of incubation at 4°C, respectively at 37°C (PBS, pH=7.2) the cell vitality (three samples from each substance) was estimated by the trypan blue exclusion test.

Morphological observations: To assess the toxic impact of the substances in native preparations, the cell integrity was determined microscopic. After reading it, to each sample was added 0.1 % trypan blue solution and the vitality of the cells was counted. The cell vitality was examined by the PI (propidium iodine)-procedure as well. Finally each cell sample was centrifuged, mixed with guinea-hen serum (10:1) and Gimsa stained smears were prepared for morphological estimations.

Propidium iodine (PI) procedure: A part of the centrifuged at 1000 rpm/5'/4°C cells (5.10^5) from each sample was washed in Ca^{2+}- and Mg^{2+}-free phosphate-buffered PBS$^-$ and fixed with 1 % glutaraldehyde/PBS. For the PI-fluorescence microscopy examination was used the method of Inanami et al. [18] and the microscopic observations were performed with "Carl Zeiss" (Germany) fluorescence microscope.

All the examined substances were proved for cytotoxicity against normal and abnormal cells by cytotoxicity screening, morphological changes and propidium iodine staining for cell death.

RESULTS AND DISCUSSION

The screening was performed with compounds **1-3** and **6-14**.

The vitality of the examined spleen lymphocytes after treatment with compounds and incubation either at 4°C or 37°C is given in Table 1. The lymphocytes from infected guinea-hens were incubated with the tested compounds and the mean survival at 4°C for the cells from infected animals, is lower than that after incubation at 37°C (Table 1). The treated lymphocytes from healthy animals show at both temperatures quite equal survival (mean value at 4 °C - 67 % and at 37 °C – 61 %).

The microscopic observations of the native cell preparations were represented in Table 2. It can be seen that in the most cases the picture was fogged by a rest from substance crystals and often the cells are agglutinated. In all cases where the middle or/and large lymphocytes disappeared (Table 2, compounds **1, 11, 12** and **14**) we consider it as a sign, that the substances influence negatively the weakly differentiated cells (like the middle or large lymphocytes).

Much better information gave to us the evaluation of the *Gimsa stained cell* preparations (Table 3). This examination shows that few substances did not damage the cells. The substance **13** destroys the cells at 37°C (compared to the results in Table 2).

Table 1. Vitality (%) of spleen lymphocytes of infected guinea-hens respectively of healthy fowls after incubation.

Subst. No	Lymphocytes of guinea-hens infected with Pts56		Lymphocytes of healthy guinea-hens	
	4°C	37°C	4°C	37°C
1	50	100	20	80
2	50	50	10	10
3	80	90	100	10
6	20	90	100	50
7	100	50	50	80
8	50	100	90	10
9	100	95	80	80
10	80	100	100	50
11	90	90	50	100
12	0	100	100	90
13	100	100	10	80
14	80	20	100	95

Table 2. Native preparations of the preincubated at 4°C / 37°C (in PBS, 24 h, 5% CO_2) spleen lymphocytes.

Substance No	Lymphocytes of guinea-hens infected with Pts56		Lymphocytes of healthy guinea-hens	
	4°C	37°C	4°C	37°C
1	single cells	S-Ly	intact cells	intact cells
2	single intact cells	M-Ly, intact	crystals; single cells	crystals; S-Ly
3	cell clusters	cell clusters; S+M-Ly	cell clusters; S+M-Ly	Agglutinated but alive cells
6	crystals; single S-Ly	alive cells without demages	crystals; single living cells	S+M+L-Ly
7	crystals; single S-Ly	intsct cells	crystals; single living cells	crystals; S-Ly
8	crystals; single S-Ly	S-Ly	opaque -not visible cells	agglutinated cells; few living
9	intact cells	cell clusters; S+M-Ly	intact cells	intact cells
10	cell clusters	cell clusters; L-Ly	cell clusters; opaque	agglutinated,but alive cells
11	crystals; single S-Ly	cell clusters; S-Ly	crystals; single cells	agglutinated cells;without L-Ly
12	crystals; S-Ly	cell clusters; S-Ly	cell clusters; no single cells	agglutination; all living cells
13	crystals; intact cells	cell clusters; L-Ly	single intact cells	agglutinated but alive cells
14	intact cells	S+M-Ly	intact cells	agglutination; many dead cells
control (only in DMSO*)	intact cells	intact cells	intact cells	single cells

S-Ly - small lymphocyte; * DMSO-dimethylsulfoxide (substance solvent); M-Ly - middle lymphocyte; L-Ly - large lymphocytes;

The results from the *proriduim iodine* staining are given in Table 4. As it was expected not all of the cells with faded chromatin and/or cytoplasm (in the Gimsa stained smears or native preparations) were dead after the treatment, only the substance **2** was toxic in all circumstances and the thiosemicarbazide **1** was toxic to the cells from infected animals after incubation at 4°C, but to the healthy cells it was effective only when the cells were incubated at 37°C.

Following the standard procedure for statistical assessment of Student, the calculations showed deviations of the results lower than 1 % ($p < 0.01$). On the base of the obtained results, it is hardly to say that the higher or lower temperatures have a definite influence on the cell vitality. Most of the studied compounds have low cytotoxic effect on the examined cells. Because the lymphocytes from healthy animals showed at both temperatures quite equal survival that fact lead us to the assumption of some suppression of important cell functions or lowering of cell metabolism in comparison to the untreated cells from healthy animals.

The lymphocytes from infected animals exhibited higher vitality after incubation at 37°C (Table 1). The substances **1**, **8**, **10** and **13** did not show any cytotoxicity against the cells from these animals. The vitality of the same cells after incubation at 4°C is also high, but after incubation with the 1,2,4-triazole **12** the cells did not survive in contrast to the same incubation by 37°C. The normal cells showed after the incubations at 4°C also a great survival. No correlation can be observed among the temperature dependent vitality after incubations of all cell lines with all studied compounds. The substance **14** is very gentle to the cells from infected fowls as well as from the healthy hens after incubation at 4°C. The same effects show substances **9** and **13**. The thiosemicarbazides **2** has a mild effect only on the cells from healthy hens and at both temperatures.

Table 3. Evaluation of spleen lymphocytes from Pts56 infected birds on smears, stained by Gimsa.

Subst.	Spleen lymphocytes after 24 h at 4°C	Spleen lymphocytes after 24 h at 37°C
1	only S-Ly	M+L-Ly; faded chromatin
2	granulocytes-eosinophils; S+M-Ly	S-Ly; destroyed cytoplasm
3	granulocytes-eosinophils; S+M-Ly	S-Ly; lymphocyte clusters
6	remained cell nuclei	faded chromatin;S+M-Ly
7	S+M-Ly	S+M-Ly, rare L-Ly
8	S+L-Ly	few cells; many crystals
9	many S-Ly, few M-Ly	only S-Ly
10	very few M-Ly	only S-Ly
11	S+M-Ly; granulocytes	S+M-Ly; deviding cells
12	many S-Ly, few M-Ly	only S-Ly
13	granulocytes-eosinophils; S+M-Ly	S+M-Ly
14	S+M-Ly	S+M+L-Ly; crystals
Control	not enough intact cells	intact cells

Table 4. PI stainings of spleen lymphocytes incubated with the substances at 4 and 37 °C.

Subst. No	Infected with Pts 56 hens		Healthy guinea hens	
	4 ^{0}C	37 ^{0}C	4 ^{o}C	37 ^{0}C
1	20% positive	NS	positive	20% positive
2	50% positive	50% positive	positive	positive
3	NS	NS	NS	70%
6	50% positive	NS	few positive	NS
7	NS	NS	few positive	NS
8	positive	few positive	NS	NS
9	NS	NS	NS	few positive
10	few positive	NS	NS	NS
11	NS	NS	few positive	NS
12	positive	NS	NS	NS
13	NS	NS	60 % positive	few positive
14	NS	positive	NS	NS
Control cells	NS	5% positive	NS	2% positive

NS] - not stained

CONCLUSIONS

The performed screening showed that the compounds **8**, **10** and **12** revealed cytotoxicity to the infected cells and not to normal. The presence of -N-NH_2 and N-C_2H_5-fragments in the structure of the compounds **8** and **12** maybe was the reason for the higher vitality of all cells at both temperatures. For our further experiments it is very important that the compounds are indifferent to normal cells but express antiviral respectively antitumor effect. The estimated toxicity indicated that the derivatives **8** and **12** could be used for further examinations because

they showed simultaneously the lowest cytotoxic activity to normal cells and the highest effect to cells from tumor-bearing animals.

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