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ARTICLE *in* EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY · SEPTEMBER 2014

Impact Factor: 3.45 · DOI: 10.1016/j.ejmech.2014.09.032 · Source: PubMed

European Journal of Medicinal Chemistry 86 (2014) 676–683

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis and antiproliferative activity of some new thieno[2,3-*d*] pyrimidin-4(3H)-ones containing 1,2,4-triazole and 1,3,4-thiadiazole moiety

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ARTICLE INFO

Article history:
Received 18 February 2014
Received in revised form
7 September 2014
Accepted 8 September 2014
Available online 10 September 2014

Keywords:
Thieno[2,3-*d*]pyrimidin-4(3H)-ones
1,2,4-Triazoles
1,3,4-Thiadiazoles
Cytotoxicity
Anticancer activity

ABSTRACT

Some new thieno[2,3-*d*]pyrimidin-4(3H)-ones containing 1,2,4-triazole and 1,3,4-thiadiazole moiety were synthesized using thieno[2,3-*d*]pyrimidin-3(4H)-yl)acetohydrazides as precursors in order to determine their cytotoxicity. Compounds **5**, **7**–**8** and **10**–**18** were evaluated for their cytotoxical effect on four cancer cell lines: human colorectal cancer cell line HT-29, breast cancer cells MDA-MB-231, cervical cancer cells HeLa, human liver carcinoma HepG2 and human normal diploid cell line Lep3. Exclusively high cytotoxic activity of compounds **8**, **16** and **17** against MDA-MB-231 cells was ascertained and the calculated IC_{50} values were $3.91 \cdot 10^{-2}$, $1.2 \cdot 10^{-3}$ and $3.74 \cdot 10^{-2}$ μM respectively. Thienopyrimidinones **10**, **15** and **17** exhibited high cytotoxicity against HT-29 cell and the IC_{50} values were in the range $1.56 \cdot 10^{-3}$ μM– 0.13 μM. To HeLa cell lines cytotoxicity demonstrated compounds **8**, **10**, **11**, **13** and **15**–**18** but the substance **13** was the most toxic with $IC_{50} - 9.5 \cdot 10^{-4}$ μM. Distinctly high antiproliferative activity of derivatives **10**, **14**–**15** and **17**–**18** was estimated against Hep G2, compound **15** showed $IC_{50} -$ 0.21 μM. Proliferative effects to Lep 3 demonstrated compounds **5**, **7**–**8**, **11**–**14**, **16**, **18** whose EC_{50} values were from 0.12 to 2.21 μM. The biological data highlighted that the nature and the position of the substituents influence both the cytotoxicity to the cancer cells and the proliferation properties to Lep3 of the tested compounds.

1. Introduction

The thieno[2,3-*d*]pyrimidines are compounds of pharmacological interest because of their similarity to the biogenic pyrimidines. The presence of a pyrimidine ring in the basic building scaffolds of DNA and RNA modules – thymine, cytosine and uracil is probably the reason for diversity of their biological activity. Many thieno[2,3-*d*]pyrimidines has been investigated for their anticancer activity. A series of thienopyrimidines was identified as a new class of compounds possessing significant antitumor activities. Among them the cytotoxic agent R-253 [*N*-cyclopropyl-2-(6-(3,5-dimethylphenyl)thieno[3,2-*d*]pyrimidin-4-yl)hydrazine carbothioamide] emerged as a potent antiproliferative agent is structurally unique and destabilizes microtubules both *in vivo* and *in vitro* [1]. Some thienopyrimidine derivatives were synthesized as antioxidant and antitumor agents and had showed significant *in vitro* cytotoxic activity against hepatocellular carcinoma (Hep G-2) compared to the reference drug Doxorubicin [2,3]. 5,6,7,8-Tetrahydrobenzothieno[2,3-*d*]pyrimidin-4(3H)-one was identified as a highly selective and potent agent displaying an IC_{50} of 91 nM toward the p21-deficient cell line and 2-amino-4-oxo-5-arylthio-substituted-6-methylthieno[2,3-*d*]pyrimidines were potent inhibitors of both thymidylate synthase and dihydrofolate reductase [4–6]. It was found a 4-morpholino-2-phenylquinazoline derivative to be selective for p110α over other PI3K isoforms and protein kinases, making it the first example of a selective PI3K p110α inhibitor [7].

On the other hand the 1,2,4-triazole heterocycle is of great value as a building block in the structure of several drug candidates. A series of 3,5-diarylsubstituted-1,2,4-triazole derivatives were synthesized and it was estimated that the compounds exhibited remarkable anticancer potential in screening tests with 60 human cancer cell lines [8]. Some 1,5-disubstituted 1,2,4-triazoles were synthesized as cis-restricted combretastatin analogs [9] and their

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http://dx.doi.org/10.1016/j.ejmech.2014.09.032
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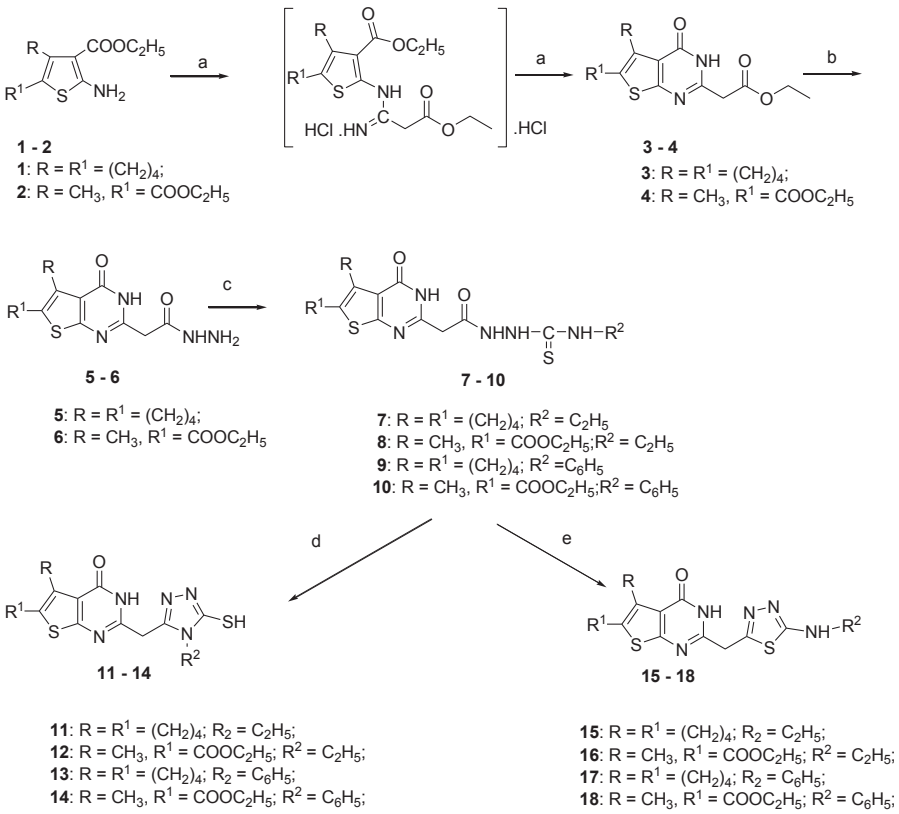

Scheme 1. Synthesis of 1,2,4-triazole and 1,3,4-thiadiazole derivatives of thieno[2,3-*d*]pyrimidin-4(3H)-ones; Regents and conditions: a) ethyl cyan acetate, dry HCl gas, ethanol; b) hydrazine hydrate, ethanol, reflux; c) isothiocyanate, ethanol, reflux; d) 10% NaOH, reflux, hydrochloric acid; e) sulfuric acid, 0 °C, NH_4OH.

effect was accompanied by apoptosis of the cells, mitochondrial depolarization, generation of reactive oxygen species, activation of caspase-3, and PARP cleavage. Diverse 1,2,4-triazoles analogs have been synthesized and the evaluation for their cytotoxicity against six human cancer cell lines revealed that some of them displayed promising activity [10]. 1,2,4-Triazole derivatives containing 1,4-benzodioxan have been screened and have showed good anti-tumor activity against HEPG2 cancer cell line [11]. The application of anastrozole and letrozole as aromatase inhibitors for the treatment of estrogen-dependent cancer as well as the anticancer properties of ribavirin led to the investigation of many 1,2,4-triazole derivatives in laboratorial conditions for their antitumor activity [12,13].

The 1,3,4-thiadiazoles are of pharmacologic interest because of their antimicrobial, antifungal [14,15], anti-acetyl- and anti-butyrylcholinesterase [16], antiviral [17] and antioxidant [18] activities. Many 1,3,4-thiadiazoles which could be seen as isosters of 5-mercapto-substituted 1,2,4-triazoles were synthesized and investigated for their anticancer properties [19–22]. Some thiadiazole compounds containing acetyl and ethoxy carbonyl groups were described as a new class of antitumor agents [23]. Having in view the known chemotherapeutic activities both of 1,2,4-triazoles and 1,3,4-thiadiazoles as anticancer agents, it was of pharmacological interest to incorporate that moieties into the parent thieno [2,3-*d*]pyrimidin-4-ones backbone structure to obtain more active and/or less toxic anticancer agents. The choice of these structures was in conformity with the fact that the thienopyrimidine heterocycle takes part in the structure of substances possessing antitumor activity.

Considering the above mentioned data and as a continuation of our previous investigation over 1,2,4-triazoles and 1,3,4-thiadiazoles and as well as thienopyrimidinones, we decided to synthesize some new thieno[2,3-*d*]pyrimidin-4-ones containing in their structure 1,2,4-triazole respectively 1,3,4-thiadiazole heterocycles in order to study their effects on four human cancer cell lines (Scheme 1).

2. Chemistry

The synthesis of thieno[2,3-*d*]pyrimidin-4(3H)-one derivatives, containing 1,2,4-triazole or 1,3,4-thiadiazole ring is illustrated and outlined in Fig. 1.

The starting 3-ethoxycarbonyl-2-amino-thiophenes **1**–**2** were synthesized according to the method, described by Gewaldt [24]. Thieno[2,3-*d*]pyrimidin-4(3H)-ones **3**–**4** were obtained by passing a stream of dry hydrogen chloride gas through a solution of the

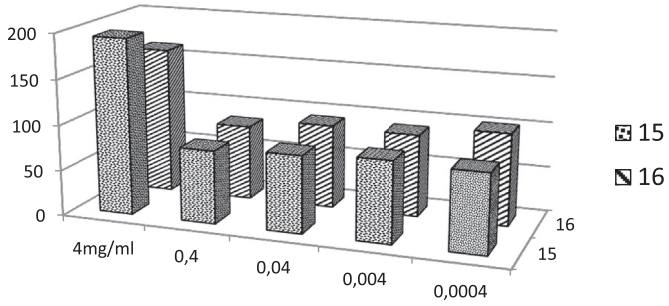

Fig. 1. Viability of HeLa cells (%) after treatment with compounds **15** and **16**.

appropriate 2-amino-3-ethoxycarbonyl-thiophene in ethyl cyanoacetate. The interaction of the esters **3**–**4** with hydrazine hydrate led to the hydrazides **5**–**6**, which by treatment with the corresponding isothiocyanates gave the semicarbazides **7**–**10** with yields of 80%. The corresponding 2-(1,2,4-triazol-3-yl)methyl-thieno[2,3-*d*]pyrimidin-4(3H)-ones **11**–**14** were synthesized by refluxing of the relevant thiosemicarbazide in water solution of sodium hydroxide and following acidifying with hydrochloric acid. The thiadiazole containing thieno[2,3-*d*]pyrimidin-4(3H)-ones **15**–**18** were obtained from compounds **7**–**8** in cooled sulfuric acid and neutralization of the obtained solution.

The structures of all new compounds were established by IR, ^{1}H NMR as well as elemental analysis. Detailed assignment of the ^{1}H NMR and some of the ^{13}C NMR spectra of the synthesized compounds is given in the Experimental part. The elemental analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values.

3. Pharmacology

Twelve of the synthesized compounds (**5**, **7**–**8** and **10**–**18**) were evaluated for their cytotoxicity to human colorectal cancer cell line HT-29, breast cancer cells MDA-MB-231, cervical cancer cells HeLa, human liver carcinoma HepG2 and human normal diploid cell line Lep3 by using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrasolium inner salt)-test [25].

4. Result and discussion

To accomplish our aims to synthesize new thienopyrimidinone derivatives we carried out a study of the reaction between the corresponding 2-aminothiophene and ethyl cyan acetate. By passing hydrogen chloride gas through a solution of corresponding 2-amino-thiophene in ethyl cyan acetate were obtained the corresponding esters as precursor for the synthesis of the target compounds. It was found that the reaction proceeds through amidine hydrochloride formation at ambient temperature. The reaction of the esters 3 and 4 with excess hydrazine hydrate in ethanol gave the acetohydrazides 5–8 which upon treatment with the appropriate isothiocyanates afforded the corresponding thiosemicarbazide derivatives in good yields – 79–83%. The known synthetic methods of 1,2,4-triazole or 1,3,4-thiadiazole fragment formation are based on two main approaches: the first approach is the transforming of thiosemicarbazides in basic medium into triazoles, and the second approach is the conversion of thiosemicarbazide in thiadiazole derivatives. The yields of the obtained triazole and thiadiazole compounds were in the range from 75 to 84% and 79–87% respectively. It should be noted that upon receiving of the products **12** and **14**, the ester group in the starting compounds undergoes hydrolysis yielding the corresponding carboxylic derivatives.

The *in vitro* screening of compounds **5**, **7**–**8** and **10**–**18** was performed in order to estimate their effects towards HT-29, MDA-MB-231, HeLa, HepG2 and Lep3 cell lines using the MTS as described in Ref. [20]. The cytotoxicity respectively proliferative effect was assessed by MTS assay, which is based on the reduction of yellow tetrazolium salt by metabolically active viable cells to a formazan product that can be measured spectrophotometrically. Hence, the intensity of the color in the solution is directly proportional to cell viability [26,27]. The bigger released amount of formazan indicates to a higher vitality of the cells (proliferation). The low vitality demonstrates a cytotoxic influence of the experimental compounds. Relative cell viability, expressed as a percentage of the untreated control (100% viability), was calculated for each concentration. The obtained results were plotted and IC_{50} and EC_{50} were calculated. The data are given in Tables 1 and 2.

The examination of the compounds showed that individual cell lines had different sensitivity towards synthesized compounds. Most of them possessed relatively high cytotoxic effect to the different tumor cell lines. Thus, compounds **10**, **15** and **17** exhibited cytotoxicity against HT-29 cell line, whereby the IC_{50} values varied between $1.56\cdot10^{-3}$ μM to 0.13 μM. In respect to Lep-3 cell line the same compounds revealed also cytotoxic effect but in concentrations, which are many times lower than those at which the compounds excited cytotoxic effect on HT-29 cells (Table 2).

Compounds **8**, **10**, **15**, **16** and **17** showed relative high cytotoxic activity against MDA-MB-231 cells. Most toxic was compound **16** possessing IC_{50} value of $1.2.10^{-3}$ μM, followed by compounds **17** and **8** with IC_{50} – $3.74\cdot10^{-2}$ and IC_{50} – $3.91\cdot10^{-2}$ μM respectively.

To HeLa cell lines cytotoxicity demonstrated the thiosemicarbazides **8** and **10**, the triazole derivatives **11** and **13** and as well as the four studied thiadiazoles **15**, **16**, **17**, **18**. The IC_{50} values of the thiadiazoles varied from 1.85 to 3.8 μM, but most toxic to HeLa cells was the triazole **13** with IC_{50} – $9.5\cdot10^{-4}$ μM. It should be pointed out that at the same time compound **13** showed proliferative activities against Lep 3 with EC_{50} – 0.46 μM. The calculated IC_{50} values of compounds **10**, **14**–**15** and **17**–**18** in test with Hep G2 were from 1.47 to 0.21 μM and most toxic was the thiadiazole **15**.

Among the thiosemicarbazides compound **10** was toxic against all cancer cell lines, used in that study, while it revealed cytotoxicity to Lep 3 in a very high concentration – EC_{50} – $1.5\cdot10^{6}$ ± 0.19 M.

The similarity in the structure of the 2-amino-1,3,4-thiadiazole and the mercapto-1,2,4-triazole ring systems presumes similar biological properties, but the data, obtained by the MTS-test accentuate that both the nature of the thienopyrimidine moiety and that of the substituents at the 4-th position of 1,2,4-triazoles as well as these at the 5-th place of the 1,3,4-thiadiazoles exert different effects not only on the cytotoxicity but also on the proliferative activity of the studied compounds. If the effects of the triazole derivatives are taken in to consideration it could be pointed out that the introduction of a phenyl substituent instead of the ethyl group at the 4-th position of the 1,2,4-triazole **13** leads to increasing of the cytotoxicity towards HeLa cells (the IC_{50} of compound **11** is 1.69 μM, while that of triazole **13** is $9.5\cdot10^{-4}$ μM). The presence of thienopyrimidine ring bearing a methyl and an ethoxycarbonyl groups in the thiophene heterocycle provoked proliferative effects of compounds **12** and **14** against HeLa cell lines independently whether an ethyl or a phenyl group is at the 4-th place of the triazole heterocycle. The biological data indicated that the nature and the position of the substituents on the 1,3,4-thiadiazole ring greatly influence both the cytotoxicity to the cancer cells and the proliferation properties to Lep3 of the tested compounds.

The contribution of the substituents in the structure of theinopyrimidine moiety as well as in the structure of 1,3,4-thiadiazole ring can be traced through the effects of compounds **15**–**16** and **17**–**18**. Compounds **15** and **16** possessed ethylamino group at 4-th place of the thiadazoles ring but compound **15** containing tetrahydrobenzothienylpyrimidinyl-methyl group in the 2-nd position showed cytotoxicity to all studied cells. At the same time the presence of ethyl 5-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxylate in the structure of **16** provoked the appearance of cytotoxicity against MDA-MB-231 and HeLa with IC_{50} $1.2\cdot10^{-3}$ and IC_{50} 3.31 μM respectively and proliferative activity towards Lep3 (Figs. 1–3).

The same relationship was observed by compounds **17** and **18**. Compound **17** was cytotoxic to all cell lines, but thiadiazole **18** showed cytotoxicity to HeLa and HepG2 and proliferative activity in regards to Lep3 (Figs. 4–6). As it can be seen from the figures the

Table 1
In vitro cytotoxicity against HeLa, Hep G2, HT-29, MDA-MB-231 and Lep 3 cells.

Comp.	$IC_{50} \pm SE$ (μM)				
	HT-29	MDA-MB-231	HeLa	Hep G2	Lep3
8		$3.91 \cdot 10^{-2} \pm 0.19$	1.7 ± 0.03	–	
10	$1.56 \cdot 10^{-3} \pm 0.02$	1.21 ± 0.17	9.3 ± 0.21	1.47 ± 0.05	$1.5 \cdot 10^{3} \pm 0.19$
11	–	–	1.69 ± 0.18	–	–
13			$9.5 \cdot 10^{-4} \pm 0.08$	$1.8 \cdot 10^{3} \pm 0.024$	–
14				0.29 ± 0.018	
15	$9.7 \cdot 10^{-2} \pm 0.21$	1.26 ± 0.41	3.8 ± 0.13	0.21 ± 0.16	0.44 ± 0.08
16		$1.2 \cdot 10^{-3} \pm 0.15$	3.31 ± 0.41		
17	0.13 ± 0.17	$3.74 \cdot 10^{-2} \pm 0.15$	1.85 ± 0.09	1.23 ± 0.06	3.17 ± 0.11
18	–	–	2.3 ± 0.08	0.88 ± 0.04	

Table 2
The proliferative activity (EC_{50}) of the studied compounds.

Comp.	$EC_{50} \pm SE$ (μM)				
	HT-29	MDA-MB-231	HeLa	Hep G2	Lep3
5	2.12 ± 0.53	1.54 ± 0.28	2.52 ± 0.15	2.18 ± 0.31	2.21 ± 0.43
7	$4 \cdot 10^{-2} \pm 0.13$	1.8 ± 0.24	$3.2 \cdot 10^{-2} \pm 0.14$	1.02 ± 0.03	0.12 ± 0.25
8	$8.9 \cdot 10^{-2} \pm 0.26$	–	–	0.19 ± 0.32	$2.5 \cdot 10^{-2} \pm 0.08$
11	2.43 ± 0.16	0.13 ± 0.09	–	2.08 ± 0.24	1.14 ± 0.38
12	0.91 ± 0.59	1.16 ± 0.26	2.34 ± 0.18	1.40 ± 0.45	1.4 ± 0.32
13	1.8 ± 0.11	1.2 ± 0.16	–	–	0.46 ± 0.12
14	0.86 ± 0.14	0.30 ± 0.20	0.120 ± 0.24	–	1.21 ± 0.11
16	0.42 ± 0.28		–	1.26 ± 0.29	0.31 ± 0.19
18	1.5 ± 0.09	$1.8 \cdot 10^{-3} \pm 0.11$	–	–	0.26 ± 0.12

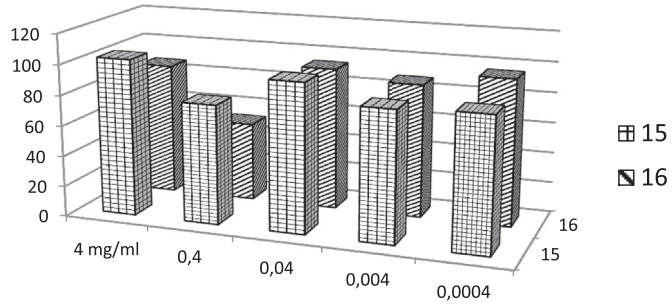

Fig. 2. Viability of Hep G2 (%) cells after treatment with compounds **15** and **16**.

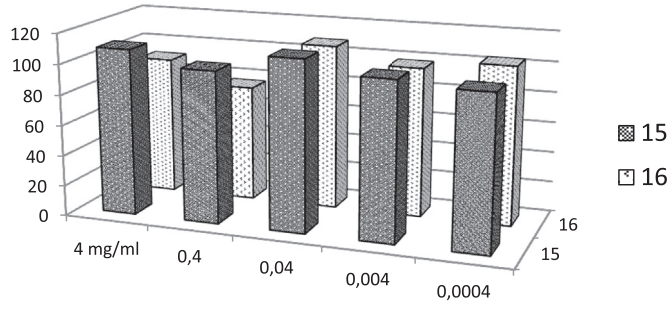

Fig. 3. Viability of Lep 3 cells (%) after treatment with compounds **15** and **16**.

vitality of all cells treated with the tested compounds do not exceed 100%. To display more precise the relationship between structure and activity of the synthesized compounds it is necessary to be carried out both further research on other derivatives and to be continued the biological research *in vivo*.

5. Conclusion

New thieno[2,3-*d*]pyrimidin-4(3H)-ones containing different substituted 1,2,4-triazoles and 1,3,4-thiadiazoles heterocycles were synthesized in good yields using the corresponding thieno[2,3-*d*]pyrimidin-3(4H)-yl)acetohydrazides as precursors under optimized reaction conditions.

The initial biological screening *in vitro* showed that the studied triazole containing compound **13** possessed relative high cytotoxicity against HeLa cells, the IC_{50} value was $9.5 \cdot 10^{-4}$ μM but towards the other cells it exhibited proliferative effects, the EC50 values varied from 0.466 to 1.8 μM. Among the thienopyrimidinones containing 1,3,4-thiadiazole ring compounds 15 and 17 revealed cytotoxicity to all used in the screening cells (IC_{50} – $3.74 \cdot 10^{-2}$–3.8 μM) while compounds16 and 18 demonstrated

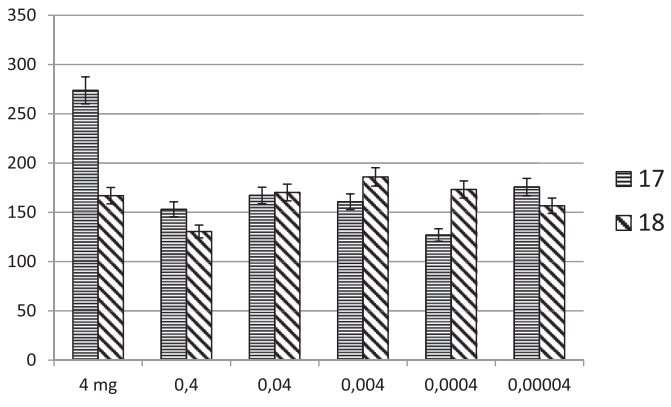

Fig. 4. Viability of HeLa cells after treatment with compounds **17** and **18**.

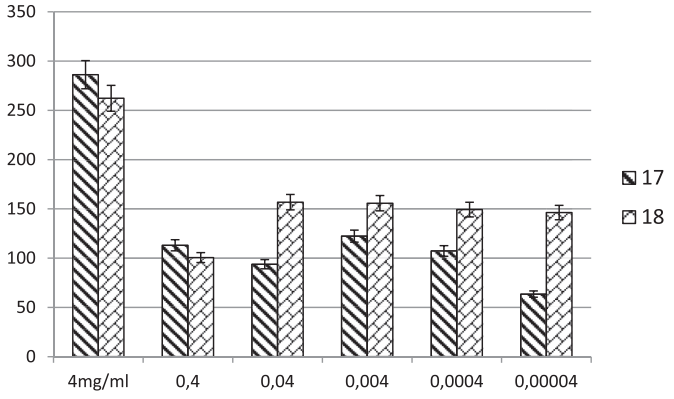

Fig. 5. Viability of Hep G2 cells after treatment with compounds **17** and **18**.

selective cytotoxicity to the cancer cell lines but showed proliferative effects on human diploid cells, the EC_{50} values were in the range 0.26–0.31 μM.

The obtained results indicated that the introduction of triazole respectively thiadiazole ring in the pyrimidinone skeleton prove the necessity for further study to estimate the features related to the antitumor potential of the tested compounds.

6. Experimental part

Melting points (mp) were determined on an Electrothermal AZ 9000 3MK4 apparatus and were uncorrected. The thin layer chromatography (TLC, Rf values) was performed on F_{254} or silica gel plates F_{254} (Merck, 0.2 mm thick) and visualization was effected with ultraviolet light. IR spectra were recorded on a Bruker Equinox 55 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 $^1H/^{13}C$ probe head. 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. General procedure for the preparation of **1**–**2**

The 2-amino-thiophenes (**1**–**2**) were synthesized by condensation of cyclohexanone or ethyl acetoacetate with ethyl cyanoacetate and sulfur in the presence of diethyl amine according to Ref. [19].

6.1.1. Ethyl 2-amino-4,5,6,7-tetrahydro-1-benzothiophene-3-carboxylate **1**

Yield – 82%; Mp – 119–121 °C, re-crystallization with ethanol; IR, (cm^{-1}): 3342, 3232 (ν NH_2); 1644.5 (ν C=O); ^1H NMR ($CDCl_3$) δ (ppm): 1.22 (t, 1H, CH_3); 1.76 (m, J^3 = 7.10, 4H, $2CH_2$); 2.45 (m, $2CH_2$); 2.66 (m, J^3 = 8.84 Hz, m 2H, $2CH_2$); 4.21 (q, 2H, CH_2); 6.84 (bs, 2H, NH_2, exchangeable with D_2O).

6.1.2. Diethyl 5-amino-3-methylthiophene-2,4-dicarboxylate

Yield – 82%; Mp – 119–121 °C, re-crystallization with ethanol; Yield – 65%; Mp – 107–109 °C, re-crystallization with ethanol; IR, (cm^{-1}): 3251.5, 3384.2 (ν NH_2); 1680.5 (ν C=O); ^1H NMR ($CDCl_3$) δ (ppm): 1.24 (dt, 6H, $2CH_3$); 2.62 (s, 3H, CH_3); 4.33 (m, J = 6.83 Hz, 4H, $2CH_2$); 6.44 (s, 2H, NH_2, exchangeable with D_2O).

6.2. General procedure for the preparation of **3**–**4**

A stream of dry hydrogen chloride gas was passed through a solution of 0.044 mol of compound **1** or **2** in ethyl cyanoacetate (0.94 mol) for 2 h at ambient temperature by stirring. At the beginning of the reaction a formation of amidine hydrochloride has been observed, which is turned later in the reaction solution, forming the thienopyrimidinone ring. The reaction solution was allowed to stand 24 h, the excess of ethyl cyan acetate was removed under reduced pressure and the thienopyrimidinone crystallized. The obtained precipitate was filtered and recrystallized with ethanol.

6.2.1. Ethyl (4-oxo-3,4,5,6,7,8-hexahydro[1]benzothieno[2,3-d] pyrimidin-2-yl)acetate **3**

Yield – 82%; Mp – 184–186 °C, re-crystallization with ethanol; Rf = 0.41, mobile phase: benzene/chloroform/ethanol – 4:1:3; IR, (cm^{-1}): 3140.4 (ν NH); 1750.5.5 (ν C=O); 1680.2 (ν C=O); ^1H NMR (DMSO-d6) δ (ppm): 1.14 (t, 3H, CH_3); 1.72 (t, 4H, $2CH_2$); 2.48 (t, J = 8.38 Hz, 2H, CH_2); 2.80 (m, J = 5.56 Hz, 2H, CH_2); 4.05 (s, 2H, CH_2); 12.36 (s, 1H, NH, exchangeable with D_2O); Analysis: Calc. for $C_{14}H_{16}N_2O_3S$: C, 57.52; H, 5.52; N, 9.58; O, 16.42; S, 10.97; Found: C, 57.54; H, 5.52; N, 9.60; O, 16.44; S, 10.95.

6.2.2. Ethyl 2-(2-ethoxy-2-oxoethyl)-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxylate **4**

Yield – 78%; Mp – 178–180 °C, re-crystallization with ethanol; Rf = 0.52, mobile phase: benzene/chloroform/ethanol – 4:1:3; IR, (cm^{-1}): 3180.2 (ν NH); 1753.5.5 (ν C=O); 1680.2 (ν C=O); ^1H NMR (DMSO-d6) δ (ppm): 1.15 (t, 3H, CH_3); 1.25 (t, 3H, CH_3); 2.76 (s, 3H, CH_3); 3.72 (s, 2H, CH_2); 4.10 (q, 2H, CH_2); 4.21 (q, 2H, CH_2); 12.67 (s, 1H, NH, exchangeable with D_2O).

Analysis: Calc. for $C_{14}H_{16}N_2O_5S$: C, 51.84; H, 4.97; N, 8.64; O, 24.66; S, 9.89; Found: C, 51.81; H, 4.94; N, 8.67; O, 24.68; S, 9.92.

6.3. General procedure for the preparation of compounds **5**–**6**

To a solution of 0.04 mol of the corresponding thieno[3,2-d] pyrimidinones **3**–**4** in 60 ml ethanol, 8.7 ml (0.16 mol) 98% hydrazine hydrate was added and the mixture was refluxed for 8 h. After cooling the obtained solid was filtered and recrystallized with ethanol.

6.3.1. 2-(4-Oxo-5,6,7,8-tetrahydro[1]benzothieno[2,3-d]pyrimidin-3(4H)-yl)acetohydrazide **5**

Yield – 90%; Mp – 278–280 °C, re-crystallization with ethanol; Rf = 0.43, mobile phase: benzene/ethanol – 8:1; IR, (cm^{-1}): 3380.2, 3183.3 (ν NH_2); 1660.2 (ν C=O); ^1H NMR (DMSO-d6) δ (ppm): 1.78 (m, 4H, $2CH_2$); 2.74(t, 2H, CH_2); 2.82 (t, 2H, CH_2); 3.83 (s, 2H, NH_2); 4.15 (s, 2H,CH_2); 9.2 (s, 1H, NH); 11.98 (bs, 1H, NH); Analysis: Calc. for $C_{12}H_{14}N_4O_2S$: C, 51.78; H, 5.07; N, 20.13; O, 11.50; S, 11.52; Found: C, 51.81; H, 5.04; N, 20.17; O, 11.54; S, 11.56.

6.3.2. Ethyl 3-(2-hydrazino-2-oxoethyl)-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxylate **6**

Yield – 80%; Mp – 350 °C (decomp.), re-crystallization with ethanol; Rf = 0.73, mobile phase: benzene/ethanol – 3:1; IR, (cm^{-1}): 3280.2, 3203.3 (ν NH_2); 1690.5 (ν C=O); 1641.6 (ν C=O); ^1H NMR (DMSO-d6) δ (ppm): 1.26 (t, J = 6.82 Hz, 3H,CH_3); 2.54 (s, 3H, CH_3); 3.98 (s.2H, NH); 4.20 (s 2H, CH_2); 4.32 (q, 2H, CH_2); 9.45 (s, 1H, NH), 12.56 (s, 1H, NH); ^{13}C NMR (DMSO-d6): 14.04 (CH_3); 47.20 (CH_3); 62.62 (CH_2); 118.27 (CH); 118.26 (9-C); 120.05 (7-C); 137.81 (8-C); 155.37 (4-C); 158.76 (6-C); 160.85 (15-C); 166.86 (2-C); 170.26 (11-C); Analysis: Calc. for $C_{12}H_{14}N_4O_4S$: C, 46.44; H, 4.55; N,

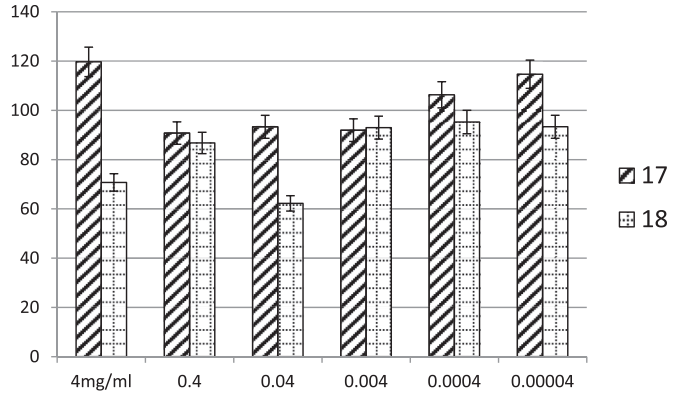

Fig. 6. Viability of Lep 3 cells after treatment with compounds **17** and **18**.

18.05; O, 20.62; S, 10.33; Found: C, 46.41; H, 4.59; N, 18.01; O, 20.60; S, 10.37.

6.4. General procedure for the preparation of compounds **7**–**10**

To a suspension of 0.013 mol of compounds **5**–**6** in 40 ml ethanol was added 0.015 mol of the respective isothiocyanate and the mixture was refluxed by stirring for 4–5 h. The thiosemicarbazides crystallized after cooling.

6.4.1. *N-Ethyl-2-[(4-oxo-3,4,5,6,7,8-hexahydro[1]benzothieno[2,3-d]pyrimidin-2-yl)acetyl]hydrazinecarbothioamide* **7**

Yield – 81%; Mp – 255–257 °C (decomp.), re-crystallization with ethanol; Rf = 0.48, mobile phase: benzene/ethanol – 2:1; IR, (cm^{-1}): 3218.1 (ν NH); 1651.5 (ν C=O); ^{1}H NMR (DMSO-d6) δ (ppm): 1.15 (t, 3H, CH_3); 1.76 (m, 4H, $2CH_2$); 2.82 (t, J = 7.14 Hz, 2H, CH_2); 2.90 (t, J = 12.40 Hz, 2H, CH_2); 3.55 (q, j = 7.30 Hz, 2H, CH_2); 4.09 (s, 2H, $2CH_2$); 12.21 (bs, 2H, NH); 13.02 (s, 1H, NH); Analysis: Calc. for $C_{15}H_{19}N_5O_2S_2$: C, 49.30; H, 5.24; N, 19.16; O, 8.76; S, 17.55; Found: C, 49.34; H, 5.26; N, 19.12; O, 8.80; S, 17.57.

6.4.2. *Ethyl 2-(2-{2-[(ethylamino)carbonothioyl]hydrazino}-2-oxoethyl)-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxylate* **8**

Yield – 79.5%; Mp –210–212 °C (decomp.), re-crystallization with ethanol; Rf = 0.53, mobile phase: benzene/ethanol – 3:1; IR, (cm^{-1}): 3380.3 (ν NH); 1740.4; 1703. 1; 1680.7 (ν C=O); ^{1}H NMR (DMSO-d6) δ (ppm): 1.14 (t, 3H, CH_3); 1.28 (t, 3H, CH_3); 2.55 (s, 3H, CH_3); 3.50 (q, J = 7.30 Hz, 2H, CH_2); 4.11 (s, 2H, CH_2); 4.32 (q, J = 6.82 Hz, 2H, CH_2); 12.15 (bs, 3H, NH); 13.18 (s, 1H, NH); Analysis: Calc. for $C_{15}H_{19}N_5O_4S_2$: C, 45.33; H, 4.82; N, 17.62; O, 16.10; S, 16.13; Found: C, 45.35; H, 4.84; N, 17.59; O, 16.14; S, 16.17.

6.4.3. *2-[(4-Oxo-3,4,5,6,7,8-hexahydro[1]benzothieno[2,3-d]pyrimidin-2-yl)acetyl]-N-phenylhydrazinecarbothioamide* **9**

Yield – 79.5%; Mp – 215–217 °C (decomp.), re-crystallization with ethanol; Rf = 0.63, mobile phase: benzene/ethanol – 4:1; IR, (cm^{-1}): 3234.6 (ν NH); 1675.6 (ν C=O); ^{1}H NMR (DMSO-d6) δ (ppm): 1.78 (t, 4H, $2CH_2$); 2.77 (t, 2H,CH_2, J = 8.0); 2.88 (t, 2H, CH_2, J = 7.15); 4.34 (s, 2H–CH_2); 6.98 (t, 1H, Bz, J = 7.37); 7.31 (m, 2H, Bz, J = 8.04); 7.48 (d, 2H, Bz, J = 7.84); 10.31 (s, 2H, NH); 11.42 (s, 1H, NH); 12.56 (s, 1H, NH); Analysis: Calc. for $C_{19}H_{19}N_5O_2S_2$: C, 55.19; H, 4.63; N, 16.94; O, 7.74; S, 15.51; Found: C, 55.23; H, 4.60; N, 16.96; O, 7.71; S, 15.48.

6.4.4. *Ethyl 2-{2-[2-(anilinocarbonothioyl)hydrazino]-2-oxoethyl}-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxylate* **10**

Yield – 83%; Mp – 257–259 °C, re-crystallization with ethanol; Rf = 0.53, mobile phase: benzene/ethanol – 3:1; IR, (cm^{-1}): 3228.4 (ν NH); 1740.3, 1704.1, 1681.4 (ν C=O); ^{1}H NMR (DMSO-d6) δ (ppm): 1.29 (t, 3H, CH_3); 2.56 (s, 3H,CH_3); 4.28 (m, 2H, CH_2–O, J = 6.83); 4.39 (s, 2H, CH_2); 7.12 (t, J = 7.37, 1H, Bz); 7.36 (m, J = 7.85, 2H, Bz); 7.59 (d, 2H, Bz); 10.34 (s, 1H, NH); 11.34 (s, 2H, NH); 12.52 (s, 1NH); Analysis: Calc. for $C_{19}H_{19}N_5O_4S_2$: C, 51.22; H, 4.30; N, 15.72; O, 14.36; S, 14.39; Found: C, 51.27; H, 4.32; N, 15.69; O, 14.32; S, 14.43.

6.5. General procedure for the preparation of compounds **11**–**14**

To 0.005 mol of relevant thiosemicarbazide were added 20 ml of 10% solution of sodium hydroxide and the solution was refluxed for 5–6 h. After completing the reaction the solution was cooled and acidified with hydrochloric acid, whereupon the target compound crystallized.

6.5.1. *2-[(4-Ethyl-5-mercapto-4H-1,2,4-triazol-3-yl)methyl]-5,6,7,8-tetrahydro[1]benzothieno[2,3-d]pyrimidin-4(3H)-one* **11**

Yield – 84%; Mp > 350 °C, re-crystallization with ethanol; Rf = 0.48, mobile phase: benzene/ethanol – 3:1; IR, (cm^{-1}): 3240.4 (ν NH); 1681.4 (ν C=O); ^{1}H NMR (DMSO-d6) δ (ppm): 1.10 (t, 3H, CH_3); 1.71 (s, 4H, $2CH_2$); 2.66 (s, 2H, CH_2); 2.80 (s, 2H, CH_2); 3.88 (q, 2H, CH_2–N); 4.15 (s, 2H, CH_2) 12.54 (s, 1H, NH); 13.64 (s1H, SH); Analysis: Calc. for $C_{15}H_{17}N_5OS_2$: C, 51.85; H, 4.93; N, 20.16; O, 4.60; S, 18.46; Found: C, 51.83; H, 4.96; N, 20.19; O, 4.58; S, 18.44.

6.5.2. *2-[(4-Ethyl-5-mercapto-4H-1,2,4-triazol-3-yl)methyl]-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxylic acid* **12**

Yield – 76.1%; Mp – 275 °C (decomp), re-crystallization with ethanol; Rf = 0.42, mobile phase: benzene/ethanol – 2:1; IR, (cm^{-1}): 2375–3433.5 (ν COOH), 3240.4 (ν NH); 1700.4 (ν C=O), 1673.7 (ν C=O); ^{1}H NMR (DMSO-d6) δ (ppm): 1.19 (t, 3H, CH_3); 2.79 (s, 3H, CH_3); 3.95 (q, 2H, CH_2); 4.22 (s, 2H, CH_2); 12.50 (s, 1H, OH); 12.81 (s, 1H, NH); 13.69 (s, 1H, SH); Analysis: Calc. for $C_{13}H_{13}N_5O_3S_2$: C, 44.43; H, 3.73; N, 19.93; O, 13.66; S, 18.25; Found: C, 44.41; H, 3.76; N, 19.91; O, 13.64; S, 18.28.

6.5.3. *2-[(5-Mercapto-4-phenyl-4H-1,2,4-triazol-3-yl)methyl]-5,6,7,8-tetrahydro[1]benzothieno[2,3-d]pyrimidin-4(3H)-one* **13**

Yield – 81%; Mp – 334–336 °C; Rf = 0.42, mobile phase: benzene/ethanol – 5:1; IR, (cm^{-1}): 3287.4 (ν NH); 1670.3; ^{1}H NMR (DMSO-d6) δ (ppm): 1.76 (t, 4H, $2CH_2$); 2.72 (t, 2H, CH_2); 2.79 (t, J = 8.40 Hz, 2H, CH_2); 3.96 (s 2H, CH_2); 7.32 (m, J = 8.04 Hz, 2H, Bz); 7.48 (m, J = 7.54 Hz, 3H, Bz); 12.22 (s, 1H, NH); 13.89 (s, 1H, SH); Analysis: Calc. for $C_{19}H_{17}N_5OS_2$: C, 57.70; H, 4.33; N, 17.71; O, 4.05; S, 16.21; Found: C, 57.66; H, 4.36; N, 17.74; O, 4.09; S, 16.18.

6.5.4. *2-[(5-Mercapto-4-phenyl-4H-1,2,4-triazol-3-yl)methyl]-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxylic acid* **14**

Yield – 81%; Mp – 316–318 °C; Rf = 0.52, mobile phase: benzene/ethanol – 4:1; IR, (cm^{-1}): 2366–3413.5 (ν COOH), 3249.5 (ν NH); 1715.2 (ν C=O), 1683 (ν C=O); ^{1}H NMR (DMSO-d6) δ (ppm): 2.75 (s, 3H, CH_3, Th); 4.02 (s, 2H, CH_2); 7.02 (s, 1H, OH); 7.33 (m, 2H, Bz, J = 7.65); 7.45 (m, 3H, Bz, J = 7.56); 12.51 (s, 1H NH); 13.92 (s, 1H, SH); Analysis: Calc. for $C_{17}H_{13}N_5O_3S_2$: C, 51.12; H, 3.28; N, 17.53; O, 12.02; S, 16.05; Found: C, 51.16; H, 3.30; N, 17.51; O, 12.06; S, 16.08.

6.6. General procedure for the preparation of compounds **15–18**

To a cooled to 0 °C 98% sulfuric acid (12 ml) was added portion wise 0.003 mol of the appropriated thiosemicarbazide. Each successive portion was added after complete dissolution of the preceding one. The reaction mixture was allowed to equilibrate to ambient temperature. The obtained solution was slowly poured in 20 ml water/ice and neutralized with ammonium hydroxide.

6.6.1. 2-{[5-(Ethylamino)-1,3,4-thiadiazol-2-yl]methyl}-5,6,7,8-tetrahydro[1]benzothieno[2,3-d]pyrimidin-4(3H)-one **15**

Yield – 84%; Mp – 166–168 °C; Rf = 0.59, mobile phase: benzene/ethanol – 4:1; IR, (cm^{-1}): 3189.5 (ν NH); 1663.4 (ν C=O); ^{1}H NMR (DMSO-d6) δ (ppm): 1.10 (t, 3H, CH_3); 1.77 (t, 2H, $2CH_2$, J = 8.04); 2.72 (t, 2H,CH_2, J = 5.54); 2.86 (t, J = 8.38 Hz, 2H, CH_2); 3.51 (t, 2H, CH_2); 3.59 (s, J = 7.13 Hz, 2H, CH_2); 9.54 (bs, 1H, NH); 10.40 (bs 1H, NH); Analysis: Calc. for $C_{15}H_{17}N_5OS_2$: C, 51.85; H, 4.93; N, 20.16; O, 4.60; S, 18.46; Found: C, 51.89; H, 4.94; N, 20.12; O, 4.66; S, 18.48.

6.6.2. Ethyl 2-{[5-(ethylamino)-1,3,4-thiadiazol-2-yl]methyl}-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxylate **16**

Yield – 87.5%; Mp – 180 °C (decomp); Rf = 0.66, mobile phase: benzene/ethanol – 3:1; IR, (cm^{-1}): 3361.5 (ν NH); 1720.2 (ν C=O), 1682.1 (ν C=O); ^{1}H NMR (DMSO-d6) δ (ppm): 1.30 (t, 6H, $2CH_3$); 2.82 (s, 3H, CH_3); 3.99 (s, 2H, CH_2); 4.31 (q, 4H, $2CH_2$); 9.33 (s, 1H, NH); 12.6 (bs, 1H, NH); Analysis: Calc. for $C_{15}H_{17}N_5O_3S_2$: C, 47.48; H, 4.52; N, 18.46; O, 12.65; S, 16.90; Found: C, 47.50; H, 4.54; N, 18.42; O, 12.63; S, 16.94.

6.6.3. 2-[(5-Anilino-1,3,4-thiadiazol-2-yl)methyl]-5,6,7,8-tetrahydro[1]benzothieno[2,3-d]pyrimidin-4(3H)-one **17**

Yield – 87.5%; Mp – 238–241 °C; Rf = 0.46, mobile phase: benzene/ethanol – 3:1; IR, (cm^{-1}): 3291.5 (ν NH); 1680.1 (ν C=O); ^{1}H NMR (DMSO-d6) δ (ppm): 1.77 (t, 4H, $2CH_2$); 2.72 (t, 2H, J = 5.54 Hz CH_2); 2.82 (t, J = 8.40 Hz, 2H, CH_2); 4.34 (s, 2H–CH_2); 6.98 (t, J = 8.04 Hz 1H, Bz); 7.31 (m, 2H, Bz); 7.48 (d, J = 7.54 Hz; 2H, Bz); 10.31 (s, 1H, NH); 12.56 (s, 1H, NH); Analysis: Calc. for $C_{19}H_{17}N_5OS_2$: C, 57.70; H, 4.33; N, 17.71; O, 4.05; S, 16.21; Found: C, 57.67; H, 4.31; N, 17.74; O, 4.08; S, 16.18.

6.6.4. Ethyl 2-[(5-anilino-1,3,4-thiadiazol-2-yl)methyl]-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxylate **18**

Yield – 79.5%; Mp – 258–260 °C; Rf = 0.51, mobile phase: benzene/ethanol – 3:1; IR, (cm^{-1}): 3331.2 (ν NH); 1742.5; 1686.1 (ν C=O); ^{1}H NMR (DMSO-d6) δ (ppm): 1.30 (t, 3H, CH_3); 2.83 (s, 2H, 1H, CH_3); 4.28 (m, 2H,CH_2–O); 4.39 (s, J = 6.83 Hz, 2H, CH_2); 7.01 (t, J = 8.05 Hz, 1H, Bz); 7.36 (m, J = 7.53 Hz, 2H, Bz); 7.59 (d, 2H, Bz); 10.34 (s, 1H, NH); 12.52 (s, NH); Analysis: Calc for $C_{19}H_{17}N_5O_3S_2$: C, 53.38; H, 4.01; N, 16.38; O, 11.23; S, 15.00; Found: C, 53.37; H, 4.05; N, 16.41; O, 11.26; S, 15.04.

7. Biological assay

The compounds were dissolved in DMSO at the concentration of 4 mg/ml. The investigation was carried out by dilution of the stock solution in ratio 1:10, 1:100, 1:1000 and 1:10,000. Samples of cells, grown in non-modified medium served as a control. After 24 h of incubation of the samples MTS colorimetric assay of cell survival was performed. The wells were treated with MTS solution and incubated for 2 h at 37 °C under 5% carbon dioxide and 95% air atmosphere. The absorbance of each well at 490 nm was read by an automatic microplate reader ("Tecan", Austria).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.09.032.

References

[1] T.L. Gururaja, D. Goff, T. Kinoshita, E. Goldstein, S. Yung, J. McLaughlin, E. Pali, J. Huang, R. Singh, S. Daniel-Issakani, Y. Hitoshi, R.D.G. Cooper, D.G. Payan, R-253 disrupts microtubule networks in multiple tumor cell lines, Clin. Cancer Res. 12 (2006) 3831–3842.
[2] M.A. Shaaban, M.M. Ghorab, H.I. Heiba, M.M. Kamel, N.H. Zaher, M.I. Mostafa, Novel thiophenes, thienopyrimidines, and triazolothienopyrimidines for the evaluation of anticancer and augmentation effects of γ-radiation, Arch. Pharm. 343 (7) (2010) 404–410.
[3] A.A. Abu-Hashem, M.F. El-Shehry, F.A. Badria, Design and synthesis of novel thiophenecarbohydrazide, thienopyrazole and thienopyrimidine derivatives as antioxidant and antitumor agents, Acta Pharm. 60 (3) (2010) 311–323.
[4] L.D. Jennings, S.L. Kincaid, Y.D. Wang, G. Krishnamurthy, C.F. Beyer, J.P. McGinnis, M. Miranda, C.M. Discafani, S.K. Rabindran, Parallel synthesis and biological evaluation of 5,6,7,8-tetrahydrobenzothieno[2,3-*d*]pyrimidin-4(3H)-one cytotoxic agents selective for p21-deficient cells, Bioorg. Med. Chem. Lett. 15 (21) (2005) 47231–47235.
[5] Y.D. Wang, S. Johnson, D. Powell, J.P. McGinnis, M. Miranda, S.K. Rabindran, Inhibition of tumor cell proliferation by thieno[2,3-d]pyrimidin-4(1H)-one-based analogs, Bioorg. Med. Chem. Lett. 15 (16) (2005) 3763–3766.
[6] A. Gangjee, Y. Qiu, W. Li, R.L. Kisliuk, Potent dual thymidylate synthase and dihydrofolate reductase inhibitors: classical and nonclassical 2-amino-4-oxo-5-arylthio-substituted-6-methylthieno[2,3-*d*]pyrimidine antifolates, J. Med. Chem. 51 (18) (2008) 5789–5797.
[7] M. Hayakawa, H. Kaizawa, H. Moritomo, T. Koizumi, T. Ohishi, M. Okada, M. Ohta, S. Tsukamoto, P. Parker, P. Workman, M. Waterfield, Synthesis and biological evaluation of 4-morpholino-2-phenylquinazolines and related derivatives as novel PI3 kinase p110α inhibitors, Bioorg. Med. Chem. 14 (20) (2006) 6847–6858.
[8] O. Bekircan, B. Kahveki, M. Küçük, Synthesis and anticancer evaluation of some new unsymmetrical 3,5-diaryl-4H-1,2,4-triazole derivatives, Turk. J. Chem. 30 (2006) 29–40.
[9] R. Romagnoli, P.G. Baraldi, O. Cruz-Lopez, C. Lopez Cara, M.-D. Carrion, A. Brancale, E. Hamel, L. Chen, R. Bortolozzi, G. Basso, G. Viola, Synthesis and antitumor activity of 1,5-disubstituted 1,2,4-triazoles as cis-restricted combretastatin analogues, J. Med. Chem. 53 (10) (2010) 4248–4258.
[10] D. Kumar, M.K. Narayanam, K.-H. Chang, K. Shah, Synthesis of novel indolyl-1,2,4-triazoles as potent and selective anticancer agents, Chem. Biol. Drug Des. 77 (3) (2011) 182–188.
[11] Y.-P. Hou, J. Sun, Z.-H. Pang, P.-C. Lv, D.-D. Li, L. Yan, H.-J. Zhang, E.X. Zheng, J. Zhao, H.-L. Zhu, Synthesis and antitumor activity of 1,2,4-triazoles having 1,4-benzodioxan fragment as a novel class of potent methionine amino peptidase type II inhibitors, Bioorg. Med. Chem. 19 (20) (2011) 5948–5954.
[12] Y.A. Al-Soud, N.A. Al-Masoudi, A. El-Rahman, S. Ferwanah, Synthesis and properties of new substituted 1,2,4-triazoles: potential antitumor agents, Bioorg. Med. Chem. 11 (8) (2003) 1701–1708.
[13] D. Sunil, P. Shetty, Synthesis, characterization and anticancer activity of 1,2,4-triazolo[3,4-b]-1,3,4-thiadiazoles on Hep G2 cell lines, Der Pharma Chem. 1 (2) (2009) 19–26.
[14] Y. Luo, Sh Zhang, Z.-J. Liu, W. Chen, J. Fu, Q.-F. Zeng, H.-L. Zhu, Synthesis and antimicrobical evaluation of a novel class of 1,3,4-thiadiazole: derivatives bearing 1,2,4-triazolo[1,5-a]pyrimidine moiety, Eur. J. Med. Chem. 64 (2013) 54–61.
[15] G.L. Almajan, S.F. Barbuceanu, E.R. Almajan, C. Draghici, G. Saramet, Synthesis, characterization and antibacterial activity of some triazole Mannich bases carrying diphenylsulfone moieties, Eur. J. Med. Chem. 44 (2009) 3083–3089.
[16] A. Skrzypek, J. Matysiak, A. Niewiadomy, M. Bajda, P. Szymański, Synthesis and biological evaluation of 1,3,4-thiadiazole analogues as novel AChE and BuChE inhibitors, Eur. J. Med. Chem. 62 (2013) 311–319.
[17] Z. Chen, W. Xu, K. Liu, S. Yang, H. Fan, P.S. Bhadury, D.Y. Hu, Y. Zhang, Synthesis and antiviral activity of 5-(4-chlorophenyl)-1,3,4-thiadiazole sulfonamides, Molecules 15 (12) (2010) 9046–9056.
[18] C. Kus, G.A. Kilcigil, S. Ozbey, F.B. Kaynak, M. Kaya, T. Coban, B. Can-Eke, Synthesis and antioxidant properties of novel N-methyl-1,3,4-thiadiazol-2-amine and 4-methyl-2H-1,2,4-triazole-3(4H)-thione derivatives of benzimidazole class, Bioorg. Med. Chem. 16 (2008) 4294–4303.
[19] D. Kumar, N.M. Kumar, U.H. Chang, K. Shah, Synthesis and anticancer activity of 5-(3-indolyl)-1,3,4-thiadiazoles, Eur. J. Med. Chem. 45 (10) (2010) 4664–4668.
[20] W. Rzeski, J. Matysiak, M. Kandefer-Szerszeń, Anticancer, neuroprotective activities and computational studies of 2-amino-1,3,4-thiadiazole based compound, Bioorg. Med. Chem. 15 (9) (2007) 3201–3207.
[21] M.N. Noolvi, H.M. Patel, N. Singh, A.K. Gadad, S.S. Cameotra, A. Badiger, Synthesis and anticancer evaluation of novel 2-cyclopropylimidazo[2,1-b][1,3,4]-thiadiazole derivatives, Eur. J. Med. Chem. 46 (9) (2011) 4411–4418.

[22] A.T. Taher, H.H. Georgey, H.I. El-Subbagh, Novel 1,3,4-heterodiazole analogues: synthesis and *in-vitro* antitumor activity, Eur. J. Med. Chem. 47 (2012) 445–451.
[23] K. Sancak, Y. Ünver, M. Er, Synthesis of 2-acylamino, 2-aroylamino and ethoxycarbonyl imino-1,3,4-thiadiazoles as antitumor agents, Turk. J. Chem. 31 (2007) 125–134.
[24] K. Gewald, E. Schinke, H. Böttcher, Heterozyklen aus CH-aciden Nitrilen. VIII. 2-Aminothiophene aus methylenaktiven Nitrilen, Carbonylverbindungen und Schwefel, Chem. Ber. 99 (1996) 94–100.
[25] CellTiter 96 non-radioactive cell proliferation assay, Technical Bulletin #TB112, Promega Corporation USA. Revised 12/99.
[26] M.V. Berridge, A.S. Tan, Characterization of the cellular reduction of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction, Arch. Biochem. Biophys. 303 (1993) 474–482.
[27] R. Jover, X. Ponsoda, J.V. Castell, M.J. Gómez-Lechón, Acute cytotoxicity of ten chemicals in human and rat cultured hepatocytes and in cell lines: correlation between in vitro data and human lethal concentrations, Toxic. Vitro 8 (1994) 47–54.