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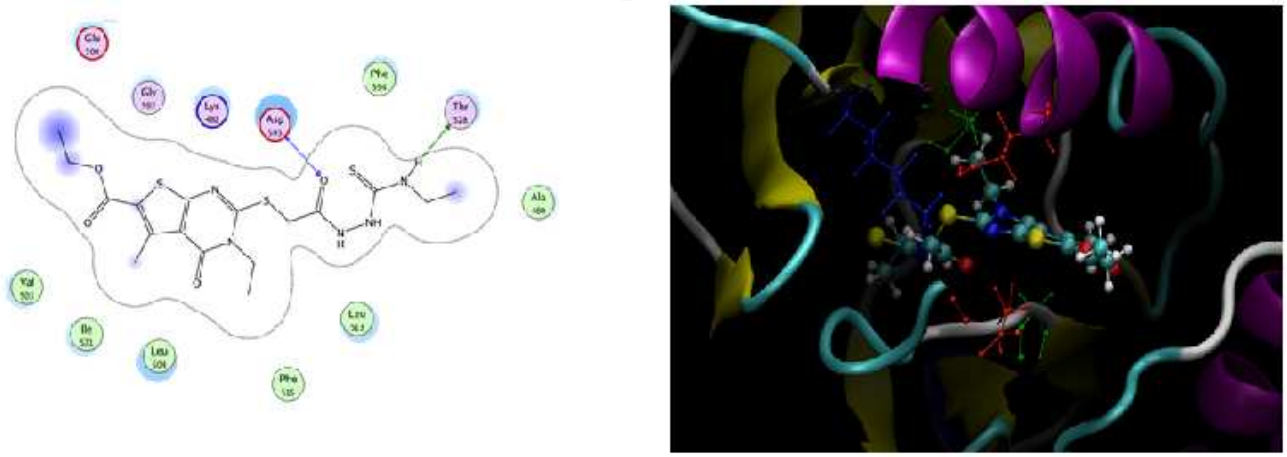
Graphical abstract



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Synthesis, anticancer activity and photostability of novel 3-ethyl-2-mercapto-thieno[2,3-*d*]pyrimidin-4(*3H*)-ones

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Abstract

Some derivatives of 3-ethyl-2-mercapto-thieno[2,3-d]pyrimidin-4(3H)-ones were synthesized using ethyl 2-aminothiophene-3-carboxylates as precursors in order to estimate their cytotoxicity, respectively proliferative activity.

Thienopyrimidinones containing thiosemicarbazide as well as 1,3,4-thiadiazole moieties were evaluated for their cytotoxical effect on four cancer cell lines: HT-29, breast cancer cells MDA-MB-231, HeLa, HepG2 as well as human diploid cell line Lep-3. Compounds **5b**, **6a** and **6b** revealed cytotoxicity to the four studied cancer cell lines. The highst cytotoxicity against MDA-MB-31 exhibited the thiosemicarbazide **5b** with IC_{50} $2.31.10^{-4}$ μM, but most active towards HT-29 cell lines was thienopyrimidine **6c** with IC_{50} 0.001 μM. Compound **6**a showed the highest inhibitory activity with IC_{50} - 0.99 μM to human liver carcinoma *HepG2* cells and low cytotoxicity towards *Lep3* (IC_{50} =

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191 μM). The thienopyrimidine derivative linked to thiadiazole **6b** was toxic to the four studied cancer cell lines, especially to HeLa (IC50 - 0.83 μM), and besides that the compound demonstrated toxicity to Lep 3 cells at very high concentration 89.10^3 μM.

The solid-state photostability of the derivatives **5a-c** and **6a-c** was tested by irradiation with UV light. All of the studied compounds show solid-state photostability in 240 min of irradiation.

Using MOE software molecular docking of the three ligands **5b**, **6b** and **7** was accomplished into an internal pocket formed by the activation segment and the P-loop of ^{V599E}B-Raf. It was established that the binding of the ligands to ^{V599E}B-Raf promotes an inactive conformation of the enzyme.

Keywords: thieno[2,3-d]pyrimidin-4(3H)-ones, DFT, cytotoxicity, photo stability, anticancer activity, B-Raf

1. Introduction

It is well known that the pyrimidine structure is closely related to three of the four nucleobases uracil, thymine and cytosine, which fact makes pyrimidines essential building blocks of all living cells [1]. Thieno[*d*]pyrimidines are found to have broad spectrum of biological activity as anticancer [2], antibacterial [3], antiviral [4], antioxidant [5], antihistaminic [6], anti-inflammatory [7], analgesic [8, 9].

It was found that some thieno[3,2-*d*]pyrimidines are bioisosters of *lapatimib* (IC_{50} 120nM) [10] and some of them have shown better inbitiory activity (IC_{50} 11nM) towards epidermal growth factor receptor tyrosine kinase (EGFR/ErbB-2), compared to the 4-aminoquinazoline derivative – *lapatimib*.

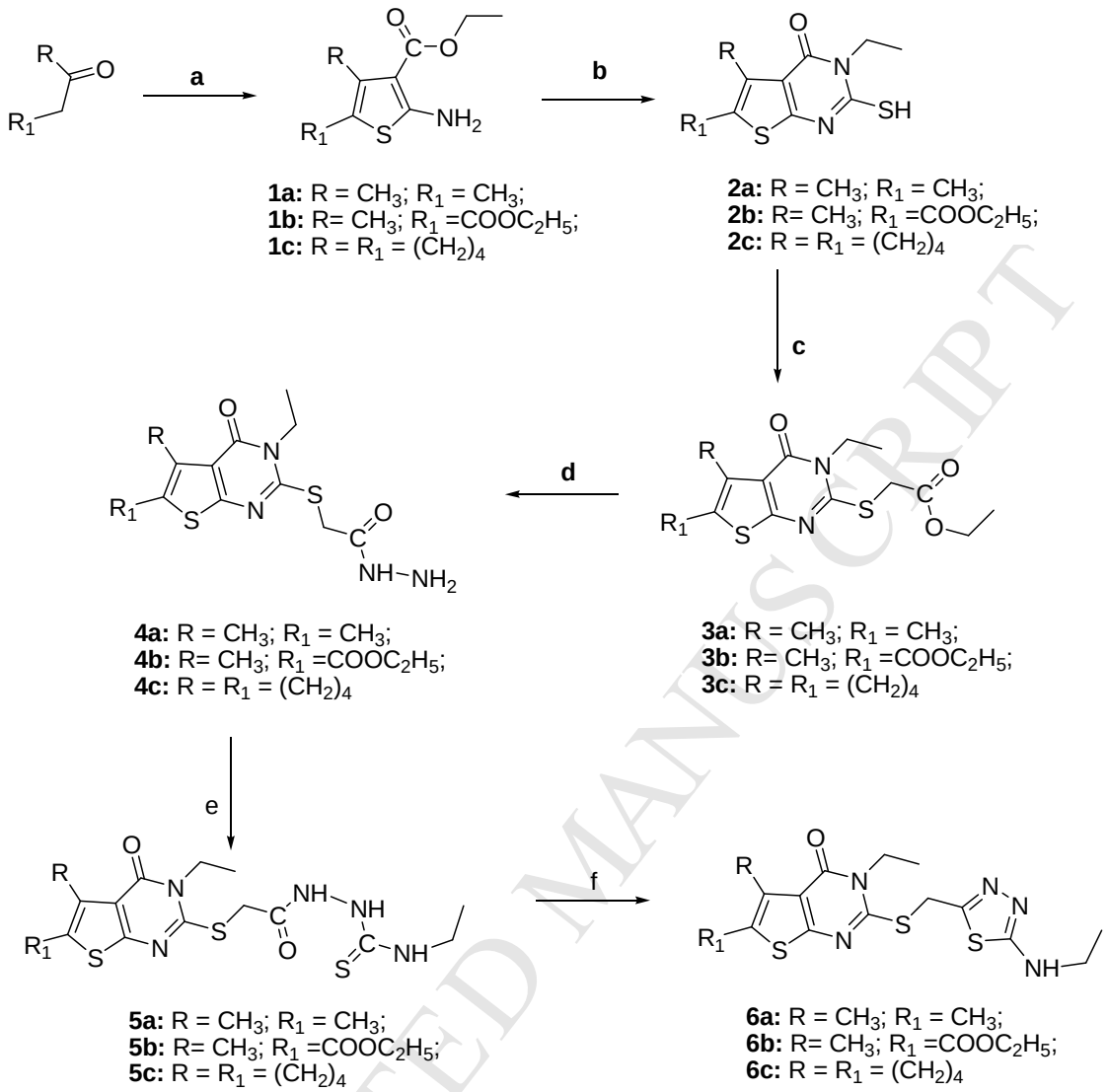
Many thieno[2,3-*d*]pyrimidines have showed significant *in vitro* cytotoxic activity against hepatocellular carcinoma (*Hep G-2*) compared to the reference drug *Doxorubicin* [11, 12]. *Wang et al.* have found that some 5,6,7,8-tetrahydrothieno[2,3-*d*]pyrimidine-4-ones preferentially affect *p21*-deficient cancer cells (IC_{50} 2.3μM) [13]. The further SAR analysis indicated that one of the important structural-activity recommendations is the presence of oxygen in C-4 position of the pyrimidine ring.

The 1,3,4-thiadiazole (TDA) derivatives are known because of their pharmacological activity as anti-inflammatory [14], antibacterial [15], antitumor [16]. The anticancer effect of 1,3,4-thiadiazoles is mainly related to the high electron-donating ability of the two nitrogen atoms to form *H*-bonds. 2-Amino-1,3,4-thiadiazoles have a variety of anticancer activity against melanoma, glioblastoma, lymphosarcoma [17]. Some thiadiazole derivatives have shown anticancer activity against *HepG-2* [18, 19] and *HeLa* cell lines compairable with *staurosporine*, resulting from *H*-bond between the nitrogen atom from the 1,3,4-thiadiazole and the amino hydrogen of *GLY505* amino acid − part of the ATP binding site of focal adhesion kinase [20]. There was reported a series of 2-amino-1,3,4-thiadiazoles that display growth inhibition in *A549* lung carcinoma cells by inhibiting ERK1/2 pathway and cell cycle progression through G_1 into S phase [21]. In addition, same TDA-based compounds are shown to possess significant anticancer activity against prostate cancer (*LnCaP*, *DU145* and *PC3*) and breast cancer cell lines (*MCF-7* and *MDA-MB-231*) [22, 23].

The mesoionic nature of thiadiazoles makes these compounds to be more able to cross cellular membranes [24]. Therefore, combining TDA with an effective anticancer adenine and guanine based pyrimidine bioisoster as thieno[2,3-*d*]pyrimidine-4-ones could lead to potential inhibiting effect on purine synthesis and less toxic anticancer agents. In the literature it has been described that the 1,3,4-thiadiazole containing 5,6,7,8-tetrahydrothieno[2,3-*d*]pyirimidine-4-ones possess anticancer activity [25, 26]. This fact has drawn our attention for further investigation of that bipharmacophoric compounds as potential anticancer drugs on four human cancer cells and one non-tumorogenic cell line.

2. Chemistry

The synthesis of *N*-ethyl-thieno[2,3-*d*]pyrimidine-4-one derivatives, containing 1,3,4-thiadiazole moiety is illustrated on *Scheme 1*.



Scheme 1 Synthesis of semicarbazide and 1,3,4-thiadiazole derivatives of thieno[2,3-d]pyrimidine-4-ones: a) ethyl cyanacetate, $HNEt_2$, S_8, ethanol; b) ethyl isothiocyanate, NaOH, DMF; c) ethyl 2-chloroacetate, NEt_3, benzene, reflux; d) hydrazine hydrate, ethanol, reflux; e) ethyl isothiocyanate, ethanol, reflux; f) 98% sulfuric acid, 0°C, NH_4OH

The 2-aminothiophene esters **1a-c** used as starting materials were synthesized by *Gewald* reaction which is a one-pot reaction method for 2-aminothiophene synthesis [27-30]. The synthesis of *N*-ethyl-2-mercapto-thieno[2,3-*d*]pyrimidine-4-ones **2a-c** was accomplished by alkaline catalized cyclocondensation of the 2-aminoesters **1a-c** with ethyl isothiocyanate. The *S*-nucleophilic substitution between the 2-mercapto-thienopyrimidine-2-ones and ethyl chloroacetate, using triethylamine as a organic base gave thioacetate derivatives **3a-c**. The interaction of the esters **3a-c** with hydrazine hydrate in absolute alcohol led to the obtaining of the hydrazides **4a-c**. The latter, by treatment with the corresponding ethyl isothiocyanate under reflux gave the semicarbazides **5a-c** with high yeilds.

The cyclocondensation to the corresponding semicarbazides **5a-c** was carried out in water solution of sodium hydroxide under reflux, followed by acidification with hydrochloric acid. The thiadiazole containing thieno[2,3-*d*]pyrimidin-4(*3H*)-ones **6a-c** were obtained from compounds **5a-c** in cooled concentrated sulfuric acid.

3. Pharmacology

The semicarbazides **5a-c** and thiadiazole containing thieno[2,3-*d*]pyrimidin-4(*3H*)-ones **6a-c** 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 cell line *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 [31].

4. Results and discussion

A series of 2-aminothiophenes has been prepared for the aim of our research via a multicomponent condensation between appropriate ketone, ethyl cyanoacetate, sulfur and diethyl amine under conditions, described by Gewald [27]. The pyrimidine ring formation of 3-ethyl-2-mercapto-thieno[2,3-*d*]pyrimidin-4(*3H*)-ones **2a-c** was performed by basic catalyzed heterocyclic cyclocondensation of the 2-aminothiophenes **1a-c** and ethyl isothiocyanate. It is known that equilibrium between the thiol and thione form exists, but in an alkaline medium the presence of the thiol form is advantageous, therefore the acidic 2-SH group of the thienopyrimidin-2-thiols was alkylated with ethyl chloroacetate in presence of triethyl amine. The reaction of esters **3a-c** with excess amount of hydrazine hydrate in absolute ethanol led to acetohydrazides **4a-c.** Within that group, the synthesis of **4c** occured with the highest yield − reaching 94%. The aforementioned compounds were treated with 12-fold access of ethyl isothiocyanate giving thiosemicarbazides **5a-c** with excellent yields from 88% to 96%. 1,3,4-Thiadiazoles **6a-c** were obtained from thiosemicarbazides **5a-c** by dehydrative cyclization in acidic medium. The yields of the final thiadiazole **6b** and **6c** were in the range from 90 to 95%.

The chemical structures of the new compounds were established by elemental analyses, IR-, ^{1}H NMR and the results are presented in the Experimental part. The elemental analyses indicated by the

symbols of the elements were within ± 0.4% of theoretical values. In the ^{1}H NMR spectra, particularly meaningful are characteristic SCH_2CO signals (singlets) of the substituded in 2 place compounds as well as the as the signals of S-CH_2 between the both heterocycles, shifted downfield due to deshielding effect of both S-atom and the CO group respectively the pyrimidine and thiadiazole rings. The chemical shift values varied in the range from 3.99 to 7.43 ppm for the esters, 4.08- 4.11 ppm for the thiosemicarbazides and from 4.45 to 4.84 ppm depending of the solvents and substituents. The labile NH protons are not characteristic and their chemical shifts depend on the water quantity in the solvent. Some ^{1}H NMR spectra are given in the Supplementary material.

The thiosemicarbazide (**5a-c**) and 1,3,4-thiadiazole (**6a-c**) thieno[2,3-*d*]pyrimidine-4-one derivatives were subjected to *in vitro* screening in order to estimate their effects towards human cancer *HT-29*, *MDA-MB-231*, *HeLa*, *HepG2* and normal human *Lep3* cell lines.

The cell proliferation was established using the MTS assay, based on the reduction of the tetrazolium salt into a coloured, aqueous soluble formazan product by mitochondrial activity of viable cells at 37°C. The released amount of formazan produced by dehydrogenase enzymes is proportional to the number of living cells in the culture and can be measured spectrophotometrically at λ_{max} = 492 nm [32]. The higher levels of formazan indicate to a higher vitality of the cells, whereas the low amount of formazan is an indicator for the cytotoxicity of the tested compounds [33, 34]. In addition, the relative cell viability, expressed as a percentage of the untreated control (100% viability) was calculated for each concentration. All data points represent an average of three independent assays. The obtained results were plotted and EC_{50} (Table 1) and IC_{50} (Table 2) were calculated. Statistical significant differences in the level of cells in both control and experimental groups were determined ($p \leq 0.05$).

Table 1 The proliferative activity (EC_{50}) of the studied compounds

Compounds	**EC_{50} ± SE (μM)**				
	HT-29	MDA-MB-231	HeLa	Hep G2	Lep3
5a	0.085 ± 0.20	8.86 ± 0.10	0.43 ± 0.03	–	–
5c	1.13 ± 0.16	9.33 ± 0.12	7.97 ± 0.72	2.72 ± 0.31	8.25 ± 0.24
6c	–	0.001 ± 0.14	9.28 ± 0.03	–	–

Table 2 In vitro cytotoxicity against HeLa, Hep G2, HT-29, MDA-MB-231 and Lep 3 cells

Compounds	**IC_{50} ± SE (μM)**				
	HT-29	MDA-MB-231	HeLa	Hep G2	Lep3
5a	–	–	–	–	0.01 ± 0.22
5b	7.38 ± 0.71	$2.31.10^{-4}$ ± 0.04	8.71 ± 0.09	8.71 ± 0.58	0.8 ± 0.18
6a	3.58 ± 0.25	9.4 ± 0.15	7.42 ± 0.22	0.99 ± 0.52	191 ± 0.03
6b	9.12 ± 0.37	2.13. ± 0.13	0.83 ± 0.18	9.51 ± 0.03	89.10^{3} ± 0.27
6c	0.001 ± 0.11	–	–	9.8 ± 0.18	9.77 ± 0.09

It can be seen that the most of the examined compounds exhibited wide inhibitory concentration range for all tested human cell lines. Among the thieno[2,3-*d*]pyrimidine-4-ones, containing thiosemicarbazide moiety, compound **5b** was toxic against all cancer cell lines, used in that study, especially against breast cancer cells *MDA-MB-231* (IC_{50} = $2,31.10^{-4}$ μM), while in the same time it revealed cytotoxicity to *Lep3* in a much higher concentration with IC_{50} = 0,8 μM.

In the group of the thienopyrimidine derivatives linked to thiadiazole ring, compound **6b** was toxic to the four studied cancer cell lines, especially to HeLa cells (IC_{50} - 0.83 μM), besides the compound demonstrated toxicity to Lep 3 cells at very high concentrations, the determined IC_{50} value was 89.10^{3} μM. The low cytotoxic profile to *Lep3* can provide high selectivity towards all four cancer cell lines at small concentrations.

Apparently, the most active against human colorectal cancer cell line *HT-29* was compound **6c** with IC_{50} 0,001 μM, followed by **6a** with IC_{50} 3.58 μM, respectively, but derivative **6c** exibits about 100-*fold* lower cytotoxicity against normal diploid cell line *Lep3*.

With respect to human liver carcinoma *HepG2* cells compound **6**a showed the highest inhibitory activity with IC_{50} - 0.99 μM and the second lowest cytotoxicity towards *Lep3* (IC_{50} = 191 μM).

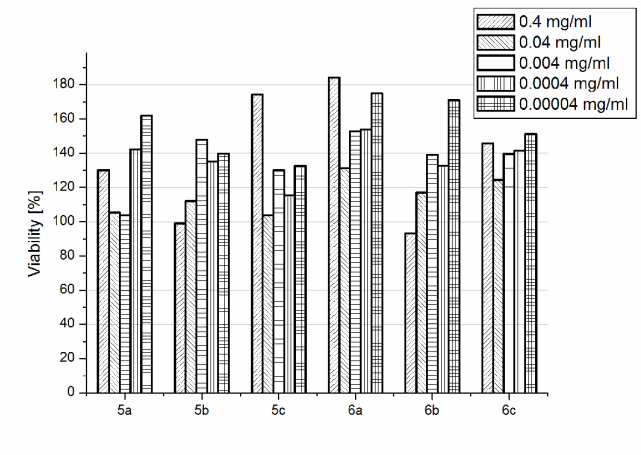


Fig. 1 Viability of Lep 3 cells (%) after treatment

In summary, it can be noted that thieno[2,3-*d*]pyirimidine-4-ones containing thiosemicarbazide (**5a-c**) and thiadiazole moiety (**6a-c**) exhibit high effect on cell proliferation towards all tested human cancer and normal cell lines, whereat compound **6b** showed comparatively much lower toxicity against normal *Lep3* cells (*Fig. 1*). This makes the thiadiazole derivative **6b** efficient anticancer agent against all tested tumor cells at low concentration, and at the same time the compound will not demonstrate any cytotoxicity upon normal human cells. It was observed that the variation of substituents at *5*-C and *6*-C position of the thiophene ring leads to a major decrease of *Lep3* cell line vitality, whereas the only significant increase (1000-*fold*) of the apoptotic effect was indicated for the more lipophilic tetrahydro-substitued compound **6c** against *MDA-MB-231* and *HT-29* cancer cells.

The term ‘photostability’ includes not only the degradation caused by exposure to light, but also processes as radical formation, energy transfer and luminescence [35]. A large number of drug substances absorb radiation in the ultraviolet and/or visible area and are thus sensitive to light. They undergo photodegradation in liquid media or in solid state on exposure to light [36]. To the photodegradation factors of drug dosage forms in the solid state belong the particle sizes, the surface area, the colour and crystal structure, the photodimerization or isomerization. There is correlation between the transparency of the products and the radiation being used. The radiation to which a sample is exposed may be reflected, scattered, transmitted or absorbed. Only the absorbed radiation participates in photodegradation. There are many results for the photodegradation of substances in

aqueous solution, but relatively few studies are done on photostability in solid state. Correlations between photochemical behaviour in solution and in solid state are not clearly established.

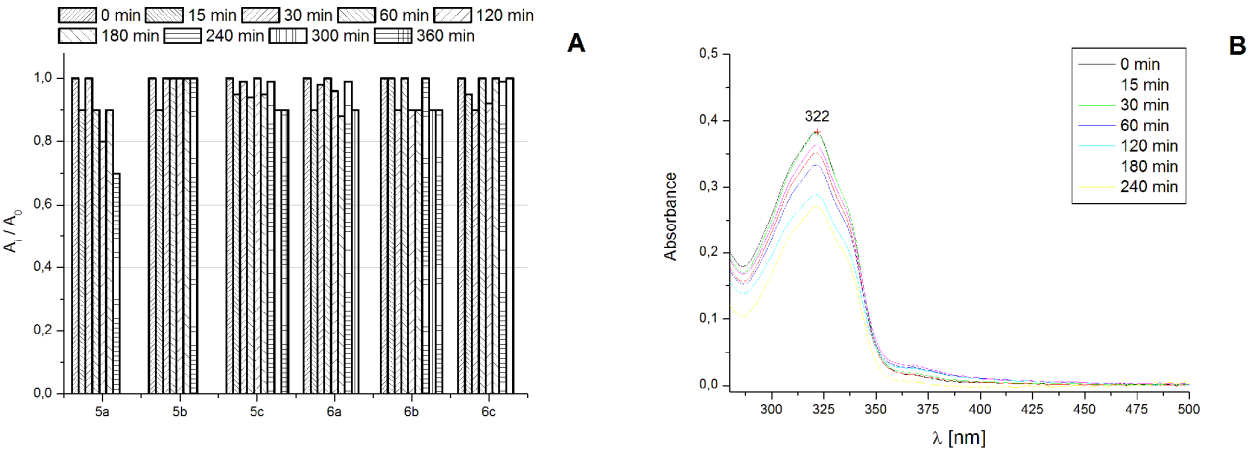


Fig. 2: (a) Solid-state photostability ratiometric analysis of **5a** (C=3,128.10^{-5}±0,125 mol/L), **5b** (C=2,185.10^{-5}±0,087 mol/L), **5c** (C=3,916.10^{-5}±0,158 mol/L), **6a** (C=5,242.10^{-5}±0,209 mol/L), **6b** (C=2,275.10^{-5}±0,091 mol/L), **6c** (C=4,089.10^{-5}±0,164 mol/L) in DMF solution; **(b)** Absorbtion spectrum of compound **5a**;

The solid-state photostability of the novel thieno[2,3-*d*]pyrimidine-4-one derivatives **5a-c** and **6a-c**, was tested by irradiation with UV light in a SUNTEST equipment. The kinetics of the compound photodegradation was monitored colorimetrically. As no changes were established in the absorption maxima (λ_A) of compound **5b-c** and **6a-c** during the irradiation, the correlation between the drug concentration and the time of irradiation was constant value (**Fig. 2a**). The 240 min irradiation of thiosemicarbazide **5a** resulted with nearly 30% photodegradation (**Fig. 2b**).

For promising pharmacological potential, the studied thieno[2,3-*d*]pyrimidine-4-ones should possess favourable pharmacokinetic properties *in vivo* i.e. sufficient bioavailability and transportation through the different membranes to the desired receptor binding site, as well as an optimal metabolization and elimination profile. Calculated molecular properties such as lipophilicity, molecular size, flexibility and presence of hydrogendonor and acceptors could provide useful information in this relation.

It can be seen from the SAR analysis (**Table 3**), made by using Molinspiration tool [37], that all of the examined compounds **5a-c** and **6a-c** respond to Lipinski's *''Rule of Five''* [38]. It appeared that compounds **6a** and **6c** possessing the one and the same low topologic polar surface area (TPSA) of

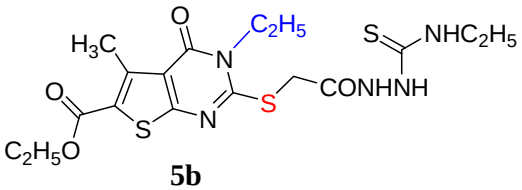
72.71 Å^2 and a equal number of hydrogen donors (N_{HD}) and acceptors (N_{HA}), were the most active against *HT-29* cell line. The low TPSA provides a sufficient intestinal absorbtion on the drug in humans [39] and the higher number of N_{HA} provides higher solubility, which explains their citotoxicity against *HT-29* and makes **6a** and **6c** promising candidates for *in vivo* analysis. The least toxic compounds against human normal diploid cell line *Lep3* **6a** and **6b** have the TPSA = 72.71 Å^2 and 99.01 Å^2 respectively.

Table 3 Calculated molecular properties of the tested compounds: partition coefficients (logP), molecular weight (MW) [g/mol], topologic polar surface area (TPSA) [Å^2], molecular volume (Vol.) [Å^3], sum of OH and NH H-bond donors (N_{HD}) and sum of O and N H-bond acceptors (N_{HA})

Compounds	***logP***	***M.W.***	***TPSA***	***Vol***	N_{HA}	N_{HD}
5a	2.14	399.57	88.05	337.77	7	3
5b	2.36	457.60	114.35	382.54	9	3
5c	2.71	425.61	88.05	361.01	7	3
6a	2.98	381.55	72.71	319.93	6	1
6b	3.21	439.59	99.01	364.70	8	1
6c	3.56	407.59	72.71	343.17	6	1
A[a)]	1.28	397.48	125.21	330.67	9	4

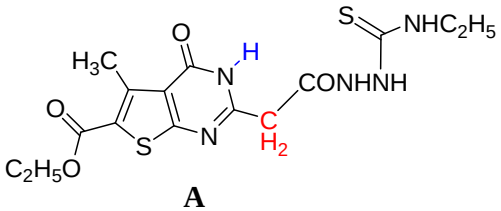
[a)] The compound is previously reported in [23]; for molecular structure see Fig. 3.

The most active to *MDA-MB-231* cells compound (**5b**) has the highest sum of H-bond donor (N_{HD}) and acceptor (N_{HA}) groups of all examined compounds as well as the highest TPSA, 114.35 Å respectively. It is well known that to show an acceptable bioavaibility one compound should have TPSA lower than 140–150 Å^2. These molecular properties coincide well with those characterizing the structurally related thieno[2,3-*d*]pyrimidine-4-one **A** (*ethyl 2-(2-{2-[(ethylamino)carbonothioyl] hydrazino}-2-oxoethyl)-5-methyl-4-oxo-3,4-dihydrothieno[2,3d]pyrimidine-6-carboxylate Fig. 3*), that shows *MDA-MB-231* selectivity and was recently reported under number **8** in [23]. It can be assumed that the high TPSA, molecular weight (M.W.) and volume (Vol.) of **5b** contribute to a better fitting with the active center, inhibiting cancer *MDA-MB-231* cell line growth. The high cytotoxicity of **5b** should be attributed to the alkylation of the amide group in the pyrimidine ring and to the replacement of the aliphatic spacer with thiomethylene group (Fig. 3).



5b

IC_{50} 0.23 nM



A

IC_{50} 39 nM

Fig. 3 Structures of MDA-MB-231 cell line growth inhibitors **5b** and **A**

Based on the structures of **5b** and **7** (Fig. S**14**), optimized at DFT B3LYP/6-311++G** level of theory [40,41], it can be presumed, that the replacement of the methylene group at *2*-nd position of the pyrimidine with sulfur spacer leads to a much flatter molecular conformation of **5b**. That fact, combined with the N-alkylation at 3-rd position, results in larger molecular volume of **5b** than **A**.

Protein tyrosine kinases are key regulators of cell function, which represent one of the largest and most functionally different gene families with particular relevance to many human diseases, including cancer. The kinases are particularly eminent in signal transduction and coordination of complex functions at the cell cycle. Beside that fact in the literature it has been reported that the highly selective carbamide derivative (*sorafenib*, **S. Fig. S15**) shows evidence for tumor regressions after 9 days of oral dosing against breast cancer *MDA-MB-231* cells [42, 43]. In addition a series of thieno[2,3-d]pyrimidines was synthesized and identified as inhibitors to the most critical isoform of the Raf family – B-Raf [44-46]. It is assumed that such cell line selectivity of the carbamide and respectively thiosemicarbazides (**5b, 6b** and **A**) share the same bioisosteric mechanism of action by affecting tumor growth through blocking two cellular processes essential for tumor development: inhibition of the MAPK pathway and inhibition of tumor angiogenesis.

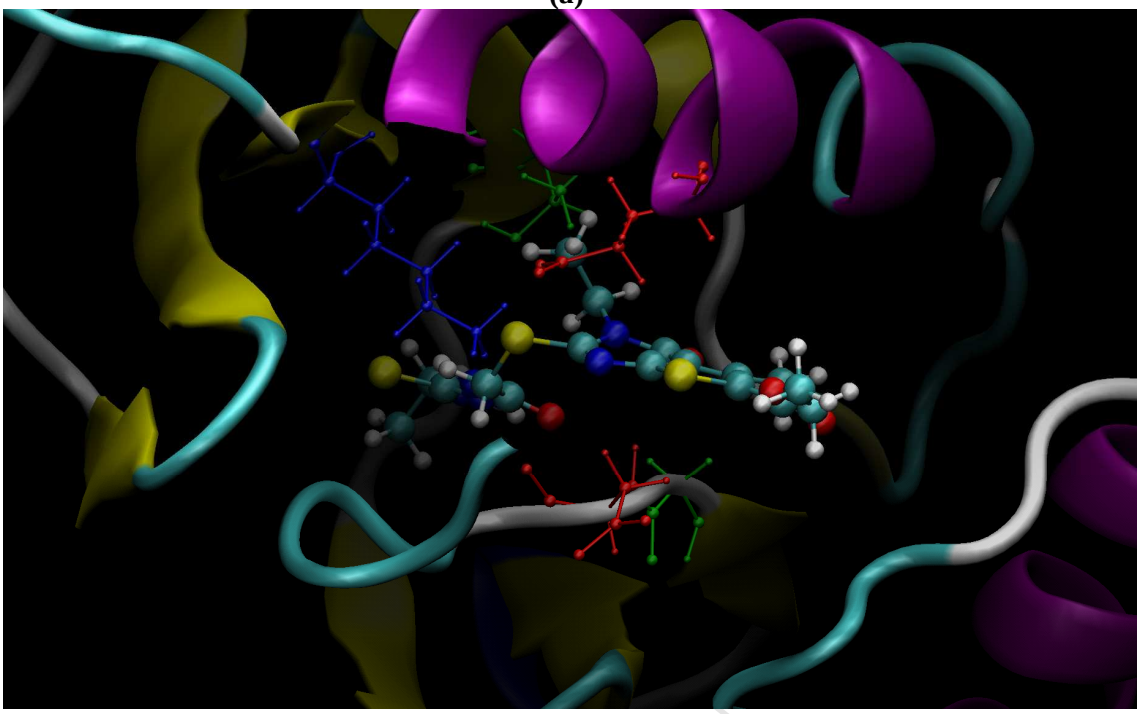
In order to clarify the possible binding modes of **5b**, **6b** and **A** with B-Raf, the compounds were docked into the crystal structures of human oncogenic ^{V599E}B-Raf in complex with sorafenib (PDB code: 1UWJ [43]) using MOE software [47].

The kinase domain of ^{V599E}B-Raf has a bilobal structure typical also for other members of the protein kinase family group [43]. Similarly to sorafenib (**Fig. S15**) the three ligands are bound to the

interfacial cleft, buried between the N and C lobes of the kinase domain. The docking conformations of **5b**, A and **6b** are shown in Fig. 4(a), 5(a) and 6(a).

In all cases, the *N*-ethyl-thieno[2,3-*d*]pyrimidine-4-one fragment occupies a pocket formed by residues Val503, Leu504, Ile571, Phe594, Lys482 and Gly592, with only the ethoxy group exposed to the solvent (Fig. 4(b), 5(b) and 6(b)). The van der Waals interactions dominate the contacts of **5b** and **7** with ^{V599E}B-Raf as it could be seen from the 2D representations of the ligands in the pocket (Fig. 4(b) and 5(b)).

(a)



(b)

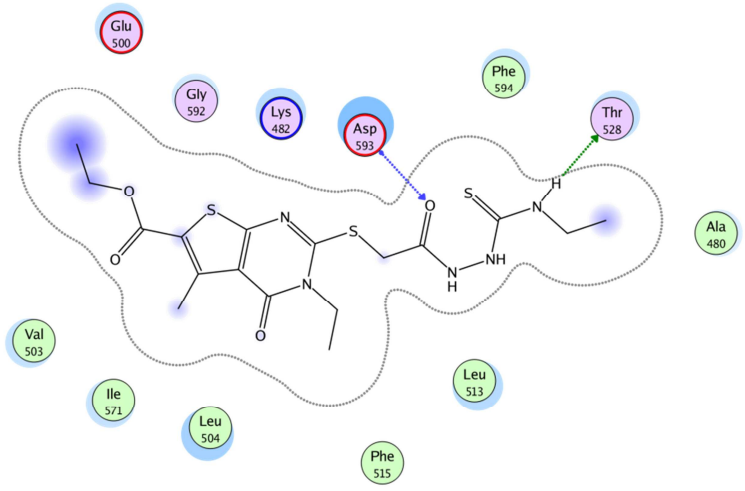


Fig. 4: (a) Docking conformation of **5b** with shown amino acid residues Thr528, Asp593, Lys482, Glu500, Gly592; **(b)** 2D representation of the interactions of **5b** with ^{V599E}B-Raf

Polar interactions to the catalytic Glu500, Gly592 from the activation segment of the DFG motif and Lys482 or Thr528 also contribute to the stabilization of the ligand-pocket binding. The thiosemicarbazides moiety of **5b** is connected via two hydrogen bonds to Thr528 and Asp593. The

other MDA-MB-231 selective thieno[2,3-*d*]pyrimidine-4-one **7** forms hydrogen bonds to Lys482 and Asp593.

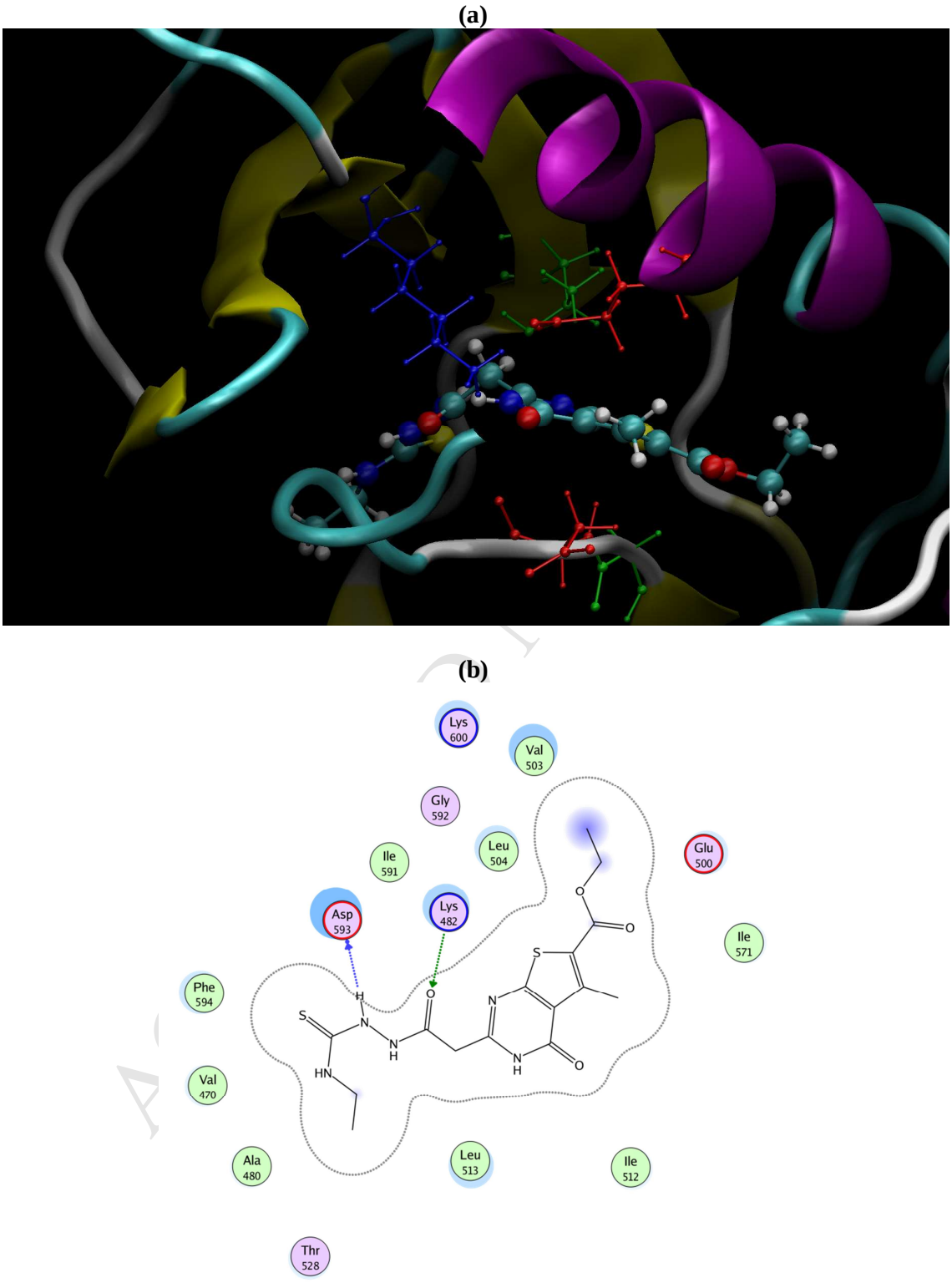
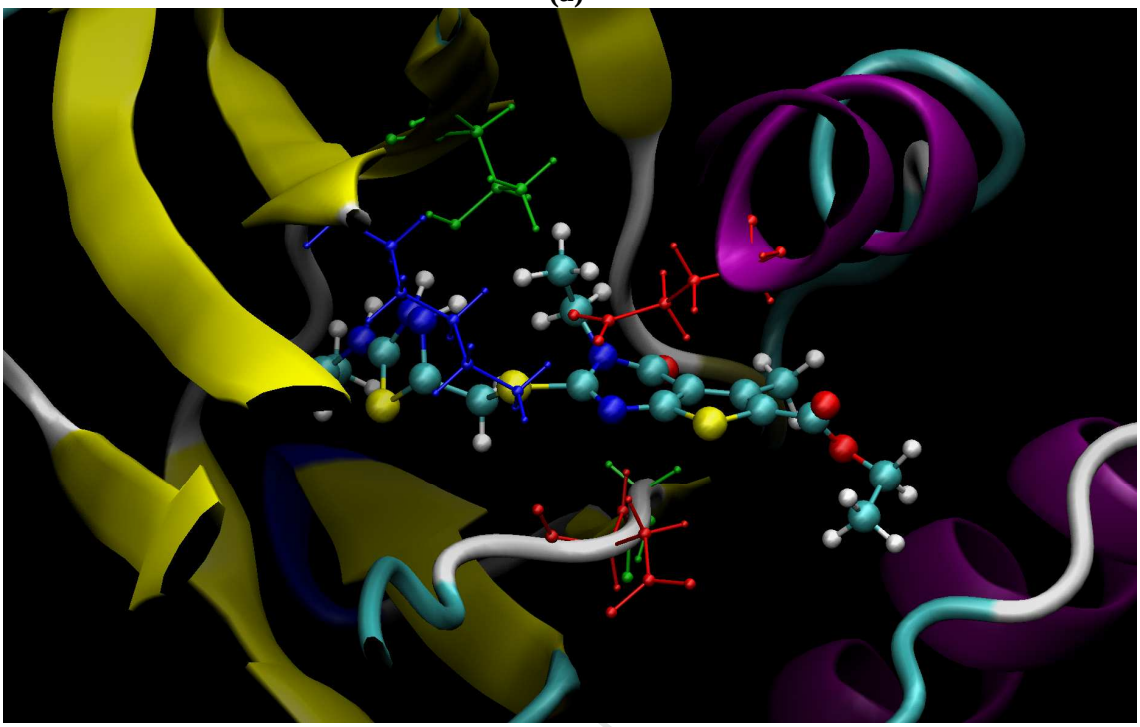


Fig. 5: (a) Docking conformation of **A** with shown amino acid residues Thr528, Asp593, Lys482, Glu500, Gly592; **(b)** 2D representation of the interactions of **A** with ^{V599E}B-Raf

In the case of **6b**, one of the N-atoms of the thiadiazole moiety acts as hydrogen bond acceptor to Thr528, while the N-ethyl substituent is oriented towards the polar Glu500 and Asp593.

(a)



(b)

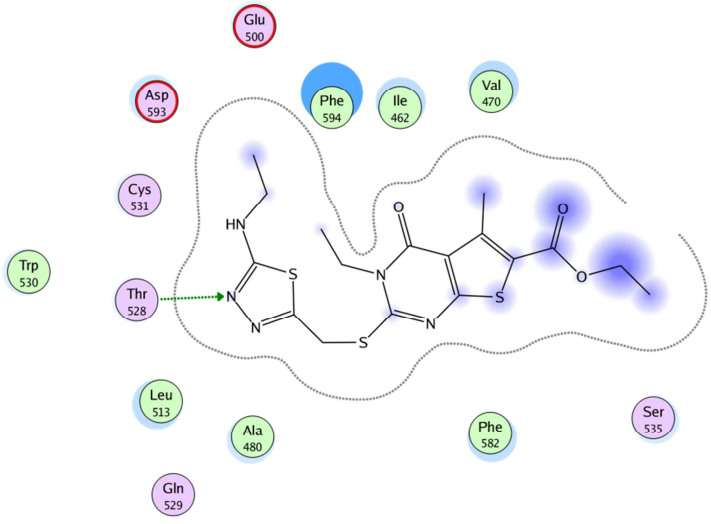


Fig. 6: (a) Docking conformation of **6b** with shown amino acid residues Thr528, Asp593, Lys482, Glu500, Gly592; **(b)** 2D representation of the interactions of **6b** with ^{V599E}B-Raf

These polar interactions along with the hydrophobic interaction to Phe594 connect the three ligands to the activation segment of the DFG motif. On the other side, as it was mentioned above a number of contacts to the catalytic residues Lys482, Glu500 is formed. In that way the binding of the ligands to ^{V599E}B-Raf promotes an inactive conformation of the enzyme by holding the activation segment of the DFG motif and hindering its phosphorylation.

5. Conclusion

A serie of eight new thieno[2,3-*d*]pyrimidin-4(*3H*)-ones containing different substituted thiosemicarbazide, 1,2,4-triazoles and 1,3,4-thiadiazoles heterocycles were synthesized using the corresponding 2-(thieno[2,3-d]pyrimidin-2-yl)thioacetohydrazides as precursors under optimized reaction conditions with good yields.

The initial biological screening *in vitro*, using MTS tetrazolium assay has showed that the studied thiosemicarbazide containing thieno[2,3-*d*]pyirimidine-4-one **5b**, connected through sulfur linker possess high cytotoxicity against *MDA-MB-231* cells. The cytotoxic effect of **5b** towards *Lep3* cells was comparatively much lower. Among the thienopyrimidinones containing 1,3,4-thiadiazole ring compounds **6b** revealed cytotoxicity to all screening cancer cells with IC_{50} from $9{,}51.10^{-5}$ μM against *Hep G2* to 0.83 μM againt *HeLa* cell line, while at the same time indicated relatively high vitality levels of *Lep3* with IC_{50} - 89.10^{3} μM. Compound **6c** demonstrated selective cytotoxicity to *HT-29* and *Hep G2* cancer cell lines, and also the 5,6-dimethyl substituted thienopyrimidone **6a** showed cytotopxicity to all four cancer cells, however both thiadiazole derivatives showed proliferative effects on human diploid cells. In contrast to **5a**, all of the examined compounds show solid-state photostability in 240 min period of irradiation.

Based on molecular docking study, the binding modes of **5b**, **6b** and a structurally related compound **A** were clarified. By association to catalytic amino acid residues Lys482, Glu500 and Gly592, Asp593 and Phe594 from the activation segment of ^{V599E}B-Raf, the ligands promote an inactive conformation of the enzyme and hinder its phosphorylation.

The obtained activity results towards *MDA-MB-231* cells indicate that the thiosemicarbazide moiety (**5b**), connected through a sulfur atom in the pyrimidinone skeleton as well as the results

exhibited by **6b** to *HeLa* and *Lep3* prove the necessity for further investigation *in vivo* to estimate the exact inhibition pathway in the cellular processes, essential for tumor development related to the antitumor potential of the tested compounds.

6. Experimental part

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 F254 or silica gel plates F254 (Merck, 0.2 mm thick) and visualization was effected with ultraviolet light. IR spectrawere recorded on a Bruker Equinox 55 spectrophotometer as potassium bromide discs. All ^{1}H-NMR spectra were recorded on a Bruker Avance DRX 250 spectrometer (Bruker, Faelanden, Switzerland) operating at 250.13 MHz and a Bruker Avance DRX 600 spectrometer (Bruker, Faelanden, Switzerland) operating at 600 MHz. 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 PerkineElmer elemental analyzer.

*6.1. General procedure of the synthesis of ethyl 2-aminothiophene-3-carboxylates **1a-c***

Diethylamine (0.1 mol) was added dropwise for 30 min to a suspension of the appropriate ketone (0.1 mol), ethyl cyanacetate 0.1 mol (11 ml) and 3.2 g sulfur in 30 ml ethanol and the reaction mixture was stirred at room temperature for about 75 min. When the synthesis was completed the mixture was cooled. The obtained 2-aminothiophene crystalized in the form of yellow powder. The precipitate was filtrated, washed with water and recrystallized from ethanol to give the 2-aminothiophene derivatives [27].

*6.1.1. Ethyl 2-amino-4,5-dimethylthiophene-3-carboxylate (**1a**):* Yield: 61%; Mp. 97–100°C [1]; IR (KBr): 3300 cm^{-1} (NH), 2920 cm^{-1} (CH_3), 2860 cm^{-1} (CH_3), 1640 cm^{-1} (C=O), 1160 cm^{-1} (C−O); Analysis: Calc. for $C_9H_{13}NO_2S$: C, 54.25; H, 6.58; N, 7.03; O, 16.06; S, 16.09; Found: C, 54.30; H, 6.55; N, 7.08; O, 16.10; S, 16.10.

*6.1.2. Diethyl 5-amino-3-methylthiophene-2,4-dicarboxylate (**1b**):* Yield: 65%; Mp. 104–106°C [1]; IR (KBr): 3300 cm^{-1} (NH), 2960 cm^{-1} (CH_3), 2860 cm^{-1} (CH_2), 1640 cm^{-1} (C=O), 1230 cm^{-1} (C−O); Analysis: Calc. for $C_{11}H_{15}NO_4S$: C, 51.35; H, 5.88; N, 5.44; O, 24.87; S, 12.46; Found: C, 51.33; H, 5.85; N, 5.52; O, 24.86; S, 12.46.

*6.1.3. Ethyl 2-amino-4,5,6,7-tetrahydrothiophene-3-carboxylate (**1c**):* Yield: 82%; Mp. 114–115°C [1]; IR (KBr): 3300 cm^{-1} (NH), 2920 cm^{-1} (CH_3), 2800 cm^{-1} (CH_2), 1640 cm^{-1} (C=O), 1260 cm^{-1} (C−O); Analysis: Calc. for $C_{11}H_{15}NO_2S$: C, 58.64; H, 6.71; N, 6.22; O, 14.20; S, 14.23; Found: C, 58.74; H, 6.73; N, 6.26; O, 14.19; S, 14.29.

*6.2. Synthesis of 3-ethyl-2-mercaptothieno[2,3-d]pyrimidin-4(3H)-ones **2a-c***

To a solution of 0.025 mol of 2-aminothiophene ***1a-c*** and 0.025 mol of NaOH in DMF (30 ml) 0.025 mol ml ethyl isothiocyanate (2.18 ml) was added dropwise. The mixture was stirred for 3h and after that poured in 750 ml water. The solution was acidified to pH~5 by use of 50% (*v/v*) acetic acid. The formed precipitate was filtered, washed with water and recrystallized from ethanol.

*6.2.1. 3-ethyl-2-mercapto-5,6-dimethylthieno[2,3-d]pyrimidin-4(3H)-one (**2a**):* Yield: 95%; Mp. 253–255°C; IR (KBr): 3250 cm^{-1} (NH), 2900 cm^{-1} (CH_3), 1660 cm^{-1} (CONH); Analysis: Calc. for $C_{10}H_{12}N_2OS_2$: C, 49.97; H, 5.03; N, 11.66; O, 6.66; S, 26.68; Found: C, 50.02; H, 5.05; N, 11.65; O, 6.71; S, 26.69.

*6.2.2. Ethyl 3-ethyl-2-mercapto-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxylate (**2b**):* Yield: 96%; Mp. 252–254°C; IR (KBr): 3195 cm^{-1} (NH), 2950 cm^{-1} (CH_3), 1740 cm^{-1} (COOEt), 1680 cm^{-1} (CONH), 1100 cm^{-1} (C−O); Analysis: Calc. for $C_{12}H_{14}N_2O_3S_2$: C, 48.30; H, 4.73; N, 9.39; O, 16.09; S, 21.49; Found: C, 48.33; H, 4.78; N, 9.41; O, 16.09; S, 21.45.

*6.2.3. 3-ethyl-2-mercapto-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4(3H)-one (**2c**):* Yield: 86%; Mp. 263–266°C [9]; IR (KBr): 3190 cm^{-1} (NH), 2915 cm^{-1} (CH_3), 2810 cm^{-1} (CH_2), 1640 cm^{-1} (CONH); ^1H NMR ($CDCl_3$): 1.35 (t, 3H, CH_3), 1.70 (m, 4H, $(CH_2)_2$), 2.48 (s, 2H, CH_2), 2.70 (s, 2H, CH_2), 3.84 (q, 2H, CH_2), 6.30 (s, 1H, NH, exchangeable with D_2O); Analysis: Calc. for $C_{12}H_{14}N_2OS_2$: C, 54.11; H, 5.30; N, 10.52; O, 6.01; S, 24.07; Found: C, 54.14; H, 5.40; N, 10.59; O, 6.01; S, 23.99.

*6.3. General procedure for the synthesis of thieno[2,3-d]pyrimidin-2-yl-thioacetates **3a-c***

A solution of 2-mercapto-thieno[2,3-d]pyrimidin-4-one ***2a-c*** (0.01 mol), ethyl 2-chloroacetate (0.01 mol) and NEt_3 (0.01 mol) in 30 ml benzene was refluxed for 4h. After the reaction was completed, the solid triethylamine hydrochloride was removed through hot filtration. The solvent was removed under reduced pressure and the obtained solid was recrystallized with methanol.

*6.3.1. Ethyl 2-((3-ethyl-5,6-dimethyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)thio) acetate (**3a**):* Yield: 81%; Mp. 91–95°C; IR (KBr): 2920 cm^{-1} (CH_3), 2880 cm^{-1} (CH_3), 2820 cm^{-1} (CH_2), 1740 cm^{-1} (COOEt), 1670 cm^{-1} (CONH), 1120 cm^{-1} (C−O); ^{1}H NMR ($CDCl_3$): 1.24 (t, 9H, $3CH_3$), 2.24 (s, 3H, CH_3), 2.30 (s, 3H, CH_3), 3.84 (s, 2H, CH_2), 4.00 (q, 4H, $2CH_2$); Analysis: Calc. for $C_{14}H_{18}N_2O_3S_2$: C, 51.51; H, 5.56; N, 8.58; O, 14.70; S, 19.65; Found: C, 51.54; H, 5.61; N, 8.56; O, 14.73; S, 19.66.

*6.3.2. Ethyl 2-((2-ethoxy-2-oxoethyl)thio)-3-ethyl-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxylate (**3b**):* Yield: 78%; Mp. 119–122°C; IR (KBr): 2990 cm^{-1} (CH_3), 2820 cm^{-1} (CH_2), 1750 cm^{-1} (COOEt), 1720 cm^{-1} (COOEt), 1690 cm^{-1} (CONH), 1120 cm^{-1} (C−O); ^{1}H NMR ($CDCl_3$): 1.35 (t, 9H, $3CH_3$), 2.91 (s, 3H, CH_3), 3.99−4.31 (dq, 6H, $3CH_2$), 4.73 (s, 2H, CH_2); Analysis: Calc. for $C_{16}H_{20}N_2O_5S_2$: C, 49.98; H, 5.24; N, 7.29; O, 20.81; S, 16.68; Found: C, 49.88; H, 5.33; N, 7.31; O, 20.71; S, 16.70.

*6.3.3. Ethyl 2-((3-ethyl-4-oxo-3,4,5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-2-yl)thio) acetate (**3c**):* Yield: 82%; Mp. 130–133°C ; IR (KBr): 2950 cm^{-1} (CH_3), 2900 cm^{-1} (CH_3), 2820 cm^{-1} (CH_2), 1740 cm^{-1} (COOEt), 1670 cm^{-1} (CONH), 1100 cm^{-1} (C−O); ^{1}H NMR (DMSO-d_6): 1.19 (t, 3H, CH_3), 1.25 (t, 3H, CH_3), 1.75 (m, 4H, $(CH_2)_2$), 2.69 (t, 2H, CH_2), 2.70 (t, 2H, CH_2), 4.05 (s, 2H, CH_2), 4.12 (t, 2H, CH_2); Analysis: Calc. for $C_{16}H_{20}N_2O_3S_2$: C, 54.52; H, 5.72; N, 7.95; O, 13.62; S, 18.19; Found: C, 54.56; H, 5.75; N, 7.97; O, 13.70; S, 18.22.

*6.4. Synthesis of thieno[2,3-d]pyrimidin-2-yl-thioacetohydrazides **4a-c***

A mixture of 0.01 mol thieno[2,3-*d*]pyrimidin-2-yl-thioacetate ***3a-c*** and 0.04 mol hydrazine hydrate in 40 ml absolute ethanol was refluxed for 8h. The reaction mixture was cooled and the formed crystaline product was filtered and recrystallized with ethanol.

*6.4.1. 2-((3-ethyl-5,6-dimethyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)thio)acetohydrazide (**4a**):* Yield: 69%; Mp. 175–178°C; IR (KBr): 3200 cm^{-1} (NH_2), 2920 cm^{-1} (CH_3), 2880 cm^{-1} (CH_2),

1660 cm^{-1} (CONH); Analysis: Calc. for $C_{12}H_{16}N_4O_2S_2$: C, 46.13; H, 5.16; N, 17.93; O, 10.24; S, 20.53; Found: C, 46.16; H, 5.26; N, 17.92; O, 10.28; S, 20.51.

*6.4.2. Ethyl 3-ethyl-2-((2-hydrazinyl-2-oxoethyl)thio)-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxylate (**4b**):* Yeild: 63%; Mp. 180–185°C; IR (KBr): 3240 cm^{-1} (NH_2), 2950 cm^{-1} (CH_3), 2920 cm^{-1} (CH_2), 1720 cm^{-1} (COOEt), 1670 cm^{-1} (CONH), 1250 cm^{-1} (C−O); ^{1}H NMR ($CDCl_3$): 1.36 (dt, 6H, $2CH_3$), 2.89 (s, 3H, CH_3), 4.01 (s, 2H, CH_2), 4.15 (q, 2H, N-CH_2CH_3), 4.38 (q, 2H, O-CH_2CH_3) 7.73 (s, 1H, NH, exchangeable with D_2O); Analysis: Calc. for $C_{14}H_{18}N_4O_4S_2$: C, 45.39; H, 4.90; N, 15.12; O, 17.28; S, 17.31; Found: C, 45.44; H, 4.91; N, 15.15; O, 17.31; S, 17.39.

*6.4.3. 2-((3-ethyl-4-oxo-3,4,5,6,7,8-tetrahydrohydrobenzo[4,5]thieno[2,3-d]pyrimidin-2-yl)thio)aceto hydrazide (**4c**):* Yield: 94%; Mp. 210–214°C; IR (KBr): 3250 cm^{-1} (NH_2), 2950 cm^{-1} (CH_3), 2880 cm^{-1} (CH_2), 2820 cm^{-1} (CH_2), 1660 cm^{-1} (CONH); Analysis: Calc. for $C_{14}H_{18}N_4O_2S_2$: C, 49.68; H, 5.36; N, 16.55; O, 9.45; S, 18.95; Found: C, 49.71; H, 5.46; N, 16.61; O, 9.38; S, 19.00.

*6.5. Synthesis of thieno[2,3-d]pyrimidin-2-yl-thiosemicarbazides **5a-c***

To a suspension of the appropriate hydrazide ***4a-c*** (0.003 mol) in 20 ml absolute ethanol ethyl isothiocyanate (0.036 mol) was added. The mixture was refluxed for 5h. After cooling the formed precipitate was filtered and recrystallized with ethanol.

*6.5.1. N-ethyl-2-(2-(((3-ethyl-5,6-dimethyl-4-oxo-3,4-dihydro-thieno[2,3-d]pyrimidin-2-yl)thio)methyl) hydrazinyl)-2-oxoethanethioamide (**5a**):* Yield: 88%; Mp. 197–200°C; UV (DMF) λ_{max}= 322 nm; IR (KBr): 3300 cm^{-1} (NH), 2920 cm^{-1} (CH_3), 2880 cm^{-1} (CH_3), 2800 cm^{-1} (CH_2), 1680 cm^{-1} (CONH); ^{1}H NMR (DMSO-d_6): 1.02 (t, 3H, CH_3), 1.26 (t, 3H, CH_3), 2.33 (s, 3H, CH_3), 2.36 (s, 3H, CH_3), 4.08 (m, 6H, $3CH_2$), 7.88 (s, 1H, NH, exchangeable with D_2O), 9.30 (s, 1H, NH, exchangeable with D_2O), 10.14 (s, 1H, NH, exchangeable with D_2O); Analysis: Calc. for $C_{15}H_{21}N_5O_2S_3$: C, 45.09; H, 5.30; N, 17.53; O, 8.01; S, 24.08; Found: C, 45.11; H, 5.40; N, 17.56; O, 8.11; S, 24.13.

*6.5.2. Ethyl 3-ethyl-2-(((2-(2-(ethylamino)-2-thioxoacetyl)hydrazinyl)methyl)thio)-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxylate (**5b**):* Yield: 91%; Mp. 182–184°C; UV (DMF) λ_{max}= 394 nm; IR (KBr): 3180 cm^{-1} (NH), 2940 cm^{-1} (CH_3), 2900 cm^{-1} (CH_3), 2880 cm^{-1} (CH_2), 1690 cm^{-1} (CONH), 1270 cm^{-1} (C−O); ^{1}H NMR (DMSO-d_6): 1.04 (t, 3H, CH_3), 1.26 (m, 6H, $2CH_3$), 2.79 (d, 3H,

CH_3), 4.00 (m, 2H, CH_2), 4.09 (s, 2H, CH_2), 4.27 (dq, 4H, $2CH_2$), 8.04 (s, 1H, NH, exchangeable with D_2O), 9.35 (d, 1H, NH, exchangeable with D_2O), 9.89 (d, 1H, NH, exchangeable with D_2O); Analysis: Calc. for $C_{17}H_{23}N_5O_4S_3$: C, 44.62; H, 5.07; N, 15.30; O, 13.99; S, 21.02; Found: C, 44.63; H, 5.11; N, 15.40; O, 13.95; S, 21.03.

*6.5.3. N-ethyl-2-(2-(((3-ethyl-4-oxo-3,4,5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-2-yl) thio)methyl)hydrazinyl)-2-oxoethanethioamide (**5c**):* Yield: 96%; Mp. 188–192°C; UV (DMF) λ_{max}= 322 nm; IR (KBr): 3200 cm^{-1} (NH), 2920 cm^{-1} (CH_3), 1670 cm^{-1} (CONH); ^{1}H NMR (DMSO-d_6): 1.02 (t, 3H, CH_3), 1.25 (m, 3H, CH_3), 1.77 (m, 4H, $(CH_2)_2$), 2.70 (d, 2H, CH_2), 2.85 (d, 2H, CH_2), 4.08 (m, 6H, $3CH_2$), 7.90 (s, 1H, NH, exchangeable with D_2O), 9.29 (s, 1H, NH, exchangeable with D_2O), 10.15 (s, 1H, NH, exchangeable with D_2O). Analysis: Calc. for $C_{17}H_{23}N_5O_2S_3$: C, 47.98; H, 5.45; N, 16.46; O, 7.52; S, 22.60; Found: C, 48.02; H, 5.39; N, 16.49; O, 7.51; S, 22.62.

*6.6. Cyclocondensation of thieno[2,3-d]pyrimidin-2-yl-thiosemicarbazides to 1,3,4-thiadiazoles **6a-c***

The thiosemicarbazide ***5a-c*** (0.001 mol) was added portionwise to 3 ml 98% sulfuric acid at 5°C by stirring. The reaction mixture was cooled and poured into ice-water and neutralized with NH_4OH. The formed precipitate was filtered and recrystalized wit methanol.

*6.6.1. 3-ethyl-2-(((5-(ethylamino)-1,3,4-thiadiazol-2-yl)methyl)thio)-5,6-dimethylthieno[2,3-d] pyrimidin-4(3H)-one (**6a**):* Yield: 63%; Mp. 208–212°C; UV (DMF) λ_{max}= 322 nm; IR (KBr): 3300 cm^{-1} (NH), 2920 cm^{-1} (CH_3), 2880 cm^{-1} (CH_3), 2800 cm^{-1} (CH_2), 1670 cm^{-1} (CONH); ^{1}H NMR (DMSO-d_6 + $CDCl_3$): 1.38 (t, 6H, $2CH_3$), 2.24 (s, 6H, $2CH_3$), 3.10 (q, 2H, CH_2), 3.86 (q, 2H, CH_2), 4.41 (s, 2H, CH_2), 6.85 (s, 1H, NH, exchangeable with D_2O); Analysis: Calc. for $C_{15}H_{19}N_5OS_3$: C, 47.22; H, 5.02; N, 18.36; O, 4.19; S, 25.21; Found: C, 47.26; H, 5.03; N, 18.44; O, 4.17; S, 25.23.

*6.6.2. Ethyl 3-ethyl-2-(((5-(ethylamino)-1,3,4-thiadiazol-2-yl)methyl)thio)-5-methyl-4-oxo -3,4-dihydro thieno[2,3-d]pyrimidine-6-carboxylate (**6b**):* Yield: 95%; Mp. 188–193°C; UV (DMF) λ_{max}= 394 nm; IR (KBr): 3420 cm^{-1} (NH), 3350 cm^{-1} (NH), 2920 cm^{-1} (CH_3), 2880 cm^{-1} (CH_3), 2820 cm^{-1} (CH_2), 1720 cm^{-1} (COOEt), 1680 cm^{-1} (CONH); ^{1}H NMR (DMSO-d_6): 1.27 (dt, 9H, $3CH_3$), 3.02 (s, 3H, CH_3), 4.01−4.29 (dq, 6H, $3CH_2$), 4.71 (s, 2H, CH_2), 8.13 (s, 1H, NH, exchangeable with D_2O);

Analysis: Calc. for $C_{17}H_{21}N_5O_3S_3$: C, 46.45; H, 4.82; N, 15.93; O, 10.92; S, 21.88; Found: C, 46.45; H, 4.88; N, 15.95; O, 10.93; S, 21.91.

*6.6.3. 3-ethyl-2-(((5-(ethylamino)-1,3,4-thiadiazol-2-yl)methyl)thio)-5,6,7,8-tetrahydrobenzo[4,5] thieno[2,3-d]pyrimidin-4(3H)-one (**6c**):* Yield: 90%; Mp. 202–204°C; UV (DMF) λ_{max}= 324 nm; IR (KBr): 3180 cm^{-1} (NH), 2920 cm^{-1} (CH_3), 2880 cm^{-1} (CH_3), 2820 cm^{-1} (CH_2), 1670 cm^{-1} (CONH); ^{1}H NMR (Pyridine-d_6): 1.27 (t, 6H, $2CH_3$), 1.66 (t, 4H, $(CH_2)_2$), 2.61 (t, 2H, CH_2), 3.05 (s, 2H, CH_2), 3.56 (q, 2H, HN-CH_2CH_3), 4.01 (q, 4H, N-CH_2CH_3), 4.84 (s, 2H, CH_2), 7.20 + 7.57 (ds, 1H, NH, exchangeable with D_2O); Analysis: Calc. for $C_{17}H_{21}N_5OS_3$: C, 50.10; H, 5.19; N, 17.18; O, 3.93; S, 23.60; Found: C, 50.08; H, 5.21; N, 17.23; O, 3.83; S, 23.60.

6.7. 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).

6.8. Photostability

The study on the photodegradation of the compounds was conducted in a solar simulator SUNTEST CPS equipment (Heraeus, Germany), supplied with an arc aircooled Xenon lamp (Hanau, 1.1 kW, 765Wm^{-2}), at ambient temperature. The irradiation was performed in solid state of the compounds. The UV–vis absorption spectra were recorded on a spectrophotometer Hewlett Packard 8452A in DMF solution at concentration 10^{-5} mol/L.

6. 9. Computational details

The most probable conformers of the studied molecules (including thione-thiol and lactam-lactime tautomerism) were constructed and energy minimized using *Avogadro*'s software [48]. Based on the energy analysis the most stable conformers were selected and optimized by density functional theory (DFT). The theoretical calculations were performed using the Gaussian 09 package [40] of

programs. Geometry and vibrational frequencies of the studied species were performed by analytical gradient technique without any symmetry constraint. The results were obtained, employing the B3LYP (Becke's three-parameter non-local exchange [40] and Lee et al. correlation [41] potentials) in conjunction with the 6-311++G** basis set. The optimized structures were further characterized by analytical computations of harmonic vibrational frequencies at the same level.

6.10. Molecular docking

Initial molecular modeling coordinates of the enzyme were taken from the crystal structure of oncogenic mutant ^{V599E}B-Raf kinase domain in complex with *sorafenib* (PDB code: 1UWJ [43]). The inhibitors were doked into an internal pocket formed by the activation segment and the P-loop of ^{V599E}B-Raf (PDB entry code 1UWJ) using MOE software [47]. Conformational search for preparation of the ligands was carried out by LowModelMD method which performs molecular dynamics perturbations along with low frequency vibrational modes with energy window 7 kCal/mol, and conformational limits of 1000. Placement of conformers was prepared according to alpha-triangle method on selected pharmacophores. Scoring of docking poses was performed by affinity dG, calculated with MMFF94x force field. VMD [49] was used to produce 3-D figures of binding modes.

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Highlights

- Synthesis of new thieno[2,3-*d*]pyrimidin-4(3H)-ones was optimized;
- Compound **6b** exibits 100-*fold* higher citotoxicity against all cancer cells than to *Lep3;*
- Compound **5b** show high citotoxicity against *MDA-MB-231* with IC_{50} - 0.23 nM;.
- Most active to HT-29 celsl was compound **6c** with IC_{50} -0,001 μM;
- No photodegradation was observed in the compounds **5b-c** and **6a-c**;
- The binding of the ligands to ^{V599E}B-Raf promotes an inactive enzyme conformation;