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Novel cyanothiouracil and cyanothiocytosine derivatives as concentration-dependent selective inhibitors of U87MG glioblastomas: Adenosine receptor binding and potent PDE4 inhibition

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ABSTRACT

Thiouracil and thiocytosine are important heterocyclic pharmacophores having pharmacological diversity. Antitumor and antiviral activity is commonly associated with thiouracil and thiocytosine derivatives, which are well known fragments for adenosine receptor affinity with many associated pharmacological properties. In this respect, 33 novel compounds have been synthesized in two groups: 24 thiouracil derivatives (**4a-x**) and 9 thiocytosine derivatives (**5a-i**). Antitumor activity of all the compounds was determined in the U87 MG glioblastoma cell line. Compound **5e** showed an anti-proliferative IC_{50} of 1.56 μM, which is slightly higher activity than cisplatin (1.67 μM). The 11 most active compounds showed no signficant binding to adenosine A_1, A_{2A} or A_{2B} receptors at 1 μM. Brain tumors express high amounts of phosphodiesterases. Compounds were tested for PDE4 inhibition, and **5e** and **5f** showed the best potency (**5e**: 3.42 μM; **5f**: 0.97 μM). Remakably, those compounds were also the most active against U87MG. However, the compounds lacked a cytotoxic effect on the HEK293 healthy cell line, which encourages further investigation.

1. Introduction

Purine and pyrimidine heterocycles are important chemical moieties that constitute structural components of nucleotides. Both of these bases themselves and their nucleoside phosphate structures have functions on various receptors and thus on physiological processes. Many of these substances themselves cannot be used as drugs, as they do not remain in circulation for a sufficient time. However, through development based on these structures, purine and pyrimidine receptors have been considered as drug targets [1]. Different pharmacological activities have been investigated on these chemical groups. Especially, effects of cyanothiouracil derivatives, such as anti-inflammatory [2], antidiabetic [3,4], antitumor [5–13] and antifungal [14,15] activities, have been discovered in some recent studies. Previously, thiouracil derivatives have been investigated on different cancer cell lines *in vitro*, but there is need to study their effects on brain tumor cells. As shown in Fig. 1, cyanothiouracil derivatives compound I, II and III showed moderate inhibition on MCF7, HOP92 and HC116 cell lines. Purine and pyrimidine bases are found to be effective on phosphodiesterases, adenosine receptors and kinases. Glioblastomas expresses high amounts of these macromolecules, so purine and pyrimidine derivatives can be considered as a potential source of inhibitors of proliferation. These studies have encouraged efforts on cyanothiouracils and cyanothiocytosines. The 4-oxo forms are preferred over hydroxy forms when representing cyanothiouracils; whereas,

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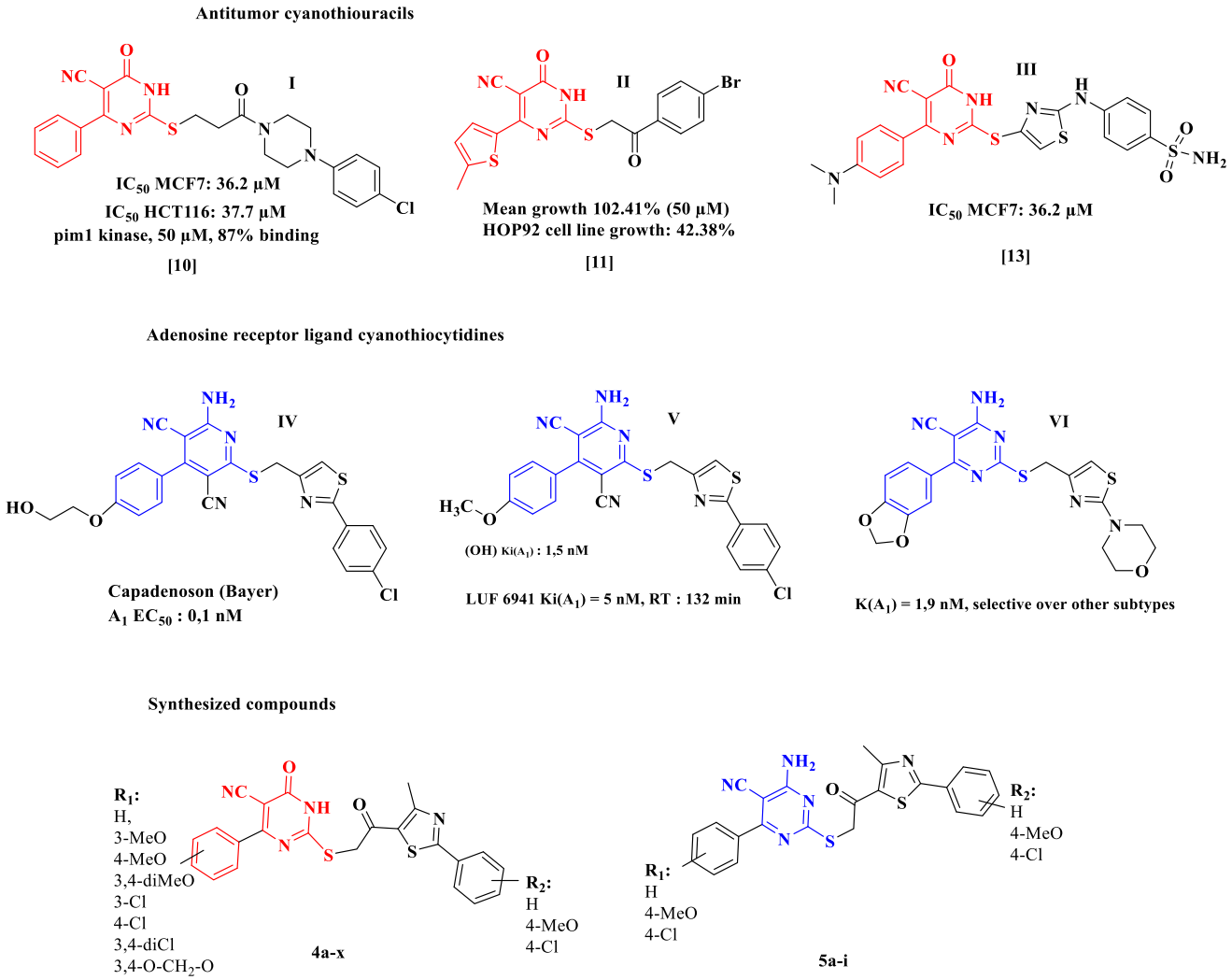


Fig. 1. Synthesized compounds and pharmacologically active molecules bearing the same fragments.

amino forms are preferred over imino forms for cyanothiocytosines, with respect to tautomeric states described for 4-oxo and 4-amino pyrimidines [16,17].

Compounds similar to cyanothiocytosine, where the 4-oxo group of thiouracil is replaced with an amino group, have mainly been investigated for adenosine (A_1, A_{2A}, A_{2B}, A_3) receptor binding affinities and pharmacological activities in such conditions as cancer, heart failure, etc. [1,18–20]. Capadenoson (IV), a selective adenosine A_1 agonist, was evaluated for heart failure but withdrawn from clinical trials due to side effects. LUF6941 (V) is a selective A_1 agonist with enhanced residence time. VI is another selective A_1 agonist with a binding Ki value of 1.9 nM. Furthermore, these derivatives have potential as antitumor agents, as they are bearing a pyrimidine core, which is commonly studied on different cell lines, but not extensively studied on brain tumor cells.

Adenosine receptor agonist and antagonist ligands typically contain a nitrogenous ring and an exocyclic amino group. This NH or NH_2 group forms a hydrogen bond with an Asn amino acid residue in the binding site of adenosine receptors, as was shown for adenosine itself in an X-ray crystallograpic structure [21]. Also, most of the early generation of adenosine agonists contain a ribose sugar that will interact with a hydrophilic region of the binding site in the center of the transmembrane helical bundle. In addition, adenosine receptor agonists and partial agonists that do not contain ribose moieties (non-ribose nucleoside analogs) have been introduced in the literature in recent years [20,22,23]. Adenosine A_1 and A_3 agonists and A_{2A} and A_{2B} antagonists show antitumor activity, with respect to their secondary messengers. Selective inhibitors of each subtype have been investigated for their antitumor potential *in vitro* and/or *in vivo*.

Glioblastomas are responsible for almost 75% of aggressive malignant brain tumors. In the United States, approximately ten thousand people are diagnosed with malignant glioma annually, and survival of only 1 year is expected for 50% of these patients [24]. U87MG (Uppsala 87 Malignant Glioma) human glioma primary brain tumor cells are from an aggressive type of brain tumor and express high levels of adenosine receptors (especially A_2 and A_3), phosphodiesterase enzymes (especially subtype 4A) and kinases, and only a few drugs are being used to treat this tumors. Temozolomide is one of the primary options, which becomes ineffective upon the development of resistance, and U87 glioblastomas may also produce resistance against cisplatin and other effective drugs [25–27]. Thus, combination therapy or discovery of novel potent molecules is required for an effective treatment [28,29]. Kinases (mainly epidermal growth factor receptor, EGFR) and phosphodiesterases are potential targets for inhibition of U87MG cell line

proliferation. Rolipram, a potent phosphodiesterase 4 inhibitor, potently inhibited U87 cell proliferation, but the mechanism of action is still unknown. It is still unclear whether the activity is related to phosphodiesterase or kinases [30–32].

In this study, a series of novel cyanothiouracil and cyanothiocytosine derivatives (**4a-x**, **5a-i**) (Table 1) were synthesized, characterized and evaluated for their antiproliferative potential against glioblastoma tumor (U87MG) and human embryonic kidney (HEK293) healthy cell lines. The 11 most potent compounds (**4d**, **4l**, **5a-i**) were subjected to adenosine A_1, A_{2A} and A_{2B} receptor binding assays at a 1 μM concentration to determine if there is an interaction with these receptors. Antitumor activity could not be associated with adenosine receptors, and subsequently the active compounds were subjected to a phosphodiesterase 4 assay and an additional MTT assay in MCF7 cells, a common breast cancer cell line that does not express high levels of adenosine receptors or the same type of phosphodiesterases as U87MG cells [33]. Briefly, we evaluated *in vitro* whether the 4-oxo and/or 4-amino type novel non-nucleoside analogs display antitumor activity, and mechanism of action was investigated in PDE4 and adenosine receptor assays.

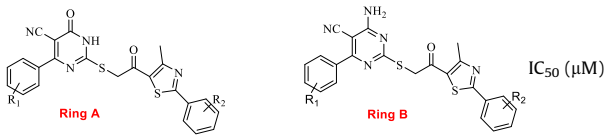
2. Results

2.1. Chemistry

There are several structures that have characteristic peaks in the FT-IR spectra of the final compounds. A C≡N triple bond stretch band was observed in all compounds in the range of 2200–2210 cm^{-1}. Bands belonging to the carbonyl group were observed in the 1600-1700 cm^{-1} range for all final compounds. In compounds with methoxy and methylenedioxy groups, characteristic C–O single bond stretch bands were observed between 1200 and 1300 cm^{-1}. First aliphatic and then aromatic C–H stretch bands were observed in the range of 2800–3000 cm^{-1}. The NH group of ring A, as seen in one tautomeric representation, could be observed in some of the IR spectra. The N–H stretch bands of the NH_2 group in the group of compounds **5a-i** were observed around 3300 cm^{-1}.

The hydrogens in the compounds can be seperated in 3 main groups as aliphatic, aromatic and those bound to heteroatoms. The first aliphatic group is a methyl group attached to the thiazole ring. Proton NMR peaks belonging to this group were observed in all

Table 1
IC_{50} (mean ± SD) values of synthesized compounds and cisplatin on HEK293 and U87MG cells.



Compound	Ring	R_1	R_2	U87MG	HEK293
4a	A	H	H	56.61 ± 0.02	93.54 ± 5.10
4b	A	3-OMe	H	59.72 ± 0.04	90.24 ± 3.63
4c	A	4-OMe	H	48.36 ± 0.01	76.24 ± 6.59
4d	A	3,4-diOMe	H	**15.33 ± 0.03**	**91.77 ± 3.29**
4e	A	3-Cl	H	28.21 ± 0.06	ND
4f	A	4-Cl	H	43.35 ± 0.05	ND
4g	A	3,4-diCl	H	65.04 ± 0.01	85,07 ± 0.33
4h	A	3,4-OCH_2O-	H	33.69 ± 0.02	ND
4i	A	H	4-OMe	57.25 ± 0.02	ND
4j	A	3-OMe	4-OMe	**15.34 ± 0.06**	**95.39 ± 5.16**
4k	A	4-OMe	4-OMe	25.27 ± 0.03	ND
4l	A	3,4-diOMe	4-OMe	**12.47 ± 0.03**	23.30 ± 3.14
4m	A	3-Cl	4-OMe	78.90 ± 0.02	ND
4n	A	4-Cl	4-OMe	45.93 ± 0.01	63.01 ± 0.46
4o	A	3,4-diCl	4-OMe	68.47 ± 0.02	45.60 ± 2.51
4p	A	3,4-OCH_2O-	4-OMe	65.28 ± 0.02	ND
4q	A	H	4-Cl	27.57 ± 0.03	ND
4r	A	3-OMe	4-Cl	40.97 ± 0.02	77.93 ± 0.41
4s	A	4-OMe	4-Cl	35.49 ± 0.03	ND
4t	A	3,4-diOMe	4-Cl	38.64 ± 0.05	ND
4u	A	3-Cl	4-Cl	40.13 ± 0.05	30.05 ± 1.92
4v	A	4-Cl	4-Cl	53.01 ± 0.01	75.07 ± 9.24
4w	A	3,4-diCl	4-Cl	83.89 ± 0.01	46.89 ± 1.72
4x	A	3,4-OCH_2O-	4-Cl	55.53 ± 0.01	81.44 ± 4.41
5a	B	H	H	28.95 ± 0.02	46.69 ± 4.65
5b	B	4-OMe	H	13.98 ± 0.03	ND
5c	B	4-Cl	H	10.80 ± 0.08	ND
5d	B	H	4-OMe	**8.54 ± 0.04**	**ND**
5e	B	4-OMe	4-OMe	**1.56 ± 0.09**	**ND**
5f	B	4-Cl	4-OMe	**4.54 ± 0.06**	**39.16 ± 1.38**
5g	B	H	4-Cl	11.42 ± 0.04	ND
5h	B	4-OMe	4-Cl	10.57 ± 0.04	ND
5i	B	4-Cl	4-Cl	**3.42 ± 0.06**	**72.96 ± 2.33**
cisplatin				**1.67 ± 0.04**	**8.44 ± 0.86**

(ND: Not determined. Means viability is higher than 50% at 100 μM).

compounds in the range of 2.6–2.7 ppm as singlets with a 3H integration. Other aliphatic hydrogens observed belong to the methylene (CH_2) group in the neighborhood of sulfur and carbonyl. The hydrogens of this group were observed for all compounds as singlets with a 2H integration in the range of 4.45–4.7 ppm. The compounds **4b-d**, **4i**, **4r-t**, **5b**, **5d-5f**, and **5h** carry a methoxy (OCH_3) group. These methyl groups attached to oxygen were observed in the range of 3.6–3.85 ppm as a singlet with 3H integration (Fig. 2). In the compounds **4d**, **4l** and **4t** carrying 3,4-dimethoxyphenyl group, two of the methoxy groups overlapped, and a singlet was observed with 6H integration. CH_2 hydrogens in compounds **4h**, **4p** and **4x** carrying methylenedioxy groups were observed in the 5.85–5.92 ppm range with 2H integration.

Aromatic hydrogens belonging to the A and B rings (Fig. 2) were observed in the range of 6.7–8.0 ppm. While the peaks in the compounds with a methoxy (OCH_3) group are approaching 6.7 ppm due to their electron donor property, in the compounds containing a Cl substituent approached 8 ppm due to the electron withdrawing effect. In 4-substituted phenyl derivatives, peaks were observed as doublet with 2H integration, representing 1–4 splitting, except for overlapping signals. Since the compounds **4f**, **4n**, **4q-x**, **5c** and **5f-i** carry 4-Cl substituents, two peaks were observed as a doublet in the aromatic region and with 2H integration in these derivatives. In some compounds, peaks could not be differentiated due to overlaps, and multiplets were observed. NH_2 or OH protons defined as X in the Figure, were not observed possibly due to the tautomeric structure. The aliphatic and aromatic hydrogen numbers were provided as observed for all compounds, and the structure of the compounds was assured.

For interpretation of ^{13}C NMR spectral results, it is possible to group the carbon atoms in compounds into several groups. The first of these is the CH_3 carbon in thiazole ring at position 4. The peak for this carbon was observed at 18.7 ppm in all compounds. It is expected that the methylene group between the sulfur and carbonyl group in the structure would be observed in the 38–40 ppm range. Since the spectra are taken in DMSO, the solvent peaks are also observed in this range in proximity to the CH_2 carbon. The peak of OCH_3 carbon was observed around 55 ppm in all compounds containing methoxy groups. In compounds containing more than one methoxy, these peaks coincided in some compounds, and it was possible to observe them separately in others. The carbon atom to which the cyano group is bound in the A ring, is expected and found in the range of 80–100 ppm. In compounds **4h**, **4p** and **4x** containing methylenedioxy groups attached to the aromatic ring, the O–CH_2–O carbon was observed in the range of 102–108 ppm. The carbon atom belonging to the CN group was observed at ~115 ppm. There are 4 aromatic rings in the compounds, two of which are phenyl and the other two are heteroaromatic rings. As expected, phenyl ring carbons were detected between 110 and 148 ppm. The carbon atoms of the heteroaromatic pyrimidine and thiazole rings were observed in the range of 150–170 ppm. These carbons can sometimes be observed overlapping due to their symmetrical state and the shading effect in three-dimensional positioning. Finally, the peak of the carbonyl group, one of the important determining groups of our compounds, was detectable in the range of 180–190 ppm. High resolution mass spectra gave the M+1 peaks accurately.

2.2. Biological activity

2.2.1. U87MG and HEK293 cellular antitumor and cytotoxic activity measurement

The compounds' antitumor and cytotoxic activity is summarized in Table 1. Accordingly, most of the compounds showed antiproliferative activity on U87MG aggresive glioblastoma. Furthermore, almost all compounds seem did not attenuate the proliferation of healthy, noncancerous human embyonic kidney (HEK) 293 cell line.

As seen in the effects of the compounds over a concentration range, the degree of inhibition increased with concentration (Table 2). At a concentration of 5 μM, compounds **5b-i** were able to reduce viability by 70%. Compounds **5b-i** at a concentration of

Table 2
% Viability values ± SD of synthesized compounds (5 μM, 10 μM, 50 μM, 100 μM) and cisplatin against U87MG tumor cells.

Compound No.	5 μM	10 μM	50 μM	100 μM
4a	92.10 ± 4.14	87.5 ± 2.12	77.5 ± 2.17	9.23 ± 1.09
4b	99.00 ± 3.33	90.2 ± 2.20	80.3 ± 4.10	21.8 ± 2.77
4c	97.30 ± 0.62	90.4 ± 0.77	52.1 ± 1.86	1.08 ± 0.45
4d	80.80 ± 6.19	69.8 ± 0.37	21.4 ± 3.52	6.48 ± 0.60
4e	86.90 ± 1.67	83.7 ± 3.58	50.6 ± 2.63	10.0 ± 1.79
4f	90.90 ± 2.11	85.2 ± 4.29	63.4 ± 3.37	22.5 ± 2.25
4g	98.60 ± 6.68	92.5 ± 2.32	75.3 ± 1.84	24.4 ± 2.11
4 h	93.40 ± 4.44	84.2 ± 2.61	42.6 ± 2.39	18.7 ± 2.53
4i	99.00 ± 3.48	90.9 ± 0.84	72.5 ± 2.83	10.9 ± 4.02
4j	71.30 ± 1.10	66.7 ± 1.54	43.2 ± 1.18	12.4 ± 0.78
4k	97.00 ± 6.31	80.3 ± 0.96	32.6 ± 2.25	11.5 ± 3.86
4l	81.20 ± 3.45	57.6 ± 3.49	22.2 ± 2.57	5.39 ± 1.22
4m	95.7 ± 3.53	90.6 ± 3.17	83.4 ± 3.72	40.6 ± 3.08
4n	102.00 ± 9.35	92.7 ± 2.95	48.0 ± 2.64	2.85 ± 0.87
4o	101.00 ± 6.46	88.6 ± 1.97	78.4 ± 3.04	31.1 ± 2.80
4p	100.00 ± 4.02	88.3 ± 2.76	83.2 ± 3.65	22.9 ± 2.28
4q	88.9 ± 3.72	83.1 ± 2.62	41.6 ± 1.47	11.4 ± 1.58
4r	96.6 ± 4.09	89.7 ± 1.45	47.6 ± 1.30	8.54 ± 2.66
4s	92.1 ± 2.73	88.3 ± 3.64	46.3 ± 3.06	16.6 ± 1.40
4t	93.4 ± 0.80	83.3 ± 3.18	59.8 ± 3.56	18.7 ± 1.54
4u	80.2 ± 5.63	95.2 ± 3.05	52.6 ± 2.77	12.5 ± 2.26
4v	99.00 ± 4.97	98.4 ± 0.59	60.0 ± 1.71	12.8 ± 3.65
4w	101.00 ± 3.46	97.1 ± 1.02	93.9 ± 1.11	36.5 ± 3.66
4x	101.00 ± 3.76	95.0 ± 3.09	68.3 ± 2.86	9.91 ± 1.20
5a	103.00 ± 3.08	93.0 ± 2.95	26.3 ± 3.17	6.48 ± 1.70
5 b	68.8 ± 3.46	60.8 ± 3.66	33.8 ± 2.51	17.2 ± 1.21
5c	62.9 ± 1.93	58.8 ± 1.24	44.3 ± 3.90	17.1 ± 2.84
5d	61.8 ± 3.38	57.8 ± 2.40	16.5 ± 1.27	5.25 ± 0.37
5e	35.6 ± 2.95	28.7 ± 2.26	13.0 ± 1.75	0.26 ± 0.45
5f	51.4 ± 1.25	44.0 ± 2.67	23.1 ± 3.02	3.90 ± 0.79
5g	71.1 ± 3.57	67.9 ± 2.28	15.2 ± 2.87	4.59 ± 1.06
5 h	69.7 ± 0.78	64.5 ± 3.08	17.8 ± 2.40	0.79 ± 0.79
5i	46.1 ± 1.95	37.3 ± 3.52	15.5 ± 2.85	1.65 ± 0.87
Cisplatin	31.7 ± 1.53	18.2 ± 1.47	6.71 ± 1.64	2.25 ± 1.86

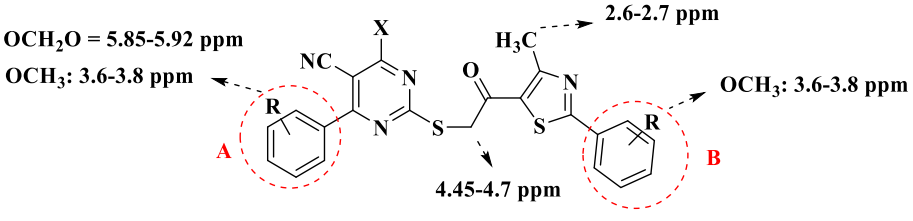


Fig. 2. *^1H NMR interpretation summary of the compounds.*

10 μM showed a modest increase compared to the 5 μM concentration, while only **4d**, **4l** and **4j** from the **4a-x** group caused <70% viability. Although compounds **4a-x** and **5a** at 50 μM showed a significantly increased antiproliferative effect, on average it remained above 45% (excluding **4d**, **4l** and **4j**), while compounds between **5d-i** reduced the viability to <20% on average. A significant increase in the efficacy of all compounds was observed at a 100 μM concentration, and compounds **4d**, **5e**, **5h** and **5i** produced an even greater effect than the standard cisplatin (Fig. 3, Table 2).

In general, compounds **5b-i** were significantly more active than the rest of series (**4a-x**) except for **4d**, **4j,** and **4l**. Unlike the **4a-x** group, these compounds (**5a-i**) carry the amino group instead of the oxo at the 4th position of the pyrimidine ring. Compound **5a** also contains an amino group at the 4 position, but its aromatic rings at both ends of the compound are unsubstituted. This compound showed reduced inhibition compared to **5b-i**. Concentration dependent viability results for all synthesized molecules are summarized in Table 2.

With respect to the effects of the compounds on the HEK293 healthy cell line, 50% inhibition was not observed for 16 of the 33 synthesized compounds even at 100 μM. The IC_{50} values of the remaining molecules were quite high, that is, the cytotoxic effects of the molecules were very low. This situation is remarkable and important in terms of antitumor drug properties. Of the compounds, in particular **5e** showed an IC_{50} of 1.56 μM against U87MG, while the IC_{50} of cisplatin was 1.67 μM (Table 1). Cisplatin also showed an IC_{50} of 8.44 μM on the healthy HEK293 cell line. Compound **5e** had no cytotoxic effect even at high concentrations. Apart from that, while **5i:** 3.42 μM, **5f:** 4.54 μM and **5d** had an IC_{50} of 8.54 μM against U87MG cells, these molecules did not show any cytotoxic effect in HEK293 cells. Although the compounds do not have significant effects in most of the series at 5 μM concentration, as seen in Figs. 3, **5b-i** compounds showed higher effects compared to the **4a-x** group. Although there was no significant increase in the effect at 10 μM, the efficiency of compound **5e** became evident and became closer to cisplatin. At a concentration of 50 μM, the active and non-active compounds were clearly delineated. Accordingly, 11 compounds, **4d**, **4l**, and **5a-i**, were considered active and were tested for adenosine receptor binding affinity.

2.2.2. MCF7 cellular antitumor activity measurement

As shown in Table 3, the compounds displayed an antiproliferative effect on MCF7 cell line. Although the IC_{50} values were considerable, the anticancer activity was not high as U87MG cell line. Different cell lines have different levels of specific proteins such as enzymes and receptors that may affect proliferation. In general, cancer cell lines typically highly expresss proteins that are considered as cancer drug targets.

Table 3
IC_{50} (mean ± SD) values of compounds and cisplatin on MCF7.

Compound	IC_{50} (μM)
4d	63.71 ± 1.92
4l	24.02 ± 0.95
5a	30.14 ± 1.62
5b	27.15 ± 0.95
5c	20.17 ± 0.75
5d	72.12 ± 2.33
5e	24.31 ± 1.95
5f	**11.25 ± 0.09**
5g	25.22 ± 1.17
5h	**10.09 ± 0.03**
5i	**12.52 ± 1.13**
Cisplatin	**2.51 ± 0.29**

The MCF7 cell line is known to express kinases and phosphodiesterases, but the range of isoforms present may change from a cell line to the other. For example, brain tumors like glioblastomas usually expresses higher amounts of PDE4B and PDE7 than MCF7 cells. The lower anticancer activity observed is possibly due to this situation. The choice of a cell line to study after U87MG will require consideration of the mechanism of action. Among the compounds tested on MCF7 cells, **5f**, **5h** and **5i** showed higher activity than the other 8 compounds.

2.2.3. Measurement of adenosine receptor binding affinity

The relationship of adenosine ligands with antitumor activity is mainly on glioblastomas. It is known that adenosine concentration (1.5 μM) in extracellular fluid of glioblastomas is lower than normal cells (3 μM). Antitumor effect studies are ongoing in all subtypes. The agonism of A_3 receptors and the antagonism of A_{2A} may provide antitumor activity, but the antitumor effect of A_3 receptor agonists is most evident in *vivo* models [34]. U87MG glioblastoma tumor cells were selected for the reasons described above to determine the pharmacological activity basis of the compounds. The degree of binding of compounds **4d**, **4l**, **5a-i** to adenosine A_1 and A_{2A} receptor subtypes was measured. The most antitumor compound **5e** additionally subjected to an A_{2B} assay to probe the basis of its activity. According to the data given in Table 4, the values are not high enough to be considered receptor blockers, at least at a fixed concentration of 1 μM. Compounds **4d**, **5a**, **5e** and **5g** are the compounds showing the most binding inhibition (8.7–10%) at the A_1 subtype. The highest degree of inhibition at the A_{2A} receptor was observed for **4d** and **4l**, with 12% and 8.5% inhibition, respectively. Compound **5e** showed 18% binding at 1 μM.

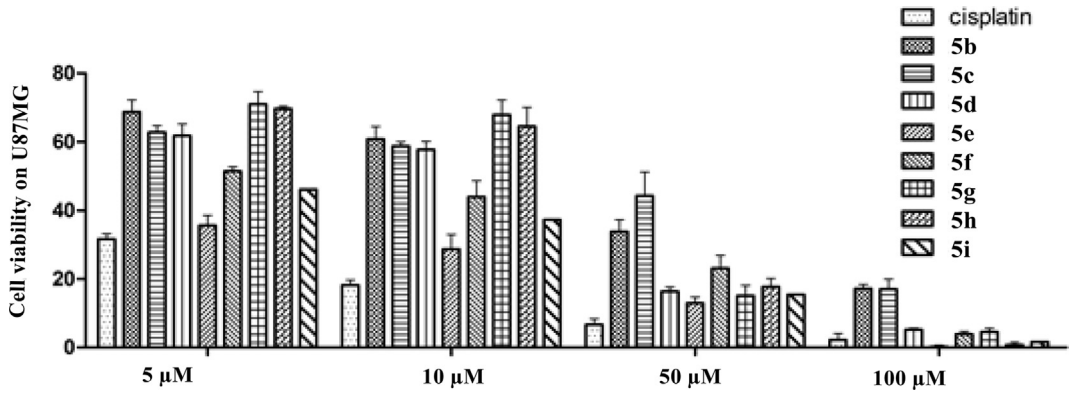


Fig. 3. Effect of increasing concentrations of **5b-i** on viability of U87MG cells.

Table 4
Inhibition of human A_1 and A_{2A} receptor radioligand binding by the synthesized substances (at 1 μM, expressed as percent of specific binding).

Cpd No.	A_1 [^{3}H]DPCPX	A_{2A} [^{3}H]ZM241385	Cpd No	A_1 [^{3}H]DPCPX	A_{2A}/A_{2B} [^{3}H]ZM241385
4d	10	12	**5e**	9.0	−18/18[a]
4l	2.5	8.5	**5f**	1.1	−7.1
5a	9.5	6.5	**5g**	8.7	−1.4
5b	−4.6	0.8	**5h**	1.2	−22
5c	−23	−9.9	**5i**	−3.7	1.8
5d	−2.8	−2.6			

[a] Inhibition of A_{2B} receptor binding by 10 μM **5e** was 49%. Only **5e** was measured at the A_{2B} receptor.

It seems that some of these values are negative values. In this radioligand binding method, negative (−) values are not meaningful and will be considered as zero. It was observed that compounds **4d**, **4l** and **5a** showed weak binding at both A_1 and A_{2A} subtypes. Among the other compounds, **5g** and **5h** showed weak binding only on A_1 receptors, **5e** showed weak binding on A_{2A} and A_{2B}, and **5b** and **5i** were found to show only weak binding on A_{2A} receptors.

Adenosine receptor binding was only evaluated at a concentration of 1 μM, and none of the compounds at this concentration showed high levels of binding. In the literature, adenosine ligands have been reported act at the nanomolar (nM) level. In addition, the A_1 receptor activities of some compounds at a concentration of 10 μM were found to be similar [35,36]. For a molecule to be considered an adenosine receptor ligand, some inhibition at the submicromolar level would be expected, so full concentration curves have not been determined. Therefore, the anticancer effect of the compounds is not associated with adenosine A_1 or A_{2A} receptor subtypes.

2.2.4. Measurement of PDE4B assay

Table 5 summarizes the IC_{50} values of the compounds tested for PDE4 inhibition. 3-Isobutyl-1-methylxanthine (IBMX), a general phosphodiesterase standard, had an IC_{50} value of 14.31 μM. Rolipram, a potent PDE4B inhibitor, showed a 0.32 μM IC_{50}, which is consistent with the literature data. Compound **5e** and **5f** showed good potency on PDE4B, with 3.15 μM and 0.97 μM IC_{50} values respectively. These inhibitory potencies are considerably high, i.e. close to the most potent PDE4B inhibitor rolipram (Fig. 4). There is a close correspondence between the most active compounds against U87MG cells and in the PDE4B assay**.** This parallelism is remarkable in the context of suggesting a basis of antitumor activity on U87MG.

When comparing the chemical structures of the compounds with U87 antitumor activity, it seems that compounds **5a-i** (thiocytosine derivatives) contain exocyclic amino groups in common. **4d**, **4l** and **4j** were the only active compounds in the cyanothiouracil series **4a-x**, and they have methoxyphenyl groups on the pyrimidine ring. Compound **4l** and **4j** also has a 4-methoxy group on the other phenyl ring attached to the thiazole. It is noteworthy that compound **5e**, which is the most active in the **5a-i** group, also carries 4-methoxyphenyl groups at both ends, parallel to the structures of **4d** and **4l**. In addition, compounds **4c**, **4n**, **5h**, which act at 100 μM, also carry a methoxy group on one of the phenyl rings. The activity of structures bearing methylenedioxy and chlorine substituents remained relatively low. The IC_{50} value of temozolomide, the drug used most frequently against glioblastoma tumors, affected the U87MG cell line only in the range of 130−171 μM [37]. From this view, our compounds are important hits as they are potent and not cytotoxic.Fig. 5.

Compounds with the highest antitumor activity did not show any parallel in adenosine A_1 or A_{2A} receptor results. The lack of correlation of adenosine receptor results and the antitumor effect precludes those receptors as the biochemical basis of the high antitumor effect. U87MG cells are in the class of glioblastomas, an important type of brain tumors. This group of tumors express phosphodiesterase 4 in particularly high amounts. Apart from that, it expresses kinase enzymes in high amounts as in all tumor types. MCF7 cells do not express PDE4 as much as U87MG. Considering the MCF7 cellular activity and phosphodiesterase enzyme results, the effect seems likely to occur through the phosphodiesterase enzyme. Compounds **5e**, **5f** and **5i** that showed the highest effect in the U87MG cell line, were similarly the most effective compounds of the series in phosphodiesterase enzyme activity.

Table 5
IC_{50} (mean ± SD) values of compounds, IBMX and rolipram on PDE4B.

Cpd	PDE4B IC_{50} (μM)
4d	>100
4l	17.11 ± 0.32
5a	>100
5b	33.14 ± 0.13
5c	32.26 ± 0.37
5d	11.17 ± 0.12
5e	**3.15 ± 0.12**
5f	**0.97 ± 0.35**
5g	22.13 ± 0.56
5h	18.11 ± 0.34
5i	24.14 ± 2.15
Rolipram	**0.32 ± 0.01**
IBMX	**14.31 ± 0.02**

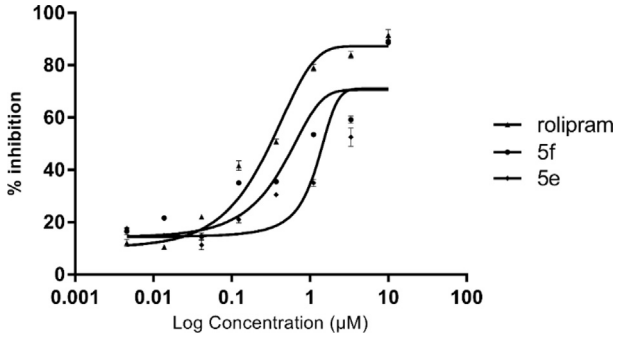


Fig. 4. Logaritmic curves of active compounds on PDE assay.

3. Conclusion

The importance of pyrimidine and thiouracil derivatives in drug development studies has been clearly demonstrated in recent studies. Developing resistance to temozolomide drug encourages the discovery of effective new drugs and the development of combination therapy for glioblastoma tumors [38,39]. Thus, the current results are relevant to others working on similar chemotypes and/or pharmacological effects.

The studies conducted within the scope of this article have been important in terms of evaluating the thiouracil and thiocytosine

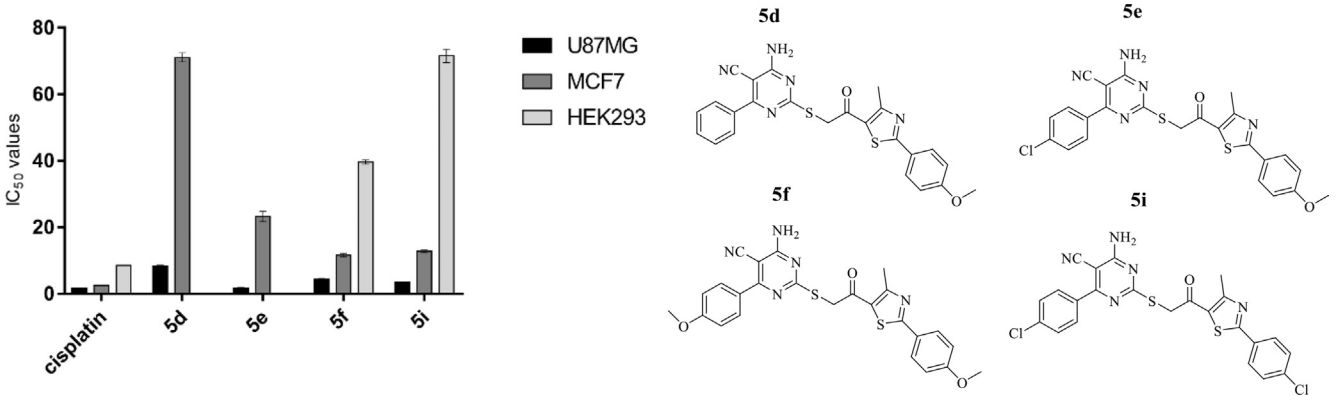


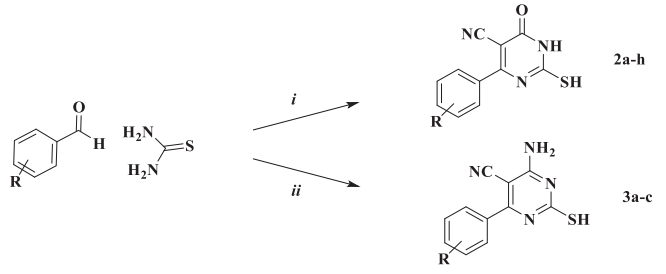
Fig. 5. Graphical representation of IC_{50} values on all tested cell lines. An HEK293 bar is not given for **5d** and **5e** because the viability was more than 50%, even at 100 μM, and therefore IC_{50} values could not be calculated.

derivatives together, investigating the biochemical bases of the antitumor effect and determining *in vitro* antitumor activity close to FDA approved drugs. In addition, further studies are planned to determine antiproliferative activity on other brain tumor cell lines, different healthy cell lines, the kinase phosphorylation levels in the presence of the active compounds, and subsequent *in vivo* experiments with a brain tumor model.

4. Experimental

4.1. Chemistry

All chemicals and biological materials were purchased from Sigma-Aldrich Chemical Corp and Honeywell Corp. All intermediate compounds were checked for their purities using thin-layer chromatography (TLC) with a Silica Gel 60 F_{254} TLC plate (Merck KGaA, Darmstadt, Germany). Melting points were measured by a Stuart Melting Point Apparatus SMP30 (Staffordshire, UK) and defined by recording the point where compound started to melt. Spectroscopic data were determined with the following instruments: Fourier transform IR (FT-IR), PerkinElmer Spectrum Two FT-IR Spectrometer (PerkinElmer, Inc, Waltham, Massachusetts, USA); ^{1}H NMR, Bruker 300- MHz UltraShield NMR Spectrophotometer (Bruker Corp, Billerica, Massachusetts, USA); and ^{13}C NMR, Bruker 75- MHz UltraShield NMR Spectrophotometer (Bruker Corp, Billerica, Massachusetts, USA) in DMSO-d_6 using standard TMS. The high resolution mass spectra of the compounds were determined on a Shimadzu 8040 LC/MS/MS ITTOF system (Shimadzu, Tokyo, Japan) using a mass spectrometer with the electron spray method (ESI).



Scheme 1. Synthesis of the compounds **1a-c** (***i***: aryl thioamide, 3-chloro-2,4-pentandione, EtOH, reflux 8 h, NaOAc; ***ii***: product of *i*, $CHCl_3$,0 °C, Br_2, r.t. 12 h, 30 min at 40 °C.

4.1.1. General synthesis of 1-(4-methyl-2-(4′-substitutedphenylthiazole-5-yl)ethane-1-one derivatives (method A)

According to the Hantzsch method, 3-chloro-2,4-pentandione is added to the hot solution of aryl thioamide in absolute ethanol (Scheme 1) and boiled for 8 h. After being controlled with TLC where the reaction is completed, it was left to cool, poured into cold water and neutralized by sodium acetate solution. After the precipiation was completed, it is taken and dried, crystallized from ethanol [40].

4.1.1.1. 1-(4-Methyl-2-phenylthiazole-5-yl)ethane-1-one.

Obtained according to Method A by the reaction of 3-chloro-2,4-pentanedione (110 mmol, 14.80 g) and thiobenzamide (100 mmol, 13.72 g). Yield: %90, m.p.: 68–69 °C, m.p. reference: 68–70 °C (Ethanol) [41].

4.1.1.2. 1-(4-Methyl-2-(4′-methoxyphenyl)thiazole-5-yl)ethane-1-one.

Obtained according to Method A by the reaction of 3-chloro-2,4-pentanedione (110 mmol, 14.80 g) and 4-methoxythiobenzamide (100 mmol, 16.72 g). Yield: 85%, m.p.: 88.8–90 °C, m.p. reference: 89 °C (Ethanol) [41].

4.1.1.3. 1-(4-Methyl-2-(4′-chlorophenyl)thiazole-5-yl)ethane-1-one.

Obtained according to Method A by the reaction of 3-chloro-2,4-pentanedione (110 mmol, 14.80 g) and 4-chlorothiobenzamide (100 mmol, 17.16 g). Yield: 90%, m.p.: 113–115 °C, m.p. reference: 114–115 °C (Ethanol) [42].

4.1.2. General synthesis of 2-bromo-1-(4-methyl-2-(4′-substitutedphenylthiazole-5-yl)ethane-1-one derivatives (1a-c)

The appropriate 1-(4-methyl-2-(4'substitutedphenylthiazole-5-yl)ethan-1-one derivative dissolved in chloroform and the reaction environment is brought to 0 °C. After 10 min of stirring, bromine solution in chloroform added dropwise. Then it is stirred at room temperature for 12 h. Then it is heated to 40 °C and stirred for 30 min for **1b** and **1c**. After being controlled with TLC, where the reaction is completed, the saturated sodium bicarbonate solution is added and stirred for 10 min. Phases are separated and the aqueous phase is re-extracted with chloroform. After organic phase is dried using sodium sulphate, the solvent is evaporated at low pressure and then the product is obtained [43]. Since the reaction was not completed in the first trials, it was determined that there was a second bromine entry when it was heated for a long time. Bromine addition should be carried out at 0 °C and the reaction should be controlled frequently, especially after heating. When the starting

material was consumed the most and a second product started to form, the reaction was terminated and the product crystallized from ethanol.

*4.1.2.1. 2-Bromo-1- (4-methyl-2-phenyl-thiazol-5-yl) ethan-1-one (**1a**).* Obtained according to Method B by reaction of 1- (4-Methyl-2-phenylthiazol-5-yl) ethan-1-one (75 mmol, 16.30 g) and bromine (Br_2) (75 mmol, 11.99 g). Yield: 76%, m.p.: 117.5–119 °C, m.p. reference: 117–118 °C (Ethanol) [44].

*4.1.2.2. 2-Bromo-1-(4-methyl-2- (4′-methoxyphenylthiazole-5-yl) ethan-1-one (**1b**).* By reaction of 1- (4-Methyl-2- (4′-methoxyphenylthiazol-5-yl) ethan-1-one (75 mmol, 18.55 g) and bromine (Br_2) (75 mmol, 11.99 g), was obtained according to Method B. Yield: 80%, m.p.: 90.2–91 °C, m.p. reference: 90–91 °C (Ethanol) [45].

*4.1.2.3. 2-Bromo-1-(4-methyl-2- (4′-chlorophenylthiazole-5-yl) ethan-1-one (**1c**).* By reaction of 1-(4-methyl-2- (4′-chlorophenylthiazole-5-yl) ethan-1-one (75 mmol, 18.88 g) and bromine (Br_2) (75 mmol, 11.99 g), was obtained according to Method B. Yield: 70%, m.p.: 117–118 °C, m.p. reference: 117–118 °C (ethanol) [45].

4.1.3. General synthesis of 2-mercapto-4-hydroxy-6-substitutedphenypyrimidine-5-carbonitrile derivatives (method C) (2a-h)

In this method, ethyl cyanoacetate was used instead of methylene ketone in the Biginelli method. In addition, the reaction is not acid catalyzed, but base catalyzed (Scheme 2). Solvents such as dimethylformamide, acetone and acetonitrile are used as solvents [44]. Potassium carbonate is most commonly used as a base. The appropriate aldehyde, thiourea and ethyl cyanoacetate were dissolved in anhydrous ethanol and potassium carbonate was added. The reaction was cooled after boiling for 16 h. The precipitated product was dissolved in 0.5 M NaOH solution. It was extracted 3 times and washed with ethyl acetate. It was then brought to pH 2 with the slow addition of a 1 M HCl and the product precipitated. The product was crystallized from ethanol [2,46,47].

*4.1.3.1. 4-Hydroxy-2-mercapto-6-phenylpyrimidine-5-carbonitrile (**2a**).* By reaction of benzaldehyde (20 mmol, 2.12 g), thiourea (20 mmol, 1.5224 g), potassium carbonate (20 mmol, 2.76 g) and ethyl cyanoacetate (20 mmol, 2.26 g), was obtained according to Method C. Yield: %75, m.p.: 299–301 °C, m.p. reference: 300–302 °C (Acetic acid) [46].

*4.1.3.2. 4-Hydroxy-2-mercapto-6-(3-methoxyphenyl)pyrimidine-5-carbonitrile (**2b**).* By reaction of 3-methoxybenzaldehyde (20 mmol, 2.72 g), thiourea (20 mmol, 1.5224 g), potassium carbonate (20 mmol, 2.76 g) and ethyl cyanoacetate (20 mmol, 2.26 g), obtained according to Method C. Yield: 78%, m.p.: 246–247 °C, m.p. reference: 244 °C (Ethanol) [48].

*4.1.3.3. 4-Hydroxy-2-mercapto-6-(4-methoxyphenyl)pyrimidine-5-carbonitrile (**2c**).* By reaction of 4-methoxybenzaldehyde (20 mmol, 2.72 g), thiourea (20 mmol, 1.5224 g), potassium carbonate (20 mmol, 2.76 g) and ethyl cyanoacetate (20 mmol, 2.26 g), obtained according to Method C. Yield: 70%, m.p.: 280–282 °C, m.p.: 281–283 °C (Ethanol) [49].

*4.1.3.4. 4-(3,4-dimethoxyphenyl)-6-hydroxy-2-mercaptopyrimidine-5-carbonitrile (**2d**).* By reaction of 3,4-dimethoxybenzaldehyde (20 mmol, 3.32 g), thiourea (20 mmol, 1.5224 g), potassium carbonate (20 mmol, 2.76 g) and ethyl cyanoacetate (20 mmol, 2.26g), obtained according to Method C. Yield: 80%, m.p.: 281–283 °C, m.p. reference: 280–282 °C (Acetic acid) [50].

*4.1.3.5. 4-(3-chlorophenyl)-6-hydroxy-2-mercaptopyrimidine-5-carbonitrile (**2e**).* By reaction of 3-chlorobenzaldehyde (20 mmol, 2.81 g), thiourea (20 mmol, 1.5224 g), potassium carbonate (20 mmol, 2.76 g) and ethyl cyanoacetate (20 mmol, 2.26 g), obtained according to Method C. Yield: 65%, m.p.: 227–228 °C, m.p. reference: 229 °C [51].

*4.1.3.6. 4-(4-chlorophenyl)-6-hydroxy-2-mercaptopyrimidine-5-carbonitrile (**2f**).* By reaction of 4-chlorobenzaldehyde (20 mmol, 2.81 g), thiourea (20 mmol, 1.5224 g), potassium carbonate (20 mmol, 2.76 g) and ethyl cyanoacetate (20 mmol, 2.26 g), obtained according to Method C. Yield: 85%, m.p.: 269.6–271 °C, m.p. reference: 270–271 °C (Acetic acid) [46].

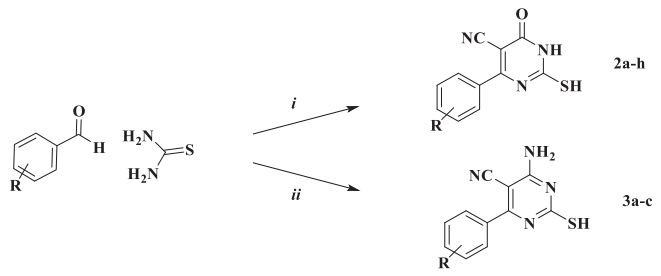
*4.1.3.7. 4-(3,4-Dichlorophenyl)-6-hydroxy-2-mercaptopyrimidine-5-carbonitrile (**2g**).* By reaction of 3,4-dichlorobenzaldehyde (20 mmol, 3.50 g), thiourea (20 mmol, 1.5224 g), potassium carbonate (20 mmol, 2.76 g) and ethyl cyanoacetate (20 mmol, 2.26 g), obtained according to Method C. Yield: 77%, m.p.: 267–269 °C, m.p. reference: 268 °C [52].

*4.1.3.8. 4-(3,4-methylenedioxyphenyl)-6-hydroxy-2-mercaptopyrimidine-5-carbonitrile (**2h**).* By reaction of 3,4-methylenedioxybenzaldehyde (20 mmol, 3.00 g), thiourea (20 mmol, 1.5224 g), potassium carbonate (20 mmol, 2.76 g) and ethyl cyanoacetate (20 mmol, 2.26 g), obtained according to Method C. Yield: 87%, m.p.: 250.5–252 °C, m.p. reference: 248–250 °C (Ethanol) [50].

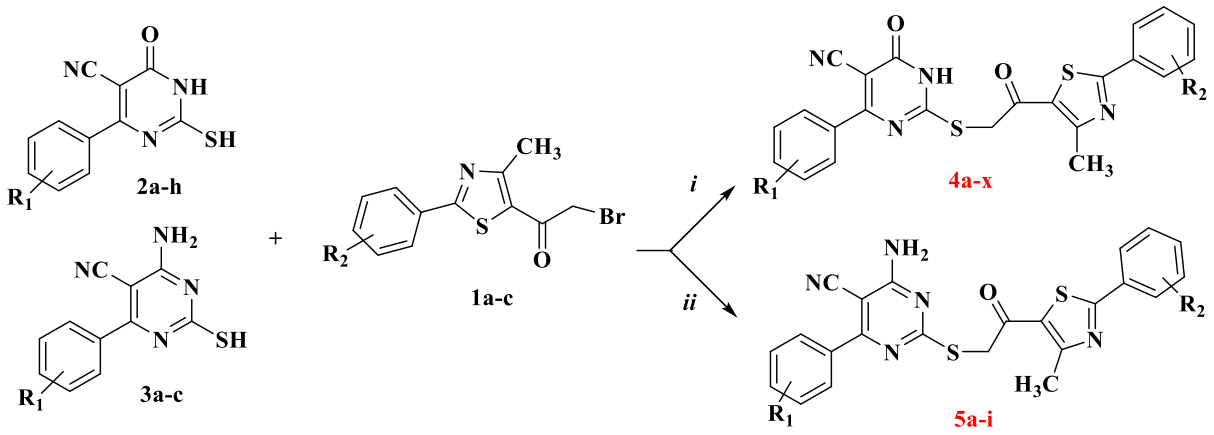
4.1.4. General synthesis of compounds 4a-x (method D)

2a-h derivatives (100 mmol) and thiazolyl acetylbromide compounds (**1a-c**) (110 mmol) are dissolved in acetone and potassium carbonate (100 mmol) was added. The reaction was mixed for 12 h at room temperature (Scheme 3). If it was not completed in this time, the reaction was boiled for 1 h. After the reaction was completed, it was poured into cold water. The product was expected to be precipitated thoroughly. In the meantime, thorough solubility of potassium carbonate should be ensured in water. The precipitated product was filtered away. Compounds are crystallized from 80% DMSO (Dimethyl sulfoxide) - 20% water mixture. The resulted products are kept in vacuum survey seizing phosphorus pentaoxide (P_2O_5) at 45 °C for overnight and ensured complete drying before analysis [15,53].

*4.1.4.1. 2-((2-(4-methyl-2-phenylthiazol-5-yl)-2-oxoethyl)thio)-6-oxo-4-phenyl-1,6-dihydropyrimidine-5-carbonitrile (**4a**).* Yield: 17.21% m.p.: 210 °C (decomposed). FT-IR υ_{max} (cm^{-1}): 3380.8 (NH),



Scheme 2. Synthesis of the compounds **2a**-**h** and **3a**-**c** (***i***: aldehyde, thiourea, K_2CO_3, ethyl cyanoacetate, EtOH, reflux 16 h; ***ii***: aldehyde, thiourea, K_2CO_3, malononitrile, EtOH, reflux 5 h).



Scheme 3. Synthesis of the compounds **4a-x** and **5a-i** (***i*: 2a-h, 1a-c**, acetone, K_2CO_3, r.t. 12 h, reflux 1 h, ***i*: 3a-c, 1a-c**, acetone, K_2CO_3, r.t. 8 h, reflux 1 h).

3056–2914 (C–H), 2205 (C≡N), 1680.33 (C=O), 1555–1462 (C=C, C=N). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.70 (3H, s, CH_3), 4.52 (2H, s, CH_2), 7.29–7.37 (3H, m, Ar), 7.54 (3H, m, Ar), 7.65 (2H, d, *J:* 6.57 Hz, Ar), 7.98 (2H, d, *J:* 7.77 Hz, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.75 (CH_3), 39.18 (CH_2), 90.02 (C–CN), 119.72, 127.08, 128.37, 128.50, 129.89, 130.38, 130.77, 131.97, 132.58, 137.51, 159.44, 167.41, 169.03, 170.28, 188.04 (C=O). HRMS (M + H): For $C_{23}H_{16}N_4O_2S_2$ Calcd: 445.0787, found: 445.0781.

*4.1.4.2. 4-(3-methoxyphenyl)-2-((2-(4-methyl-2-phenylthiazol-5-yl)-2-oxoethyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (**4b**).* Yield: 8.92% m.p.: 283 °C (decomposed). FT-IR υ_{max} (cm^{-1}): 3397.8 (NH), 3056.5–2899 (C–H), 2206.6 (C≡N), 1675 (C=O), 1548–1446 (C=C, C=N), 1235.85 (C–O). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.70 (3H, s, CH_3), 3.66 (3H, s, OCH_3), 4.50 (2H, s, CH_2), 6.94–6.95 (1H, m, Ar), 7.22–7.26 (3H,m, Ar), 7.50–7.56 (3H, m, Ar), 7.97–8.00 (2H, m, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.72 (CH_3), 39.16 (CH_2), 55.46 (OCH_3), 90.04 (C–CN), 114.04, 115.79, 120.02, 120.77, 120.97, 126.54, 126.84, 127.05, 129.44, 129.68, 129.85, 130.64, 131.92, 132.62, 139.07, 159.26, 159.40, 167.10, 168.96, 188.16 (C=O). HRMS (M + H): For $C_{24}H_{18}N_4O_3S_2$ calcd: 475.0893, found: 475.0890.

*4.1.4.3. 4-(4-methoxyphenyl)-2-((2-(4-methyl-2-phenylthiazol-5-yl)-2-oxoethyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (**4c**).* Yield: 10.89% m.p.: 124.3 °C. IR υ_{max} (cm^{-1}): 3394.5 (NH), 3000–2836 (C–H), 2206.6 (C≡N), 1671.6 (C=O), 1543.13–1413 (C=C, C=N), 1253.63 (C–O). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.71 (3H, s, CH_3), 3.67 (3H, s, OCH_3), 4.45 (2H, s, CH_2), 6.85 (2H, d, *J:* 8.62 Hz, Ar), 7.52–7.56 (3H, m, Ar), 7.65 (2H, d, *J:* 8.56 Hz, Ar), 7.98–8.01 (2H, m, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.76 (CH_3), 39.16 (CH_2), 55.53 (OCH_3), 88.90 (C–CN), 113.63, 120.80, 127.05, 129.87, 130.01, 130.21, 130.86, 131.92, 132.62, 159.36, 160.81, 166.60, 168.92, 170.78, 170.85, 188.52 (C=O). HRMS (M + H): For $C_{24}H_{18}N_4O_3S_2$ calcd: 475.0893, found: 475.0885.

*4.1.4.4. 4-(3,4-dimethoxyphenyl)-2-((2-(4-methyl-2-phenylthiazol-5-yl)-2-oxoethyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (**4d**).* Yield: 39.17% m.p.: 244 °C. FT-IR υ_{max} (cm^{-1}): 2977.8–2899 (C–H), 2206.6 (C≡N), 1658.5 (C=O), 1568.75–1416.32 (C=C, C=N), 1255.71 (C–O). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.70 (3H, s, CH_3), 3.70 (6H, m, OCH_3), 4.49 (2H, s, CH_2), 6.88 (1H, d, *J:* 8.47 Hz, Ar), 7.32–7.38 (2H, m, Ar), 7.48–7.53 (3H, m, Ar), 7.96 (2H, d, *J:* 7.80 Hz, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.75 (CH_3), 39.42 (CH_2), 55.78 (OCH_3), 55.84 (OCH_3), 89.09 (C–CN), 111.24, 112.18, 120.85, 121.65, 127.02, 129.83, 130.21, 130.55, 131.91, 132.57, 148.42, 150.65, 159.50, 166.65, 168.98, 170.82, 170.99, 188.36 (C=O). HRMS (M + H): For $C_{25}H_{20}N_4O_4S_2$ calcd: 505.0999, found: 505.0988.

*4.1.4.5. 4-(3-chlorophenyl)-2-((2-(4-methyl-2-phenylthiazol-5-yl)-2-oxoethyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (**4e**).* Yield: 61.86% m.p.: 231 °C. FT-IR υ_{max} (cm^{-1}): 3374.8 (NH), 2990.9–2902.3 (C–H), 2203.3 (C≡N), 1673.95 (C=O), 1560.23–1423.97 (C=C, C=N). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.70 (3H, s, CH_3), 4.47 (2H, s, CH_2), 7.37–7.41 (2H, m, Ar), 7.51–7.54 (3H, m, Ar), 7.55–7.64 (2H, m, Ar), 7.96–7.98 (2H, m, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.75 (CH_3), 39.44 (CH_2), 89.87 (C–CN), 120.09, 127.05, 128.12, 129.82, 129.96, 130.28, 131.89, 132.64, 133.34, 139.87, 159.39, 165.57, 169.00, 170.41, 171.35, 188.21 (C=O). HRMS (M + H): For $C_{23}H_{15}ClN_4O_2S_2$ calcd: 479.0398, found: 479.0391.

*4.1.4.6. 4-(4-chlorophenyl)-2-((2-(4-methyl-2-phenylthiazol-5-yl)-2-oxoethyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (**4f**).* Yield: 70.36% m.p.: 314 °C. FT-IR υ_{max} (cm^{-1}): 3371.6 (NH), 2987.6–2895.7 (C–H), 2209.8 (C≡N), 1658.5 (C=O), 1583–1417.96 (C=C, C=N). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.68 (3H, s, CH_3), 4.43 (2H, s, CH_2), 7.35 (2H, dd, *J:* 6.72 Hz, *j:* 1.86 Hz, Ar), 7.51–7.53 (3H, m, Ar), 7.65 (2H, dd, *J:* 6.70 Hz, *j:* 1.88 Hz, Ar), 7.93–7.96 (2H, m, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.72 (CH_3), 39.37 (CH_2), 89.75 (C–CN), 120.18, 127.02, 128.42, 129.83, 130.18, 130.76, 131.89, 132.54, 134.91, 136.58, 159.42, 166.14, 168.98, 170.64, 171.36, 188.45 (C=O). HRMS (M + H): For $C_{23}H_{15}ClN_4O_2S_2$ calcd: 479.0386, found: 479.0386.

*4.1.4.7. 4-(3,4-dichlorophenyl)-2-((2-(4-methyl-2-phenylthiazol-5-yl)-2-oxoethyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (**4g**).* Yield: 61.20% m.p.: 231.5 °C. FT-IR υ_{max} (cm^{-1}): 3384.7 (NH), 2990.9–2902.3 (C–H), 2209.8 (C≡N), 1661.8 (C=O), 1567.83–1420.31 (C=C, C=N). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.69 (3H, s, CH_3), 4.45 (2H, s, CH_2), 7.48–7.56 (3H, m, Ar), 7.59 (1H, s, Ar), 7.66 (2H, dd, *J:* 8.4 Hz, *j:* 2.04 Hz, Ar), 7.75–7.80 (1H, m, Ar), 7.94–7.97 (2H, m, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.75 (CH_3), 39.40 (CH_2), 89.87 (C–CN), 119.94, 127.02, 128.46, 128.82, 129.80, 130.19, 130.53, 130.64, 130.70, 131.45, 131.87, 132.59, 132.89, 138.23, 159.36, 164.52, 168.99, 170.34, 171.50, 188.26 (C=O). HRMS (M + H): For $C_{23}H_{14}Cl_2N_4O_2S_2$ calcd: 513.0008, found: 513.0011.

*4.1.4.8. 4-(benzo[d] [1,3]dioxol-5-yl)-2-((2-(4-methyl-2-phenylthiazol-5-yl)-2-oxoethyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (**4h**).* Yield: 23.64% m.p.: 240.8 °C. FT-IR υ_{max} (cm^{-1}): 3594.7 (NH), 2977.8–2915.4 (C–H), 2209.8 (C≡N), 1648.64 (C═O), 1503.41–1415.91 (C═C, C═N), 1254.60, 1242.78 (C–O). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.67 (3H, s, CH_3), 4.76 (2H, s, CH_2), 5.85 (2H, s, CH_2), 6.85 (1H, d, *J*: 8.23 Hz, Ar), 7.16, (1H, s, Ar), 7.36 (1H, dd, *J:* 8.19 Hz, *j:* 1.61 Hz, Ar), 7.52–7.59 (3H, m, Ar), 7.99 (2H, dd, *J:* 7.52 Hz, *j:* 1.58 Hz, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.74 (CH_3), 39.18 (CH_2), 102.22 (O–CH2–O), 92.01 (C–CN), 108.26, 108.83, 116.55, 124.29, 127.08, 129.20, 129.86, 130.48, 132.07, 132.52, 147.77, 150.55, 159.83, 161.94, 165.38, 166.59, 169.55, 186.51 (C═O). HRMS (M + H): $C_{24}H_{16}N_4O_2S_2$ calcd: 489.0686, found: 489.0685.

*4.1.4.9. 2-((2-(2-(4-methoxyphenyl)-4-methylthiazol-5-yl)-2-oxoethyl)thio)-6-oxo-4-phenyl-1,6-dihydropyrimidine-5-carbonitrile (**4i**).* Yield: 82.93% m.p.: 288 °C. FT-IR υ_{max} (cm^{-1}): 3404.4 (NH), 3004–2872.7 (C–H), 2209.8 (C≡N), 1672.11 (C═O), 1553.10–1411.21 (C═C, C═N), 1265.51 (C–O). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.68 (3H, s, CH_3), 3.84 (3H, s, CH_3), 4.45 (2H, s, CH_2), 7.08 (2H, d, *J:* 8.88 Hz, Ar), 7.32–7.35 (3H, m, Ar), 7.66 (2H, dd, *J:* 7.8 Hz, j: 1.65 Hz, Ar), 7.93 (2H, d, *J:* 8.83 Hz, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.77 (CH_3), 39.15 (CH_2), 55.95 (OCH_3), 89.73 (C–CN), 115.23, 120.40, 125.35, 128.33, 128.45, 128.84, 129.77, 130.11, 137.91, 159.46, 162.34, 167.33, 169.02, 170.58, 171.07, 188.11 (C═O). HRMS (M + H): For $C_{24}H_{18}N_4O_3S_2$ calcd: 475.0893, found: 475.0884.

*4.1.4.10. 4-(3-methoxyphenyl)-2-((2-(2-(4-methoxyphenyl)-4-methylthiazol-5-yl)-2-oxoethyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (**4j**).* Yield: 36.75% m.p.: 212.5 °C. FT-IR υ_{max} (cm^{-1}): 3322.3 (NH), 2977.8–2902.3 (C–H), 2213.1 (C≡N), 1670.63 (C═O), 1562.11–1442.88 (C═C, C═N), 1257.20, 1236.30 (C–O). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.68 (3H, s, CH_3), 3.68 (3H, s, OCH_3), 3.85 (3H, s, CH_3), 4.46 (2H, s, CH_2), 6.93–6.97 (1H, m, Ar), 7.08 (2H, dd, *J:* 6.84 Hz, j: 2.07 Hz, Ar), 7.22–7.26 (3H, m, Ar), 7.94 (2H, dd, *J:* 6.81 Hz, *j:* 2.08 Hz, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.75 (CH_3), 39.16 (CH_2), 55.45 (OCH_3), 55.96 (OCH_3), 89.74 (C–CN), 113.97, 115.22, 115.71, 120.41, 120.77, 125.39, 128.84, 129.42, 129.68, 139.31, 159.26, 159.43, 162.33, 167.04, 168.99, 170.57, 171.04, 188.08 (C═O). HRMS (M + H): For $C_{25}H_{20}N_4O_4S_2$ calcd: 505.0999, found: 505.0985.

*4.1.4.11. 4-(4-methoxyphenyl)-2-((2-(2-(4-methoxyphenyl)-4-methylthiazol-5-yl)-2-oxoethyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (**4k**).* Yield: 58.55% m.p.: 242.9 °C. FT-IR υ_{max} (cm^{-1}): 3680 (NH), 2971.2–2840 (C–H), 2203.3 (C≡N), 1642.1 (C═O), 1606–1416.86 (C═C, C═N), 1288.33, 1242.77 (C–O). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.68 (3H, s, CH_3), 3.69 (3H, s, OCH_3), 3.84 (3H, s, CH_3), 4.43 (2H, s, CH_2), 6.85 (2H, d, *J:* 8.89 Hz, Ar), 7.07 (2H, d, *J:* 8.88 Hz, Ar), 7.66 (2H, d, *J:* 8.83 Hz, Ar), 7.93 (2H, d, *J:* 8.82 Hz, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.79 (CH_3), 39.16 (CH_2), 55.53 (OCH_3), 56.00 (OCH_3), 88.86 (C–CN), 113.65, 115.23, 120.82, 125.36, 128.82, 129.83, 130.03, 130.19, 159.45, 160.84, 162.35, 166.56, 169.00, 170.92, 188.28 (C═O). HRMS (M + H): For $C_{25}H_{20}N_4O_4S_2$ calcd: 505.0999, found: 505.0986.

*4.1.4.12. 4-(3,4-dimethoxyphenyl)-2-((2-(2-(4-methoxyphenyl)-4-methylthiazol-5-yl)-2-oxoethyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (**4l**).* Yield: 48.37% m.p.: 243.4 °C. FT-IR υ_{max} (cm^{-1}): 3673.5 (NH), 2977.8–2836.6 (C–H), 2206.6 (C≡N), 1642.1 (C═O), 1606–1417.63 (C═C, C═N), 1255.24, 1228.20 (C–O). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.68 (3H, s, CH_3), 3.71 (6H, s, OCH_3), 3.84 (3H, s, CH_3), 4.47 (2H, s, CH_2), 6.90 (1H, d, *J:* 8.45 Hz, Ar), 7.06 (2H, d, *J:* 8.90 Hz, Ar), 7.35 (1H, d, *J:* 8.33 Hz, Ar), 7.37–7.38 (1H, m, Ar), 7.92 (2H, d, *J:* 8.87 Hz, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.77 (CH_3), 39.15 (CH_2), 55.77 (OCH_3), 55.86 (OCH_3), 55.94 (OCH_3), 89.03 (C–CN), 111.26, 112.17, 115.21, 120.89, 121.64, 125.33, 128.81, 129.60, 130.26, 148.42, 150.63, 159.52, 162.35, 166.61, 169.04, 170.81, 170.90, 188.12 (C═O). HRMS (M + H): For $C_{26}H_{22}N_4O_5S_2$ calcd: 535.1104, found: 535.1084.

*4.1.4.13. 4-(3-chlorophenyl)-2-((2-(2-(4-methoxyphenyl)-4-methylthiazol-5-yl)-2-oxoethyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (**4m**).* Yield: 83.67% m.p.: 245 °C. FT-IR υ_{max} (cm^{-1}): 3676.8, 3532.4 (NH), 2990.9–2908.8 (C–H), 2213.1 (C≡N), 1670.98 (C═O), 1606–1406.68 (C═C, C═N), 1259.41 (C–O). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.68 (3H, s, CH_3), 3.84 (3H, s, CH_3), 4.46 (2H, s, CH_2), 7.07 (2H, brs, Ar), 7.42 (2H, brs, Ar), 7.65 (2H, brs, Ar), 7.92 (2H, brs, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.78 (CH_3), 39.13 (CH_2), 55.97 (OCH_3), 89.90 (C–CN), 115.20, 119.95, 125.39, 127.06, 128.16, 128.85, 129.69, 130.05, 130.30, 133.36, 139.76, 159.44, 162.32, 165.56, 169.08, 170.29, 171.31, 187.88 (C═O). HRMS (M + H): For $C_{24}H_{17}ClN_4O_3S_2$ calcd: 509.0503, found: 509.0493.

*4.1.4.14. 4-(4-chlorophenyl)-2-((2-(2-(4-methoxyphenyl)-4-methylthiazol-5-yl)-2-oxoethyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (**4n**).* Yield: 51.98% m.p.: 278 °C. FT-IR υ_{max} (cm^{-1}): 3371.6 (NH), 2971.2–2902.3 (C–H), 2213.1 (C≡N), 1652 (C═O), 1606–1411.64 (C═C, C═N), 1257.08 (C–O). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.67 (3H, s, CH_3), 3.83 (3H, s, CH_3), 4.42 (2H, s, CH_2), 7.07 (2H, d, *J:* 8.88 Hz, Ar), 7.38 (2H, d, *J:* 8.54 Hz, Ar), 7.67 (2H, d, *J:* 8.55 Hz, Ar), 7.91 (2H, d, *J:* 8.83 Hz, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.76 (CH_3), 39.13 (CH_2), 55.98 (OCH_3), 89.69 (C–CN), 115.21, 120.23, 125.32, 128.44, 128.82, 129.79, 130.22, 134.88, 136.65, 159.46, 162.33, 166.09, 169.03, 170.52, 171.33, 188.19 (C═O). HRMS (M + H): For $C_{24}H_{17}ClN_4O_3S_2$ calcd: 509.0503, found: 509.0488.

*4.1.4.15. 4-(3,4-dichlorophenyl)-2-((2-(2-(4-methoxyphenyl)-4-methylthiazol-5-yl)-2-oxoethyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (**4o**).* Yield: 36.22% m.p.: 243 °C. FT-IR υ_{max} (cm^{-1}): 3565.2, 3378.1 (NH), 2977.8–2836.6 (C–H), 2209.8 (C≡N), 1665 (C═O), 1556.59–1410.08 (C═C, C═N), 1262.69 (C–O). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.68 (3H, s, CH_3), 3.84 (3H, s, CH_3), 4.44 (2H, s, CH_2), 7.06 (2H, d, *J:* 8.91 Hz, Ar), 7.60 (1H, d, J: 8.38 Hz, Ar), 7.68 (1H, dd, *J:* 8.4 Hz, *j:* 2.03 Hz, Ar), 7.81 (1H, d, *J:* 1.98 Hz, Ar), 7.91 (2H, dd, *J:* 6.81 Hz, *j:* 2.01 Hz, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.78 (CH_3), 39.14 (CH_2), 55.97 (OCH_3), 89.82 (C–CN), 115.19, 119.97, 125.36, 128.49, 128.82, 129.73, 130.21, 130.66, 131.45, 132.87, 138.28, 159.40, 162.31, 164.48, 169.06, 170.26, 171.50, 188.00 (C═O). HRMS (M + H): For $C_{24}H_{16}Cl_2N_4O_3S_2$ calcd: 543.0114, found: 543.0101.

*4.1.4.16. 4-(benzo[d] [1,3]dioxol-5-yl)-2-((2-(2-(4-methoxyphenyl)-4-methylthiazol-5-yl)-2-oxoethyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (**4p**).* Yield: 44.09% m.p.: 216 °C. FT-IR υ_{max} (cm^{-1}): 2899 (C–H), 2213.1 (C≡N), 1650 (C═O), 1643.63 (C═O), 1538.68–1429.88 (C═C, C═N), 1251.39 (C–O). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.66 (3H, s, CH_3), 3.85 (3H, s, OCH_3), 4.59 (2H, s, CH_2), 5.92 (2H, s, CH_2), 6.86 (1H, d, *J:* 8.21 Hz, Ar), 7.08, (2H, d, *J:* 8.86 Hz, Ar), 7.17 (1H, d, *J:* 1.65 Hz, Ar), 7.32 (1H, dd, *J:* 8.18 Hz, *j:* 1.73 Hz, Ar), 7.93 (2H, d, *J:* 8.78 Hz, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.73 (CH_3), 39.09 (CH_2), 55.54 (OCH_3), 89.82 (C–CN), 120.30, 128.33, 128.43, 128.78, 129.97, 130.12, 131.25, 131.41, 136.52, 137.83, 159.35, 167.38, 167.48, 170.22, 170.88, 188.35 (C═O). HRMS (M + H): For $C_{25}H_{18}N_4O_5S_2$ calcd: 519.0791, found: 519.0779.

4.1.4.17. 2-((2-(2-(4-chlorophenyl)-4-methylthiazol-5-yl)-2-oxoethyl)thio)-6-oxo-4-phenyl-1,6-dihydropyrimidine-5-carbonitrile

(***4q***). Yield: 53.49% m.p.: 160 °C. FT-IR υ_{max} (cm^{-1}): 3365 (NH), 2964.6–2912.1 (C–H), 2206.6 (C≡N), 1670.81 (C=O), 1654.91 (C=O) 1535–1432.94 (C=C, C=N). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.70 (3H, s, CH_3), 4.46 (2H, s, CH_2), 7.28–7.38 (3H, m, Ar), 7.59–7.64 (4H, m, Ar), 8.01 (2H, d, *J:* 8.60 Hz, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.73 (CH_3), 39.09 (CH_2), 90.01 (C–CN), 120.30, 128.33, 128.43, 128.78, 129.97, 130.12, 131.25, 131.41, 136.52, 137.83, 159.35, 167.38, 167.48, 170.22, 170.88, 188.35 (C=O). HRMS (M + H): For $C_{23}H_{15}ClN_4O_2S_2$ calcd: 479.0398, found: 479.0387.

*4.1.4.18. 2-((2-(2-(4-chlorophenyl)-4-methylthiazol-5-yl)-2-oxoethyl)thio)-4-(3-methoxyphenyl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (**4r**).* Yield: 21.41% m.p.: 195 °C (decomposed). FT-IR υ_{max} (cm^{-1}): 3381.4 (NH), 2994.2–2908.8 (C–H), 2206.6 (C≡N), 1675.57 (C=O), 1551.61–1433.78 (C=C, C=N), 1231.89 (C–O). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.69 (3H, s, CH_3), 3.67 (3H, s, OCH_3), 4.46 (2H, s, CH_2), 6.91–6.95 (1H, m, Ar), 7.18–7.25, (3H, m, Ar), 7.61 (2H, d, *J:* 8.53 Hz, Ar), 8.01 (2H, d, *J:* 8.52 Hz, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.67 (CH_3), 39.72 (CH_2), 55.47 (OCH_3), 105.06 (C–CN), 113.97, 115.65, 120.30, 120.74, 128.77, 129.40, 129.93, 131.49, 136.48, 159.23, 159.33, 167.09, 188.35 (C=O). HRMS (M + H): For $C_{24}H_{17}ClN_4O_3S_2$ calcd: 509.0503, found: 509.0494.

*4.1.4.19. 2-((2-(2-(4-chlorophenyl)-4-methylthiazol-5-yl)-2-oxoethyl)thio)-4-(4-methoxyphenyl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (**4s**).* Yield: 32.05% m.p.: 215 °C. FT-IR υ_{max} (cm^{-1}): 3338.1 (NH), 2963.7–2838.9 (C–H), 2201.6 (C≡N), 1669.02 (C=O), 1537.92–1470 (C=C, C=N), 1253.44 (C–O). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.70 (3H, s, CH_3), 3.66 (3H, s, OCH_3), 4.49 (2H, s, CH_2), 6.83 (2H, d, *J:* 8.83 Hz, Ar), 7.59–7.64 (4H, m, Ar), 8.00 (2H, d, *J:* 8.57 Hz, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.74 (CH_3), 39.35 (CH_2), 55.50 (OCH_3), 89.56 (C–CN), 113.66, 128.76, 129.67, 129.96, 130.13, 131.32, 131.38, 136.54, 159.39, 161.02, 166.68, 167.58, 188.22 (C=O). HRMS (M + H): For $C_{24}H_{17}ClN_4O_3S_2$ calcd: 509.0503, found: 509.0486.

*4.1.4.20. 2-((2-(2-(4-chlorophenyl)-4-methylthiazol-5-yl)-2-oxoethyl)thio)-4-(3,4-dimethoxyphenyl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (**4t**).* Yield: 48.59% m.p.: 243 °C. FT-IR υ_{max} (cm^{-1}): 3568 (NH), 3091.8–2842.2 (C–H), 2205 (C≡N), 1653.76 (C=O), 1556.74–1433.31 (C=C, C=N), 1298.61, 1257.95 (C–O). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.70 (3H, s, CH_3), 3.69 (6H, s, OCH_3), 4.49 (2H, s, CH_2), 6.89 (1H, d, *J:* 8.42 Hz, Ar), 7.29–7.34 (2H, m, Ar), 7.59 (2H, d, *J:* 8.55 Hz, Ar), 7.99 (2H, d, *J:* 8.55 Hz, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.70 (CH_3), 39.72 (CH_2), 55.80 (OCH_3), 89.20 (C–CN), 111.24, 112.18, 120.67, 121.62, 128.75, 129.93, 130.18, 131.11, 131.42, 136.52, 148.41, 150.64, 159.37, 166.69, 167.52, 170.45, 188.35 (C=O). HRMS (M + H): For $C_{25}H_{19}ClN_4O_4S_2$ calcd: 539.0609, found: 539.0612.

*4.1.4.21. 4-(3-chlorophenyl)-2-((2-(2-(4-chlorophenyl)-4-methylthiazol-5-yl)-2-oxoethyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (**4u**).* Yield: 50.71% m.p.: 245 °C. FT-IR υ_{max} (cm^{-1}): 3397.3 (NH), 2980.1–2904.6 (C–H), 2214.8 (C≡N), 1671.69 (C=O), 1580.10–1434.03 (C=C, C=N). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.69 (3H, s, CH_3), 4.50 (2H, s, CH_2), 7.37–7.42 (2H, m, Ar), 7.58–7.66 (4H, m, Ar), 8.00 (2H, d, *J:* 8.60 Hz, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.74 (CH_3), 39.08 (CH_2), 85.43 (C–CN), 127.10, 128.16, 128.79, 129.94, 130.22, 130.34, 131.13, 131.44, 133.35, 136.52, 159.38, 165.65, 170.40, 188.42 (C=O). HRMS (M + H): For $C_{23}H_{14}Cl_2N_4O_2S_2$ calcd: 513.0008, found: 513.0010.

*4.1.4.22. 4-(4-chlorophenyl)-2-((2-(2-(4-chlorophenyl)-4-methylthiazol-5-yl)-2-oxoethyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (**4v**).* Yield: 70.51% m.p.: 287 °C. FT-IR υ_{max} (cm^{-1}): 3370 (NH), 2990.9–2908.8 (C–H), 2209.8 (C≡N), 1668.95 (C=O), 1537.86–1396.57 (C=C, C=N). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.69 (3H, s, CH_3), 4.47 (2H, s, CH_2), 7.36 (2H, d, *J:* 8.59 Hz, Ar), 7.57–7.64 (4H, m, Ar), 7.98 (2H, d, *J:* 8.58 Hz, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.74 (CH_3), 39.08 (CH_2), 85.43 (C–CN), 127.10, 128.16, 128.79, 129.94, 130.22, 130.34, 131.12, 131.44, 133.35, 136.5158, 159.38, 165.65, 170.38, 188.36 (C=O). HRMS (M + H): For $C_{23}H_{14}Cl_2N_4O_2S_2$ calcd 513.0008, found: 513.0008.

*4.1.4.23. 2-((2-(2-(4-chlorophenyl)-4-methylthiazol-5-yl)-2-oxoethyl)thio)-4-(3,4-dichlorophenyl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (**4w**).* Yield: 68.84% m.p.: 258 °C. FT-IR υ_{max} (cm^{-1}): 3370 (NH), 2990.9–2908.8 (C–H), 2213.1 (C≡N), 1665 (C=O), 1567.59–1432.79 (C=C, C=N). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.70 (3H, s, CH_3), 4.44 (2H, s, CH_2), 7.57–7.66 (4H, m, Ar), 7.77 (1H, d, *J:* 1.90 Hz, Ar), 7.98 (2H, dd, *J:* 6.6 Hz, *j:* 1.98 Hz, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.70 (CH_3), 39.72 (CH_2), 89.90 (C–CN), 119.90, 128.47, 128.74, 129.91, 130.17, 130.64, 131.26, 131.40, 131.44, 132.80, 136.48, 138.29, 159.24, 164.56, 167.48, 170.06, 171.37, 188.34 (C=O). HRMS (M + H): For $C_{23}H_{13}Cl_3N_4O_2S_2$ calcd: 548.0220, found: 548.0220.

*4.4.5.24. 4-(benzo[d] [1,3]dioxol-5-yl)-2-((2-(2-(4-chlorophenyl)-4-methylthiazol-5-yl)-2-oxoethyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (**4x**).* Yield: 65.26% m.p.: 291.1 °C. FT-IR υ_{max} (cm^{-1}): 3667 (NH), 2990.9–2905.9 (C–H), 2223 (C≡N), 1675 (C=O), 1642 (C=O), 1550–1373.04 (C=C, C=N), 1248.26 (C–O). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.65 (3H, s, CH_3), 4.74 (2H, s, CH_2), 5.87 (2H, s, CH_2), 6.85 (1H, d, *J:* 8.23 Hz, Ar), 7.13 (1H, d, *J:* 1.67 Hz, Ar), 7.35 (1H, dd, *J:* 8.26 Hz, *j:* 1.64 Hz, Ar), 7.60 (2H, d, *J:* 8.60 Hz, Ar), 7.98 (2H, d, *J:* 8.57 Hz, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.68 (CH_3), 39.73 (CH_2), 92.40 (C–CN), 102.26 (O–CH_2–O), 108.27, 108.80, 116.48, 124.29, 128.77, 129.15, 129.93, 130.92, 131.33, 136.68, 147.76, 150.55, 159.81, 161.86, 165.30, 166.56, 168.11, 186.49 (C=O). HRMS (M + H): For $C_{24}H_{15}ClN_4O_4S_2$ calcd: 523.0296, found: 523.0288.

4.1.5. General synthesis of 4-amino-2-mercapto-6-substitutedphenylpyrimidine-5-carbonitrile derivatives (method E) (3a-c)

Appropriate aldehyde, thiourea and malononitrile were dissolved in anhydrous ethanol and potassium carbonate was added. The reaction was controlled with TLC after boiling for 5 h (ethyl acetate 1:2 petroleum ether). The precipitated product was washed with ethanol. The raw product was mixed with hot water and filtered. After the filtrate cools, it was acidified with acetic acid. It was taken and washed with water. Crystallized from ethanol.

*4.1.5.1. 4-Amino-2-mercapto-6-phenylpyrimidine-5-carbonitrile (**3a**).* It was synthesized by Method E, using benzaldehyde (98.00 mmol, 10.4 g), thiourea (98.00 mmol, 7.46 g), potassium carbonate (98.00 mmol, 13.54 g) and malondinitrile (98.00 mmol, 6.47 g). Yield: 55%, m.p.: 255 °C, No reference value.

*4.1.5.2. 4-Amino-6-(4-methoxyphenyl)-2-mercaptopyrimidine-5-carbonitrile (**3b**).* It was synthesized by Method E, using 4-methoxybenzaldehyde (132.21 mmol, 18 g), thiourea (132.21 mmol, 10.06 g), potassium carbonate (132.21 mmol, 36.65 g) and malondinitrile (132.21 mmol, 8.73 g). Yield: 35%, m.p.: 261–262 °C, m.p. reference: 258–260 °C (N,N-DMF) [54].

*4.1.5.3. 4-Amino-6-(4-chlorophenyl)-2-mercaptopyrimidine-5-carbonitrile (**3c**).* It was synthesized by Method E, using 4-chlorobenzaldehyde (177.85 mmol, 25 g), thiourea (177.85 mmol,

13.54 g), potassium carbonate (177.85 mmol, 24.58 g) and malondinitrile (177.85 mmol, 11.75 mmol). Yield: 45%, m.p.: 268–269 °C, m.p. reference: 268–270 °C (1,4-dioxane) [54].

*4.1.6. General synthesis of compounds **5a-i** (method F)*

3a-c derivatives (50 mmol) and thiazolyl acetylbromide compounds 1a-c (55 mmol) are dissolved in acetone and then potassium carbonate (50 mmol) was added. The reaction was mixed for 12 h at room temperature. If it was not completed in this time, the reaction was boiled for 1 h. After the reaction was completed, it was poured into cold water. The product was expected to be precipitated thoroughly. In the meantime, thorough solubility of potassium carbonate should be ensured in water. The precipitated product was filtered away. Compounds are crystallized from ethanol twice. The resulted products are kept in vacuum survey seizing phosphorus pentaoxide (P_2O_5) at 45 °C for overnight and ensured complete drying before analysis [15,53].

*4.1.6.1. 4-Amino-2-((2-(4-methyl-2-phenylthiazol-5-yl)-2-oxoethyl) thio)-6-phenylpyrimidine-5-carbonitrile (**5a**).* Yield: 41.04% m.p.: 196 °C. FT-IR υ_{max} (cm^{-1}): 3676.8 (NH), 3299 (NH), 3145–2901.05 (C–H), 2216.4 (C≡N), 1638.8 (C=O), 1525.27–1393.71 (C=C, C=N). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.69 (3H, s, CH_3), 4.64 (2H, s, CH_2), 7.32–7.37 (2H, m, Ar), 7.42 (1H, d, *J:* 7.25 Hz, Ar), 7.54–7.58 (3H, m, Hz, Ar), 7.68 (2H, d, *J:* 7.07 Hz, Ar), 7.98 (2H, dd, *J:* 7.22 Hz, *j:* 1,78 Hz, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.79 (CH_3), 39.17 (CH_2), 83.37 (C–CN), 116.52, 127.09, 128.65, 128.79, 129.90, 130.81, 131.43, 132.03, 132.54, 136.11, 159.59, 163.73, 167.71, 169.2, 172.61, 187.31 (C=O). HRMS (M + H): For $C_{23}H_{17}N_5OS_2$ calcd: 444.0947, found: 444.0940.

*4.1.6.2. 4-Amino-6-(4-methoxyphenyl)-2-((2-(4-methyl-2-phenylthiazol-5-yl)-2-oxoethyl)thio)pyrimidine-5-carbonitrile (**5b**).* Yield: 23.52% m.p.: 218 °C. FT-IR υ_{max} (cm^{-1}): 3689.9–3663.6 (NH_2), 3378, 3309.2, (NH), 3168–2901.11 (C–H), 2213.1 (C≡N), 1671.6 (C=O), 1648.7 (C=O), 1541.13–1394.06 (C=C, C=N), 1251.39 (C–O). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.67 (3H, s, CH_3), 3.85 (3H, s, CH_3), 4.62 (2H, s, CH_2), 7.08 (2H, d, *J:* 8.01 Hz, Ar), 7.40 (3H, m, Ar), 7.69 (2H, d, *J:* 6.71 Hz, Ar), 7.94 (2H, d, *J:* 8.08 Hz, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.83 (CH_3), 39.45 (CH_2), 56.01 (OCH_3), 85.08 (C–CN), 115.27, 116.54, 125.29, 128.66, 128.81, 128.88, 131.46, 136.12, 159.64, 162.43, 163.73, 167.68, 172.65, 187.07 (C=O). HRMS (M + H): For $C_{24}H_{19}N_5O_2S_2$ calcd: 474.1053, found: 474.1047.

*4.1.6.3. 4-Amino-6-(4-chlorophenyl)-2-((2-(4-methyl-2-phenylthiazol-5-yl)-2-oxoethyl)thio)pyrimidine-5-carbonitrile (**5c**).* Yield: 28.20% m.p.: 231 °C. FT-IR υ_{max} (cm^{-1}): 3397.3, 3325 (NH_2), 3223.2 (NH), 2990–2894.7 (C–H), 2214.8 (C≡N), 1669.52 (C=O), 1525.40–1400 (C=C, C=N). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.69 (3H, s, CH_3), 4.62 (2H, s, CH_2), 7.34–7.43 (3H, m, Ar), 7.61 (2H, d, *J:* 8.62 Hz, Ar), 7.66 (2H, d, *J:* 7.06 Hz, Ar), 7.98 (2H, d, *J:* 8.61 Hz, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.75 (CH_3), 39.17 (CH_2), 83.39 (C–CN), 116.50, 128.65, 128.77, 129.97, 131.25, 131.36, 131.41, 136.10, 136.63, 159.57, 163.71, 167.73, 167.79, 172.57, 187.35 (C=O). HRMS (M + H): For $C_{23}H_{16}ClN_5OS_2$ calcd: 478.0534, found: 478.0534.

*4.1.6.4. 4-Amino-2-((2-(2-(4-methoxyphenyl)-4-methylthiazol-5-yl)-2-oxoethyl)thio)-6-phenylpyrimidine-5-carbonitrile (**5d**).* Yield: 46.38% m.p.: 206 °C. FT-IR υ_{max} (cm^{-1}): 3670.2 (NH_2), 3407, 3225.6 (NH), 2987.53–2900 (C–H), 2209.8 (C≡N), 1675 (C=O), 1579.8–1315.72 (C=C, C=N), 1249.42 (C–O). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.70 (3H, s, CH_3), 3,64 (3H, s, OCH_3), 4.62 (2H, s, CH_2), 6.85 (2H, d, *J:* 8.89 Hz, Ar), 7.54–7.56 (3H, m, Ar), 7.68 (2H, d, *J:* 8.84 Hz, Ar), 7.99 (2H, dd, *J:* 7.35 Hz, *j:* 1.85 Hz, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.82 (CH_3), 39.17 (CH_2), 55.64 (OCH_3), 82.38 (C–CN), 114.02, 116.87, 127.07, 128.20, 129.91, 130.62, 130.91, 132.04, 132.54, 159.58, 161.92, 163.88, 166.88, 169.26, 172.34, 187.48 (C=O). HRMS (M + H): For $C_{24}H_{19}N_5O_2S_2$ calcd: 474.1053, found: 474.1050.

*4.1.6.5. 4-Amino-6-(4-methoxyphenyl)-2-((2-(2-(4-methoxyphenyl)-4-methylthiazol-5-yl)-2-oxoethyl)thio)pyrimidine-5-carbonitrile (**5e**).* Yield: 64.28% m.p.: 199.3 °C. FT-IR υ_{max} (cm^{-1}): 3667 (NH_2), 3476.6, 3235.5 (NH), 2987.51–2901.05 (C–H), 2206.6 (C≡N), 1665 (C=O), 1530.15–1393.63 (C=C, C=N), 1250.25 (C–O). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.68 (3H, s, CH_3), 3.67 (3H, s, OCH_3), 3,85 (3H, s, OCH_3), 4.60 (2H, s, CH_2), 6.87 (2H, d, *J:* 8.96 Hz, Ar), 7.08 (2H, d, *J:* 8.93 Hz, Ar), 7.69 (2H, d, *J:* 8.90 Hz, Ar), 7.95 (2H, d, *J:* 8.86 Hz, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.86 (CH_3), 39.17 (CH_2), 55.67 (OCH_3), 56.03 (OCH_3), 82.34 (C–CN), 114.03, 115.27, 116.89, 125.29, 128.22, 128.87, 129.93, 130.64, 159.65, 161.94, 162.44, 163.88, 166.85, 169.33, 172.39, 187.23 (C=O). HRMS (M + H): For $C_{25}H_{21}N_5O_3S_2$ calcd: 504.1159, found: 504.1150.

*4.1.6.6. 4-Amino-6-(4-chlorophenyl)-2-((2-(2-(4-methoxyphenyl)-4-methylthiazol-5-yl)-2-oxoethyl)thio)pyrimidine-5-carbonitrile (**5f**).* Yield: 48.48% m.p.: 216.5 °C. FT-IR υ_{max} (cm^{-1}): 3670.2 (NH_2), 3328.9, 3181.2 (NH), 2987.54–2901.04 (C–H), 2209.8 (C≡N), 1658.5 (C=O), 1513.68–1371.62 (C=C, C=N), 1249.71 (C–O). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.70 (3H, s, CH_3), 3.67 (3H, s, OCH_3), 4.62 (2H, s, CH_2), 6.85 (2H, d, *J:* 8.96 Hz, Ar), 7.60–7.68 (4H, m, Ar), 8.01 (2H, d, *J:* 8.60 Hz, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.78 (CH_3), 39.17 (CH_2), 55.66 (OCH_3), 82.42 (C–CN), 114.02, 116.85, 128.21, 128.78, 129.98, 130.60, 131.37, 136.64, 159.53, 161.91, 163.86, 166.92, 167.81, 187.53 (C=O). HRMS (M + H): For $C_{24}H_{18}ClN_5O_2S_2$ calcd: 508.0663, found: 508.0638.

*4.1.6.7. 4-Amino-2-((2-(2-(4-chlorophenyl)-4-methylthiazol-5-yl)-2-oxoethyl)thio)-6-phenylpyrimidine-5-carbonitrile (**5g**).* Yield: 88.79% m.p.: 228 °C. FT-IR υ_{max} (cm^{-1}): 3420.3, 3334.9 (NH_2), 3233 (NH), 3000–2898 (C–H), 2211.5 (C≡N), 1637.40 (C=O), 1521.65–1493.19 (C=C, C=N). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.69 (3H, s, CH_3), 4.62 (2H, s, CH_2), 7.40 (2H, d, *J:* 8.60 Hz, Ar), 7.54–7.57 (3H, m, Ar), 7.67 (2H, d, *J:* 8.58 Hz, Ar), 7.98 (2H, dd, *J:* 7.64 Hz, *j:* 1.93 Hz, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.82 (CH_3), 39.09 (CH_2), 83.46 (C–CN), 116.35, 127.06, 128.76, 129.92, 130.60, 130.93, 132.06, 132.47, 134.85, 136.28, 159.53, 163.56, 166.62, 169.25, 172.70, 187.49 (C=O). HRMS (M + H): For $C_{23}H_{16}ClN_5OS_2$ calcd: 478.0558, found: 478.0573.

*4.1.6.8. 4-Amino-2-((2-(2-(4-chlorophenyl)-4-methylthiazol-5-yl)-2-oxoethyl)thio)-6-(4-methoxyphenyl)pyrimidine-5-carbonitrile (**5h**).* Yield: 31.72% m.p.: 247 °C. FT-IR υ_{max} (cm^{-1}): 3670.2(NH_2), 3325.6, 3164.8 (NH), 2987.52–2900.47 (C–H), 2216.4 (C≡N), 1652 (C=O), 1518.50–1373.97 (C=C, C=N), 1248.10 (C–O). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.70 (3H, s, CH_3), 3.65 (3H, s, OCH_3), 4.61 (2H, s, CH_2), 6.84 (2H, d, *J:* 8.91 Hz, Ar), 7.61 (2H, d, *J:* 8.61 Hz, Ar), 7.65 (2H, d, *J:* 8.88 Hz, Ar), 8.01 (2H, d, *J:* 8.61 Hz, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.81 (CH_3), 39.09 (CH_2), 55.61 (OCH_3), 82.38 (C–CN), 113.99, 116.89, 128.18, 128.77, 129.99 130.60, 131.33, 131.42, 136.64, 159.51, 161.86, 163.83, 166.92, 167.81, 172.31, 187.57 (C=O). HRMS (M + H): For $C_{24}H_{18}ClN_5O_2S_2$ calcd: 508.0663, found: 508.0664.

*4.1.6.9. 4-Amino-6-(4-chlorophenyl)-2-((2-(2-(4-chlorophenyl)-4-methylthiazol-5-yl)-2-oxoethyl)thio)pyrimidine-5-carbonitrile (**5i**).* Yield: 8.60% m.p.: 230 °C. FT-IR υ_{max} (cm^{-1}): 3673.5 (NH_2), 3430.6, 3335.5 (NH), 2987.50–2899 (C–H), 2219.7 (C≡N), 1661.8 (C=O),

1518.24–1368.60 (C=C, C=N). ^{1}H NMR (300 MHz) DMSO-d_6) δ (ppm): 2.69 (3H, s, CH_3), 4.61 (2H, s, CH_2), 7.40 (2H, d, *J:* 8.60 Hz, Ar), 7.61 (2H, d, *J:* 8.62 Hz, Ar), 7.65 (2H, d, *J:* 8.59 Hz, Ar), 7.99 (2H, d, *J:* 8.62 Hz, Ar). ^{13}C NMR (75 MHz) DMSO-d_6) δ (ppm): 18.75 (CH_3), 39.17 (CH_2), 87.84 (C–CN), 116.28, 128.76, 129.97, 130.56, 131.34, 134.86, 136.25, 136.62, 159.49, 163.58, 166.66, 167.79, 172.69, 187.52 (C=O). HRMS (M + H): For $C_{23}H_{15}Cl_2N_5OS_2$ calcd: 512.0168, found: 512.0151.

4.2. Biological activity

4.2.1. Cellular U87MG antitumor MTT activity measurement

4.2.1.1. Subcultures of the cells. HEK293 cells were maintained in Dulbecco's Modified Eagle medium (Gibco, UK) supplemented with 10% (v/v) fetal bovine serum (Gibco, UK), 10.000 U/mL penicillin, 10.000 μg/mL of streptomycin. The human glioblastoma cell lines U87MG were cultured in Dulbecco's Modified Eagle's medium/high glucose (Gibco, UK) supplemented with 10% heat inactivated fetal bovine serum (Gibco, UK), 1% (v/v) antibiotic-antimycotics solution (10.000 U/mL penicillin, 10.000 μg/mL streptomycin and 0.25 μg/mL amphotericin B; Gibco, UK), and 1% (v/v) non-essential amino acids (Gibco, UK) at 37 °C in a humidified 5% CO_2 atmosphere. MCF7 cells were maintained in Dulbecco's Modified Eagle medium-Nutrient Mixture F-12 (Gibco, UK) supplemented with 10% (v/v) fetal bovine serum (Gibco, UK), 10.000 U/mL penicillin, 10.000 μg/mL of streptomycin and %10 L-glutamine. The cells were passaged every 3 days [55].

4.2.1.2. Cytotoxic and anticancer activity. Cytotoxic activities of the tested compounds were determined by cell proliferation analysis using the standard 3- (4,5-dimethylthiazole-2 yl) −2,5-diphenyltetrazolium bromide (MTT) assay [55–57]. In order to determine the toxic and proliferative effects of compounds, cells were seeded in 96-well plates add a density of 5.10^4 (HEK293 human embryonic kidney cell line (ATCC, USA), 4.10^4 (MCF7), 10^5 (U87MG) and per well 24 h prior to treatment. The compounds to be tested were first dissolved in DMSO (less than 1%) and diluted with DMEM medium for use in MTT assay. After 24 and 48 h incubation times, the medium on the cells was removed, washed with D-PBS and 30 μL of MTT was added from stock solution (5 mg/mL, prepared in D-PBS). At the end of the 4 h incubation period, 150 μL DMSO was added and the culture vessel was shaken at room temperature for homogeneity of the solution. The absorbance of the solution at a wavelength of 540 nm was measured in a microplate reader (EON, BioTek Instruments Inc.). Three independent experiments were performed at least [58,59].

% Viability = (Absorbance experiment group) / (Absorbance control) × 100%

4.2.2. Measuring adenosine receptor binding inhibition

4.2.2.1. Cell culture and membrane preparation. The degree of binding of the synthesized compounds to adenosine A_1 and A_{2A} receptor subtypes was tested. For this purpose, competitive attachment experiments NIH (American National Institute of Health) NIDDK unit. CHO cells (Chinese Hamster Ovary cells) producing recombinant hA_1 (human adenosine A_1) receptor and HEK293 (human embryonic kidney cell line) cells producing $hA_{2A}AR$ (human adenosine A_{2A}) receptor, 10% fetal bovine serum in DMEM: F12 (1: 1) mixture, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 μmol/mL glutamine. Cells are seeded by trypsination. After homogenization and suspension, cells are centrifuged at 500 g for 10 min and the precipitate formed was suspended in 50 mM tris HCl. The suspension was homogenized for 10 s with an electrical homogenizer and immediately centrifuged at 20 kg for 20 min at 4 °C. The resulting pellets are resuspended in 3 U/mL adenosine deaminase buffer and stored at −80 °C until the time of use.

4.2.2.2. Measuring A_1 and A_{2A} adenosine receptor binding inhibition. 50 μL each of 1 μM concentrations of test compounds and standard [^{3}H]DPCPX radioligand (2 nM, PerkinElmer, Boston, MA) for hA_1AR binding measurement with A_1AR-expressing CHO cell membranes (40 μg/tube) 50 mM Tris- Incubate for 60 min at 25 °C in a total volume of 200 μL in HCl buffer (pH: 7.4; $MgCl_2$, 10 mM) [60,61].

For measurement of $A_{2A}AR$ and $A_{2B}AR$ binding, 50 μL each of 1 μM concentrations of test compounds and [^{3}H]ZM241385 concentrations to 1 and 15 nM for a2a and a2b binding, respectively. The mixture was incubated for 60 min at 25 °C in a total volume of 200 μL in 50 mM Tris-HCl buffer (pH: 7.4; $MgCl_2$, 10 mM). The reaction was terminated by filtration through GF/B filters. Filters used for A_1 and A_{2A} measurement are placed in scintillation vials containing 5 mL of Hydrofluor scintillation buffer and counted with the help of a PerkinElmer Tricarb 2810 TR Liquid Scintillation counter [62].

4.2.3. Phosphodiesterase enzyme (PDE) inhibition assay

Experiments were carried out following the instructions provided by the "PDE Activity Assay Kit" (ab139460, Abcam, Cambdrige, UK) using the modified method detailed by Araiz et al. [63]. Stock solutions of the compounds were prepared with DMSO (Less than 1%). Recombinant human PDE4 was incubated with 0.5 μM 5′-cGMP and 10 μL 5′-nucleotidase under 30 °C for 90 min. The kit based on the sequential hydrolysis of cyclic nucleotides by PDE4 and 5′-nucleotidase. The released phosphate by enzymatic cleavage was directly proportional to PDE4 activity. The reaction was terminated by incubation with termination buffer under room temperature for 20 min. OD 620 nm were measured on a plate reader to determine the amount of GMP generated. For measurement of IC_{50}, 6 concentrations of inhibitors were used under the substrate concentration the suitable enzyme concentration. The PDE4B and PDE4D inhibitor rolipram and PDE inhibitor IBMX were used as a positive control.

4.2.4. Statistical analysis

Data are presented as mean ± standard deviation. All experiments were performed at least in triplicates. Statistical comparisons were performed through the Student's t-test or one-way ANOVA followed by Tukey test, for 2 or more experimental groups, respectively. Statistical significance was set at $p < 0.05$. Statistical analysis and artwork were performed using Graph Pad Prism 7.0d (Graph Pad Software, La Jolla, USA). Specific tests are detailed in the figure legends.

Declaration of competing interest

There is not any conflict of interest by the authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2020.113125.

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