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Original article

Synthesis, anticancer activity and effects on cell cycle profile and apoptosis of novel thieno[2,3-d]pyrimidine and thieno[3,2-e] triazolo [4,3-c]pyrimidine derivatives

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ABSTRACT

Motivated by the widely reported anticancer activity of thieno[2,3-d]pyrimidines a series of 24 new 2-substitutedhexahydrocycloocta[4,5] thieno[2,3-d]pyrimidines with different substituents at C-4 position and hexahydrocycloocta[4,5]thieno[3,2-e]-1,2,4-triazolo[4,3-c]pyrimidines were synthesized. The anticancer activity of 17 compounds were evaluated by National Cancer Institute (USA) using a two stage process utilizing 59 different human tumor cell lines representing leukemia, melanoma, cancers of lung, colon, central nervous system (CNS), ovary, kidney, prostate as well as breast. Compound **9c** showed broad spectrum potent anticancer activity in nano molar to micro molar range against 56 human tumor cell lines with GI_{50} less than 10 μM ranging from 0.495 to 5.57 μM, also it is worth mentioning that compound **9c** had the marked highest selectivity against the two cell lines T-47D and MDA-MB-468 belonging to breast cancer with GI_{50} = 0.495 and 0.568 μM respectively, and its effect was further studied on cell cycle progression and induction of apoptosis in the MDA-MB-468 cell line. Results showed that compound **9c** induced cell cycle arrest at G2/M phase and also, showed accumulation of cells in pre-G1 phase which may result from, degradation or fragmentation of the genetic materials indicating a possible role of apoptosis in compound **9c**-induced cancer cell death and cytotoxicity and verifying this compound as promising selective anticancer lead. Compound **6c** was selective against K-562, SR and MOLT-4 cell lines belonging to leukemia showing growth inhibition percentages 86.38, 65.76 and 60.40 at a single dose test, at the same time it showed lethal activity against HOP-92 representing non-small cell lung cancer. Interestingly, leukemia SR, CNS cancer SNB-75 and renal cancer UO-31 cell lines proved to be sensitive to compound **6d** with growth inhibition percentages 52.86, 50.94 and 53.99 respectively. Additionally, compound **6d** demonstrated lethal activity to HOP-92 belonging non-small cell lung cancer.

1. Introduction

Cancer is a major health problem acting as a global killer, so identifying new chemical substances which may act as leads for discovering new potent antitumor agents is critically desirable. In this study we are interested in the widely reported anticancer activity of thieno[2,3-d]pyrimidine derivatives via different mechanisms [1–20] and the presence of several potent marketed anticancer drugs such as gefitinib (Iressa™) [21], erlotinib (Tarceva™) [22] and tandutinib (MLN518) (phase II clinical trials) [23] (Fig. 1) containing 4-substituted quinazoline core taking into consideration that the thieno[2,3-d]pyrimidine core was evaluated as bioisostere of quinazoline core. Moreover, recently the tricyclic system, cycloalkylthieno[2,3-d]pyrimidine was found to possess potent antitumor activity [4,5,13,24]. In addition, several reports supported the anticancer activity of cycloalkyl thieno[2,3-d]pyrimidines fused

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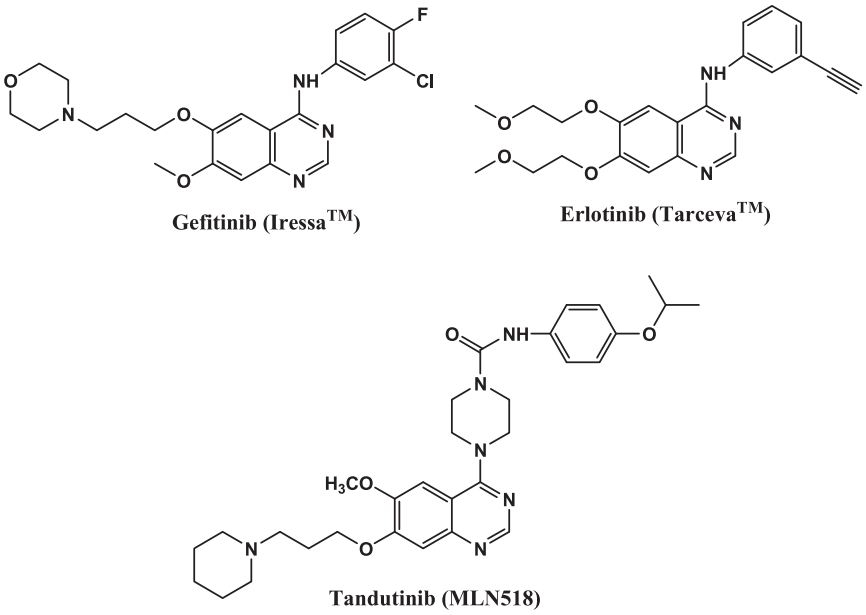


Fig. 1. Examples of 4-substituted quinazoline compounds are potent anticancer drugs.

with triazole ring [25,26]. We have previously synthesized various hexahydrocycloocta[4,5]thieno[2,3-d]pyrimidines which showed remarkable potent anticancer activity [18–20,27]. In the same direction, and in an effort to find more potent selective thieno[2,3-d] pyrimidine lead as anticancer agent, we synthesized new hexahydrocycloocta[4,5]thieno[2,3-d]pyrimidines having different substituents at C-2 position and different groups at position number 4. Also, it was considered to synthesize several hexahydrocycloocta [4,5]thieno[3,2-e]-1,2,4-triazolo[4,3-c]pyrimidines hypothesizing that the potency of the thieno[2,3-d]pyrimidine core might be enhanced by adding different aryl groups at C-2 position or by adding a triazole moiety. The anticancer activity of these new compounds was evaluated against a panel of 59 human tumor cell lines provided by National Cancer Institute (USA). For the most potent compound, the effect on the normal cell cycle profile and induction of apoptosis were performed in the MDA-MB-468 cell line in order to investigate its mechanism of action.

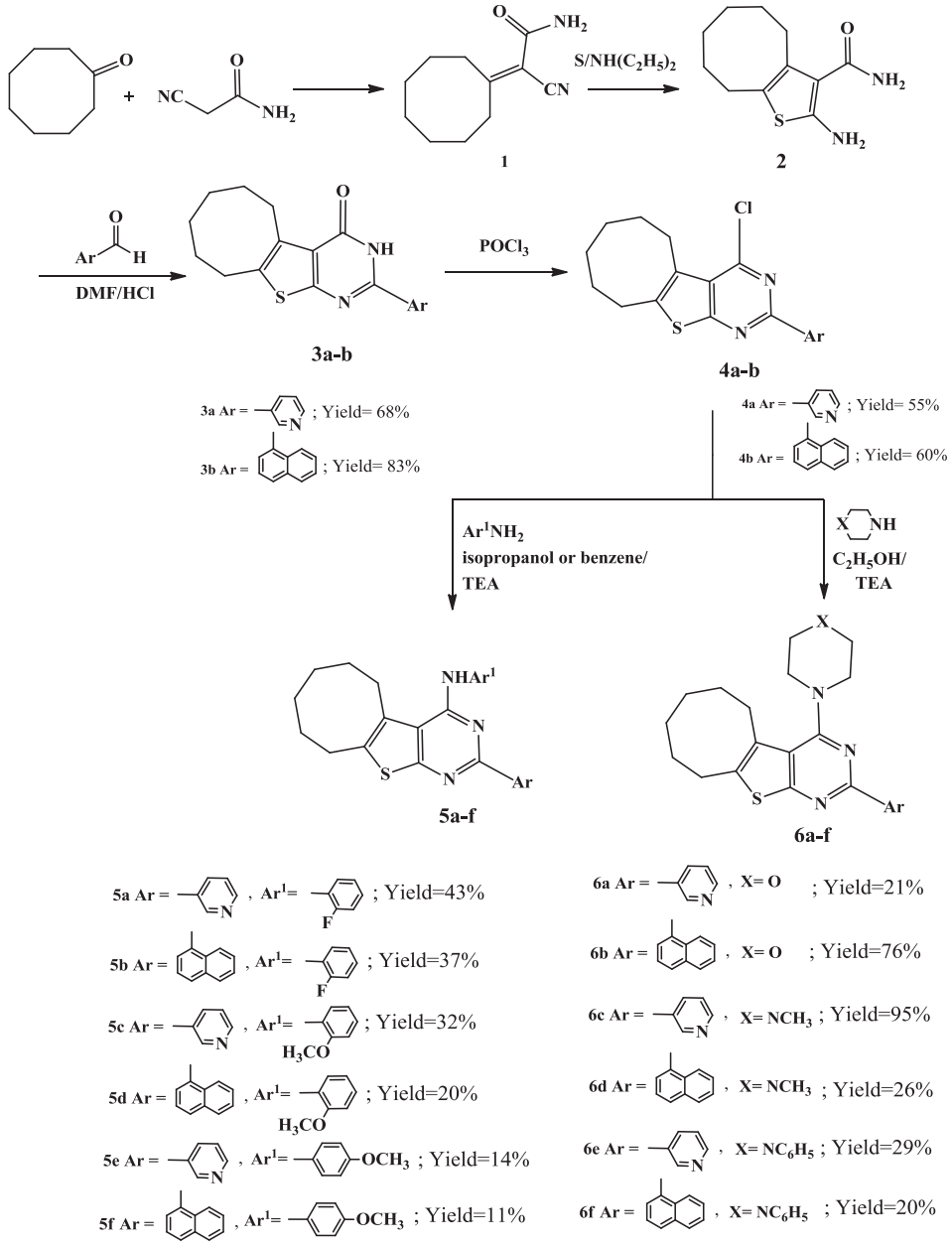
2. Results and discussion

2.1. Chemistry

The synthesis of the target compounds is outlined in Schemes 1 and 2. Our primary starting material 2-amino-4,5,6,7,8,9-hexahydro-cycloocta[4,5]thiophene-3-carboxamide (**2**) was prepared through two steps following the method of Arya [28]. Reacting compound **2** with the appropriate aromatic aldehyde in dry dimethylformamide in the presence of concentrated hydrochloric acid afforded the 2-substituted hexahydrocycloocta[4,5] thieno[2,3-d]pyrimidin-4(3H)-ones **3a**–**b** in good yields. The IR spectra of **3a**–**b** revealed the presence of an absorption band at 3175 and 3120 cm^{-1} belonging to NH group, in addition to another absorption band at 1655 and 1647 cm^{-1}; sequentially to prove the presence of C=O group. Further evidence was obtained from ^1H NMR spectra that showed exchangeable singlet signals at δ 12.68 corresponding to NH protons. Compounds **4a**–**b** were synthesized through refluxing of **3a**–**b** with phosphorus oxychloride. The IR spectra of **4a**–**b** showed the disappearance of the NH and C=O absorption bands. Moreover, the ^1H NMR spectra demonstrated the disappearance of NH signal which ascertains the success of chlorination.

Stirring compounds **4a**–**b** with the appropriate aniline at room temperature afforded compounds **5a**–**f** in yields ranging from 11% to 43%. The IR spectra of these compounds demonstrated absorption bands at 3400–3433 cm^{-1} indicating the presence of NH group. The ^1H NMR spectra also revealed the NH exchangeable signals in the range of δ 9.51–10.60 ppm.

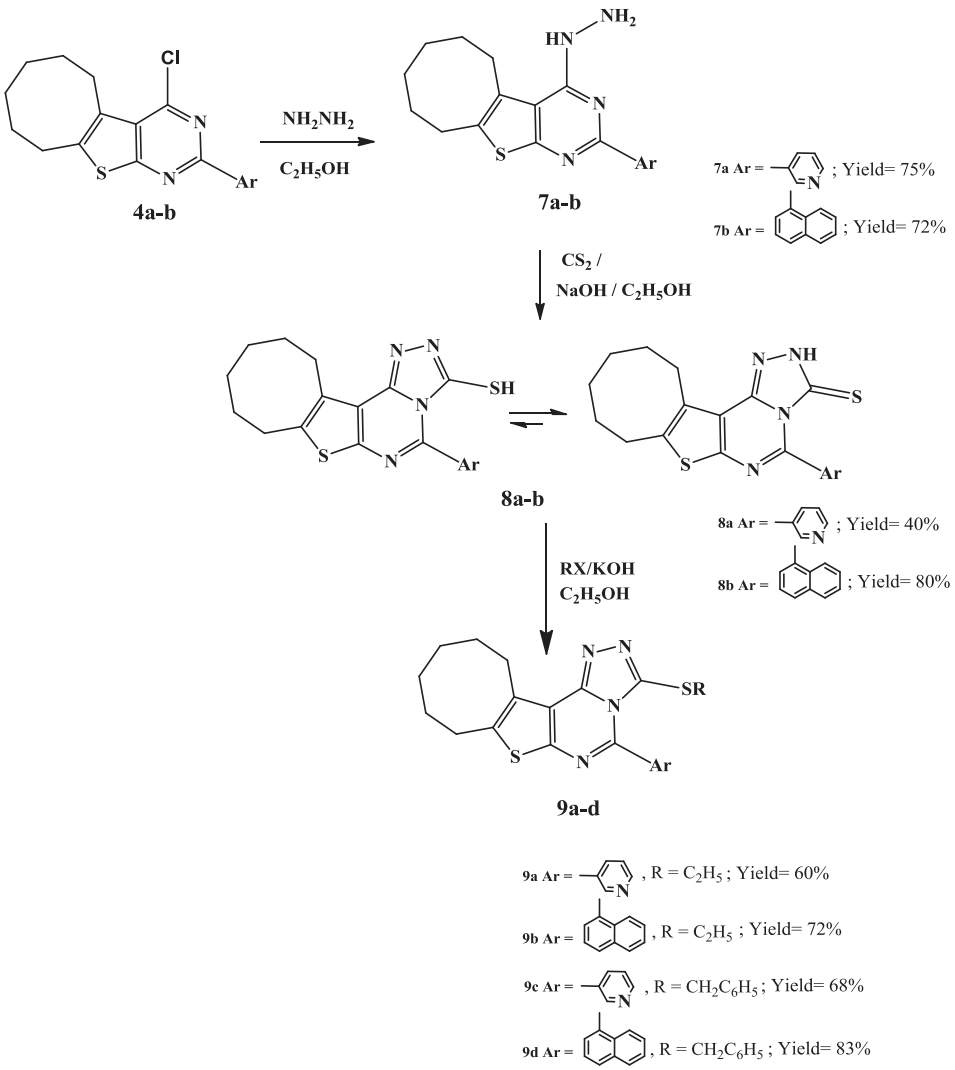
The 4-substitutedaminothieno[2,3-d]pyrimidines **6a**–**f** were obtained through reacting compounds **4a**–**b** with the appropriate secondary amine in absolute ethanol in the presence of triethylamine. The ^1H NMR spectra revealed the presence of expected signals corresponding to the different N substituted groups. The required 4-hydrazinyl thienopyrimidines **7a**–**b** were obtained in high yields by refluxing **4a**–**b** with excess hydrazine hydrate in absolute ethanol. The IR spectra showed two absorption bands in the ranges 3295–3358 and 3150–3250 cm^{-1} indicating the presence of NH and NH_2. The ^1H NMR spectra of these compounds displayed the presence of exchangeable singlet signals corresponding to NH_2 and NH protons. Refluxing **7a**–**b** with carbon disulphide in ethanolic sodium hydroxide gave the thieno[3,2-e]-1,2,4-triazolo[4,3-c]pyrimidines **8a** and **8b** in 40% and 80% respectively. The IR spectra of **8a** and **8b** showed an absorption band at 3421 and 3431 cm^{-1}, respectively indicating the presence of NH group. In addition to the presence of an absorption band at 3060 and 3056 cm^{-1} indicating the presence of SH group. Moreover the presence of C=S group was predicted by the presence of an absorption band at 1182 and 1180 cm^{-1}. The ^1H NMR spectra of **8a** and **8b** also showed the SH exchangeable singlet signals at δ 14.53 and 14.38, respectively. The 3-substitutedthiothieno[3,2-e]-1,2,4-triazolo[4,3-c]pyrimidines **9a**–**d** were achieved in yields between 46% and 83% by the reaction of the appropriate alkyl halide with **8a**–**b** in ethanolic potassium hydroxide. The ^1H NMR spectra of these compounds revealed the disappearance of the signal of SH group and appearance of the expected signals corresponding to the S-substituted groups which are indicative for the success of alkylation.



Scheme 1. The synthetic path and reagents for the preparation of the target compounds **3**–**6**.

2.2. Growth inhibition against a panel of 59 human tumor cell lines

In this study, 17 newly synthesized compounds were selected by National Cancer Institute (USA) for anticancer evaluation. In a first screening, the selected compounds were evaluated at a single dose (10^{-5} M) against 59 different human tumor cell lines, representing leukemia, melanoma and cancers of lung, colon, central nervous system (CNS), ovary, kidney, prostate as well as breast. The growth inhibition percentages obtained from the single dose test for compounds **3a**&**b**, **4a**&**b**, **5c**, **5e**, **5f**, **6a**–**f** and **9a**–**d** are shown in Tables 1 and 2. Compound **9c** showed potent growth inhibition against almost all of human cancer cell lines so it was evaluated against 59 cell lines at 5 dose concentration levels. The relationship between percentage growth and $\log_{10}$ of sample concentration was plotted to obtain $\log_{10}$ GI_{50} (concentration required for 50% inhibition of cell growth). The percentage growth at each concentration and GI_{50} of compound **9c** are shown in Table 3. The results are presented graphically in Figs. 2 and 3. The *in vitro* results showed that compound **9c** has potent broad spectrum anticancer activity against almost all human cancer cell lines with GI_{50} in nano molar to micro molar range between 0.495 and 5.57 μM. It worth mentioning that it had the marked highest selectivity against the two cell lines (T-47D) and (MDA-MB-468) belonging to breast cancer with GI_{50} 0.495 and 0.568 μM respectively. Compound **6c** was selective against K-562, SR and MOLT-4 cell lines belonging to leukemia showing growth inhibition percentages 86.38, 65.76 and 60.40 at a single dose test, at the same time it showed lethal activity against HOP-92 respecting non-small lung cancer. Interestingly, leukemia SR, CNS cancer SNB-75 and renal cancer UO-31 cell lines proved to be sensitive to compound **6d** with growth inhibition percentages 52.86, 50.94 and 53.99 respectively. Additionally, compound 6d demonstrated lethal activity to HOP-92 belonging



Scheme 2. The synthetic path and reagents for the preparation of the target compounds **7**–**9**.

non-small cell lung cancer. The antitumor activity correlation of the newly synthesized compounds showed that the thieno[2.3-d]pyrimidine derivatives **3**, **4**, **5** bearing carbonyl, chloro or aniline moieties at 4-position did not show remarkable anticancer activity. Introduction of 4-methylpiperazinyl group at C-4 position in compounds **6c** and **6d** resulted in an improved potent anticancer activity. Regarding the thieno[3,2-e]-1,2,4-triazolo[4,3-c]pyrimidines it was found that compound **9c**, the most remarkable broad spectrum antitumor agent of this study, has a 3-pyridyl moiety at C-5 position and a benzylthio group at position 3.

In the present work we can conclude:

1 The substituents at C-4 position in thieno[2.3-d]pyrimidines appeared to have a remarkable effect on the anticancer activity. Compounds with 4-methylpiperazinyl moiety showed potent anticancer activity, while those with carbonyl, chloro or aniline moieties were inactive.
2 In the thieno[3,2-e]-1,2,4-triazolo[4,3-c]pyrimidines the substituent at position 3 showed an effect on the activity since it was found that compound with 3-benzylthio group exhibited the most potent anticancer activity.
3 The aryl group at position 5 in thienotriazolopyrimidines has a considerable effect on the antitumor activity since it was observed that the best anticancer activity was obtained by compound bearing 3-pyridyl moiety.

2.3. Cell cycle analysis and detection of apoptosis

The most active compound **9c** was selected to be further studied regarding to its effects on cell cycle progression and induction of apoptosis in the MDA-MB-468 cell line. The MDA-MB-468 cells were incubated with GI_{50} concentration of compound **9c** for 24 h and its effect on the normal cell cycle profile and induction of apoptosis was analyzed. Exposure of MDA-MB-468 cells to Compound **9c** resulted in an interference with the normal cell cycle distribution of this cell line. Compound **9c** induced a significant increase in the percentage of cells at pre-G1 and G2/M phases by 3 and 1.2 folds respectively, compared to control. Such increase was accompanied by a significant reduction in the percentage of cells at the G1 and S phases of the cell cycle. Accumulation of cells in pre-G1 phase which was confirmed by the presence of a sub-G1 peak in the cell cycle profile analysis may result from degradation or fragmentation of the genetic materials indicating a possible role of apoptosis in compound **9c**-induced cancer cell death and cytotoxicity. While the accumulation of the cells in G2/M phase may result from G2 arrest (Figs. 4 and 5).

Table 1
Growth inhibition percentages obtained from the single dose (10^{-5} M) test.

Panel/cell line	Compound								
	3a	**3b**	**4a**	**4b**	**5c**	**5e**	**5f**	**6a**	**6b**
Leukemia									
CCRF-CEM	–	24.95	16.64	7.95	5.54	–	−0.76	15.09	3.08
HL-60(TB)	–	5.54	−2.09	−6.66	0.64	2.25	−1.15	–	−13.63
K-562	1.28	14.56	−1.46	−1.30	−15.73	36.80	6.72	32.00	−15.31
MOLT-4	–	36.61	10.04	7.96	−2.16	−2.06	11.45	–	2.75
RPMI-8226	–	9.08	3.12	3.04	23.20	25.77	0.86	37.64	−11.99
SR	16.16	22.47	12.39	2.70	−8.14	2.39	9.93	17.12	5.19
Non-small cell lung cancer									
A549/ATCC	25.03	18.95	5.17	3.06	−5.78	14.33	−5.45	21.45	2.30
HOP-62	0.95	6.59	2.46	0.10	−19.21	14.42	−2.73	3.21	−1.05
HOP-92	–	36.92	13.77	–	15.00	–	−1.24	–	–
NCI-H226	−7.58	9.90	1.40	−0.08	−0.44	5.23	6.14	9.46	6.17
NCI-H23	−2.46	10.97	0.52	3.93	1.66	14.06	7.28	6.19	−6.76
NCI-H322M	−16.27	−5.53	−1.58	4.23	−9.01	−9.04	−29.66	−5.16	−9.51
NCI-H460	11.50	5.08	−6.66	0.14	−2.14	4.34	−3.97	4.88	−3.69
NCI-H522	32.17	18.87	14.30	10.63	−0.94	17.79	5.83	40.20	8.77
Colon cancer									
COLO 205	−8.45	0.89	−6.93	−7.02	−33.82	−6.25	−17.14	15.90	−14.37
HCC-2998	−5.27	−7.84	−7.05	−5.19	0.87	5.71	−8.94	12.64	−6.18
HCT-116	41.81	15.72	2.94	3.91	−11.26	12.16	11.50	21.60	−8.60
HCT-15	6.62	23.06	1.11	3.39	4.30	29.16	3.58	22.85	−0.21
HT29	12.50	4.20	−0.35	−3.92	−7.01	0.45	−4.22	29.90	−1.26
KM12	7.33	1.67	−15.78	−3.14	−5.83	24.87	−18.01	9.11	−9.27
SW-620	3.51	12.77	−5.55	0.11	−11.70	21.63	−1.94	−3.32	−8.17
CNS cancer									
SF-268	4.77	11.80	4.29	0.79	−10.08	14.72	−7.38	8.30	−1.75
SF-295	−1.58	–	7.08	−0.23	9.22	–	2.11	10.59	1.86
SF-539	3.78	5.76	3.38	0.99	8.54	14.58	0.54	6.18	1.14
SNB-19	−4.61	−7.74	3.84	−4.16	−5.36	8.15	−9.26	23.84	−4.11
SNB-75	5.64	29.55	8.69	13.45	23.47	11.31	13.45	39.39	14.76
U251	7.79	25.33	8.71	10.87	−4.98	10.42	1.66	31.45	9.02
Melanoma									
LOX IMVI	26.68	8.41	0.00	4.92	1.05	28.05	7.62	5.20	−4.59
MALME-3M	4.07	10.41	−3.16	2.51	−48.55	−39.52	−8.29	−8.02	2.96
M14	2.24	−2.52	−6.85	1.39	−13.79	5.82	0.56	10.30	−9.62
MDA-MB-435	−9.14	17.01	2.69	−4.84	−1.75	8.44	−4.68	3.07	−10.86
SK-MEL-2	12.89	3.53	−5.44	−1.65	−15.26	−10.87	−2.16	25.99	−3.83
SK-MEL-28	−12.68	1.05	−4.00	−2.72	−0.45	−4.25	−7.66	21.23	−4.51
SK-MEL-5	−2.89	4.92	3.17	2.85	0.80	9.20	2.21	38.77	5.98

Table 1 (*continued*)

Panel/cell line	Compound								
	3a	**3b**	**4a**	**4b**	**5c**	**5e**	**5f**	**6a**	**6b**
UACC-257	−6.46	7.24	6.85	0.66	−1.14	0.50	−4.67	7.53	−10.31
UACC-62	−5.68	8.03	8.67	11.34	−5.25	32.06	7.05	26.04	3.40
Ovarian cancer									
IGR-OV1	−6.83	−2.87	−7.17	−7.47	−19.98	−7.00	−18.89	2.21	−11.95
OVCAR-3	−17.11	–	–	–	−13.51	−19.06	–	7.70	–
OVCAR-4	−18.66	11.66	2.87	−1.29	4.39	3.43	−11.56	31.30	−5.99
OVCAR-5	3.90	3.13	−1.58	−5.00	−0.39	5.96	−7.92	3.71	−3.14
OVCAR-8	12.41	3.89	−2.68	4.10	−2.70	6.40	−8.23	4.47	−1.45
NCI/ADR-RES	10.79	11.72	−6.15	−8.52	−0.58	21.02	−3.07	9.12	−9.42
SK-OV-3	6.08	−2.38	−2.14	−2.04	−17.86	−6.64	−10.30	4.54	−11.29
Renal cancer									
786-0	2.43	1.40	−1.24	−4.61	−5.12	10.75	2.00	20.88	−2.96
A498	0.47	11.40	5.89	3.95	4.18	15.63	−28.85	22.26	−2.21
ACHN	6.16	4.07	−4.95	0.49	0.84	31.48	−0.79	18.05	−0.17
CAKI-1	5.17	–	6.17	−3.13	−3.13	34.79	1.27	28.30	0.85
RXF 393	7.11	12.52	−16.18	−3.02	−3.47	4.54	−14.99	27.96	4.05
SN12C	6.42	3.17	0.42	−2.01	−2.01	10.67	5.91	25.03	0.55
TK-10	−1.32	−5.08	−3.20	−16.17	−7.02	−15.45	−17.34	7.66	−15.69
UO-31	0.07	9.38	−0.79	4.31	−16.47	30.64	−6.49	22.20	−1.44
Prostate cancer									
PC-3	–	23.04	5.26	4.71	8.59	26.80	−4.15	28.01	5.05
DU-145	10.75	2.46	−8.99	−8.26	−8.00	5.40	−13.28	−5.89	−18.54
Breast cancer									
MCF7	13.82	25.46	7.29	7.75	2.73	6.04	18.92	30.67	8.20
MDA-MB-231/ATCC	9.40	21.31	2.30	3.06	−6.42	30.97	−3.21	36.00	3.26
MDA-MB-468	2.16	4.29	−8.79	−3.24	−12.88	−2.49	−3.62	19.96	−3.57
HS 578T	13.00	4.69	0.22	7.65	−15.71	38.70	5.76	8.69	2.10
BT-549	16.99	13.06	4.63	3.68	−9.19	−1.84	−5.67	39.47	4.96
T-47D	5.83	5.23	4.94	−7.53	0.07	9.60	−8.48	27.43	4.86

3. Conclusion

Hexahydrocyclooctathieno[2,3-d]pyrimidine core represent novel and promising lead for the design and synthesis of potent anticancer agents. The thienotriazolopyrimidine derivative **9c** showed broad spectrum potent anticancer activity in nano molar to micro molar range against 56 human tumor cell lines with GI_{50} ranging from 0.495 to 5.57 μM, also it is worth mentioning that compound **9c** had the marked highest selectivity against the two cell lines T-47D and MDA-MB-468 belonging to breast cancer with $GI_{50} = 0.495$ and 0.568 μM respectively, and its effect was further studied on cell cycle progression and induction of apoptosis in the MDA-MB-468 cell line. Results showed that compound **9c** induced cell cycle arrest at G2/M phase and also, showed accumulation of cells in pre-G1 phase which may result from degradation or fragmentation of the genetic materials indicating a possible role of apoptosis in compound **9c**-induced cancer cell death and cytotoxicity and furthermore verifying this compound as promising selective anticancer lead. Other compounds such as **6c** and **6d** showed good anticancer activity.

Table 2
Growth inhibition percentages obtained from the single dose (10^{-5} M) test.

Panel/cell line	Compound							
	6c	**6d**	**6e**	**6f**	**9a**	**9b**	**9c**	**9d**
Leukemia								
CCRF-CEM	20.39	24.82	11.37	11.74	0.00	8.25	74.81	3.42
HL-60(TB)	12.80	8.44	1.11	−8.02	−1.90	−14.09	96.00	−25.12
K-562	86.38	14.68	−0.86	−8.30	−8.27	−18.13	78.25	−20.37
MOLT-4	60.40	25.97	6.74	−0.22	−0.95	0.33	–	−11.29
RPMI-8226	19.45	20.71	11.05	4.76	−4.71	2.63	79.92	1.88
SR	65.76	52.86	−18.38	3.24	−14.12	1.66	53.51	−8.38
Non-small cell lung cancer								
A549/ATCC	8.85	4.49	8.45	−3.28	3.04	4.85	63.25	−7.87
HOP-62	14.54	24.60	−10.70	1.55	8.77	−3.15	8.68	−12.74
HOP-92	102.98	100.42	23.16	39.43	–	13.57	–	21.96
NCI-H226	6.93	25.57	10.14	17.38	4.87	7.57	32.91	−2.03
NCI-H23	6.27	7.81	0.46	4.46	3.24	−4.64	45.26	−7.02
NCI-H322M	0.67	−4.22	−18.55	1.54	−13.12	−17.82	0.29	−16.57
NCI-H460	1.97	8.60	−0.78	0.84	−4.59	−6.39	80.67	−5.79
NCI-H522	−2.93	11.25	−1.71	10.58	13.84	8.02	93.74	0.88
Colon cancer								
COLO 205	−2.27	8.26	−13.26	−5.61	1.62	−4.29	79.53	−11.56
HCC-2998	43.33	4.16	15.01	−9.26	3.05	−9.27	53.47	−10.13
HCT-116	14.69	32.13	−3.04	6.37	3.28	3.22	70.73	−9.16
HCT-15	6.77	8.15	0.19	3.73	−0.86	2.41	5.83	1.20
HT29	8.72	28.80	−0.16	1.96	5.65	1.41	77.55	−6.31
KM12	−0.09	1.21	1.10	−7.64	−2.57	−8.74	65.55	−13.88
SW-620	−2.26	16.20	−5.15	6.00	−8.97	0.28	44.65	−13.62
CNS cancer								
SF-268	19.68	18.02	−2.27	9.95	−7.39	1.66	43.02	−9.63
SF-295	24.42	–	–	1.82	–	2.28	54.95	−3.92
SF-539	6.65	14.02	−3.59	4.05	2.27	5.05	20.28	−6.53
SNB-19	−1.40	8.50	2.03	−2.62	−0.61	−3.03	53.53	−5.08
SNB-75	32.60	50.94	−7.85	31.78	−0.47	9.65	25.91	8.32
U251	27.74	33.56	6.27	3.17	7.91	10.45	83.22	−3.52
Melanoma								
LOX IMVI	21.37	4.93	−2.60	9.25	−1.36	−2.84	39.92	0.34
MALME-3M	−5.90	18.65	−25.68	5.84	−19.76	−0.07	83.78	−7.74
M14	−1.23	6.82	−8.43	8.87	−7.37	−2.84	54.96	−4.20
MDA-MB-435	1.57	6.74	−0.11	−2.10	−16.06	−5.40	80.88	−0.93
SK-MEL-2	−14.12	−6.06	−2.74	1.47	5.50	1.41	83.47	−8.63
SK-MEL-28	0.26	1.91	2.53	−5.22	0.03	−10.49	50.80	−12.54
SK-MEL-5	12.13	14.73	9.00	1.98	0.65	1.22	145.97	−0.96
UACC-257	−2.48	5.58	−5.25	−6.60	−4.21	4.86	64.96	−8.55
UACC-62	−3.12	12.44	5.09	9.61	6.68	12.94	65.13	−1.38
Ovarian cancer								
IGR-OV1	−2.33	27.37	−18.62	3.09	−22.71	−9.33	23.04	−11.98
OVCAR-3	4.23	–	−2.99	–	−18.94	–	63.59	–
OVCAR-4	19.24	16.23	0.92	0.47	−4.80	−11.43	72.70	−17.31
OVCAR-5	−3.93	9.78	2.14	8.24	8.11	−0.64	4.79	−6.52
OVCAR-8	4.94	6.99	4.06	3.63	−0.54	−3.12	79.72	−7.26
NCI/ADR-RES	10.67	12.62	1.94	−0.46	6.62	−9.91	−0.38	−9.74
SK-OV-3	2.76	10.78	−13.03	−3.81	−0.91	−10.96	28.84	−13.19
Renal cancer								
786-0	7.21	12.33	−0.47	−6.73	−8.79	−2.89	26.37	−4.05
A498	0.70	5.77	5.70	−11.90	−4.30	0.21	41.47	−9.79
ACHN	10.41	17.27	2.20	7.35	2.79	−2.05	26.26	−12.37
CAKI-1	13.25	–	8.61	19.21	−12.24	6.70	28.38	9.66
RXF 393	20.56	39.08	2.80	−21.93	3.59	−7.88	16.75	−5.42
SN12C	9.19	24.08	3.56	3.97	−0.06	−3.02	43.48	−4.62
TK-10	−7.88	−13.10	−5.42	−17.29	−14.09	−13.29	27.77	−7.27
UO-31	25.04	53.99	2.10	30.06	7.73	−4.88	5.18	−0.09
Prostate cancer								
PC-3	33.29	43.26	16.12	11.96	−1.66	9.36	90.99	1.90
DU-145	1.13	−2.32	−12.56	−10.68	−12.51	−7.88	43.59	−16.54
Breast cancer								
MCF7	11.84	31.79	4.71	0.18	8.91	17.34	62.38	−6.30
MDA-MB-231/ATCC	21.09	38.19	2.68	12.19	−0.40	5.54	28.62	2.60
MDA-MB-468	14.69	13.86	−5.01	4.04	−15.92	−4.47	13.75	−2.81
HS 578T	4.68	8.09	−11.50	1.25	−9.44	3.18	58.86	−1.98
BT-549	22.60	32.84	12.16	10.34	14.55	11.79	86.02	−3.54
T-47D	5.76	22.68	5.09	−9.87	−0.42	−6.08	99.87	−4.60

Table 3
Concentrations required for 50% inhibition of cell growth (GI_{50}) for compound **9c**.

Panel/cell line	Percentage growth at each concentration (log10)					GI_{50} (μM)	Log_{10} GI_{50}
	−8	−7	−6	−5	−4		
Leukemia							
CCRF-CEM	95	92	52	10	−41	1.11	−5.96
HL-60(TB)	93	81	79	−31	−56	1.84	−5.74
K-562	100	88	79	1	−52	2.34	−5.63
MOLT-4	99	95	93	3	−60	3.00	−5.52
RPMI-8226	102	98	67	5	−47	1.87	−5.73
SR	100	93	85	–	−64	2.59	−5.59
Non-small cell lung cancer							
A549/ATCC	99	99	83	9	−65	2.80	−5.55
HOP-62	91	86	89	6	−58	2.96	−5.53
HOP-92	82	79	68	−11	−71	1.70	−5.77
NCI-H226	85	106	72	–	−60	2.03	−5.69
NCI-H23	96	98	95	−8	−76	2.74	−5.56
NCI-H322M	92	92	99	33	−78	5.57	−5.25
NCI-H460	104	101	92	4	−75	2.97	−5.53
NCI-H522	83	90	63	−26	−74	1.39	−5.86
Colon cancer							
COLO 205	101	91	79	−72	−73	1.55	−5.81
HCC-2998	91	98	105	17	−75	4.21	−5.38
HCT-116	102	97	86	4	−76	2.77	−5.56
HCT-15	95	94	96	26	−87	4.51	−5.35
HT29	101	98	88	3	−66	2.78	−5.56
KM12	97	95	84	2	−79	2.60	−5.59
SW-620	97	96	87	11	−80	3.09	−5.51
CNS cancer							
SF-268	97	95	102	23	−70	4.57	−5.34
SF-295	93	93	86	6	−75	2.80	−5.55
SF-539	100	92	97	10	−85	3.51	−5.45
SNB-19	97	98	97	19	−70	3.96	−5.40
SNB-75	83	81	84	1	−89	2.58	−5.59
U251	96	91	73	6	−74	2.20	−5.66
Melanoma							
LOX IMVI	94	94	95	3	−68	3.08	−5.51
MALME-3M	92	92	101	−14	−57	2.76	−5.56
M14	98	91	94	18	−60	3.75	−5.43
MDA-MB-435	98	90	86	–	−91	2.61	−5.58
SK-MEL-2	98	107	83	−24	−77	2.05	−5.69
SK-MEL-28	105	95	87	16	−92	3.35	−5.47
SK-MEL-5	97	89	74	−98	−87	1.37	−5.86
UACC-257	94	100	77	−5	−76	2.12	−5.67
UACC-62	98	95	74	−12	−79	1.91	−5.72
Ovarian cancer							
IGR-OV1	96	94	94	23	−59	4.18	−5.38
OVCAR-3	101	100	85	9	−84	2.86	−5.54
OVCAR-4	97	88	73	1	−87	2.08	−5.68
OVCAR-5	101	96	91	32	−100	4.99	−5.30
OVCAR-8	98	98	85	8	−61	2.86	−5.54
NCI/ADR-RES	101	99	102	67	−49	14.1	−4.85
SK-OV-3	98	90	91	−2	−83	2.76	−5.56
Renal cancer							
786-0	103	93	97	13	−72	3.63	−5.44
A498	89	86	77	–	−97	2.24	−5.65
ACHN	98	91	93	2	−89	2.98	−5.53
CAKI-1	90	83	83	22	−90	3.43	−5.46
RXF 393	94	98	83	8	−64	2.79	−5.55
SN12C	98	95	91	5	−70	2.99	−5.52
TK-10	95	97	108	8	−75	3.83	−5.42
UO-31	89	87	92	19	−86	3.73	−5.43
Prostate cancer							
PC-3	97	96	78	3	−82	2.36	−5.63
DU-145	103	100	87	18	−94	3.42	−5.47
Breast cancer							
MCF7	95	88	84	3	−65	2.64	−5.58
MDA-MB-231/ATCC	97	92	83	−14	−75	2.18	−5.66
HS 578T	100	99	87	24	−29	3.84	−5.42
BT-549	100	94	94	−4	−76	2.80	−5.55
T-47D	95	71	41	6	−56	0.495	−6.31
MDA-MB-468	99	88	38	−24	−80	0.568	−6.25

4. Experimental

4.1. Chemistry

4.1.1. General

Melting points were obtained on a Griffin apparatus and were uncorrected. Microanalyses for C, H and N were carried out at the Microanalytical center, Cairo University. IR spectra were recorded on a Shimadzu 435 spectrometer, using KBr discs. ^{1}H NMR and ^{13}C NMR spectra were performed on joel NMR FXQ-300 MHz and joel NMR FXQ-400 MHz spectrometers, using TMS as the internal standard. Mass spectra were recorded on a GCMP-QP1000 EX Mass spectrometer. Progress of the reactions were monitored by TLC using precoated aluminum sheet silica gel MERCK 60F 254 and was visualized by UV lamp.

4.1.2. General procedure for the preparation of 2-aryl-5,6,7,8,9,10-hexahydrocycloocta[4,5]thieno[2,3-d]pyrimidin-4(3H)-ones (**3a**–**b**)

A mixture of o-amino amide derivative **2** (2.25 g, 0.01 mol) and the appropriate aromatic aldehyde (0.03 mol) in dry dimethylformamide (25 mL) containing concentrated hydrochloric acid (0.2 mL) was refluxed for 24 h. The mixture was cooled, filtered and the precipitate was crystallized from the appropriate solvent.

*4.1.2.1. 2-(3-Pyridyl)-5,6,7,8,9,10-hexahydrocycloocta[4,5]thieno[2,3-d]pyrimidin-4(3H)-one (**3a**).* mp > 300 °C (ethanol); yield 68%; IR (KBr) ν_{max}: 3175 (NH), 1655 (C═O) cm^{-1}; ^{1}H NMR (DMSO-d$_6$): δ 1.20–1.25 (m, 2H, CH_2), 1.35–1.42 (m, 2H, CH_2), 1.53–1.65 (m, 4H, 2 CH_2), 2.85–2.91 (m, 2H, CH_2), 3.04–3.10 (m, 2H, CH_2), 7.50–7.53 (t, 1H, J = 5.0 Hz, pyridyl H), 8.38 (d, 1H, J = 5.0 Hz, pyridyl H), 8.68 (d, 1H, J = 5.0 Hz, pyridyl H), 9.18 (s, 1H, pyridyl H) and 12.68 (s, 1H, NH, D_2O exchangeable) ppm; MS [*m*/*z*, %]: 311 [M•$^+$, 54.78]. Anal. Calcd for $C_{17}H_{17}N_3OS$ (311.40): C, 65.57; H, 5.50; N, 13.49. Found: C, 65.66; H, 5.53; N, 13.61.

*4.1.2.2. 2-(1-Naphthyl)-5,6,7,8,9,10-hexahydrocycloocta[4,5]thieno[2,3-d] pyrimidin-4(3H)-one (**3b**).* mp > 266–267 °C (n-butanol); yield 83%; IR (KBr) ν_{max}: 3120 (NH), 1647 (C═O) cm^{-1}; ^{1}H NMR (DMSO-d$_6$): δ 1.25–1.30 (m, 2H, CH_2), 1.40–1.45 (m, 2H, CH_2), 1.60–1.70 (m, 4H, 2 CH_2), 2.82–2.90 (m, 2H, CH_2), 3.03–3.10 (m, 2H, CH_2), 7.51–7.58 (m, 2H, naphthyl H), 7.59–7.61 (m, 1H, naphthyl H), 7.73 (d, 1H, J = 7.6 Hz, naphthyl H), 8.0–8.04 (m, 1H, naphthyl H), 8.07 (d, 2H, J = 7.6 Hz, naphthyl H) and 12.68 (s, 1H, NH, D_2O exchangeable) ppm; ^{13}C NMR (DMSO-d$_6$): δ 24.95, 25.83, 25.95, 26.63, 30.37, 32.00, 121.66, 125.50, 125.56, 126.84, 127.60, 128.53, 128.83, 130.68, 130.99, 131.32, 133.60, 134.22, 135.75 ppm; MS [*m*/*z*, %]: 360 [M•$^+$, 100]. Anal. Calcd for $C_{22}H_{20}N_2OS$ (360.47): C, 73.30; H, 5.59; N, 7.77. Found: C, 73.41; H, 5.62; N, 7.90.

4.1.3. General procedure for the preparation of 2-aryl-4-chloro-5,6,7,8,9,10-hexahydrocycloocta[4,5]thieno[2,3-d]pyrimidines (**4a**–**b**)

Phosphorus oxychloride (15 mL) was added to the respective thienopyrimidone **3a**–**b** (0.003 mol) and the mixture was refluxed for 1 h. The reaction mixture was concentrated under reduced pressure then poured into ice cold water (100 mL). The precipitated product was filtered, washed with water (2 × 10 mL),dried and crystallized from ethanol.

*4.1.3.1. 4-Chloro-2-(3-pyridyl)-5,6,7,8,9,10-hexahydrocycloocta[4,5]thieno[2,3-d]pyrimidine (**4a**).* mp 140–141 °C; yield 55%; IR (KBr) ν_{max}: 1629 (C═N), 1546 (C═C) cm^{-1}; ^{1}H NMR (DMSO-d$_6$): δ 1.05–1.15 (m, 2H, CH_2), 1.31–1.40 (m, 2H, CH_2), 1.50–1.55 (m, 4H, 2CH_2), 2.80–2.90 (m, 2H, CH_2), 2.95–3.05 (m, 2H, CH_2), 8.06–8.09 (t, 1H, J = 5.0 Hz, pyridyl H), 8.82 (d, 1H, J = 5.0 Hz, pyridyl H), 9.10

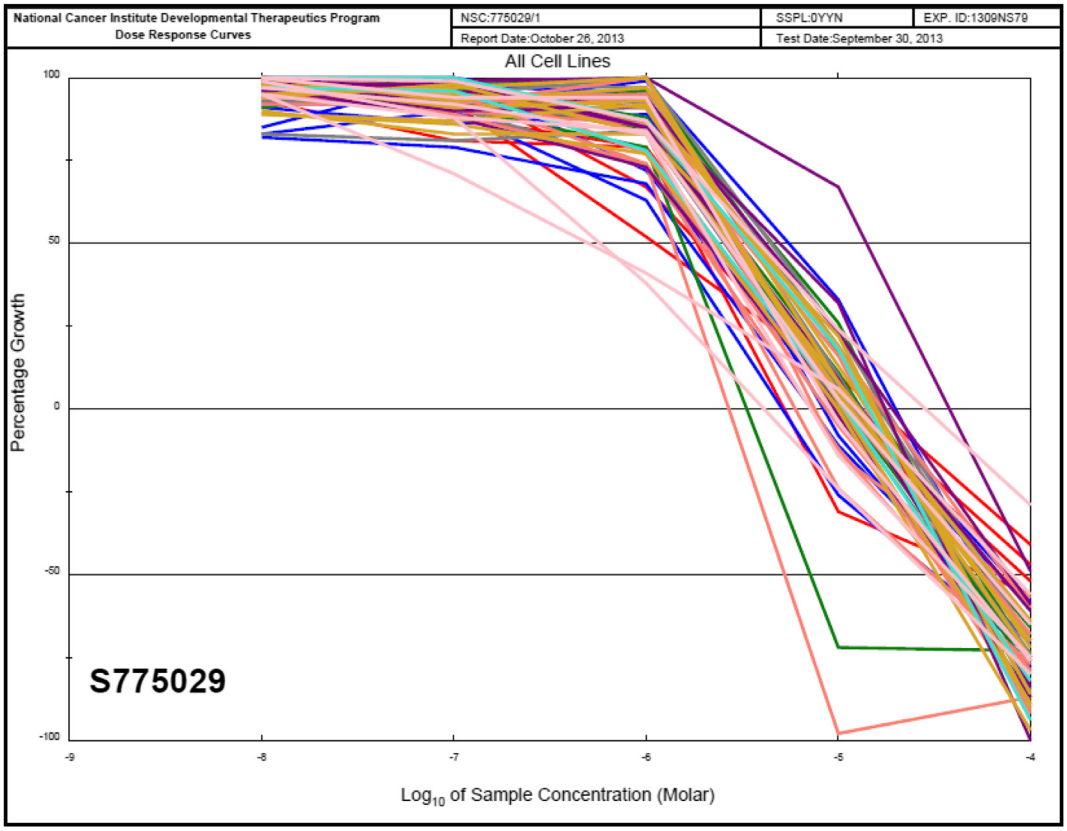


Fig. 2. Dose response curves for 59 cell lines showing the percentage growth against sample concentrations (5 concentrations) for compound **9c**.

(d, 1H, $J = 5.0$ Hz, pyridyl H) and 9.36 (s, 1H, pyridyl H) ppm; MS [*m/z*, %]: 331[M + 2$^{\bullet+}$, 38.45], 330 [M + 1$^{\bullet+}$, 23.09], 329 [M$^{\bullet+}$, 100]. Anal. Calcd for $C_{17}H_{16}ClN_3S$ (329.85): C, 61.90; H, 4.89; N, 12.74. Found: C, 61.97; H, 4.93; N, 12.89.

*4.1.3.2. 4-Chloro-2-(1-naphthyl)-5,6,7,8,9,10-hexahydrocycloocta [4,5]thieno[2,3-d]pyrimidine (**4b**).* mp 110–112 °C; yield 60%; IR (KBr) ν_{max}: 1620 (C═N), 1554 (C═C) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 1.23–1.33 (m, 2H, CH_2), 1.42–1.51 (m, 2H, CH_2), 1.61–1.82 (m, 4H, 2 CH_2), 3.02–3.06 (t, 2H, $J = 6.0$ Hz, CH_2), 3.16–3.22 (t, 2H, $J = 6.0$ Hz, CH_2), 7.55–7.64 (m, 3H, naphthyl H), 8.00–8.03 (m, 1H, naphthyl H),8.06–8.13 (m, 2H, naphthyl H) and 8.68–8.74 (m, 1H, naphthyl H) ppm; MS [*m/z*, %]: 380 [M + 2$^{\bullet+}$, 26.85], 378 [M$^{\bullet+}$, 67.33]. Anal. Calcd for $C_{22}H_{19}ClN_2S$ (378.92): C, 69.73; H, 5.05; N, 7.39. Found: C, 69.82; H, 5.11; N, 7.47.

*4.1.4. General procedure for the preparation of 2-aryl-4-substituted anilino-5,6,7,8,9,10-hexahydrocycloocta [4,5]thieno[2,3-d] pyrimidines (**5a**–**f**)*

A mixture of **4a**–**b** (0.001 mol), the selected aromatic amine (0.002 mol) and triethylamine (0.36 mL, 0.003 mol) in different solvents (12 mL) was stirred for 15 h. The separated solid was filtered, dried and crystallized from the suitable solvent.

*4.1.4.1. 4-(2-Flouroanilino)-2-(3-pyridyl)-5,6,7,8,9,10-hexahydro-cyclooct[4,5]thieno[2,3-d]pyrimidine (**5a**).* mp 155–157 °C (isopropanol); yield 43%; IR (KBr) ν_{max}: 3400 (NH) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 1.26–1.28 (m, 2H, CH_2), 1.46–1.47 (m, 2H, CH_2), 1.68–1.76 (m, 4H, 2 CH_2), 2.95–2.99 (m, 2H, CH_2), 2.99–3.02 (m, 2H, CH_2), 6.48–6.55 (m, 1H, ArH), 6.80 (d, 1H,$J = 6.0$ Hz, ArH), 6.87 (d, 1H, $J = 6.0$ Hz, ArH), 6.90–7.00 (m, 2H, 1ArH and NH, D_2O exchangeable), 7.55–7.59 (dd, 1H, $J = 7.0$ Hz, pyridyl H), 8.61 (d, 1H, $J = 6.9$ Hz, pyridyl H), 8.71 (d, 1H, $J = 7.0$ Hz, pyridyl H), 9.46 (s, 1H, pyridyl H) ppm. Anal. Calcd for $C_{23}H_{21}FN_4S$ (404.50): C, 68.29; H, 5.23; N, 13.85. Found: C, 68.34; H, 5.28; N, 14.04.

*4.1.4.2. 4-(2-Flouroanilino)-2-(1-naphthyl)-5,6,7,8,9,10-hexahydro-cyclooct[4,5]thieno[2,3-d]pyrimidine (**5b**).* mp 180–182 °C (benzene); yield 37%; IR (KBr) ν_{max}: 3412 (NH) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 1.29–1.31 (m, 2H, CH_2), 1.48–1.49 (m, 2H, CH_2), 1.69–1.77 (m, 4H, 2 CH_2), 2.98–3.07 (m, 4H, 2CH_2), 7.58–7.68 (m, 5H, 4ArH and NH, D_2O exchangeable), 8.02–8.12 (m, 5H, naphthyl H) and 8.68–8.71 (m, 2H, naphthyl H) ppm. Anal. Calcd for $C_{28}H_{24}FN_3S$ (453.57): C, 74.14; H, 5.33; N, 9.26. Found: C, 74.21; H, 5.31; N, 9.56.

*4.1.4.3. 4-(2-Methoxyanilino)-2-(3-pyridyl)-5,6,7,8,9,10-hexahydro-cyclooct[4,5]thieno[2,3-d]pyrimidine (**5c**).* mp 190–191 °C (isopropanol); yield 32%; IR (KBr) ν_{max}: 3433 (NH) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 1.30–1.40 (m, 2H, CH_2), 1.42–1.55 (m, 2H, CH_2), 1.64–1.8 (m, 4H, 2 CH_2), 2.95–3.10 (m, 4H, 2CH_2), 3.73 (s, 3H, OCH_3), 6.48–6.51 (m, 2H, ArH), 6.63 (d, 1H, ArH), 6.77 (d, 1H, $J = 9$ Hz, ArH), 6.80 (d, 1H, $J = 9.0$ Hz, ArH), 7.58–7.60 (t, 1H, pyridyl H), 8.64 (d, 1H, $J = 7.8$ Hz, pyridyl H), 8.73 (d, 1H, $J = 7.8$ Hz, pyridyl H), 9.48 (s, 1H, pyridyl H) and 10.60 (br.s, 1H, NH, D_2O exchangeable) ppm; ^{13}C NMR (DMSO-d_6): δ 24.52, 25.45, 26.15, 27.78, 30.40, 31.73, 55.56, 111.00, 114.27, 116.61, 121.29, 124.46, 127.02, 129.53, 131.73, 135.63, 138.03, 143.41, 146.62, 149.32, 152.03, 153.10 ppm. Anal. Calcd for $C_{24}H_{24}N_4OS$ (416.54): C, 69.20; H, 5.81; N, 13.45. Found: C, 69.28; H, 5.85; N, 13.58.

*4.1.4.4. 4-(2-Methoxyanilino)-2-(1-naphthyl)-5,6,7,8,9,10-hexahydro-cyclooct[4,5]thieno[2,3-d]pyrimidine (**5d**).* mp 160–162 °C (benzene); yield 20%; IR (KBr) ν_{max}: 3414 (NH) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 1.20–1.29 (m, 2H, CH_2), 1.45–1.56 (m, 2H,

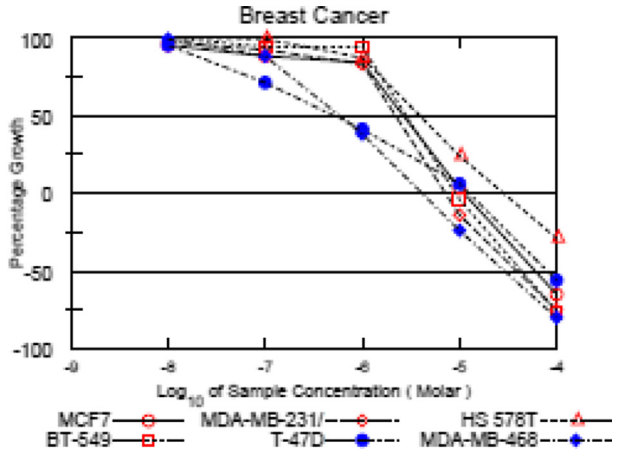


Fig. 3. Dose response curves of breast cancer cell lines, against sample concentrations (5 concentrations) for compound **9c**.

CH_2), 1.65–1.84 (m, 4H, 2 CH_2), 2.99–3.10 (m, 4H, 2CH_2), 3.73 (s, 3H, OCH_3), 6.46–6.59 (m, 2H, ArH), 6.67 (d, 1H, $J = 6.5$ Hz, ArH), 6.81 (d, 1H, $J = 6.5$ Hz, ArH), 7.58–7.66 (m, 3H, naphthyl H), 8.00–8.10 (m, 1H, naphthyl H), 8.12 (d, 1H, $J = 6.0$ Hz, naphthyl H), 8.70 (d, 2H, $J = 6.0$ Hz, naphthyl H) and 10.40 (br.s, 1H, NH, D_2O exchangeable) ppm. Anal. Calcd for $C_{29}H_{27}N_3OS$ (465.61): C, 74.81; H, 5.88; N, 9.02. Found: C, 74.88; H, 5.89; N, 9.23.

*4.1.4.5. 4-(4-Methoxyanilino)-2-(3-pyridyl)-5,6,7,8,9,10-hexahydrocyclooct[4,5]thieno[2,3-d]pyrimidine (**5e**).* mp 239–241 °C (isopropanol); yield 14%; IR (KBr) ν_{max}: 3431 (NH) cm^{-1}; ^1H NMR (DMSO-d_6): δ 1.15–1.25 (m, 2H, CH_2), 1.40–1.45 (m, 2H, CH_2), 1.60–1.77 (m, 4H, 2 CH_2), 2.95–3.05 (m, 2H, CH_2), 3.00–3.02 (m, 2H, CH_2), 3.58 (s, 3H, OCH_3), 6.52 (d, 2H $J = 9.0$ Hz, ArH), 6.61 (d, 2H, $J = 9.0$ Hz, ArH), 7.57–7.59 (dd, 1H, $J = 8.1$ Hz, pyridyl H), 8.59 (d, 1H, $J = 8.1$ Hz, pyridyl H), 8.66 (d, 1H, $J = 8.1$ Hz, pyridyl H), 9.38 (s, 1H, pyridyl H) and 9.91 (br.s, 1H, NH, D_2O exchangeable) ppm; MS [*m*/*z*, %]: 416 [$M^{\bullet+}$, 2.68]. Anal. Calcd for $C_{24}H_{24}N_4OS$ (416.54): C, 69.20; H, 5.81; N, 13.45. Found: C, 69.27; H, 5.83; N, 13.53.

*4.1.4.6. 4-(4-Methoxyanilino)-2-(1-naphthyl)-5,6,7,8,9,10-hexahydro-cyclooct[4,5]thieno[2,3-d]pyrimidine (**5f**).* mp 160–162 °C (benzene); yield 11%; IR (KBr) ν_{max}: 3431 (NH) cm^{-1}; ^1H NMR (DMSO-d_6): δ 1.21–1.38 (m, 2H, CH_2), 1.46–1.56 (m, 2H, CH_2), 1.64–1.81 (m, 4H, 2 CH_2), 2.98–3.02 (m, 2H, CH_2), 3.22–3.24 (m, 2H, CH_2), 3.72 (s, 3H, OCH_3), 6.90 (d, 2H, $J = 9.0$ Hz, ArH), 7.05 (d, 2H, $J = 9.0$ Hz, ArH),7.44–7.50 (m, 3H, naphthyl H), 7.54 (d, 1H, $J = 7.5$ Hz, naphthyl H),7.89–7.92 (m, 2H, naphthyl H) and 8.52 (d, 2H, $J = 7.5$ Hz, naphthyl H) and 9.51 (br.s, 1H, NH, D_2O exchangeable) ppm; ^{13}C NMR (DMSO-d_6): δ 24.95, 25.52, 26.19, 27.78, 30.41, 31.76, 52.30, 116.37, 120.22, 125.74, 126.02, 126.58, 126.84, 127.52, 128.53, 128.99, 130.16, 130.98, 134.22, 135.47, 143.53, 152.34, 153.92, 159.10, 159.87 ppm; MS [*m*/*z*, %]: 465 [$M^{\bullet+}$, 77.89]. Anal. Calcd for $C_{29}H_{27}N_3OS$ (465.61): C, 74.81; H, 5.84; N, 9.02. Found: C, 74.93; H, 5.83; N, 9.11.

*4.1.5. General procedure for the preparation of 2-aryl-4-substituted-amino-5,6,7,8,9,10-hexahydrocycloocta [4,5]thieno[2,3-d]pyrimidines (**6a**–**f**)*

A mixture of **4a**–**b** (0.001 mol), the selected secondary amine (0.001 mol) and triethylamine (0.36 mL, 0.003 mol) in absolute ethanol (12 mL) was stirred for 20 h, after cooling the separated solid was filtered, dried and crystallized from ethanol.

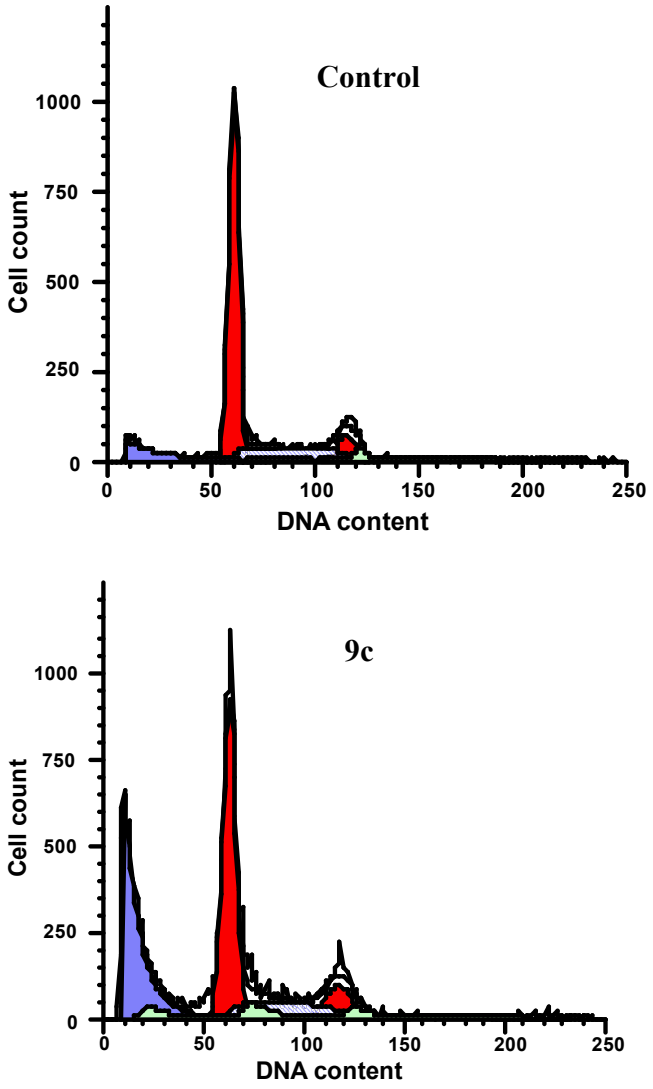


Fig. 4. Effect of compound **9c** on DNA-ploidy flow cytometric analysis of MDA-MB-468 cells. The cells were treated with compound **9c** (0.568 μM), for 24 h, and the harvested cells were subjected to Cell-cycle analysis using a FACS Calibur flow cytometer.

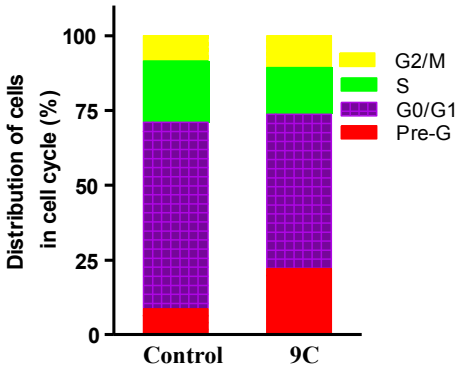


Fig. 5. Bar chart shows percentage of MDA-MB-468 cells at each phase of cell cycle in control cells and cells treated with compound **9c**.

*4.1.5.1. 4-(Morpholin-4-yl)-2-(3-pyridyl)-5,6,7,8,9,10-hexahydrocyclo-octa[4,5]thieno[2,3-d]pyrimidine (**6a**).* mp 130–132 °C; yield 21%; IR (KBr) ν_{max}: 1577 (C=N), 1546 (C=C)

cm^{-1}; ^1H NMR (DMSO-d_6): δ 1.03–1.19 (m, 2H, CH_2), 1.40–1.48 (m, 2H, CH_2), 1.54–1.62 (m, 2H, CH_2), 1.67–1.77 (m, 2H, CH_2), 2.94–3.03 (m, 4H, *J* = 6 Hz, $2CH_2$), 3.36–3.43 (m, 4H, −C***H₂***–N–C***H₂***–), 3.79–3.82 (m, 4H, −C***H₂***–O–C***H₂***–), 7.51-7.55 (dd, *J* = 7.8 Hz, 1H, pyridyl H), 8.63–8.71 (m, 2H, pyridyl H), 9.52 (s, 1H, pyridyl H) ppm. Anal. Calcd for $C_{21}H_{24}N_4OS$ (380.51): C, 66.29; H, 6.36; N, 14.72. Found: C, 66.32; H, 6.38; N, 14.90.

*4.1.5.2. 4-(Morpholin-4-yl)-2-(1-naphthyl)-5,6,7,8,9,10-hexahydrocycloocta[4,5]thieno[2,3-d]pyrimidine (**6b**).* mp 102–103 °C; yield 76%; IR (KBr) ν_{max}: 1647 (C═N), 1535 (C═C) cm^{-1}; ^1H NMR (DMSO-d_6): δ 1.19–1.24 (m, 2H, CH_2), 1.42–1.47 (m, 2H, CH_2), 1.64–1.80 (m, 4H, 2 CH_2), 3.00–3.04 (m, 2H, CH_2), 3.06–3.10 (m, 2H, CH_2), 3.60–3.63 (m, 4H, −C***H₂***–N–C***H₂***–), 3.80-3.83 (m, 4H, −C***H₂***–O–C***H₂***–), 7.56–7.61 (m, 3H, naphthyl H), 7.63 (d, 1H, *J* = 8.1 Hz, naphthyl H), 8.01–8.10 (m, 1H, naphthyl H), 8.13 (d, 1H, *J* = 6 Hz, naphthyl H), and 8.80 (d, 1H, *J* = 6 Hz, naphthyl H) ppm. Anal. Calcd for $C_{26}H_{27}N_3OS$ (429.58): C, 72.69; H, 6.34; N, 9.78. Found: C, 72.65; H, 6.37; N, 9.67.

*4.1.5.3. 4-(4-Methylpiperazin-1-yl)-2-(3-pyridyl)-5,6,7,8,9,10-hexahydrocycloocta[4,5]thieno[2,3-d]pyrimidine (**6c**).* mp 125–127 °C; yield 95%; IR (KBr) ν_{max}: 1612 (C═N), 1516 (C═C) cm^{-1}; ^1H NMR (DMSO-d_6): δ 1.03–1.13 (m, 2H, CH_2), 1.36–1.48 (m, 2H, CH_2), 1.51–1.62 (m, 2H, CH_2), 1.63–1.71 (m, 2H, CH_2), 2.66 (s, 3H, N–CH_3), 2.90–3.05 (m, 8H, $2CH_2$ and −C***H₂***–N(CH_3)–C***H₂***–), 3.42-3.50 (m, 4H, −C***H₂***–N–C***H₂***–), 7.51–7.55 (dd, 1H, *J* = 7.8 Hz, pyridyl H), 8.64 (d, 2H, pyridyl H) and 9.51 (s, 1H, pyridyl H) ppm. Anal. Calcd for $C_{22}H_{27}N_5S$ (393.55): C, 67.14; H, 6.92; N, 17.80. Found: C, 67.31; H, 6.91; N, 17.94.

*4.1.5.4. 4-(4-Methylpiperazin-1-yl)-2-(1-naphthyl)-5,6,7,8,9,10-hexa-hydrocycloocta[4,5]thieno[2,3-d]pyrimidine (**6d**).* mp 115–117 °C; yield 26%; IR (KBr) ν_{max}: 1629 (C═N), 1548 (C═C) cm^{-1}; ^1H NMR (DMSO-d_6): δ 1.10–1.20 (m, 2H, CH_2), 1.40–1.48 (m, 2H, CH_2), 1.54–1.61 (m, 2H, CH_2), 1.64–1.73 (m, 2H, CH_2), 2.54 (s, 3H, N–CH_3), 2.90–3.00 (m, 2H, CH_2), 3.01–3.09 (m, 2H, CH_2), 3.29–3.40 (m, 4H, −C***H₂***–N(CH_3)–C***H₂***–), 3.41–3.52 (m, 4H, −C***H₂***–N–C***H₂***–), 7.54–7.64 (m, 3H, naphthyl H), 7.98–8.02 (t, 2H, *J* = 7.5 Hz, naphthyl H), 8.12 (d, 1H, *J* = 7.5 Hz, naphthyl H), and 8.81 (d, 1H, *J* = 7.5 Hz naphthyl H) ppm. Anal. Calcd for $C_{27}H_{30}N_4S$ (442.62): C, 73.27; H, 6.83; N, 12.66. Found: C, 73.35; H, 6.81; N, 12.82.

*4.1.5.5. 4-(4-Phenylpiperazin-1-yl)-2-(3-pyridyl)-5,6,7,8,9,10-hexahydrocycloocta [4,5]thieno[2,3-d]pyrimidine (**6e**).* mp 220–222 °C; yield 29%; IR (KBr) ν_{max}: 1596 (C═N), 1543 (C═C) cm^{-1}; ^1H NMR (DMSO-d6): δ 1.18–1.21 (m, 2H, CH_2), 1.38–1.48 (m, 2H, CH_2), 1.56-1.62 (m, 2H, CH_2), 1.66–1.75 (m, 2H, CH_2), 2.95–3.01 (t, 2H, CH_2), 3.03–3.06 (t, 2H, CH_2), 3.30–3.40 (m, 4H, −C***H₂***–N(C_6H_5)–C***H₂***–), 3.55–3.62 (m, 4H, −C***H₂***–N–C***H₂***–), 6.80–6.84 (t, 1H, *J* = 7.2 Hz, ArH), 6.96–6.99 (t, 2H, *J* = 7.2 Hz,ArH), 7.22–7.27 (m, 2H, *J* = 7.2 Hz, ArH), 7.51–7.55 (dd, 1H, *J* = 6.2 Hz, pyridyl H), 8.68 (d, 2H, *J* = 6.2 Hz, pyridyl H) and 9.54 (s, 1H, pyridyl H) ppm; ^{13}C NMR (DMSO-d_6): δ 24.98, 25.42, 26.03, 27.49, 31.20, 31.76, 42.89, 45.67, 48.52, 50.81, 116.15, 116.44, 119.20, 119.76, 120.31, 124.12, 129.71, 130.16, 133.19, 135.29, 139.56, 149.38, 150.55, 151.30, 155.22, 162.60, 168.56 ppm; MS [*m*/*z*, %]: 455 [M•$^+$, 64.04] and 440 [M•$^+$−CH_3, 59.65]. Anal. Calcd for $C_{27}H_{29}N_5S$ (455.62): C, 71.18; H, 6.42; N, 15.37. Found: C, 71.25; H, 6.48; N, 15.51.

*4.1.5.6. 2-(1-Naphthyl)-4-(4-phenylpiperazin-1-yl)-5,6,7,8,9,10-hexa-hydrocycloocta [4,5]thieno[2,3-d]pyrimidine (**6f**).* mp 195–196 °C; yield 20%; IR (KBr) ν_{max}: 1598 (C═N), 1560 (C═C) cm^{-1}; ^1H NMR (DMSO-d6): δ 1.15–1.21 (m, 2H, CH_2), 1.40–1.49 (m, 2H, CH_2), 1.63–1.75 (m, 4H, 2 CH_2), 2.99-3.04 (m, 2H, CH_2), 3.12–3.20 (m, 6H, CH_2 and −C***H₂***–N(C_6H_5)–C***H₂***–), 3.30-3.40 (m, 4H, −C***H₂***–N–C***H₂***–), 6.82–6.87 (t, 1H *J* = 7.5 Hz, ArH), 6.96–7.02 (d, 2H, *J* = 7.5 Hz, ArH), 7.21–7.29 (t, 2H, *J* = 7.5 Hz, ArH), 7.55–7.66 (m, 3H, naphthyl H), 8.01-8.06 (m, 2H, *J* = 7.5 Hz, naphthyl H), 8.15 (d, 1H, *J* = 7.5 Hz, naphthyl H) and 8.85 (d, 1H, *J* = 6 Hz, naphthyl H) ppm; MS [*m*/*z*, %]: 504 [M•$^+$, 37.60]. Anal. Calcd for $C_{32}H_{32}N_4S$ (504.69): C, 76.15; H, 6.39; N, 11.10. Found: C, 76.32; H, 6.47; N, 11.23.

*4.1.6. General procedure for the preparation of 2-aryl-4-hydrazinyl-5,6,7,8,9,10-hexahydrocycloocta[4,5]thieno[2,3-d]pyrimidines (**7a**–**b**)*

A mixture of the selected chloro derivative **4a**–**b** (0.002 mol) and hydrazine hydrate (99%, 0.62 g, 0.012 mol) in absolute ethanol (20 mL) was refluxed for 6 h. The reaction mixture was then cooled and the precipitate was filtered, dried and crystallized from ethanol.

*4.1.6.1. 4-Hydrazinyl 2-(3-pyridyl) -5,6,7,8,9,10-hexahydrocycloocta-[4,5]thieno[2,3-d]pyrimidine (**7a**).* mp 277–278 °C; yield 75%; IR (KBr) ν_{max}: 3358,3250 (NH/NH_2) cm^{-1}; ^1H NMR (DMSO-d6): δ 1.19–1.22 (m, 2H, CH_2), 1.38–1.46 (m, 2H, CH_2), 1.60–1.70 (m, 4H, $2CH_2$), 2.89–2.94 (m, 2H, CH_2), 3.03–3.09 (m, 2H, CH_2), 7.00 (br.s, 2H, NH_2, D_2O exchangeable), 7.02 (s, 1H, NH, D_2O exchangeable), 7.48–7.55 (m, 1H, pyridyl H), 8.41 (d, 1H, *J* = 6 Hz, pyridyl H), 8.61 (d, 1H, *J* = 6 Hz, pyridyl H) and 9.45 (s, 1H, pyridyl H) ppm; MS [*m*/*z*, %]: 325 [M•$^+$, 100] and 309 [M$^+$−NH_2, 68]. Anal. Calcd for $C_{17}H_{19}N_5S$ (325.43): C, 62.74; H, 5.88; N, 21.52. Found: C, 62.82; H, 5.94; N, 21.68.

*4.1.6.2. 4-Hydrazinyl 2-(1-naphthyl) -5,6,7,8,9,10-hexahydrocycloocta[4,5]thieno[2,3-d]pyrimidine (**7b**).* mp 200–202 °C; yield 72%; IR (KBr) ν_{max}: 3295,3150 (NH/NH_2) cm^{-1}; ^1H NMR (DMSO-d6): δ 1.20–1.25 (m, 2H, CH_2), 1.42–1.49 (m, 2H, CH_2), 1.65–1.75 (m, 4H, 2 CH_2), 2.90–2.94 (t, 2H, *J* = 6.0 Hz, CH_2), 3.06–3.10 (t, 2H, *J* = 6.0 Hz, CH_2), 4.80 (s, 2H, NH_2, D_2O exchangeable), 7.53–7.58 (m, 3H, naphthyl H), 7.62 (d, 1H, *J* = 7.5 Hz, naphthyl H), 7.97–8.00 (t, 1H, *J* = 7.5 Hz, naphthyl H), 8.03 (s, 1H, NH, D_2O exchangeable), 8.12 (d, 1H, *J* = 7.5 Hz, naphthyl H) and 8.83 (d, 1H, *J* = 7.5 Hz, naphthyl H) ppm; MS [*m*/*z*, %]: 374 [M•$^+$, 75.73], 358 [M•$^+$−NH_2] and 343 [M•$^+$−$NHNH_2$, 100]. Anal. Calcd for $C_{22}H_{22}N_4S$ (374.50): C, 70.56; H, 5.92; N, 14.96. Found: C, 70.63; H, 5.97; N, 15.13.

*4.1.7. General procedure for the preparation of 5-aryl-3-sulfanyl-8,9,10,11,12,13-hexahydrocycloocta[4,5]thieno[3,2-e]-1,2,4-triazolo-[4,3-c]pyrimidines (**8a**–**b**)*

To a warmed ethanolic sodium hydroxide solution, prepared by dissolving sodium hydroxide (0.08 g, 0.002 mol) in absolute ethanol (10 mL) compounds **7a**–**b** (0.002 mol) and excess carbon disulphide (2 mL) were added. The reaction mixture was refluxed in a water bath at 80 °C for 10 h, then cooled, poured into ice cold water (20 mL), neutralized by dilute acetic acid. The precipitate was filtered, dried and crystallized from ethanol.

*4.1.7.1. 5-(3-Pyridyl) -3-sulfanyl-8,9,10,11,12,13-hexahydrocycloocta [4,5]thieno[3,2-e]-1,2,4-triazolo[4,3-c]pyrimidines (**8a**).* mp 230–231 °C; yield 40%; IR (KBr) ν_{max}: 3421(NH), 3060 (SH), 1182 (C═S) cm^{-1}; ^1H NMR (DMSO-d6): δ 1.23–1.40 (m, 2H, CH_2), 1.41–1.50 (m, 2H, CH_2), 1.61–1.80 (m, 4H, $2CH_2$), 2.87–2.91 (t, 2H, *J* = 6.0 Hz, CH_2), 3.00–3.07 (t, 2H, *J* = 6.0 Hz, CH_2), 7.45–7.56 (m, 1H, pyridyl H), 8.04 (d, 1H, *J* = 6.0 Hz, pyridyl H), 8.44 (d, 1H, *J* = 6.0 Hz, pyridyl H), 9.21 (s, 1H, pyridyl H) and 14.53 (s, 1H, SH, D_2O exchangeable) ppm; MS [*m*/*z*, %]: 367 [M•$^+$, 28.42]. Anal. Calcd for $C_{18}H_{17}N_5S_2$ (367.49): C, 58.83; H, 4.66; N, 19.06. Found: C, 58.94; H, 4.64; N, 19.13.

*4.1.7.2. 5-(1-Naphthyl)-3-sulfanyl-8,9,10,11,12,13-hexahydrocycloocta[4,5]thieno[3,2-e]-1,2,4-triazolo[4,3-c]pyrimidines (**8b**).* mp 202–204 °C; yield 80%; IR (KBr) ν_{max}: 3431 (NH), 3056 (SH), 1180 (C=S) cm^{-1}; ^1H NMR (DMSO-d6): δ 1.40–1.45 (m, 2H, CH_2), 1.46–1.55 (m, 2H, CH_2), 1.64–1.75 (m, 2H, CH_2),1.76–1.82 (m, 2H, CH_2), 3.01–3.04 (t, 2H, J = 6.0 Hz, CH_2), 3.19–3.23 (t, 2H, J = 6.0 Hz, CH_2), 7.42–7.63 (m, 5H, naphthyl H), 7.97 (d, 1H, J = 8.4 Hz, naphthyl H), 8.03 (d, 1H, J = 8.1 Hz, naphthyl H) and 14.38 (br.s, 1H, SH, D_2O exchangeable) ppm. Anal. Calcd for $C_{23}H_{20}N_4S_2$ (416.56): C, 66.32; H, 4.84; N, 13.45. Found: C, 66.39; H, 4.89; N, 13.58.

*4.1.8. General procedure for the preparation of 5-aryl-3-(substituted-thio)-8,9,10,11,12,13-hexahydrocycloocta[4,5]thieno[3,2-e]-1,2,4-triazolo[4,3-c]pyrimidines (**9a**–**d**)*

Ethyl iodide or benzyl chloride (0.0025 mol) was added to **8a**–**b** (0.0025 mol), dissolved in a solution of potassium hydroxide (0.14 g, 0.0025 mol) in absolute ethanol (6 mL). The reaction mixture was heated under reflux for 10 h, cooled, diluted with ice cold water (50 mL) and the separated solid was filtered, dried and crystallized from ethanol.

*4.1.8.1. 3-(Ethylthio) 5-(3-pyridyl) -8,9,10,11,12,13-hexahydrocycloocta[4,5]thieno[3,2-e]-1,2,4-triazolo[4,3-c]pyrimidine (**9a**).* mp 232–234 °C; yield 60%; IR (KBr) ν_{max}: 1653 (C=N) cm^{-1}; ^1H NMR (DMSO-d6): δ 1.20–1.26 (m, 2H, CH_2), 1.28–1.33 (t, 3H, J = 7.2 Hz, $CH_2C\boldsymbol{H_3}$), 1.40–1.51 (m, 2H, CH_2), 1.59–1.71 (m, 4H, $2CH_2$), 2.73–2.80 (t, 2H, CH_2), 2.82–2.90 (t, 2H, CH_2), 3.14–3.18 (q, 2H, J = 7.2 Hz, $C\boldsymbol{H_2}CH_3$), 7.56–7.60 (m, 1H, 3-pyridyl H), 8.41 (d, 1H, 3-pyridyl H), 8.68 (d, 1H, 3-pyridyl H) and 9.18 (s, 1H, 3-pyridyl H) ppm. Anal. Calcd for $C_{20}H_{21}N_5S_2$ (395.54): C, 60.73; H, 5.35; N, 17.71. Found: C, 60.89; H, 5.39; N, 17.92.

*4.1.8.2. 3-(Ethylthio) 5-(1-naphthyl)-8,9,10,11,12,13-hexahydrocycloocta[4,5]thieno[3,2-e]-1,2,4-triazolo[4,3-c]pyrimidine (**9b**).* mp 90–92 °C; yield 46%; IR (KBr) ν_{max}: 1608 (C=N) cm^{-1}; ^1H NMR (DMSO-d6): δ 1.25–1.30 (t, 3H, J = 7.5 Hz, $CH_2C\boldsymbol{H_3}$), 1.35–1.42 (m, 2H, CH_2), 1.45–1.55 (m, 2H, CH_2), 1.70–1.80 (m, 2H, CH_2), 1.81–1.85 (m, 2H, CH_2), 3.05–3.09 (m, 4H, J = 7.2 Hz, $2CH_2$), 3.30–3.40 (q, J = 7.5 Hz, 2H, $C\boldsymbol{H_2}CH_3$), 7.48–7.50 (m, 1H, naphthyl H), 7.59–7.62 (m, 1H, naphthyl H), 7.67–7.70 (m, 1H, naphthyl H), 7.85 (d, 1H, naphthyl H), 7.95 (d, 1H, naphthyl H) 8.07 (d, 1H, J = 8.1 Hz, naphthyl H) and 8.10–8.30 (t, 1H, naphthyl H) ppm; MS [m/z, %]: 444 [$M^{\bullet+}$, 100], 429 [$M^{\bullet+}-CH_3$, 32.30] and 416 [$M^{\bullet+} - C_2H_5^+$ 56.14]. Anal. Calcd for $C_{25}H_{24}N_4S_2$ (444.61): C, 67.53; H, 5.44; N, 12.60. Found: C, 67.59; H, 5.47; N, 12.72.

*4.1.8.3. 3-(Benzylthio)-5-(3-pyridyl)-8,9,10,11,12,13-hexahydrocycloocta[4,5]thieno[3,2-e]-1,2,4-triazolo[4,3-c]pyrimidine (**9c**).* mp 160–161 °C; yield 68%; IR (KBr) ν_{max}: 1608 (C=N) cm^{-1}; ^1H NMR (DMSO-d6): δ 1.30–1.40 (m, 2H, CH_2), 1.41-1.51 (m, 2H, CH_2), 1.68–1.80 (m, 4H, $2CH_2$), 3.01-3.09 (m, 2H, CH_2), 3.28–3.36 (m, 2H, CH_2), 4.54 (s, 2H, $C\boldsymbol{H_2}$–S–), 7.22–7.34 (m, 3H, J = 7.2 Hz, ArH), 7.51 (d, 2H, J = 7.5 Hz, ArH), 7.63-7.67 (t, 1H, J = 7.8 Hz, pyridyl H), 8.70 (d, 1H, J = 8.1 Hz, pyridyl H), 8.78 (d, 1H, J = 7.8 Hz, pyridyl H) and 9.49 (s, 1H, pyridyl H) ppm; ^{13}C NMR (DMSO-d_6): δ 24.93, 25.77, 26.27, 26.66, 29.78, 32.68, 36.77, 113.56, 124.37, 128.01, 128.85, 129.03, 129.22, 129.37, 130.75, 133.36, 135.39, 136.14, 137.21, 148.49, 153.14, 161.48 ppm. Anal. Calcd for $C_{25}H_{23}N_5S_2$ (457.61): C, 65.62; H, 5.07; N, 15.30. Found: C, 65.79; H, 5.13; N, 15.48.

*4.1.8.4. 3-(Benzylthio)-5-(1-naphthyl)-8,9,10,11,12,13-hexahydrocycloocta[4,5]thieno[3,2-e]-1,2,4-triazolo[4,3-c]pyrimidine (**9d**).* mp 153–156 °C; yield 83%; IR (KBr) ν_{max}: 1602 (C=N) cm^{-1}; ^1H NMR (DMSO-d6): δ 1.30–1.41 (m, 2H, CH_2), 1.42–1.51 (m, 2H, CH_2), 1.67–1.78 (m, 2H, CH_2), 1.79–1.88 (m, 2H, CH_2), 3.03–3.07 (t, 2H, J = 6.0 Hz, CH_2), 3.31–3.35 (m, 2H, J = 6.0 Hz, CH_2), 4.29 (s, 2H, $C\boldsymbol{H_2}$–S–), 7.17–7.21 (t, 2H, ArH), 7.23 (d, J = 6.9 Hz, 2H, ArH), 7.45–7.50 (t, 1H, J = 6.9 Hz, ArH), 7.58–7.63 (t, 1H, J = 7.2 Hz, naphthyl H), 7.67–7.74 (m, 3H, J = 7.8 Hz, naphthyl H), 7.92 (d, 1H, J = 7.2 Hz, naphthyl H) and 8.09 (d, 1H, J = 7.8 Hz, naphthyl H), 8.20 (d, 1H, J = 7.5 Hz, naphthyl H) ppm. Anal. Calcd for $C_{30}H_{26}N_4S_2$ (506.68): C, 71.11; H, 5.17; N, 11.06. Found: C, 71.23; H, 5.22; N, 11.19.

4.2. Measurement of anticancer activity

Anticancer activity screening of the newly synthesized compounds was measured *in vitro* utilizing 59 different human cancer cell lines provided by US National Cancer Institute according to previously reported standard procedure [29–31] as follows:

Cells were seeded into 96 well microtiter plates in a density of 5000–1000 cell/100 μL/well. Cells then were incubated at 37 °C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to addition of experimental drugs.

After 24 h, two plates of each cell line were fixed *in situ* with TCA, to represent a measurement of the cell population for each cell line at the time of drug exposure (Tz). Experimental drugs were solubilized in dimethyl sulfoxide (DMSO) at 400-fold the desired final maximum test concentration and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate was thawed and diluted to twice the desired final maximum test concentration with complete medium containing 50 μg/mL gentamicin. Additional four, 10-fold or ½ log serial dilutions were made to provide a total of five drug concentrations plus control. Aliquots of 100 μL of these different drug dilutions were added to the appropriate microtiter wells containing 100 μL of medium, resulting in the required final drug concentrations.

Following drug addition, the plates were incubated for an additional 48 h at 37 °C, 5% CO_2, 95% air, and 100% relative humidity. For adherent cells, the assay is terminated by the addition of cold TCA. Cells were fixed *in situ* by the gentle addition of 50 μL of cold 50% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4 °C. The supernatant was discarded, and the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (100 μL) at 0.4% (w/v) in 1% acetic acid was added to each well, and plates were incubated for 10 min at room temperature. After staining, unbound dye was removed by washing five times with 1% acetic acid and the plates were air dried. Bound stain was subsequently solubilized with 10 mM trizma base, and the absorbance was obtained using an automated plate reader at a wavelength of 515 nm. For suspension cells, we used the same methodology except that the assay was terminated by fixing settled cells at the bottom of the wells by gently adding 50 μL of 80% TCA (final concentration, 16% TCA). Using the seven absorbance measurements [time zero, (Tz), control growth, (C), and test growth in the presence of drug at the five concentration levels (Ti)], the percentage growth was calculated at each of the drug concentrations levels. Percentage growth inhibition is calculated as:

$$[(Ti - Tz)/(C - Tz)] \times 100 \text{ for concentrations for which } Ti \geq Tz$$

$$[(Ti - Tz)/Tz] \times 100 \text{ for concentrations for which } Ti < Tz.$$

For each experimental agent. Growth inhibition of 50% (GI_{50}) is calculated from $[(Ti - Tz)/(C - Tz)] \times 100 = 50$, which is the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation, however, if the effect is not reached or is exceeded, the value for that parameter is expressed as greater or less than the maximum or minimum concentration tested.

4.3. Cell cycle analysis

4.3.1. Cell culture

Human breast cancer cell line MDA-MB-468 cells, were obtained from the National Cancer Institute (Cairo, Egypt). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 mg/mL of streptomycin, 100 units/mL of penicillin and 10% of heat-inactivated fetal bovine serum in a humidified, 5% (v/v) CO_2 atmosphere at 37 °C.

4.3.2. DNA-flow cytometry analysis

MDA-MB-468 cells at a density of 4×10^6 cell/T 75 flask were exposed to compound **9c** at 0.568 μM concentration for 24 h. The cells then were collected by trypsinization, washed with phosphate buffered saline (PBS) and fixed in ice-cold absolute alcohol. Thereafter, cells were stained using Cycle TESTTM PLUS DNA Reagent Kit (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. Cell cycle distribution was determined using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA).

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