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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis and anticancer activities of novel (tetrahydrobenzo [4,5] thieno [2,3-d] pyrimidine-4-yl)-pyrolidine-2-carboxylic acid derivatives

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Abstract Cancer chemotherapy has been one of the major medical advances in the last few decades. The medications involved in treatment have a narrow therapeutic index, and often the responses produced are only just palliative as well as unpredictable. In contrast, targeted therapy such as Tyrosine kinases inhibitors, that has been introduced in recent years is directed against cancer-specific molecules and signaling pathways with some side effects Therefore, we studied these class of molecules to obtain new candidates of remarkable activities and minimal side effects. We herein reported SAR driven synthesis of 16 novel molecules and their anticancer profiles in specifically targeted human Breast cell line MCF-7 and Human Colon Cancer Cell Line HCT-15. Compounds **7e** and **8f** showed descent antitumor activities when compared to Doxorubicine ($GI_{50} < 0.1$) for human Breast cell line MCF-7. The synthesized analogs were ensured by spectral and elemental analysis.

Keywords Thieno pyrimidine · Proline · Gewald reaction · MCF-7 · HCT-15 · Antitumor activities

Introduction

Cancer disease has major health issue on mankind around the globe. The relative rate of death by cancer is very high even in the developed countries. Surprisingly, among all malignant cancer, breast cancer is leading in second position. Alcohol-related cirrhosis, and nonalcoholic fatty liver disease allied with obesity, is a major cause of liver cancer cases in US and another western countries.

Clinically, cancer is known as a malignant neoplasm and is a broad group of various diseases all involving unregulated cell growth. Rapid and uncontrolled cell growth leads to malignant tumors and occupy the nearby parts of the body. The cancer may also spread to more distant parts of the body through the lymphatic system or blood stream. There are over 200 different known cancers that afflict humans. Cancer can develop in almost any organ or tissue, such as the lung, colon, breast, skin, bones, or nerve tissue. Cancer can be detected in a number of ways including presence of certain signs and symptoms, screening test, or medical imaging. Once a possible cancer is detected, it is diagnosed by microscopic examination of a tissue sample. Cancer is usually treated with chemotherapy, radiation therapy, and surgery.

Tyrosine kinases are orally active, small molecules that have a favorable safety profile and can be easily combined with other forms of chemotherapy or radiation therapy. Several tyrosine kinase inhibitors (TKIs) such as *gefitinib* (Paez et al., 2004) (Fig. 1) *erlotinib* (Higgins et al., 2004) (Fig. 1) and *lapatinib* (Johnston and Leary, 2006) (Fig. 1) have gained market approval worldwide. TKIs are thus an important new class of targeted therapy that interfere with specific cell signaling pathways and thus allow target specific therapy for selected malignancies.

Pyrimidine and its fused ring system is present in cytosine, adenine, guanine, and thiamine, which form a part of ribonucleic acid, deoxyribonucleic acid, vitamins and coenzymes, and other purines. A pyrimidine nucleus fused with another heterocycle act as a core moiety in the

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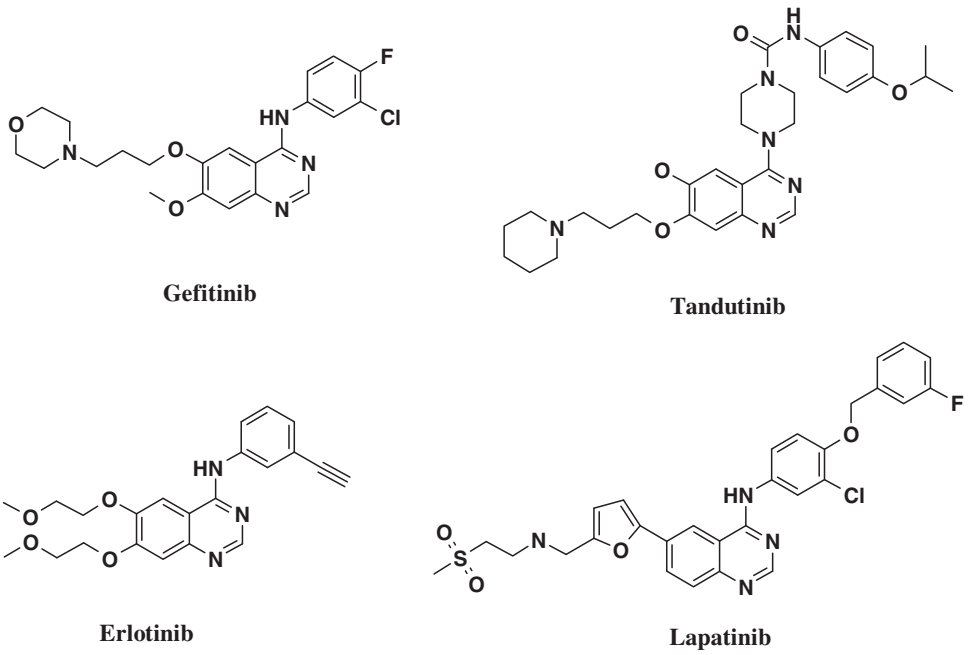


Fig. 1 Representative examples of anticancer quinazoline compounds

discovery of bioactive molecules (Adepu et al., 2012). Hydrazide and their heterocyclic products show evidence of diverse biological activities including antibacterial, antifungicidal, analgesic, antituberculosis, anticancer, anti-inflammatory properties (Shiradkar et al., 2007; Gursoy and Guzeldemirci-Ulusoy, 2007; Gemma et al., 2006). In addition the thieno [2,3-d] pyrimidine core, which is evaluated as a bioisostere of the quinazoline core, was used in the mechanism-based design and synthesis of new antitumor agents (Habib et al., 2013).

A number of thieno [2,3-d] pyrimidine derivatives with different substituent at C-2 and C-4 positions were found to exert potential antitumor activity (Pedeboscq et al., 2010; Beckers et al., 2012). It is observed from the literature that C-4 position must contain a hetero atom such as primary nitrogen [Gefitinib (*Iressa*)] (Fig. 1) or secondary nitrogen containing side chains on the quinazoline moiety [Tandutinib (*MLN518*)] (Fig. 1) to have antitumor potency. From the literature study it was observed that fusion of five, six, or seven member cycloalkyl lipophilic moieties conserved the antitumor activity (Amr et al., 2006; Pedeboscq et al., 2010; Aponte et al., 2010; Golub et al., 2011; Lou et al., 2011) as exhibited by the 4-sustituted 5,6,7,8-tetrahydrobenzo [4,5]thieno [2,3-d] pyrimidine **6**.

In our current manuscript we have prepared a series of new thieno [2,3-d] pyrimidines by introducing a five member cycloalkyl ring containing secondary nitrogen (L-proline) at C-4 position and synthesized pharmacophoric moieties of interest such as amides and hydrazides that proved to contribute to antitumor activities such as **7e** and **8f**.

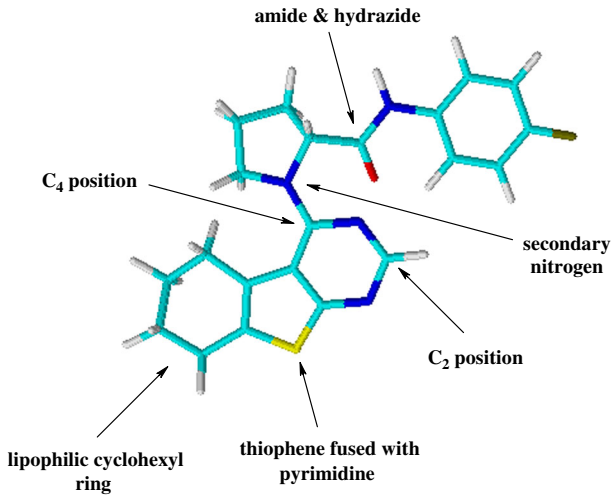


Fig. 2 Essential elements of synthesized compounds

Tandutinib (Fig. 1) consist of C-4 substituted piperazine attached to the quinazoline moiety and formed urea derivative with the propoxy aniline.

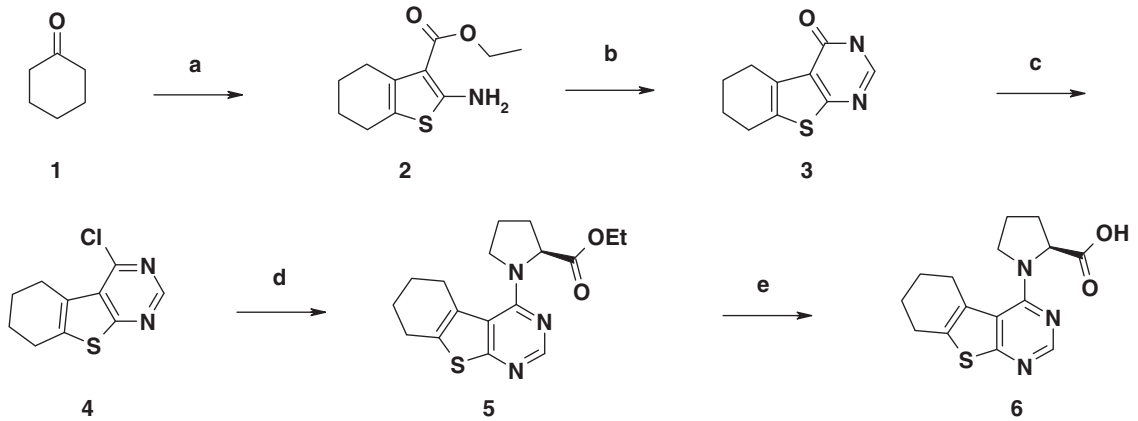
We design the molecules after modifying the C-4 substitution by secondary nitrogen containing amino acid L-proline and used the substituted anilines like gefitinib, lapatinib and also substituted piperazine side chains to form terminal amides replacing urea. (Fig. 2)

Hydrazide derivatives were also prepared to compare its relationship with amide derivatives. We studied the structural modifications of thieno [2,3-d] pyrimidine by aiming to obtain the new candidates of remarkable antitumor activities.

Chemistry

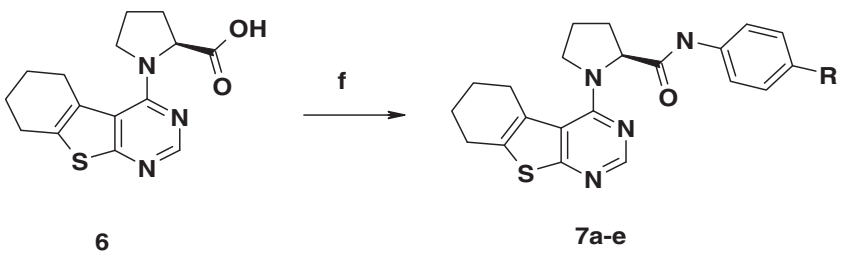
The targeted compounds were prepared from acid intermediate **6** as outlined in Scheme 1. The starting material ethyl-2-amino-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate **2** was prepared by following the method of Gewald (Gewald, 1965; Sabnis et al., 1999) via the reaction of cyclohexanone **1** and sulfur with ethyl cynoacetate in the presence of morpholine. Cyclization of **2** to **3** is adopted using the reported reaction condition by refluxing it in formamide (Nirogi et al., 2011). The desired 4-chloro derivative **4** was obtained via the reaction **3** with phosphorous oxychloride in reflux (Perspicace et al., 2009). L-Proline ethyl ester was introduced by replacing chloro group of thieno [2,3-d] pyrimidine **4** in methanol to obtain the ester intermediate **5**. Tetrahydrobenzo [4,5]thieno [2,3-d] pyrimidine-4yl-pyrrolidine-2-carboxylic acid **6** was obtained after the alkaline hydrolysis of ester intermediate **5** by using lithium hydroxide.

^{1}H NMR spectrum of **2** revealed the presence of triplet signal at δ 1.33 ppm and quartet signal at δ 4.25 ppm corresponds to ethyl group of ester, and singlet of two protons at δ 5.94 ppm corresponds to amino group. Similarly, ^{1}H NMR spectrum of **3** was identified by the broad singlet at δ 12.31 and singlet at δ 8.00 ppm of the pyrimidin-4(3H)-one. Infrared (IR) spectrum of **3** showed bands at 3363, 3383, and 1639 cm^{-1} corresponding to the NH and C=O groups, respectively. Chloro intermediate **4** was assigned by the shifted single signal to δ 8.72 ppm. Crude ester intermediate **5** was used directly for hydrolysis reaction, its formation was confirmed by the ESI-MS spectrum showing ES-MS: *m/z* 332.3 $(M+H)^+$. Acid intermediate **6** shows a characteristic triplet signal at δ 4.80 ppm corresponds to chiral proton of L-proline and pyrimidine proton at δ 8.32 ppm. Novel amides containing substituted anilines **7a–e** and substituted piperazines **8a–f** were prepared by coupling selected amines with the acid core **6**. Hydrazide derivatives containing aromatic hydrazide **9a–e** were also prepared from **6**. Secondary amines such as substituted piperazines and primary amines such as substituted anilines were selected to evaluate the structure activity relationship among the novel analog. Hydrazide derivatives containing



Scheme 1 Synthetic route for *1-(5,6,7,8-tetrahydro-benzo[4,5]thieno [2,3-d]pyrimidin-4-yl)-pyrrolidine-2-carboxylic acid* (**6**). Reagents and solvents: **a** ethyl cynoacetate, sulphur, morpholine, EtOH, Reflux; **b** formamide; **c** $POCl_3$ **d** L-proline ethyl ester hydrochloride, Et_3N, MeOH **e**. LiOH.H2O, THF: H_2O

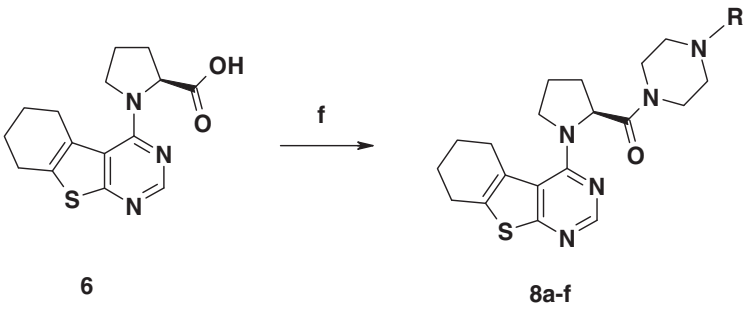
Scheme 2 Synthetic route for *1-(5,6,7,8-tetrahydro-benzo[4,5] thieno[2,3-d]pyrimidin-4-yl)-pyrrolidine-2-carboxylic acid phenylamides* (**7a–e**)



f. R-Anilines, EDC.HCl/HOBt, DMF, RT

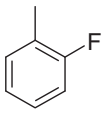
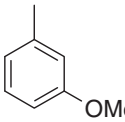
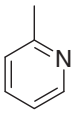
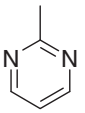
Compound	7a	7b	7c	7d	7e
R	H	CH_3	OCH_3	Cl	F

Scheme 3 Synthetic route for *Piperazin-1-yl-[1-(5,6,7,8-tetrahydro-benzo[4,5]thieno [2,3-d]pyrimidin-4-yl)-pyrrolidin-2-yl]-methanone* (**8a–f**)

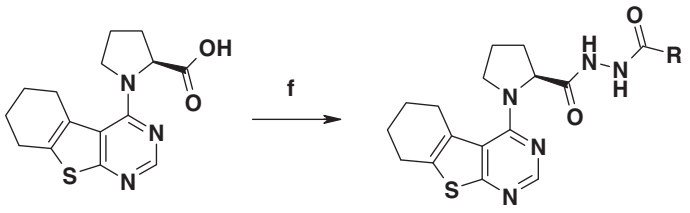


6 8a-f

f. R-Piperazines, EDC.HCl/HOBt, DMF, RT

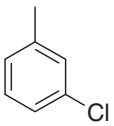
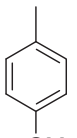
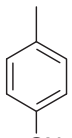
Compound	8a	8b	8c	8d	8e	8f
R	CH_3	Ph				

Scheme 4 Synthetic route for *1-(5,6,7,8-tetrahydro-benzo[4,5] thieno[2,3-d]pyrimidin-4-yl)-pyrrolidine -2-carboxylic acid N'-aryl-hydrazide* (**9a–e**)



6 9a-e

f. R-Hydrazides, EDC.HCl/HOBt, DMF, RT

Compound	9a	9b	9c	9d	9e
R					

aromatic hydrazide were also studied for anticancer activities. (Schemes 2, 3, and 4)

Material and methods

Experimental

All the raw materials were obtained commercially and used without further purification. IR spectra were recorded on MIRacle10 shimadzu IR Affinity-1 instrument. ^{1}H NMR spectra were recorded using $CDCl_3$ and DMSO-d_6 as solvent with tetramethylsilane as an internal standard on Varian 400-MHz instruments. Elecronspray ionization-mass spectra were recorded on LC-MS/MS Waters (Aquity) TQ detector instrument, Elemental analysis is reported from Vario Micro Elementar instrument. Melting points were taken on Veego melting point apparatus model VMP-D.

*Ethyl 2-amino-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (**2**)*

To a mixture of cyclohexanone (49 g, 0.5 mol), ethyl 2-cyanoacetate (56 g, 0.5 mol), and sulphur (16 g, 0.5 mol) in 150 ml of ethanol was added morpholine (44 g, 0.5 mol). The mixture was stirred for 8 h at room temperature. The reaction mixture was diluted with water and the precipitate was collected by filtration and recrystallized from ethanol. Compound **2** as yellow solid (62 g, 55 %); m.p. = 115 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.94(s, 2H, Ar–$\underline{NH_2}$), 4.25

(q, 2H, $J = 7$ Hz, –O–C<u>H</u>$_2$–), 2.69–2.60 (m, 2H, –CH$_2$–C<u>H</u>$_2$–CH$_2$–), 2.49–2.43(m, 2H, –CH$_2$–C<u>H</u>$_2$–CH$_2$–), 1.76–1.66 (m, 4H, –CH$_2$–C<u>H</u>$_2$–CH$_2$–), 1.33(t, 3H, $J = 7$ Hz, –OCH$_2$–C<u>H</u>$_3$); ES-MS: *m/z* 226.3 $(M+H)^+$.

*5,6,7,8-Tetrahydrobenzo [4,5]thieno [2,3-d] pyrimidin-4 (3H)-one (**3**)*

The mixture of compound **2** (35 g, 0.16 mol) in 150 ml of formamide was heated at 180 °C for 4 h and cooled down. The mixture was poured into 200 ml water and filtered. The solid was collected and recrystallized from ethanol. Compound **3** was obtained as a yellow solid (25 g, 75 %); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 12.31 (br s, 1H, –CO–N<u>H</u>–), 8.00 (s, 1H, =C<u>H</u>–N–), 2.85–2.88 (m, 2H, –CH$_2$–C<u>H</u>$_2$–CH$_2$–), 2.72–2.75 (m, 2H, –CH$_2$–C<u>H</u>$_2$–CH$_2$–), 1.75–1.82 (m, 4H, –CH$_2$–C<u>H</u>$_2$–CH$_2$–); ES-MS: *m/z* 207.2 $(M+H)^+$.

*4-Chloro-5,6,7,8-tetrahydrobenzo [4,5]thieno[2,3-d] pyrimidin-4(3H)-one (**4**)*

A suspension of compound **3** (25 g, 0.12 mol) in 150 ml of $POCl_3$ was heated at reflux for 2 h. $POCl_3$ was removed at reduced pressure and the residue was poured onto ice and filtered. The solid was washed with water and dried. Compound **4** was obtained as a brown solid (23 g, 85 %); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.72 (s, 1H), 3.10–3.12 (m, 2H, –CH$_2$–C<u>H</u>$_2$–CH$_2$–), 2.88–2.90 (m, 2H, –CH$_2$–C<u>H</u>$_2$–CH$_2$–), 1.75–1.92–1.95 (m, 4H, –CH$_2$–C<u>H</u>$_2$–CH$_2$–); ES-MS: *m/z* 225.3 $(M+H)^+$.

*1-(5,6,7,8-Tetrahydrobenzo[4,5]thieno[2,3-d] pyrimidine-4-yl)-pyrolidine-2-carboxylic acid ethyl ester (**5**)*

To a clear solution of compound **4** (20 g, 0.089 mol) in methanol 200 ml was added L-proline ethyl ester hydrochloride (16 g, 0.089 mol) and triethylamine (27 ml, 0.267 mol) , and the reaction mixture was stirred for 3 h. Methanol was removed under reduced pressure and residue was taken in EtOAc, washed with water, 1 N HCl solution in water and saturated $NaHCO_3$ solution in water. Collected organic layer was dried over Na_2SO_4 and removed under reduced pressure to yield ester intermediate as a yellowish gel (25 g, 84 %) **5**. This was used without further purification. ES-MS: *m/z* 332.3 $(M+H)^+$.

*1-(5,6,7,8-Tetrahydrobenzo[4,5]thieno[2,3-d] pyrimidine-4-yl)-pyrolidine-2-carboxylic acid (**6**)*

A suspension of ester compound **5** (25 g, 0.075 mol) in 225 ml THF and 25 ml water was added to lithium hydroxide monohydrate (4.2 g, 0.11 mol) at 0 °C, and the reaction mixture was stirred for 12 h. THF was distilled out under vacuum and to the remaining aqueous residue was added 1 N HCl solution in water to adjust the solution pH = 4, the solid was precipitated out. The solid was filtered and dried to obtain compound **6** as a yellowish solid (16 g, 70 %); IR (KBr) ν_{max} 3458, 3253, 2939, 1708, 1589, 1553, 1456, 1338, 1219, 1060, 920, 769 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.23(s, 1H, pyrimidine–<u>H</u>), 4.80 (t, 1H, $J = 8$ Hz, –N–<u>CH</u>–CO), 3.82–3.89 (m, 2H, –N–C<u>H</u>$_2$–CH$_2$–), 2.80–2.86 (m, 4H, –CH$_2$–C<u>H</u>$_2$–C<u>H</u>$_2$–), 1.75–1.98 (m, 6H, –CH$_2$–C<u>H</u>$_2$–CH$_2$–), 1.49–1.50 (m, 2H, CH$_2$–C<u>H</u>$_2$–CH$_2$–); ES-MS: *m/z* 302.2 $(M+H)^-$.

*1-(5,6,7,8-Tetrahydrobenzo[4,5]thieno[2,3-d] pyrimidine-4-yl)-pyrolidine-2-carboxylic acid phenyl-amide (**7a**)*

To a solution of 1-(5,6,7,8-tetrahydro-benzo[4,5]thieno[2,3-d] pyrimidine-4-yl)-pyrolidine-2-carboxylic acid **6** (1 g, 0.0033 mol) in DMF (5 ml) was added EDC.HCl (0.95 g, 0.0050 mol) and aniline (0.306 g, 0.0033 mol) followed by the HOBt (0.443 g, 0.0033 mol). The reaction mixture was stirred for 6 h and quenched with water (50 ml); the brown solid that came out was filtered, dried, and washed with diethyl ether to give compound **7a** as an off-white solid (0.82 g, yield = 66 %); m.p. = 127–129 °C; IR (KBr) ν_{max} 3264, 2931, 1674, 1538, 1522, 1450, 1217, 1149, 1037, 851 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 10.0 (s, 1H, –CO–N<u>H</u>–), 8.21 (s, 1H, pyrimidine–<u>H</u>), 7.59 (d, $J = 8$ Hz, 2H, Ar–<u>H</u>), 7.27 (t, 2H, $J = 8$ Hz, Ar–<u>H</u>), 7.0–7.02 (t, 1H, $J = 7.6$ Hz, Ar–<u>H</u>), 4.96 (t, 1H, $J = 7.6$ Hz, N–C<u>H</u>–CO), 3.92–3.96 (m, 1H, –Ar–C<u>H</u>$_2$–CH$_2$–), 3.69 (t, 1H, $J = 8$ Hz, –N–C<u>H</u>$_2$–CH$_2$–), 2.85–3.01 (m, 4H, –CH$_2$–C<u>H</u>$_2$–CH$_2$–), 2.33–2.38 (m, 1H, –CH$_2$–C<u>H</u>$_2$–CH$_2$–), 1.72–1.98 (m, 6H, –CH$_2$–C<u>H</u>$_2$–CH$_2$–), 1.49–1.51 (m, 1H, –CH$_2$–C<u>H</u>$_2$–CH$_2$–); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 22.3, 22.9, 25.3 , 25.4, 28.6, 29.8, 53.8, 62.6, 117.2, 119.3, 119.3, 123.1, 127.8, 128.6, 128.6, 131.8, 139.1, 150.4, 157.5, 167.1, 170.7; ES-MS: *m/z* 379.3 $(M+H)^+$; anal. calcd. for $C_{21}H_{22}N_4OS$; C, 66.64; H, 5.86; N, 14.80; found: C, 66.61; H, 5.89; N, 14.82.

*1-(5,6,7,8-Tetrahydrobenzo[4,5]thieno[2,3-d] pyrimidine-4-yl)-pyrolidine-2-carboxylic acid p-tolylamide (**7b**)*

Brownish solid (0.85 g, yield = 66 %); m.p. = 136–138 °C; IR (KBr) ν_{max} 3234, 2918, 1664, 1530, 1508, 1436, 1210, 1146, 1037, 843 cm^{-1} ; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 9.9 (s, 1H, –CO–N<u>H</u>–), 8.12 (s, 1H, pyrimidine–<u>H</u>), 7.46 (d, $J = 9.6$ Hz, 2H, Ar–<u>H</u>), 7.08 (d, 2H, $J = 8.4$ Hz, Ar–<u>H</u>), 4.95 (t, 1H, $J = 7.2$ Hz, –N–C<u>H</u>–CO), 3.89–3.94 (m, 1H, Ar–C<u>H</u>$_2$–CH$_2$–), 3.68 (t, $J = 7.6$ Hz, 1H, –N–C<u>H</u>$_2$–CH$_2$–), 2.85–2.97 (m, 4H, –C<u>H</u>$_2$–C<u>H</u>$_2$–CH$_2$–), 2.32–2.35 (m, 1H, –CH$_2$–C<u>H</u>$_2$–CH$_2$–), 2.0 (s, 3H, –C<u>H</u>$_3$), 1.75–1.98 (m, 6H, –C<u>H</u>$_2$–C<u>H</u>$_2$–C<u>H</u>$_2$–), 1.47–1.51 (m, 1H, –CH$_2$–C<u>H</u>$_2$–CH$_2$–);

^{13}C NMR (DMSO-d_6, 100 MHz) δ 20.4, 22.3, 22.9 , 25.4, 25.8, 28.6, 29.5, 53.8, 62.5, 117.2, 119.3, 119.3, 127.8, 128.9, 128.9, 131.8, 131.9, 136.6, 150.4, 157.5, 167.1, 170.5; ES-MS: *m/z* 393.3 $(M+H)^+$; anal. calcd. for $C_{22}H_{24}N_4O_2S$; C, 64.68; H, 5.92; N; 13.71; found: C, 64.66; H, 5.91; N, 13.74.

*1-(5,6,7,8-Tetrahydrobenzo[4,5]thieno[2,3-d] pyrimidine-4-yl)-pyrolidine-2-carboxylic acid (4-methoxy-phenyl)-amide (**7c**)*

Yellowish solid (0.9 g, yield = 67 %); m.p. = 135–137 °C; IR (KBr) ν_{max} 3264, 2948, 1674, 1532, 1516, 1442, 1216, 1152, 1057, 841 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 9.82(s, 1H, –CO–NH–), 8.20 (s, 1H, pyrimidine–H), 7.45–7.47 (m, 2H, Ar–H), 6.82–6.84 (m, 2H, Ar–H), 4.92 (t, 1H, –N–CH–CO), 3.84–3.87 (m, 1H), 3.66–3.67 (s, 1H), 3.68 (s,3H, –OCH$_3$), 2.83–2.89 (m, 4H, –CH$_2$–CH$_2$–CH$_2$–), 2.53–2.56 (m, 1H, –CH$_2$–CH$_2$–CH$_2$–), 1.72–1.89 (m, 6H, –CH$_2$–CH$_2$–CH$_2$–), 1.48–1.49 (m, 1H, –CH$_2$–CH$_2$–CH$_2$–); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 22.2, 22.8, 25.3 , 25.8, 28.5, 29.5, 53.8, 55.1, 62.4, 113.7, 113.7, 117.3, 120.8, 120.8, 127.8, 131.7, 132.2, 150.3, 155.1, 157.5, 167.1, 170.2; ES-MS: *m/z* 408.3 $(M+H)^+$; anal. calcd. for $C_{22}H_{24}N_4O_2S$; C, 64.68; H, 5.92; N; 13.71; found: C, 64.66; H, 5.91; N, 13.74.

*1-(5,6,7,8-Tetrahydrobenzo[4,5]thieno[2,3-d] pyrimidine-4-yl)-pyrolidine-2-carboxylic acid (4-chloro-phenyl)-amide (**7d**)*

0.92 g, yield = 67 %; m.p. = 139–141 °C; IR (KBr) ν_{max} 3265, 2945, 1668, 1530, 1512, 1446, 1210, 1145, 1060, 835 cm^{-1} ; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 10.01(s, 1H, –CO–NH–), 8.21 (s, 1H, pyrimidine–H), 7.62 (d, 2H, J = 7.2 Hz, Ar–H), 7.33 (d, 2H, J = 9.2 Hz Ar–H), 4.94 (t, 1H, J = 8 Hz, –N–CH–CO), 3.92–3.94 (m, 1H, Ar–CH$_2$–CH$_2$–), 3.70 (t, J = 7.2 Hz, 1H, –N–CH$_2$–CH$_2$–), 2.74–2.96 (m, 5H, –CH$_2$–CH$_2$–CH$_2$–), 2.0–2.35 (m, 1H, –CH$_2$–CH$_2$–CH$_2$–), 1.87–2.0 (m, 5H, –CH$_2$–CH$_2$–CH$_2$–), 1.47–1.50 (m, 1H, , –CH$_2$–CH$_2$–CH$_2$–); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 21.4, 22.3, 22.9 , 25.4, 25.7, 28.6, 29.6, 53.8, 62.5, 115.4, 117.8, 119.3, 127.4, 127.9, 132.8, 136.6, 150.2, 157.5, 159.4, 167.1, 170.5; ES-MS: *m/z* 413.4 $(M+H)^+$; anal. calcd. for $C_{21}H_{21}ClN_4OS$; C, 61.08; H, 5.13; N; 13.57; found: C, 61.09; H, 5.17; N, 13.54.

*1-(5,6,7,8-Tetrahydrobenzo[4,5]thieno[2,3-d] pyrimidine-4-yl)-pyrolidine-2-carboxylic acid (4-fluoro-phenyl)-amide (**7e**)*

Brownish solid (0.85 g, yield = 65 %); m.p. = 131–133 °C; IR (KBr) ν_{max} 3261, 2943, 1668, 1527, 1510, 1448, 1215, 1143, 1058, 833 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 10.05(s, 1H, –CO–NH–), 8.21 (s, 1H, pyrimidine–H), 7.59 (d, J = 4.8 Hz, 2H, Ar–H), 7.11 (t, J = 8.4 Hz, 2H, Ar–H), 4.94 (t, 1H, –N–CH–CO, J = 8.8 Hz), 3.91–3.94 (m, 1H, Ar–CH$_2$–CH$_2$), 3.71 (t, 1H, J = 7.6 Hz, –N–CH$_2$–CH$_2$–), 2.85–2.97 (m, 4H, –CH$_2$–CH$_2$–CH$_2$–), 2.32–2.36 (m, 1H, –CH$_2$–CH$_2$–CH$_2$–), 1.74–1.98 (m, 6H, –CH$_2$–CH$_2$–CH$_2$–), 1.50–1.51 (m, 1H, –CH$_2$–CH$_2$–CH$_2$–); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 22.3, 22.8, 25.3, 25.8, 28.5, 29.8, 53.8, 62.5, 115.0, 115.3, 117.3, 120.9, 121.0, 127.8, 131.8, 135.5, 150.4, 157.5, 159.0, 167.1, 170.6 ; ES-MS: *m/z* 397.3 $(M+H)^+$; anal. calcd. for $C_{21}H_{21}FN_4OS$; C, 63.62; H, 5.34; N, 14.13; found: C, 63.66; H, 5.37; N, 14.11.

*(4-Methyl-piperazine-1-yl)-[1-(5,6,7,8-tetrahydro-benzo [4,5]thieno[2,3-d] pyrimidine-4-yl)-pyrolidine-2-yl]-methanone (**8a**)*

To a solution of 1-(5,6,7,8-tetrahydro-benzo [4,5]thieno [2,3-d] pyrimidine-4-yl)-pyrolidine-2-carboxylic acid **6** (1 g, 0.0033 mol) in DMF (5 ml) was added EDC.HCl (0.950 g, 0.0050 mol) and 1-methyl piperazine (0.331 g, 0.0033 mol) followed by the HOBt (0.443 mg, 0.0033 mol). The reaction mixture was stirred for 6 h and was quenched with water (50 ml), solid that comes out was filtered, dried, and washed with diethyl ether to give compound **8a** as a yellowish solid, (0.80 g, yield = 62 %); m.p. = 80–82 °C, IR (KBr) ν_{max} 3418, 2905, 1635, 1548, 1548, 1431, 1361, 1230, 948, 742 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.20 (s, 1H, pyrimidine–H), 5.38 (t, 1H, N–CH–CO), 3.51–3.56 (m, 4H, –CH$_2$–CH$_2$–CH$_2$–), 3.28–3.31 (m, 2H, –CH$_2$–CH$_2$–CH$_2$–), 2.72–2.83 (m, 4H, –CH$_2$–CH$_2$–CH$_2$–), 2.62–2.68 (m, 4H, –CH$_2$–CH$_2$–CH$_2$–), 2.31 (m, 3H, –CH$_3$), 1.94–1.99 (m, 5H, –CH$_2$–CH$_2$–CH$_2$–), 1.72–1.82 (m, 3H, –CH$_2$–CH$_2$–CH$_2$–), 1.45–1.48 (m, 1H, –CH$_2$–CH$_2$–CH$_2$–); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 22.3, 23.5, 24.2, 25.8, 28.6, 28.6, 43.6, 47.3, 47.6, 49.2, 48.8, 54.6, 54.6, 58.2, 127.6, 131.5, 145.7, 155.9, 167.1, 170.4; ES-MS: *m/z* 386.1 $(M+H)^+$; anal. calcd. for $C_{20}H_{27}N_5OS$; C, 62.31; H, 7.06; N, 18.17; found: C, 62.33; H, 7.03; N, 18.21.

*(4-Phenyl-piperazine-1-yl)-[1-(5,6,7,8-tetrahydro-benzo [4,5]thieno[2,3-d] pyrimidine-4-yl)-pyrolidine-2-yl]-methanone (**8b**)*

Yellowish solid (0.80g, yield = 62 %); m.p. = 86–88 °C; IR (KBr) ν_{max} 3428, 2915, 2843, 1665, 1578, 1542, 1428, 1359, 1228, 948, 768 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.16 (s, 1H, pyrimidine–H), 7.23 (t, 2H, J = 8.4Hz, Ar–H), 6.96 (s, 1H, Ar–H), 6.98 (d, 1H, J = 8.4Hz, Ar–H), 6.82 (t, 1H, J = 8.4Hz, Ar–H), 5.38 (t, 1H, J = 8Hz, N–CH–CO), 3.81–3.86 (m, 3H, –CH$_2$–CH$_2$–CH$_2$–), 3.67–3.71 (m, 1H, –CH$_2$–CH$_2$–CH$_2$–), 3.60 (br s, 2H, –CH$_2$–CH$_2$–CH$_2$–),

3.28–3.31 (m, 2H, –CH_2–$C\underline{H}_2$–CH_2–). 3.14–3.17 (m, 2H, –CH_2–$C\underline{H}_2$–CH_2–), 3.09 (br s, 1H, –CH_2–$C\underline{H}_2$–CH_2–), 2.83–2.88 (m, 3H, –CH_2–$C\underline{H}_2$–CH_2–). 2.31–2.32 (m, 1H, –CH_2–$C\underline{H}_2$–CH_2–), 1.94–1.99 (m, 3H, –CH_2–$C\underline{H}_2$–CH_2–), 1.72–1.99 (m, 3H, –CH_2–$C\underline{H}_2$–CH_2–), 1.45–1.48 (m, 1H, –CH_2–$C\underline{H}_2$–CH_2–); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 22.3, 22.8, 25.3 , 25.8, 28.6, 28.6, 41.5, 44.9, 48.5, 48.8, 58.2, 58.2, 115.8, 116.8, 119.3, 127.6, 128.9, 128.9, 131.5, 131.6, 150.4, 150.8, 156.9, 167.1, 170.2; ES-MS: *m/z* 448.4 $(M+H)^+$; anal. calcd. for $C_{25}H_{29}N_5OS$; C, 67.09; H, 6.53; N, 15.65; found: C, 67.07; H, 6.57; N, 15.67.

*[4-(2-Fluoro-phenyl)-piperazine-1-yl)-[1-(5,6,7,8-tetrahydro-benzo[4,5]thieno[2,3-d] pyrimidine-4-yl)-pyrolidine-2-yl]-methanone (**8c**)*

Yellow solid (1.2 g, yield = 62 %), m.p. = 83–85 °C; IR (KBr) ν_{max} 3435, 2938, 2863, 1660, 1598, 1546, 1438, 1357, 1224, 968, 788 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.19 (s, 1H, pyrimidine–H), 7.00–7.18 (m, 4H, Ar–H), 5.37 (t, 1H, *J* = 8Hz, N–C$\underline{H}$–CO), 3.82–3.90 (m, 3H), 3.72 (t, 3H, *J* = 6.8Hz, –N–$C\underline{H}_2$–CH_2–), 3.03–3.14 (m, 3H, –CH_2–$C\underline{H}_2$–CH_2–), 2.83–2.94 (m, 5H, –$C\underline{H}_2$–$C\underline{H}_2$–CH_2–). 2.32 (br s, 1H, –CH_2–$C\underline{H}_2$–CH_2–), 1.94–1.98 (m, 3H, –CH_2–$C\underline{H}_2$–CH_2–), 1.76–1.81 (m, 3H, –CH_2–$C\underline{H}_2$–CH_2–), 1.47–1.49 (m, 1H, –CH_2–$C\underline{H}_2$–CH_2–); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 22.3, 22.8, 25.3 , 25.9, 28.6, 40.2, 41.7, 45.2, 50.2, 50.7, 58.3, 58.3, 115.9, 116.1, 119.6, 119.6, 122.7, 122.8, 124.9, 127.7, 131.8, 150.4, 156.9, 167.1, 170.2; ES-MS: *m/z* 466.4 $(M+H)^+$; anal. calcd. for $C_{25}H_{28}FN_5OS$; C, 64.49; H, 6.06; N, 15.04; found: C, 64.51; H, 6.08; N, 15.07.

*[4-(3-Methoxy-phenyl)-piperazine-1-yl)-[1-(5,6,7,8-tetrahydro-benzo[4,5]thieno[2,3-d] pyrimidine-4-yl)-pyrolidine-2-yl]-methanone (**8d**)*

Brownish solid (900 mg, yield = 57 %); m.p. = 89–91 °C; IR (KBr) ν_{max} 3415, 2918, 2859, 1657, 1589, 1526, 1438, 1351, 1214, 978, 789 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.22 (s, 1H, pyrimidine–H), 6.98–7.18 (m, 2H, Ar–H), 6.38–6.42 (m, 2H, Ar–H), 5.37 (t, 1H, *J* = 8.4 Hz, –N–C$\underline{H}$–CO–), 3.85–3.91 (m, 3H, –CH_2–$C\underline{H}_2$–CH_2–), 3.62–3.72 (m, 3H, –CH_2–$C\underline{H}_2$–CH_2–), 3.83 (s, 3H, –O$C\underline{H}_3$) 3.01–3.12 (m, 3H, –CH_2–$C\underline{H}_2$–CH_2–), 2.83–2.94 (m, 5H, –CH_2–$C\underline{H}_2$–CH_2–). 2.38 (br s, 1H, –CH_2–$C\underline{H}_2$–CH_2–), 1.94–1.98 (m, 3H, –CH_2–$C\underline{H}_2$–CH–), 1.76–1.80 (m, 3H, –CH_2–$C\underline{H}_2$–CH_2–), 1.47–1.49 (m, 1H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 22.3, 23.5, 23.5, 24.2, 25.3 , 25.9, 28.6, 40.2, 42.7, 45.2, 50.2, 50.7, 55.8, 58.3, 115.9, 118.1, 119.6, 122.7, 124.9, 127.7, 131.8, 150.4, 156.9, 161.6, 167.1, 170.2; ES-MS: *m/z* 478.2 $(M+H)^+$; anal. calcd. for $C_{26}H_{31}N_5O_2S$; C, 65.38; H, 6.54; N, 14.66; found: C, 65.37; H, 6.58; N, 14.69.

*(4-Pyridine-2-yl-piperazine-1-yl)-[1-(5,6,7,8-tetrahydro-benzo[4,5]thieno[2,3-d] pyrimidine-4-yl)-pyrolidine-2-yl]-methanone (**8e**)*

Yellowish solid (850 mg, yield = 57 %); m.p. = 90–92 °C; IR (KBr) ν_{max} 3425, 2927, 2856, 1653, 1587, 1527, 1439, 1348, 1228, 985, 799 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.39 (d, 2H, *J* = 8.4Hz, Ar–H), 8.18 (s, 1H, Ar–H), 7.44(s, 1H, Ar–H), 6.66–6.68 (m, 2H, Ar–H), 5.37 (t, 1H, *J* = 8.4Hz, –N–C$\underline{H}$–CO–), 3.73–3.89 (m, 7H, –$C\underline{H}_2$–$C\underline{H}_2$–$C\underline{H}_2$–), 3.54 (br s, 2H, –CH_2–$C\underline{H}_2$–CH_2–), 2.83–2.89 (m, 4H, –CH_2–$C\underline{H}_2$–$C\underline{H}_2$–), 2.35–2.38 (m, 1H, –CH_2–$C\underline{H}_2$–CH_2–). 1.91–2.01 (m, 3H, –CH_2–$C\underline{H}_2$–CH_2–), 1.78–1.82 (m, 3H, –CH_2–$C\underline{H}_2$–CH_2–), 1.45–1.49 (m, 1H, –CH_2–$C\underline{H}_2$–CH_2–); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 22.3, 22.8, 25.3 , 25.9, 28.6, 28.6,28.6, 42.3, 41.7, 43.2, 44.7, 53.4, 58.3, 110.4, 116.9, 126.4, 132.5, 151.6, 156.9, 157.9, 157.9, 163.2, 167.1, 170.5; ES-MS: *m/z* 448.3 $(M+H)^+$; anal. calcd. for $C_{24}H_{28}N_6OS$; C, 64.26; H, 6.29; N, 18.73; found: C, 64.23; H, 6.23; N, 18.75.

*(4-Pyrimidine-2-yl-piperazine-1-yl)-[1-(5,6,7,8-tetrahydro-benzo[4,5]thieno[2,3-d] pyrimidine-4-yl)-pyrolidine-2-yl]-methanone (**8f**)*

Yellowish solid (1.3 g, yield = 87 %); m.p. = 88–90 °C ; IR (KBr) ν_{max} 3421, 2931, 2854, 1651, 1585, 1529, 1442, 1355, 1226, 981, 796 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.39 (d, 2H, *J* = 2 Hz, Ar–H), 8.18 (s, 1H, Ar–H), 6.67 (d, 1H, *J* = 4.8 Hz, Ar–H), 5.37 (t, 1H, *J* = 8.4 Hz, –N–C$\underline{H}$–CO–), 3.71–3.91 (m, 8H, –CH_2–$C\underline{H}_2$–CH_2–), 3.54 (br s, 2H, –CH_2–$C\underline{H}_2$–CH_2–), 2.83–2.89 (m, 4H, –CH_2–$C\underline{H}_2$–CH_2–), 2.31–2.35 (m, 1H, –CH_2–$C\underline{H}_2$–CH_2–), 1.91–2.01 (m, 3H, –CH_2–$C\underline{H}_2$–CH_2–), 1.70–1.82 (m, 3H, –CH_2–$C\underline{H}_2$–CH_2–), 1.45–1.49 (m, 1H, –CH_2–$C\underline{H}_2$–CH_2–); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 22.3, 22.8, 25.3 , 25.9, 28.6, 28.6, 40.2, 41.5, 43.2, 44.7, 53.4, 58.3, 110.4, 116.9, 127.7, 131.5, 150.4, 156.9, 157.9, 157.9, 161.1, 167.1, 170.5; ES-MS: *m/z* 450.3 $(M+H)^+$; anal. calcd. for $C_{23}H_{27}N_7OS$; C, 61.45; H, 6.05; N, 21.81; found: C, 61.47; H, 6.08; N, 21.83.

*3-Chloro-benzoic acid N′-[1-(5,6,7,8,-tetrahydro-benzo [4,5]thieno[2,3-d] pyrimidine-4-yl)-pyrrolidine-2-carbonyl]-hydrazide (**9a**)*

To a solution of 1-(5,6,7,8-tetrahydro-benzo[4,5]thieno[2,3-d] pyrimidine-4-yl)-pyrolidine-2-carboxylic acid **6** (1 g, 0.0033 mmol) in DMF (5 ml) was added EDC.HCl (0.950 g, 0.0050 mol) and 3-chloro-benzoic acid hydrazide

(0.561 g, 0.0033 mol) followed by the HOBt (0.443 g, 0.0033 mol). The reaction mixture was stirred for 6 h and quenched with water (50 ml); the off-white color solid that came out was filtered, dried, and washed with diethyl ether to give compound **9a** (1.3 g, yield = 87 %); m.p. = 115–117 °C; IR (KBr) ν_{max} 3228, 3064, 2935, 1716, 1647, 1531, 1446, 1323, 1236, 1138, 964, 742 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 10.45 (s, 1H, –NH–NH–), 10.05 (s, 1H, –NH–NH–), 8.26 (s, 1H, pyrimidine–H), 7.88 (d, 1H, J = 1.6 Hz, Ar–H), 7.81 (d, 1H, J = 8 Hz, Ar–H), 7.62–7.64 (m, 1H, Ar–H), 7.50–7.54 (m, 1H, Ar–H), 4.95 (t, 1H, J = 8 Hz, –N–CH–CO–), 3.90–3.94 (m, 1H, $–CH_2–CH_2–CH_2–$), 3.65–3.69 (m,1H, $–CH_2–CH_2–CH_2–$), 3.11–3.15 (m, 2H, $–CH_2–CH_2–CH_2–$), 2.33–2.40 (m, 2H, $–CH_2–CH_2–CH_2–$), 1.95–2.02 (m, 4H, $–CH_2–CH_2–CH_2–$), 1.74–1.79 (m, 4H, $–CH_2–CH_2–CH_2–$); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 23.3, 23.8, 25.3, 25.8, 28.6, 28.6, 52.4, 62.9, 115.9, 116.1, 122.8, 124.9, 127.7, 131.8, 132.5, 134.3, 135.2 151.4, 158.7, 164.3, 167.1, 170.2; ES-MS: m/z 455.3 $(M+H)^+$; anal. calcd. for $C_{22}H_{22}ClN_5O_2S$: C, 57.95; H, 4.86; N, 15.36; found: C, 57.93; H, 4.84; N, 15.33.

*4-Methoxy-benzoic acid N′-[1-(5,6,7,8,-tetrahydro-benzo [4,5]thieno[2,3-d] pyrimidine-4-yl)-pyrrolidine-2-carbonyl]-hydrazide (**9b**)*

1.1 g, yield = 74 %; m.p. = 89–91 °C; IR (KBr) ν_{max} 3225, 3066, 2938, 1718, 1650, 1533, 1448, 1323, 1236, 1137, 962, 740 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 10.20 (s,1H, –NH–NH–), 9.95 (s, 1H, –NH–NH), 8.14 (s, 1H, Ar–H), 7.85 (d, 2H, J = 8 Hz, Ar–H), 7.01 (d, 2H, J = 8.4 Hz, Ar–H), 4.93–4.97 (t, 1H, J = 8 Hz –N–CH–CO–), 3.95 (br s, 1H, $–CH_2–CH_2–CH_2–$), 3.80 (s, 3H), 3.77–3.78 (m, 1H, $–CH_2–CH_2–CH_2–$), 3.06–3.10 (m, 2H), 2.87 (br s, 3H, $–CH_2–CH_2–CH_2–$), 2.40 (br s, 1H, $–CH_2–CH_2–CH_2–$), 1.77–2.03 (m, 6H, $–CH_2–CH_2–CH_2–$); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 23.2, 23.8, 25.6, 25.9, 28.3, 28.6, 52.3, 55.9, 62.9, 114.5, 116.1, 123.1, 124.7, 126.8, 127.7, 132.8, 133.5, 151.4, 158.7, 162.2, 164.3, 167.1, 170.2; ES-MS: m/z 451.3 $(M+H)^+$; anal. calcd. for $C_{23}H_{25}N_5O_3S$: C, 61.18; H, 5.58; N, 15.51; found: C, 61.15; H, 5.55; N, 15.49.

*4-Cyno-benzoic acid N′-[1-(5,6,7,8,-tetrahydro-benzo[4,5] thieno[2,3-d] pyrimidine-4-yl)-pyrrolidine-2-carbonyl]-hydrazide (**9c**)*

Yellowish solid (0.9 g, yield = 61 %); m.p. = 117–119 °C; IR (KBr) ν_{max} 3228, 3069, 2978, 1738, 1670, 1553, 1468, 1343, 1246, 1139, 972, 748 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 10.59 (s, 1H, –NH–NH–), 10.02 (s, 1H, NH–NH–), 8.26 (s, 1H, Ar–H), 7.96–8.08 (m, 4H, Ar–H), 4.95 (t, 1H, J = 8 Hz, –N–CH–CO–), 3.90–3.96 (m, 1H, $–CH_2–CH_2–CH_2–$), 3.67 (t, 1H, J = 8 Hz, $–CH_2–CH_2–CH_2–$), 3.11–3.14 (m, 1H, $–CH_2–CH_2–CH_2–$), 2.85 (br s, 3H, $–CH_2–CH_2–CH_2–$), 2.67–2.74 (m, 1H, $–CH_2–CH_2–CH_2–$), 2.32–2.40 (m, 1H, $–CH_2–CH_2–CH_2–$); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 23.3, 24.2, 25.6, 25.9, 29.5, 28.6, 52.3, 62.9, 115.8, 116.1, 123.1, 124.7, 126.8, 127.7, 128.2, 132.8, 133.3, 152.3, 157.8, 162.2, 164.3, 167.1, 170.2; ES-MS: m/z 446.2 $(M+H)^+$; anal. calcd. for $C_{23}H_{22}N_6O_2S$: C, 61.87; H, 4.97; N, 18.82; found: C, 61.83; H, 4.94; N, 18.81.

*Furan-2-carboxylic acid N′-[1-(5,6,7,8,-tetrahydro-benzo [4,5]thieno[2,3-d] pyrimidine-4-yl)-pyrrolidine-2-carbonyl]-hydrazide (**9d**)*

Brownish solid (1.1 g, yield = 84 %); m.p. = 118–120 °C; IR (KBr) ν_{max} 3238, 3080, 2938, 1741, 1674, 1553, 1469, 1345, 1248, 1141, 976, 758 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 10.22 (s, 1H, –NH–NH–); 9.89 (s, 1H, –NH–NH), 8.22 (s, 1H, Ar–H), 7.86 (s, 1H, Ar–H), 7.23–7.225 (m, 1H, Ar–H), 6.68 (br s, 1H, Ar–H), 4.84 (t, 1H, J = 8Hz, –N–CH–CO–), 3.91–3.93 (m, 1H, $–CH_2–CH_2–CH_2–$), 3.73–3.76 (m, 1H, $–CH_2–CH_2–CH_2–$), 3.13–3.17 (m, 2H, $–CH_2–CH_2–CH_2–$), 2.95–2.98 (m, 2H, $–CH_2–CH_2–CH_2–$), 2.58–2.62 (m, 2H, $–CH_2–CH_2–CH_2–$), 2.25–2.45 (m, 3H, $–CH_2–CH_2–CH_2–$), 1.85–2.05 (m, 3H, $–CH_2–CH_2–CH_2–$); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 23.3, 23.5, 25.4, 25.7, 28.2, 28.5, 52.3, 55.9, 62.9, 111.7, 113.5, 126.8, 132.8, 146.1, 148.3, 157.3, 158.7, , 164.3, 167.1, 170.2; ES-MS: m/z 412.2 $(M+H)^+$; anal. calcd. for $C_{20}H_{21}N_5O_3S$: C, 58.38; H, 5.14; N, 17.02; found: C, 57.34; H, 5.12; N, 17.01.

*Thiophene-2-carboxylic acid N′-[1-(5,6,7,8,-tetrahydro-benzo[4,5]thieno[2,3-d] pyrimidine-4-yl)-pyrrolidine-2-carbonyl]-hydrazide (**9e**)*

Yellowish solid (1.2 g, yield = 85 %); m.p. = 122–124 °C; IR (KBr) ν_{max} 3239, 3075, 2931, 1751, 1678, 1557, 1471, 1346, 1249, 1140, 978, 753 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 10.18 (s, 1H, –NH–NH–), 9.85 (s, 1H, –NH–NH), 8.22 (s, 1H, Ar–H), 7.66 (s, 1H, Ar–H), 7.27–7.29 (m, 1H, Ar–H), 6.70 (br s, 1H, Ar–H), 4.81–4.83 (m, 1H, –N–CH–CO–), 3.91–3.93 (m,1H, $–CH_2–CH_2–CH_2–$), 3.73–3.76 (m, 1H, $–CH_2–CH_2–CH_2–$), 3.15–3.18 (m, 2H, $–CH_2–CH_2–CH_2–$), 2.95–2.98 (m, 2H, $–CH_2–CH_2–CH_2–$), 2.58–2.62 (m, 2H, $–CH_2–CH_2–CH_2–$), 2.25–2.46 (m, 3H, $–CH_2–CH_2–CH_2–$), 1.89–2.03 (m, 3H, $–CH_2–CH_2–CH_2$); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 23.3, 23.5, 25.4, 25.7, 28.2, 28.5, 52.3, 55.9, 62.9, 123.1, 124.7, 126.8, 127.7, 132.8, 133.5, 158.7, 162.2, 164.3, 167.1, 170.2; ES-MS: m/z 428.2 $(M+H)^+$; anal. calcd. for $C_{20}H_{21}N_5O_2S_2$: C, 56.19; H, 4.95; N, 16.38; found: C, 56.16; H, 4.94; N, 16.35.

Biology

Antitumor activity

All the prepared target compounds **7a**–**7e**, **8a**–**8f**, and **9a**–**9e** were screened for their antitumor activities against breast MCF-7 cell line and colon HCT-15 cell lines at Anti-Cancer Drug screening facility, Tata memorial centre, Navi Mumbai.

Experimental procedure for Sulforhodamine B (SRB) assay (Vichai and Kirtikara, 2006) The cell lines were grown in RPMI 1640 medium containing 10 % fetal bovine serum and 2 mM L-glutamine. For present screening experiment, cells were inoculated into 96-well microtiter plates in 90 µL at 5000 cells per well. After cell inoculation, the microtiter plates were incubated at 37 °C, 5 %CO_2, 95 % air and 100 % relative humidity for 24 h prior to addition of experimental drugs. Experimental drugs were solubilized in appropriate solvent to prepare stock of 10^{-2} concentration. At the time of experiment four 10-fold serial dilutions were made using complete medium. Aliquots of 10 µl of these different drug dilutions were added to the appropriate microtiter wells already containing 90 µl of medium, resulting in the required final drug concentrations.

After compound addition, plates were incubated at standard conditions for 48 h and assay was terminated by the addition of cold TCA. Cells were fixed in situ by the gentle addition of 50 µl of cold 30 % (w/v) TCA (final concentration, 10 % TCA) and incubated for 60 min at 4 °C. The supernatant was discarded; the plates were washed five times with tap water and air dried. SRB solution (50 µl) at 0.4 % (w/v) in 1 % acetic acid was added to each of the wells, and plates were incubated for 20 min at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing five times with 1 % acetic acid. The plates were air dried. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on an Elisa plate reader at a wavelength of 540 nm with 690 nm reference wavelength.

Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percent growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells * 100. Using the six absorbance measurements [time zero (Tz), control growth (C), and test growth in the presence of drug at the four concentration levels (Ti)], the percentage growth was calculated at each of the drug concentration levels. The dose response parameters were calculated for each test article. Growth inhibition of 50 % (GI_{50}) was calculated from [(Ti−Tz)/(C −Tz)] × 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. The drug concentration resulting in total growth inhibition (TGI) was calculated from Ti = Tz. The LC_{50} (concentration of drug resulting in a 50 % reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of cells following treatment is calculated from [(Ti−Tz)/Tz] × 100 = −50.

Values were calculated for each of these three parameters if the level of activity was reached; however, if the effect was not reached or was exceeded, the values for that parameter were expressed as greater or less than the maximum or minimum concentration tested.

GI_{50}	Growth inhibition of 50 % (GI50) calculated from [(Ti−Tz)/(C −Tz)] × 100 = 50, drug concentration resulting in a 50 % reduction in the net protein increase
TGI	Drug concentration resulting in TGI will calculated from Ti = Tz
LC_{50}	Concentration of drug resulting in a 50 % reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of 50 % cells following treatment is calculated from [(Ti−Tz)/Tz] × 100 = −50.

All tested compounds showing **TGI > 100** and **LC_{50} > 100** for both the breast MCF-7 and colon HCT-15 cell lines.

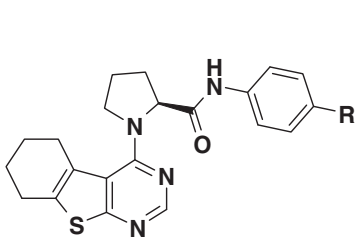
Results and discussion

A substituted aniline derivatives often improves the activity of different pharmacophores and hence plays an important role for various SAR studies. It was also found that these substituted aniline derivatives could be potential anticancer agents [Gefitinib (*Iressa*)] Fig. 1. With the above facts, here we have designed and prepared five novel compounds, (**7a**–**e**) (Table 1).

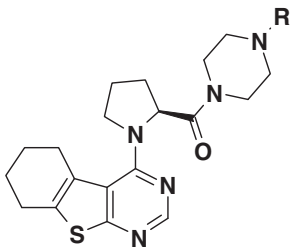
The variation was brought across acid core **6** with five different anilines to get the desired derivatives (**7a**–**e**). In the above series, 4-Fluoro aniline compound **7e** has shown remarkable inhibitory activity in MCF-7 cell line (GI_{50} < 0.1 µmol/L) and good inhibitory activity in HCT-15 cell line (GI_{50} = 56.9 µmol/L). All the other aniline derivatives showed very poor inhibitory activity in HCT-15 cell line. 4-Chloro aniline **7d** revealed inferior activity in MCF-7 cell line than the 4-Fluoro aniline compound **7e**. 4-Methoxy aniline **7c** found to show better activity than 4-Methyl aniline **7b** in MCF-7 cell line, for HCT-15 cell line both are equally inferior. Here, electron withdrawing group found to improving the potential of inhibiting the anticancer activities in MCF-7 cell line; therefore it was observed that compound **7e** and compound **7c** are superior to compound **7d** and compound **7b**, respectively.

Only compound **7d** and compound **7e** were showed notable contribution among the compounds **7a**–**e** in Human Colon Cancer Cell Line HCT-15. In conclusion, between

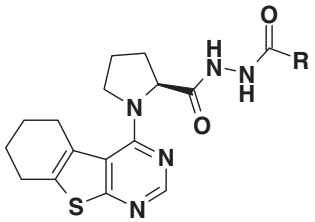
Table 1 GI_{50} of the tested compounds against human tumor cell line MCF-7 and HCT-15



7a-e



8a-f



9a-e

Compounds	R	GI_{50} (μmol/L)	
		Breast MCF-7	Colon HCT-15
7a	H	>100	>100
7b	CH_3	78.5	>100
7c	OCH_3	61.4	>100
7d	Cl	32.2	71.8
7e	F	**<0.1**	56.9
8a	CH_3	>100	>100
8b	Ph	24.0	67.3
8c	2-Fluoro phenyl	20.8	55.2
8d	3-Methoxy phenyl	68.7	>100
8e	Pyridine	20.3	71.8
8f	Pyrimidine	**<0.1**	63.5
9a	3-Chloro phenyl	>100	>100
9b	4-Methoxy phenyl	88.7	>100
9c	4-Cyno phenyl	>100	>100
9d	2-Furan	71.8	>100
9e	2-Thiophene	>100	>100
Reference	***Doxorubicine***	**<0.1**	**<0.1**

the two studied cell lines, compound **7e** was found to be potent for MCF-7 cell line.

Secondary nitrogen side chains on the quinazoline moiety [Tandutinib (*MLN518*)] Fig 1 exhibits antitumor potency when piperazine was coupled with aniline, to get the terminal urea. In order to enhance the activity, here we have modified our synthetic approach and accordingly prepared piperazine 4-substituted amide derivatives **8a–f** from same acid core **6**.

In our SAR study, 4-pyridinyl piperazine compound **8e**, showed intermittent activities in both the cell lines correspond to 4-pyrimidinyl piperazine compound **8f** and 4-phenyl piperazine compound **8b**. Insertion of the nitrogen atom in the aromatic ring attached to the piperazine ring increases the activity of compound **8b** from ($GI_{50} = 24.0$ μmol/L) to compound **8e** ($GI_{50} = 20.3$ μmol/L) for MCF-7 cell line and the trend continues still increase in inhibitory activity reaches to remarkable ($GI_{50} < 0.1$ μmol/L) value in compound **8f**. Alkyl substituted piperazine derivative **8a** could not make to contribute towards the inhibitory activity as compare to aryl substituted piperazine derivatives **8b**, **8c**, and **8d**. Between the substituted phenyl piperazine derivatives 4-(2-fluoro phenyl) piperazine compound **8c** and 4-(3-methoxy phenyl) piperazine compound **8d**, compound **8c** is more superior to compound **8d**. Electron withdrawing group increases inhibitory activity of **8c** and it is also comparable to compound **8d** in MCF-7 cell line.

For Human Colon Cancer Cell Line HCT-15 activities it was found that compound **8c** ($GI_{50} = 55.7$ μmol/L) is the good inhibitor among the **8a–f**. Compound **8b** ($GI_{50} = 67.3$ μmol/L) is inferior to **8c**, because the electron withdrawing fluoro group at second position of the phenyl ring was present in **8c**. Compound **8e** and compound **8f** followed the activity trend as they were followed for the MCF-7 cell line. Compound **8f** was superior to compound **8e** in HCT-15 cell line (Fig. 3).

Hydrazide side chains attached to the acid **6** was observed very poorly in showing anticancer activities in

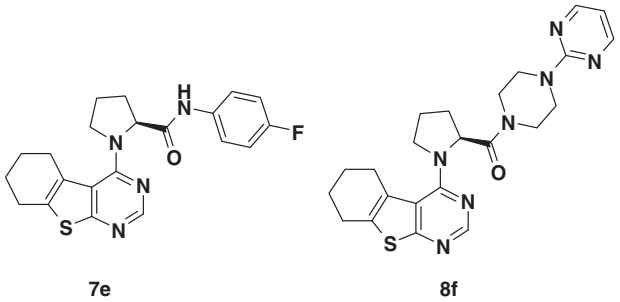


Fig. 3 Representative examples of primary evolutionary tumor cell inhibitors with $GI_{50} < 0.1$ in MCF-7 cell line

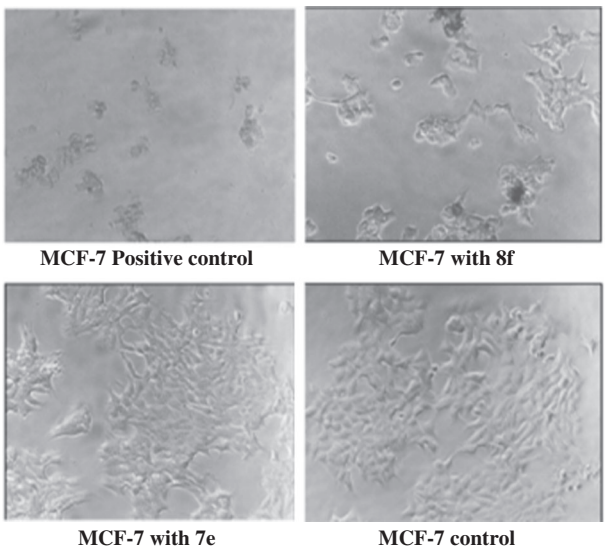


Fig. 4 Images of anticancer activities of **7e** and **8f**

Human Colon Cancer Cell Line HCT-15. All the synthesized compounds **9a**–**e** was reportedly showed ($GI_{50} > 100$ µmol/L). Compound **9b** ($GI_{50} = 88.7$ µmol/L) and compound **9d** ($GI_{50} = 71.8$ µmol/L) exhibiting the anticancer activities for the human Breast Cancer MCF-7 cell line. Compound **9b** contains 4-methoxy benzoic acid hydrazide side chain and compound **9d** was with 2-Furoic acid hydrazide side chain. Compounds **9b** and **9d** have an oxygen atom present in hydrazide analog and it might be resemblance to phenolic and ethereal type of oxygen containing Erlotinib (Fig. 1) to show some activities (Fig. 4).

Between the three series of compounds containing substituted anilines **7a**–**7e**, substituted piperazine **8a**–**8f** and hydrazide side chains **9a**–**9e**, it was found that secondary substituted piperazine derivatives are exhibiting good inhibitory activities in both the MCF-7 and HCT-15 cell lines. Primary substituted anilines resulted in improved activities in MCF-7 cell line than HCT-15 cell line and hydrazide compounds were very poor in both the MCF-7 and HCT-15 cell lines.

Conclusion

A series of novel tetrahydrobenzo [4,5]thieno [2,3-d] pyrimidine-4yl-pyrrolidine-2-carboxamides were synthesized by a facile six step procedure. Their structures were characterized by spectral and elemental analysis. The preliminary bioassay results imply that some of the compounds exhibit first-rate tumor cell inhibitor activity against human Breast MCF-7 and Colon HCT-15 cell lines. Compound **7e** and compound **8f** were the new finding from this research work and it will be studied further in near future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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