MEDICINAL CHEMISTRY RESEARCH

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ORIGINAL RESEARCH

Synthesis, biological screening and molecular docking studies of novel 4,6-pyrimidine derivatives as EGFR-TK inhibitors

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Abstract Novel 4, 6-disubstituted pyrimidine derivatives (5–16) were synthesized in four steps starting from 2,4-dichloropyrimidine and screened for their cytotoxicity using brine shrimp (*Artemia Salina*) lethality bioassay. The compounds such as 6, 11, 14 and 15 were found to be more toxic. The compounds were also studied for in vitro anticancer properties using six different cancer cell lines viz SIHA, PANC-1, MDA-MB-231, IMR-32, DU145 and A549. The compound 14 was effective inhibitor of SIHA and DU145, whereas compound 16 in Panc 1 and A549, compound 7 in MDA-MB-231 and compound 6 in IMR 32 respectively. Molecular docking studies were carried out using an X-ray crystallographic structure of epidermal growth factor receptor tyrosine kinase to explore the possible mode of action of compounds as epidermal growth factor receptor tyrosine kinase inhibitors.

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Introduction

Pyrimidine derivatives were found to possess a wide range of biological activities such as antifungal, anti-tubercular, antibacterial (Ahluwalia and Madhu, 1996; Singh et al., 2005; Shamroukh et al., 2005; Pasha et al., 2005), antiviral (Lin et al., 1988; Holy et al., 2002; Rakesh et al., 2002; Joule et al., 1995), anticancer (Skibo et al., 2002), anti-oxidant (Vidal et al., 2000), and anti-inflammatory (Alam et al., 2013). Four-substituted and 4,6-disubstituted pyrimidines bonded to a saturated heterocyclic unit at C-2 position of pyrimidine ring and related compounds have attracted much attention as potent 5-HT2A receptor ligands, including atypical antipsychotic, antidepressants, anxiolytics (Mokrosz et al., 1997; Glennon et al., 1997; Miyamoto et al., 2000), dual EGFR/ErbB-2 kinase inhibitors (Li et al., 2012), as potent HIV-1 NNRTIs (Yu et al., 2009), and TRPV1 antagonists (Stec et al., 2008). Some of the anticancer agents such as fluorouracil, gemcitabine, capecitabine cytrabine, cladribine and bleomycin had pyrimidine ring (Fig. 1).

Epidermal growth factor receptor tyrosine kinase (EGFR-TK) is one of the important therapeutic targets in human cancers. EGFR belongs to the ErbB family of TK proteins which includes four members: ErbB1/HER1, ErbB-2/Neu/HER2, ErbB-3/HER3 and ErbB-4/HER4 (Normanno et al., 2003). The participation of the EGFR family of TKs in cancer proliferation suggests that an inhibitor, which blocks the TK activity of the entire EGFR family, could have substantial therapeutic potential (Mendelsohn and Baselga, 2000). Gefitinib, erlotinib, lapatinib, canceratinib, PD 153035, AG 1295, AG 1385, etc. are examples of EGFR-TKIs, a biological target for human cancer. Owing to the significant biological activities of pyrimidine nucleus, we report herein the synthesis of
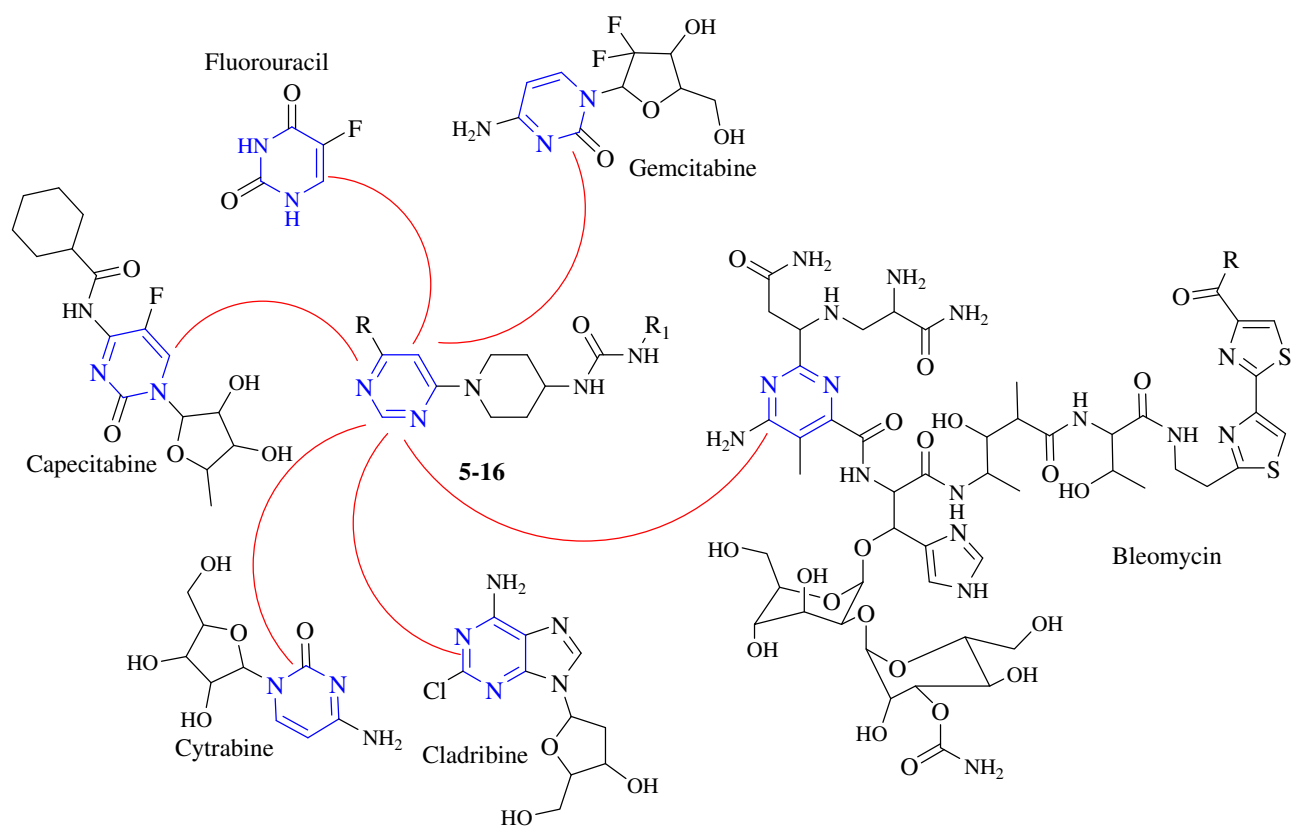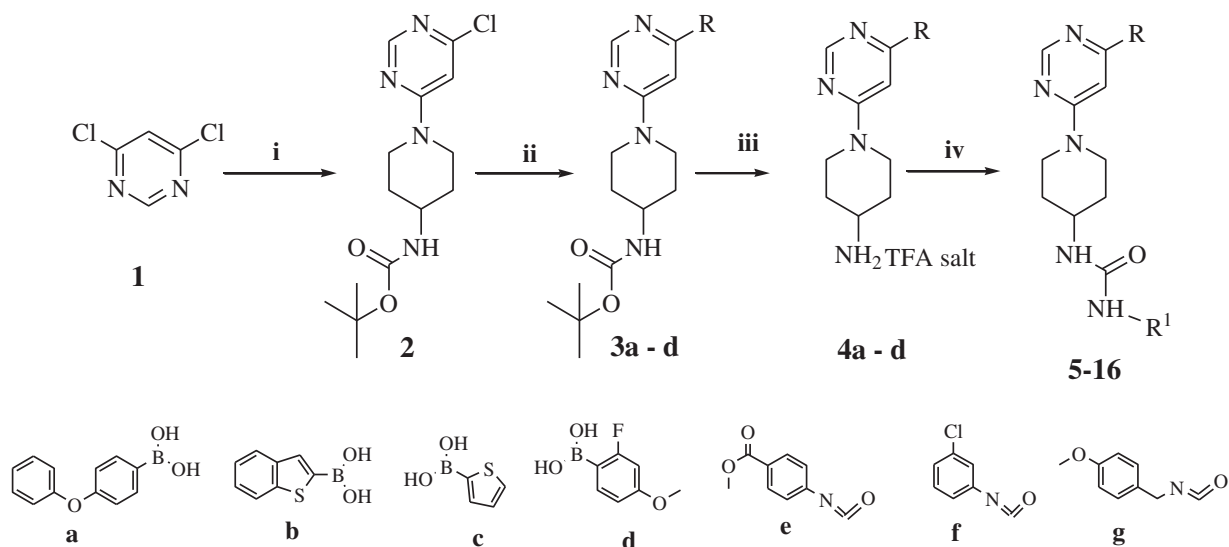
Fig. 1 Reported structures of some anticancer agents having pyrimidine ring and the synthesized title compounds (5–16)

Scheme 1 Synthesis of 4,6-disubstituted pyrimidine derivatives (5–16). *Reagents and conditions i* 4-N-Boc-Amio piperidine, DIPEA, THF, rt, 4h; *ii* a–d, Pd2(dba)3, X-Phos, DIPEA, RT, 4h; *iii* TFA, DCM, rt, 10 min; *iv* e–f, DIPEA, reflux, 10 min
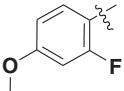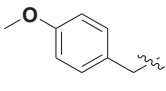
some novel 4,6-disubstituted pyrimidine derivatives **5–16** (Scheme 1) derived starting from 2,4-dichloropyrimidine and subsequently screened for their cytotoxicity properties. Further, these compounds were evaluated for their anticancer activity on six cancer cells, including SIHA (cervical

cancer), PANC-1 (pancreatic cancer), MDA-MB-231 (breast cancer), IMR 32 (neuroblastoma), DU 145 (prostate cancer) and A549 (lung adenocarcinoma). Although several EGFR-TKIs have been clinically validated for the treatment of cancer patients, yet the search for new active molecules

Table 1 Synthesis of 4,6-pyrimidine derivatives (**5–16**)

Compound	R	R^1	Molecular formula	M.P (°C)	Yield (%)
5			$C_{30}H_{29}N_5O_4$	177–180	90
6			$C_{28}H_{26}ClN_5O_2$	111–112	95
7			$C_{30}H_{31}N_5O_3$	148–150	93
8			$C_{26}H_{25}N_5O_3S$	188–192	93
9			$C_{24}H_{22}ClN_5OS$	236–238	94
10			$C_{26}H_{27}N_5O_2S$	206–209	93
11			$C_{22}H_{23}N_5O_3S$	188–191	94
12			$C_{20}H_{20}ClN_5OS$	175–177	91
13			$C_{22}H_{25}N_5O_2S$	173–175	93
14			$C_{25}H_{26}FN_5O_4$	153–154	95
15			$C_{23}H_{23}ClFN_5O_2$	168–171	92

Table 1 continued

Compound	R	R^1	Molecular formula	M.P (°C)	Yield (%)
16			$C_{25}H_{28}FN_5O_3$	163–164	95

against EGFR-TK is still continuing. Hence we have selected EGFR-TK as putative target for molecular docking studies and explored the binding mode of our ligands with active site of EGFR-TK.

Results and discussion

Chemistry

The synthesis of 4,6-disubstituted pyrimidine derivatives **5–16** is shown in Scheme 1. The reaction of 2,4-dichloropyrimidine (**1**) with *tert*-butylpiperidin-4-ylcarbamate in the presence of diisopropylamine at room temperature afforded pyrimidine chloride derivative **2**. Suzuki coupling of chloride intermediate **2** with various boronic acids (**a–d**) was carried out at room temperature in presence of $Pd_2(dba)_3$, X-Phos, diisopropylamine in tetrahydrofuran (THF) to obtain corresponding compounds **3a–d** in 85–95 % yield. De-BOC of **3a–d** was done by using trifluroacetic acid (TFA) at 0 °C for 10 min to isolate compounds **4a–4d** as their respective TFA salts. The reaction of compounds **4a–d** with isocyanates (**e–f**) in the presence of *N,N*-diisopropylethylamine (Hunig's base) with reflux for 10 min, resulted in urea derivatives **5–16** (Table 1). The yield of the products varied from 90 to 95 %.

All the synthesized compounds were well characterized by ^{1}H & ^{13}C-NMR, FT-IR and mass spectrometry. IR data of compounds **5–16** showed a strong –C=O stretching band around 1615–1657 cm^{-1} and a strong -NH stretching band at 3295–3338 cm^{-1}. All final compounds have strong absorption around 3060–3435 cm^{-1} and around 1590 cm^{-1}, which are evidence for aromatic C–H and aromatic C–C bonds respectively. All these compounds have shown a singlet in the range of δ 8.5–8.9 ppm of corresponding pyrimidine nuclei and another singlet in the range of δ 8.4–8.6 ppm of corresponding urea linkage (–NH proton), and signals for some protons in the range of δ 3.2–4.5 ppm,

corresponding piperidine nuclei. These data clearly confirm the presence of specific functional groups present in final synthesized compounds.

In vitro biological activity

The compounds **5–16** (up to 300 μg/mL concentrations) were tested for cytotoxicity using brine shrimp lethality assay (Meyer et al., 1982) and compared with the activity of podophyllotoxin (Table 2). The compounds **6**, **11**, **14** and **15** were showed ED_{50} values of 3.50, 3.69, 3.78 and 4.49 μg/mL, respectively and displayed equipotent activity with respect to the standard Podophyllotoxin (ED_{50} = 3.69 μg/mL). The compounds **5** (ED_{50} = 27.86 μg/mL), compound **10** (ED_{50} = 39.49 μg/mL), compound **16** (ED_{50} = 39.64 μg/mL) were found to be exhibited moderate cytotoxicity. The remaining analogs in the series were less toxic.

Among the tested analogs, pyrimidine ring flanked by 4-phenoxyphenyl **6** (with R^1=3-chlorophenyl on piperidine ring), thiophene **11** (with R^1=4-methylbenzoate on piperidine ring) and 2-fluoro-4-methoxyphenyl **14** and **16** (with R^1=4-methylbenzoate and 3-chloro phenyl on piperidine ring respectively) were exhibited cytotoxicity like Podophyllotoxin. The exact mechanism of cytotoxic activity between 4-phenoxyphenyl (with R^1=3-chlorophenyl on piperidine ring), thiophene (with R^1=4-methylbenzoate on piperidine ring) and 2-fluoro-4-methoxyphenyl (with R^1=4-methylbenzoate and 3-chlorophenyl on piperidine ring) substitution on the pyrimidine/piperidine ring was not established earlier, so further we made an attempt to find out structural activity relationship (SAR) with cytotoxicity and anticancer activities.

The compounds were further evaluated for anticancer activity in six cancer cell lines (SIHA, PANC-1, MDA-MB-231, IMR-32, DU145, and A549). Three response parameters such as growth inhibitory activity (GI_{50}), total growth inhibition (TGI), and lethal concentration (LC_{50}) were calculated for each cell line, using Doxorubicin and

Table 2 Brine shrimp lethality data of compounds **5–16**

Compound	R	R^1	ED$_{50}$ (µg/mL)
5	4-phenoxyphenyl	methyl 4-(…)benzoate	27.86
6	4-phenoxyphenyl	3-chlorophenyl	3.50
7	4-phenoxyphenyl	4-methoxybenzyl	42.46
8	benzo[b]thiophen-2-yl	methyl 4-(…)benzoate	40.52
9	benzo[b]thiophen-2-yl	3-chlorophenyl	43.79
10	benzo[b]thiophen-2-yl	4-methoxybenzyl	39.49
11	thiophen-2-yl	methyl 4-(…)benzoate	3.69
12	thiophen-2-yl	3-chlorophenyl	44.23
13	thiophen-2-yl	4-methoxybenzyl	43.28
14	3-fluoro-4-methoxyphenyl	methyl 4-(…)benzoate	3.78
15	3-fluoro-4-methoxyphenyl	3-chlorophenyl	4.49
16	3-fluoro-4-methoxyphenyl	4-methoxybenzyl	39.64
Podophyllotoxin	–	–	3.69

Table 3 In vitro anticancer activity of pyrimidine-piperidine derivatives (**5–16**)

Compound	SIHA GI$_{50}$	SIHA TGI	SIHA LC$_{50}$	PANC 1 GI$_{50}$	PANC 1 TGI	PANC 1 LC$_{50}$	MDA-MB-231 GI$_{50}$	MDA-MB-231 TGI	MDA-MB-231 LC$_{50}$	IMR 32 GI$_{50}$	IMR 32 TGI	IMR 32 LC$_{50}$	DU 145 GI$_{50}$	DU 145 TGI	DU 145 LC$_{50}$	A549 GI$_{50}$	A549 TGI	A549 LC$_{50}$
5	40.0 ± 0.07	>100	>100	>100	>100	>100	0.7 ± 0.03	>100	>100	3.1 ± 0.07	8.3 ± 0.03	22.2 ± 0.6	1.0 ± 0.06	>100	>100	0.3 ± 0.09	>100	>100
6	0.38 ± 0.01	>100	>100	>100	>100	>100	0.44 ± 0.09	10.5 ± 0.1	>100	0.65 ± 0.03	4.6 ± 0.2	33.3 ± 0.1	0.17 ± 0.02	>100	>100	0.32 ± 0.04	>100	>100
7	12.1 ± 0.6	>100	>100	>100	>100	>100	0.2 ± 0.01	3.1 ± 0.09	49.5 ± 0.7	2.1 ± 0.04	15.1 ± 0.09	>100	4.3 ± 0.08	16.0 ± 0.5	58.7 ± 0.09	1.0 ± 0.05	51.5 ± 0.3	>100
8	1.5 ± 0.1	31.6 ± 0.5	>100	0.58 ± 0.03	14.7 ± 0.08	>100	1.1 ± 0.03	6.4 ± 0.05	36.3 ± 0.5	18.2 ± 0.4	>100	>100	1.2 ± 0.06	17.3 ± 0.2	>100	1.1 ± 0.03	47.6 ± 0.07	>100
9	0.45 ± 0.08	>100	>100	3.3 ± 0.04	50.6 ± 0.1	>100	2.4 ± 0.03	10.4 ± 0.06	44.8 ± 0.06	0.86 ± 0.06	8.0 ± 0.2	>100	4.1 ± 0.08	56.2 ± 0.3	>100	5.1 ± 0.07	>100	>100
10	40.4 ± 0.6	>100	>100	3.3 ± 0.1	24.6 ± 0.3	>100	12.8 ± 0.3	21.1 ± 0.09	34.7 ± 0.03	8.7 ± 0.4	>100	>100	9.0 ± 0.06	>100	>100	4.6 ± 0.1	>100	>100
11	20.0 ± 0.5	>100	>100	>100	>100	>100	2.4 ± 0.05	40.7 ± 0.1	>100	38.1 ± 0.7	>100	>100	1.8 ± 0.1	35.3 ± 0.4	>100	>100	>100	>100
12	19.9 ± 0.05	>100	>100	3.8 ± 0.05	14.8 ± 0.07	>100	2.0 ± 0.03	>100	>100	>100	>100	>100	5.0 ± 0.08	>100	>100	4.8 ± 0.1	22.8 ± 0.09	>100
13	2.0 ± 0.03	>100	>100	0.5 ± 0.01	9.7 ± 0.06	>100	>100	>100	>100	33.3 ± 0.8	>100	>100	2.5 ± 0.01	>100	>100	0.82 ± 0.03	25.8 ± 0.04	>100
14	0.22 ± 0.04	>100	>100	0.97 ± 0.03	>100	>100	>100	>100	>100	4.2 ± 0.01	22.1 ± 0.8	>100	0.1 ± 0.02	1.3 ± 0.01	>100	1.4 ± 0.02	21.5 ± 0.04	>100
15	0.41 ± 0.09	>100	>100	>100	>100	>100	>100	>100	>100	4.1 ± 0.08	>100	>100	13.7 ± 0.05	>100	>100	>100	>100	>100
16	1.0 ± 0.03	>100	>100	0.47 ± 0.04	1.6 ± 0.01	>100	7.6 ± 0.06	1.6 ± 0.01	>100	>100	>100	>100	2.5 ± 0.04	>100	>100	0.11 ± 0.01	4.3 ± 0.06	>100
Doxorubicin	<0.01	0.17 ± 0.01	1.9 ± 0.02	<0.01	0.29 ± 0.05	3.2 ± 0.1	<0.01	0.32 ± 0.04	3.2 ± 0.05	<0.01	0.12 ± 0.03	3.2 ± 0.1	0.09 ± 0.001	0.32 ± 0.02	5.4 ± 0.13	<0.01	0.23 ± 0.02	4.2 ± 0.09
Combretastatin	<0.01	0.23 ± 0.03	2.0 ± 0.01	<0.01	0.34 ± 0.01	3.5 ± 0.1	<0.01	0.22 ± 0.03	3.5 ± 0.02	<0.01	0.24 ± 0.02	2.4 ± 0.3	0.031 ± 0.002	0.25 ± 0.01	3.9 ± 0.2	<0.01	0.34 ± 0.012	3.5 ± 0.1

GI$_{50}$ growth inhibitory activity, *TGI* total growth inhibition, *LC$_{50}$* lethal concentration

Fig. 2 The binding mode of ligands (5–16) with the EGFR-TK active site
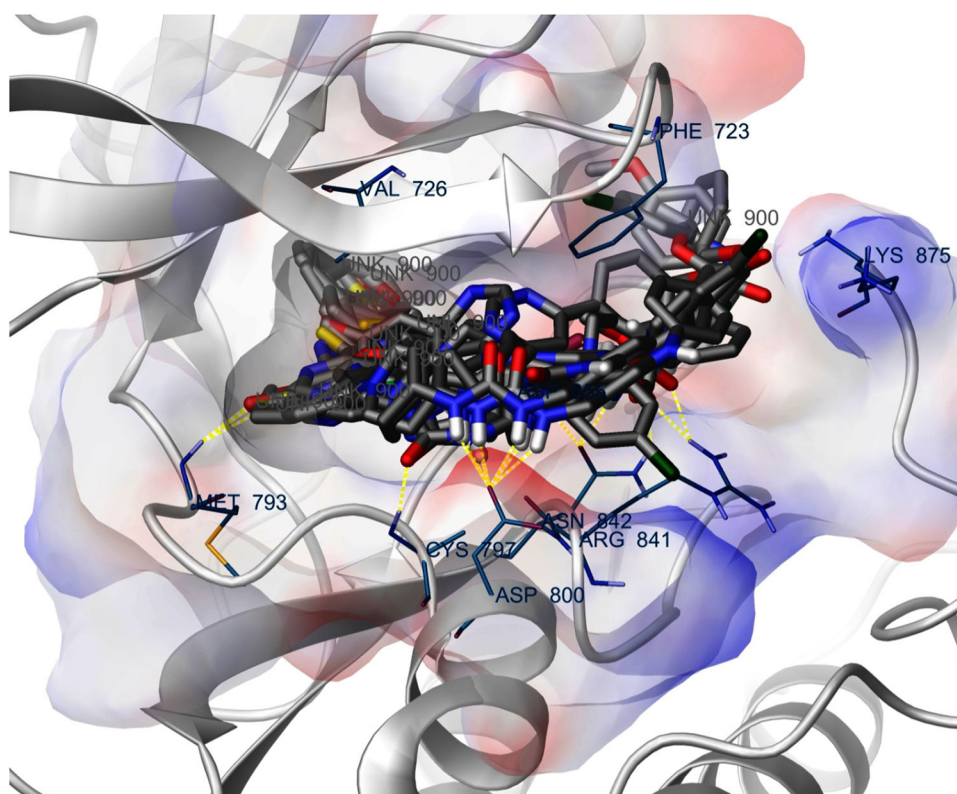
Combretastatin as positive controls and data presented in Table 3. Different concentrations of compounds were studied for growth inhibition in cancer cell lines, and found to be exhibited various levels of growth arrest vary from cell line to cell line. The compound **14** showed better efficacy in inhibiting cell proliferation of cervical cancer and prostate cancer, followed by the compound **6** with GI_{50} range from 0.1 to 0.4 µM, the compound **14** required 1.3 µM for TGI in DU145 but more than 100 µM in SIHA. The compound **16** was showed maximum growth inhibition in PANC 1 as well A549 with GI_{50} of 0.47 and 0.11 µM, TGI of 1.6 and 4.3 µM respectively. The compound **7** was most effective inhibitor of MDA-MB-231 with GI_{50} of 0.2 and TGI of 3.1 µM whereas compound **6** was highly potent inhibitor of IMR 32 with GI_{50} of 0.65 and TGI of 4.6 µM correspondingly. Even though positive controls such as Doxorubicin and Combretastatin were highly potent growth inhibitors than tested compounds but exhibited severe toxicity with LC_{50} range of 1–5 µM. In the present study, all the compounds from **5** to **16** were less toxic than Doxorubicin and Combretastatin which might give a better therapeutic index in the treatment of cancer, which is very promising for this new group of compounds.

SAR developed from the anticancer data showed that pyrimidine derivatives having R (substitution) with 2-fluoro-4-methoxyphenyl responsible for maximum activity followed by 4-phenoxyphenyl and 2-benzthiazole while R^1 (substitution) with 4-methoxyphenyl showed maximum activity followed by 4-methylbenzoate and 3-chlorophenyl.

Molecular docking studies

EGFR-TKs are believed to have an essential role in the instigation and the growth of the numerous cancers (Mostafa et al., 2013). Moreover compounds bearing pyrimidine rings were also reported as inhibitors of EGFR-TKs and few with molecular docking to understand the key interactions (Mao et al., 2013). Guided by these facts, we carried out the molecular docking studies to explore the possible mode of action of compounds **5–16** as EGFR-TKase inhibitors (Fig. 2). The X-ray crystallographic structure of EGFR-TK (PDB Code: 2J5F) was utilized for the current docking studies (Gangjee et al., 2013). The binding site of the co-crystallized ligand (N-[4-(3-bromophenylamino) quinazolin-6-yl] acrylamide) is well defined by the hydrophobic cavity lines by Lys745, Glu762, Met766, Leu788, Thr790, Met793, Cys797 and Thr854 (Blair et al., 2007; Ahsan et al., 2013). All the molecules were docked against EGFR-TKase (PDB Code: 2J5F) using XP-Glide-5.0 implemented in Maestro-8.5. Docked poses of the molecules were carefully analyzed
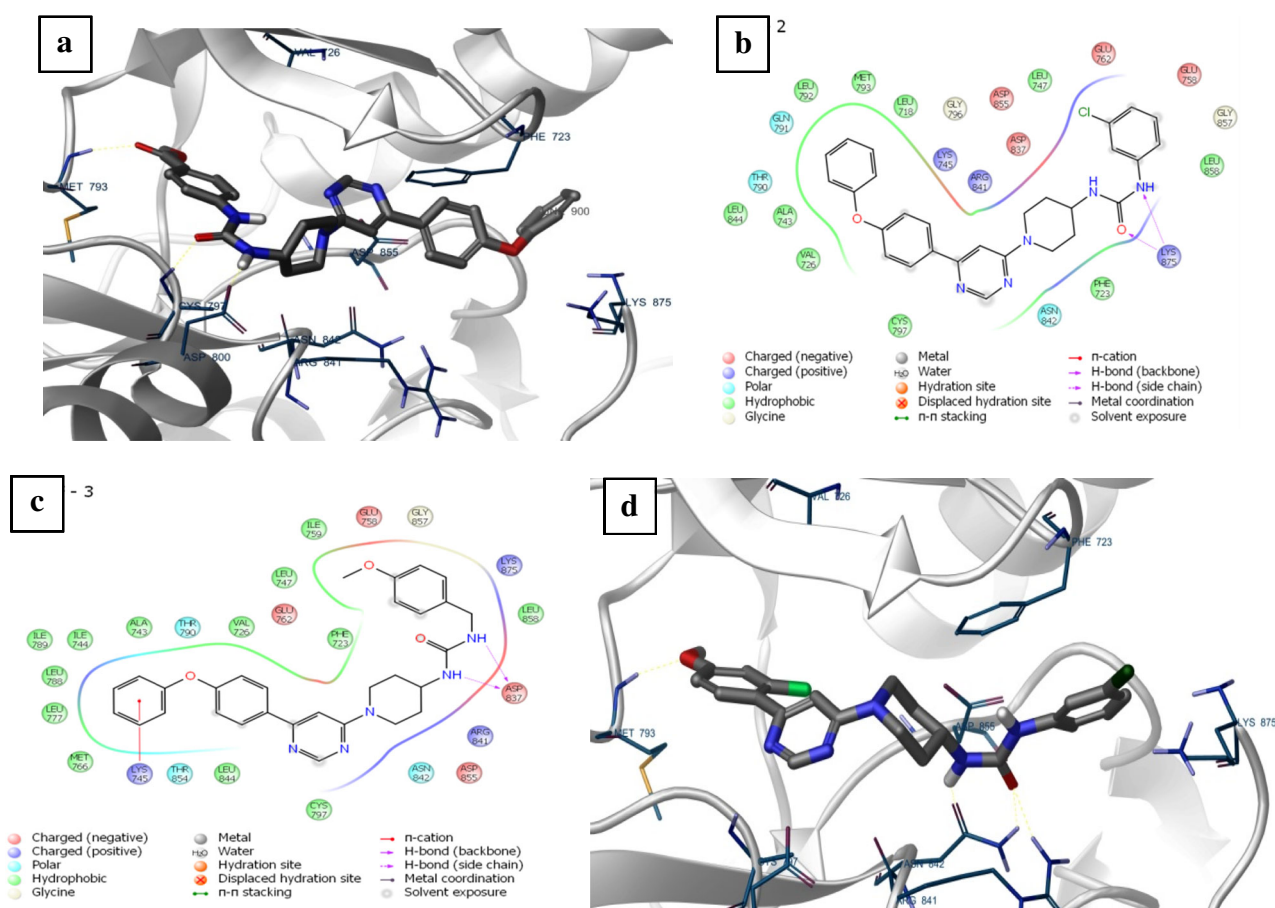
Fig. 3 3D Binding mode of compounds against EGFR-TK active site: **a** Compound **5 b**. Compound **6 c** Compound **7**, **d** Compound **9**

to understand the crucial interaction responsible for the activity and found that the compound **5** and **6** were displayed interactions with EGFR-TK. Both **5** and **6** were exhibited hydrophobic interaction with Leu792 and Met793 and might be crucial for anticancer activity. The other interactions of compound **5** were as followed, H-bonding interaction with Cys797 and a π–π interaction with Phe723 (Fig. 3a), and compound **6**, displayed two H-bonding interaction with Lys875 (Fig. 3b). The compounds **7**, **8** and **9** were displayed hydrophobic interaction with Leu777 and Leu778 (Fig. 3c, d). The ATP binding pocket of EGFR-TK is considerably hydrophobic, thus hydrophobic interactions plays an important role in affinity toward EGFR-TK. Moreover it was proposed that at least two hydrogen bonds are required for small molecules to act as EGFR-TK inhibitor (Zuccotto et al., 2010). In the present study, compounds **5**, **6**, **7**, **8** and **9** might be showed hydrophobic interactions towards ATP binding pocket to explored as EGFR-TK inhibitors. All the other compounds were exhibited unusual interactions with EGFR-TK and might be specific for other targets. Attempts are in progress to find out SAR with therapeutic targets in cancer.

Conclusion

A series of novel 4, 6-disubstituted pyrimidine derivatives (**5–16**) were synthesized with good yields and were screened for their in vitro cytotoxicity and anticancer activity. The present study revealed that four compounds (**5**, **11**, **14** and **15**) were potent cytotoxic molecules in brine shrimp lethality assay. The compounds were also studied for in vitro anticancer properties using six different cancer cell lines viz SIHA, PANC-1, MDA-MB-231, IMR-32, DU145 and A549. The compound **14** was effective inhibitor of SIHA and DU145, whereas compound **16** in Panc 1 and A549, compound **7** in MDA-MB-231 and compound **6** in IMR 32 respectively. Molecular docking studies were carried out using an X-ray crystallographic structure of EGFR-TK to explore the possible mode of action of compounds as EGFR-TK inhibitors. Among potent anticancer compounds in the present study, compound **6** was found to be exhibited hydrophobic interaction with Leu792 and Met793 whereas compound **7** was with Leu777 and 778. This binding pattern with EGFR-TK might play a crucial role in its inhibition and anticancer activities. The present work provides

strong incentive for further development of 4,6-dis-ubstituted pyrimidine derivatives as potential antitumor agents for the treatment of metastatic cancer.

Materials and methods

All the chemicals and solvents used were purchased either from Fluka or Merck. All the reagents were of analytical grade. Thin layer chromatography (TLC) was performed on the E. Merck AL silica gel 60 F254 plates and visualized under UV light. The IR spectra were recorded on a Perkin Elmer FT-IR spectrometer. The ^{1}H NMR spectra were recorded in CDCl$_3$ on a Varian EM-360 spectrometer operating at 400 MHz. The ^{13}C NMR spectra recorded in CDCl$_3$ on a Varian EM-360 spectrometer operating at 100 MHz. The chemical shifts were reported in δ (ppm) using TMS as an internal standard. The mass spectra were recorded on Agilent ion trap MS. All the appropriate boronic acids (**a–d**) and isocyanates (**e–f**) used for the preparation of **5–16** were purchased from commercial sources. All computational analysis were carried out on a Dell Precision T3400n workstation with Intel core 2 quad processor, 8 GB RAM, 500 GB hard disk, running on the operating system Red Hat Enterprise 5.0 Linux (RHEL 5.0) platform. Molecular docking simulations were carried out using Protein preparation wizard, Ligprep and Glide module implemented in Maestro-8.5 (Schrödinger LLC).

Synthesis of tert-butyl 1-(6-chloropyrimidin-4-yl) piperidin-4-ylcarbamate (2)

To a solution of *tert*-butylpiperidin-4-ylcarbamate (4 g, 20 mmol) in THF (80 mL) was added *N,N*-diisopropylethyla-mine (3.87 g, 30 mmol) followed by 2,4-dichloropyrimidine (2.95 g, 20 mmol). The resulting mixture was stirred at room temperature for 4 h. The reaction mixture was eva-porated under reduced pressure and then water was added. The resulted off-white solid was filtered and dried in vacuum to afford compound **2** (Yield: 5.6 g, 92 %, off-white solid); ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.30 (s, 1H), 6.96 (s, 1H), 6.87 (d, 1H, $J = 8.0$ Hz), 4.27 (brs, 2H), 3.55 (brs, 1H), 3.08 (t, 2H, $J = 7.0$ Hz), 1.79 (d, 2H, $J = 6.0$ Hz), 1.38 (s, 9H), 1.33–1.23 (m, 2H). EI MS: *m/z* (rel. abund.%): 313.0 (M$^+$, 100).

General method for the synthesis of compounds (3a–d)

To a solution of compound **2** (1 g, 3.2 mmol) in THF (15 mL) was added sequentially corresponding boronic acid (**a–d**, 4.8 mmol), DIPEA (6.4 mmol) followed by Pd$_2$(dba)$_3$ (10 mol%) and X-Phos (10 mol%) under argon. The resul-tanting reaction mixture was stirred at room temperature for

4 h. TLC indicated complete consumption of starting material. The reaction mixture was diluted with water (5 mL) and extracted with ethyl acetate (15 mL), the combined organic layer was washed with water (3 × 5 mL) followed by brine solution. The organic layer was dried over anhy-drous sodium sulphate, filtered and concentrated under reduced pressure, to obtain the crude compounds. The crude compounds were purified by column chromatography (Silica gel: 100–200 mesh, elluant: 1:4 of ethyl acetate: *n*-hexane) to isolate compounds **3a–d** (Yield: 95, 90, 85, 88 % respectively).

tert-Butyl 1-(6-(4-phenoxyphenyl)pyrimidin-4-yl) piperidin-4-ylcarbamate (3a)

^{1}H NMR (400 MHz, DMSO-d_6): δ 1.33-1.28 (m, 2 H), 1.39 (s, 9H), 1.81 (d, 2H, $J = 4.0$ Hz), 3.07 (t, 2H, $J = 5.0$ Hz), 3.56 (brs, 2H), 4.48 (d, 2H, $J = 12.0$ Hz), 6.87 (d, 1H, $J = 8.0$ Hz), 7.10 (t, 4H, $J = 2.0$ Hz), 7.21 (t, 1H, $J = 12.0$ Hz), 7.28 (s, 1H), 7.45 (t, 2H, $J = 4.0$ Hz), 8.19 (d, 2H, $J = 4.0$ Hz), 8.53 (s, 1H); EI MS: *m/z* (rel. abund.%) 447.2 (M$^+$, 100).

Isopropyl 1-(6-(benzo[b]thiophen-2-yl)pyrimidin-4-yl) piperidin-4-ylcarbamate (3b)

^{1}H NMR (400 MHz, DMSO-d_6): δ 1.35–1.30 (m, 2 H), 1.39 (s, 9H), 1.84 (d, 2H, $J = 3.0$ Hz), 3.10 (t, 2H, $J = 6.0$ Hz), 3.59 (brs, 1H), 4.46 (d, 2H, $J = 12.0$ Hz), 6.89 (d, 1H, $J = 8.0$ Hz), 7.44–7.39 (m, 2H), 7.50 (s, 1H), 7.90–7.87 (m, 1H), 8.01–7.99 (m,1H), 8.40 (s, 1H),8.50 (s, 1H); EI MS: *m/z* (rel. abund.%) 411.0 (M$^+$, 100).

tert-Butyl 1-(6-(thiophen-2-yl)pyrimidin-4-yl)piperidin-4-ylcarbamate (3c)

^{1}H NMR (400 MHz, DMSO-d_6): δ1.35-1.27 (m, 2H), 1.39 (s, 9H), 1.81 (d, 2H, $J = 4.0$ Hz), 3.07 (t, 2H, $J = 6.0$ Hz), 3.56 (brs, 1H), 4.42 (d, 2H, $J = 6.0$ Hz), 6.88 (d, 1H, $J = 8.0$ Hz), 7.20 (t, 1H, $J = 8.0$ Hz), 7.28 (s, 1H),7.69 (d, 1H, $J = 4.0$ Hz), 8.02 (d, 1 H, $J = 4.0$ Hz), 8.41 (s, 1H); EI MS: *m/z* (rel. abund.%) 361.2 (M$^+$, 100).

tert-Butyl 1-(6-(2-fluoro-4-methoxyphenyl)pyrimidin-4-yl)piperidin-4-ylcarbamate (3d)

^{1}H NMR (400 MHz, DMSO-d_6): δ 1.35–1.27 (m, 2 H), 1.38 (s, 9H), 1.81 (d, 2H, $J = 4.0$ Hz), 3.09 (t, 2H, $J = 7.0$ Hz), 3.56 (brs, 1H), 3.79 (s, 3H), 4.34 (d, 2H, $J = 6.0$ Hz), 6.87 (d, 1H, $J = 8.0$ Hz), 7.07–7.03 (m, 1H),7.11 (s, 1H), 7.28 (t, 1H, $J = 10.0$ Hz), 7.42 (m,1H), 8.58 (s, 1H); EI MS: *m/z* (rel. abund.%) 403.2 (M$^+$, 100).

General method for the synthesis of compounds (4a–d)

A mixture of compound 3a–d (10 mmol), trifluroacetic acid (5 vol) was stirred at 0 °C to room temperature for 10 min. The reaction mixture was evaporated under vacuum and dried to obtain the compounds, 4a–d as TFA salts and were used in the next step without further purification.

1-(6-(4-Phenoxyphenyl)pyrimidin-4-yl)piperidin-4-amine (4a)

^{1}H NMR (300 MHz, D$_2$O): δ 1.76 (t, 2 H, $J = 5.5$ Hz), 2.23–2.20 (m, 3H), 3.24–3.16 (t, 1H, $J = 12.0$ Hz), 3.46 (t, 1H, $J = 12.0$ Hz), 3.68 (t, 1H, $J = 14.0$ Hz), 4.41 (d, 1H, $J = 12.0$ Hz), 5.16 (d, 1H, $J = 18.0$ Hz), 7.21 (t, 4H, $J = 2.2$ Hz), 7.32 (t, 1H, $J = 7.5$ Hz), 7.51 (t, 2H, $J = 3.5$Hz), 7.79 (d, 2H, $J = 4.5$ Hz), 8.61 (s, 1H); EI MS: m/z (rel. abund.%) 347.2 (M$^+$, 100).

1-(6-(Benzo[b]thiophen-2-yl)pyrimidin-4-yl)piperidin-4-amine (4b)

^{1}H NMR (300 MHz, D$_2$O): δ 1.75–1. 68 (m, 2H), 2.30 (d, 2H, $J = 4.5$ Hz), 3.32–3.28 (m, 2H), 3.67 (brs, 1H), 3.67 (brs, 1H), 4-45–4.43 (m, 2H), 7.04 (s, 1H), 7.58–7.49 (m, 2H), 8.01–8.98 (m, 2H), 8.07 (s, 1H), 8.52 (s, 1H); EI MS: m/z (rel. abund.%) 311.2 (M$^+$, 100).

1-(6-(Thiophen-2-yl)pyrimidin-4-yl)piperidin-4-amine (4c)

^{1}H NMR (300 MHz, D$_2$O): δ 1.51–1.40 (m, 2H), 2.01 (d, 2H, $J = 4.5$ Hz), 3.10 (t, 2H, $J = 6.0$ Hz), 3.38 (brs, 1H), 4.55 (d, 2H, $J = 3.0$ Hz), 7.24 (t, 1H, $J = 4.5$ Hz), 7.34 (s, 1H), 7.77 (d, 1H, $J = 3.0$ Hz), 8.04 (d, 1H, $J = 3.0$ Hz), 8.51 (s, 2H); EI MS: m/z (rel. abund.%) 261.2 (M$^+$, 100).

1-(6-(2-Fluoro-4-methoxyphenyl)pyrimidin-4-yl)piperidin-4-amine (4d)

^{1}H NMR (300 MHz, D$_2$O): δ 1.78–1.63 (m, 2H), 2.25 (d, 2H, $J = 6.0$ Hz), 3.23 (t, 1H, $J = 13.0$ Hz), 3.40 (t, 1H, $J = 6.0$ Hz), 3.66–3.55 (m, 1H), 3.83 (s, 3H), 4.35 (d, 1H, $J = 15.0$ Hz), 5.15 (d, 1H, $J = 6.0$ Hz), 7.31–7.14 (m, 4.0 Hz), 8.62 (s, 1H); EI MS: m/z (rel. abund.%) 303.2.

General method for the synthesis of compounds (5–16)

To a stirred mixture of compounds 4a–4d (10 mmol) in N,N-diisopropylethylamine (10 Vol) was added appropriate isocyanates (e–f, 10 mmol) and refluxed for 10 min. The reaction mixture was evaporated under reduced pressure and diluted with water to obtain crude compounds (5–16).

The crude compounds were purified by column chromatography (Silica gel: 100–200 mesh, elluant: 1:1, ethylacetate: n-hexane) to obtain compounds 5–16 as off-white solids. Yields of the products varied from 90 to 95 %.

1-(4-Methyl benzoate)-3-(1-(6-(4-phenoxyphenyl) pyrimidin-4-yl)piperidin-4-yl)urea (5)

IR (KBr): ν_{max}1651 (C=O), 3326 (–NH) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.44–1.39 (m, 2H), 1.94 (d, 2H, $J = 4.5$ Hz), 3.22–3.16 (m, 2H), 3.79 (s, 3H), 3.82–3.81 (m, 1H), 4.40 (brs, 2H), 6.40 (d, 1H, $J = 6.0$ Hz), 7.10 (d, 3H, $J = 2.0$ Hz), 7.22 (t, 1H, $J = 6.0$ Hz), 7.30 (brs, 1H), 7.46 (t, 2H, $J = 3.0$ Hz), 7.52 (d, 2H, $J = 4.5$ Hz), 7.84 (d, 2H, $J = 3.0$ Hz), 8.18 (d, 2H, $J = 3.0$ Hz), 8.57–8.56 (m, 1H), 8.80 (s, 1H); ^{13}C NMR (400 MHz, DMSO-d_6 + TFA): δ 165.96, 161.65, 159.55, 158.89, 157.00, 155.84, 154.06, 145.13, 131.11, 130.37, 130.18, 128.92, 124.11, 121.64, 119.27, 118.02, 116.69, 97.94, 61.65, 46.32, 42.58, 31.52; EI MS: m/z (rel. abund.%) 524.2 (M$^+$, 100); analy. found for C$_{30}$H$_{29}$N$_5$O$_4$–C 68.86, H 5.54, N 13.58, O 12.02 (%).

1-(3-Chlorophenyl)-3-(1-(6-(4-phenoxyphenyl) pyrimidin-4-yl)piperidin-4-yl)urea (6)

IR (KBr): ν_{max}1657 (–C=O), 3331 (–NH) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.41–1.37 (m, 2H), 1.92 (d, 2H, $J = 3.0$ Hz), 3.21 (t, 2H, $J = 4.5$ Hz), 3.83–3.80 (m, 1H), 4.42 (d, 2H, $J = 4.5$ Hz), 6.31 (d, 1H, $J = 6.0$ Hz), 6.93(d, 1H, $J = 3.0$ Hz), 7.10 (t, 4H, $J = 1.5$ Hz), 7.25–7.16 (m, 3H), 7.32 (s, 1H), 7.46 (t, 2H, $J = 3.7$ Hz), 7.67 (t, 1H, $J = 1.5$ Hz), 8.21 (d, 2 H, $J = 3.0$ Hz), 8.57 (d, 2H, $J = 4.5$Hz); ^{13}C NMR (400 MHz, DMSO-d_6): δ 161.88, 160.99 158.55, 158.02, 155.96, 154.23, 141.95, 133.06, 132.34, 130.19, 130.15, 128.71, 123.97, 120.58, 119.16, 117.99, 116.94, 115.97, 97.62, 46.42, 42.36, 31.52; EI MS: m/z (rel. abund.%) 500.1 (M$^+$, 100); analy. found for C$_{28}$H$_{26}$ClN$_5$O$_2$–C 67.46, H 5.04, N 14.20, O 6.20, Cl 7.10 (%).

1-(4-Methoxybenzyl)-3-(1-(6-(4-phenoxyphenyl) pyrimidin-4-yl)piperidin-4-yl)urea (7)

IR (KBr): ν_{max}1620 (–C=O), 3295 (–NH) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.33–1.25 (m, 2H), 1.86 (d, 2H, $J = 4.0$ Hz), 3.16 (t, 2H, $J = 6.0$ Hz), 3.73 (s, 3H), 3.74–3.73 (m, 1H), 4.13 (d, 2H, $J = 2.0$ Hz), 4.38 (d, 2H, $J = 6.0$ Hz), 5.95 (d, 1H, $J = 8.0$ Hz), 6.15 (t, 1H, $J = 6$Hz), 6.87 (d, 2H, $J = 4.0$ Hz), 7.10 (t, 4H, $J = 2.0$ Hz), 7.21–7.15 (m, 3H), 7.27 (s, 1H), 7.45 (t, 2H, $J = 4.0$ Hz), 8.20 (d, 2H, $J = 4.0$ Hz), 8.53 (s, 1H); ^{13}C NMR (400 MHz, DMSO-d_6): ð 161.07, 160.76, 159.39, 159.01, 158.63, 158.49, 158.26, 157.87, 155.48, 152.06, 151.70, 132.93, 130.59, 130.19, 128.64, 125.03, 124.72, 120.10, 118.35, 117.11, 114.22,

113.90, 99.49, 55.16, 46.13, 42.63, 32.32; EI MS: *m/z* (rel. abund.%) 510.1 (M$^+$, 100); analy. found for C$_{30}$H$_{31}$N$_5$O$_3$–C 70.60, H 6.11, N 13.79, O 9.50 (%).

1-(4-Methyl benzoate)-3-(1-(6-(benzo[b]thiophen-2-yl) pyrimidin-4-yl)piperidin-4-yl) urea (8)

IR (KBr): ν_{max} 1641 (–C=O), 3310 (–NH) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.45–1.37 (m, 2H), 1.95 (d, 2H, *J* = 4.0 Hz), 3.26 (t , 2H, *J* = 6.0 Hz), 3.79 (s, 3H), 3.85–3.83 (m, 1H), 4.43–4.40 (d, 2H, *J* = 3.0 Hz), 6.40–6.38 (d, 1H, *J* = 8.0 Hz), 7.43–7.40 (m, 2H), 7.54–7.50 (t, 2H, *J* = 4.0 Hz), 7.84–7.82 (d, 2H, *J* = 4.0 Hz), 7.90–7.88 (m, 1H), 8.01–7.99 (m, 1H), 8.42 (s, 1H), 8.52 (s, 1H), 8.81 (s, 1H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 165.49, 161.41, 158.07, 156.84, 154.03, 145.08, 143.33, 140.06, 139.89, 130.33, 125.57, 124.76, 124.34, 123.44, 122.69, 121.66, 116.69, 97.18, 51.65, 46.40, 42.41, 31.50; EI MS: *m/z* (rel. abund.%) 488.1 (M$^+$, 100); analy. found for C$_{26}$H$_{25}$N$_5$O$_3$S–C 64.15, H 5.06, N 14.37, O 9.88, S 6.54 (%).

1-(1-(6-(Benzo[b]thiophen-2-yl)pyrimidin-4-yl) piperidin-4-yl)-3-(3-chlorophenyl)urea (9)

IR (KBr): ν_{max} 1639 (–C=O), 33140 (–NH) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.44–1.36 (m, 2H), 1.95 (d, 2H, *J* = 6.0 Hz), 3.24 (t, 2H, 6.0 Hz), 3.83–3.82 (m, 1H), 4.43 (d, 2H, *J* = 4.0 Hz), 6.32 (d, 1H, *J* = 8.0 Hz), 6.94 (d, 1H, *J* = 8.0 Hz), 7.25–7.16 (m, 2H), 7.43–7.40 (m, 2H), 7.54 (s, 1H), 7.67 (s, 1H), 7.90–7.88 (m,1H), 8.01–7.99 (m, 1H), 8.41 (s, 1H), 8.52 (s, 1H), 8.58 (s, 1H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 161.44, 158.15, 156.97, 154.26, 143.49, 141.98, 140.07, 139.90, 133.09, 130.2, 125.54, 124.74, 124.34, 123.37, 122.67, 120.60, 116.97, 115.99, 97.15, 46.41, 42.42, 31.56; EI MS: *m/z* (rel. abund.%) 464.1 (M$^+$, 100); analy. found for C$_{24}$H$_{22}$ClN$_5$OS–C 62.14; H 4.76; N 15.11; O 3.43; S 3.93; Cl 7.63 (%).

1-(4-Methoxybenzyl)-3-(1-(6-(benzo[b]thiophen-2-yl) pyrimidin-4-yl)piperidin-4-yl)urea (10)

IR (KBr): ν_{max} 1625 (–C=O), 3293 (–NH) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.35–1.27 (m, 2H), 1.89 (d, 2H, *J* = 3.0 Hz), 3.20 (t, 2H, *J* = 6.0 Hz), 3.72 (s, 3H), 3.74 (m, 1H), 4.14 (d, 1H, *J* = 4.0 Hz), 4.40 (d, 2H, *J* = 6.0 Hz), 5.97 (d, 1H, *J* = 8.0 Hz), 6.17 (t, 1H, *J* = 6.0 Hz), 6.88 (d, 2H, *J* = 4.0 Hz), 7.18 (d, 2H, *J* = 4.0 Hz),7.42 (d, 2H, *J* = 4.0 Hz), 7.51 (s, 1H), 7.89–7.87 (m, 2H), 8.01–7.99 (m, 1H), 8.40 (s, 1H), 8.50 (s, 1H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 161.50, 158.22, 158.11, 157.40, 156.99, 143.50, 140.11, 139.96, 132.77, 128.40, 125.63, 124.83, 124.42, 123.42, 122.75, 113.67, 97.19, 55.07, 46.50, 42.59, 32.00; EI MS: *m/z* (rel. abund.%) 474.1 (M$^+$, 100); analy. found for

C$_{26}$H$_{27}$N$_5$O$_2$S–C 65.91, H 5.78, N 14.80, O 6.72, S 6.80 (%).

1-(4-Methyl benzoate)-3-(1-(6-(thiophen-2-yl)pyrimidin-4-yl)piperidin-4-yl)urea (11)

IR (KBr): ν_{max} 1650 (–C=O), 3316 (–NH) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.43–1.35 (m, 2H), 1.94 (d, 2 H, *J* = 6.0 Hz), 3.21 (t, 2H, *J* = 6.0 Hz), 3.80 (s, 3H), 3.83–3.82 (m, 1H), 4.39 (d, 2H, *J* = 6.0 Hz), 6.39 (d, 1H, *J* = 4.0 Hz), 7.20–7.18 (t, 1H, *J* = 8.0 Hz), 7.31 (s, 1H), 7.52 (d, 2H, *J* = 4.0 Hz), 7.70–7.69 (d, 1H, *J* = 4.0 Hz), 7.84 (d, 2H, *J* = 4.0 Hz), 8.04 (d, 1H, *J* = 4.0 Hz), 8.44 (s, 1H), 8.90 (s, 1H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 165.96, 161.54, 158.03, 156.99, 154.02, 145.08, 143.22, 130.33, 129.37, 128.34, 126.78, 121.67, 116.71, 95.95, 51.65, 46.41, 42.33, 31.51. EI MS: *m/z* (rel. abund.%) 438.1 (M$^+$, 100); analy found for C$_{22}$H$_{23}$N$_5$O$_3$S - C 60.36, H 5.33, N 15.98, O 10.96, S 7.37 (%).

1-(3-Chlorophenyl)-3-(1-(6-(thiophen-2-yl)pyrimidin-4-yl)piperidin-4-yl)urea (12)

IR (KBr): ν_{max} 1633 (–C=O), 3296 (–NH) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.41–1.34 (m, 2H), 1.93 (d, 2H, *J* = 6.0 Hz), 3.20 (t, 2H, *J* = 6.0 Hz), 3.81 (brs, 1H), 4.39 (d, 2H, *J* = 6.0 Hz), 6.31 (d, 1H, *J* = 8.0 Hz), 6.94 (d, 1H, *J* = 8.0 Hz), 7.25–7.16 (m, 3H), 7.31 (s, 1H), 7.70–7.66 (m, 3H), 8.03 (d, 1H, *J* = 4.0 Hz), 8.43 (s, 1H), 8.58 (s, 1H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 161.62, 158.10, 157.06, 154.35, 143.24, 142.00, 133.16, 130.30, 129.46, 128.44, 126.86, 120.73, 117.05, 116.09, 96.02, 46.47, 42.46, 31.61; EI MS: *m/z* (rel. abund.%) 414.1 (M$^+$, 100); analy. found for C$_{20}$H$_{20}$ClN$_5$OS–C 58.05, H 4.87, N 16.90, O 3.89, S 7.74, Cl 8.55 (%).

1-(4-Methoxybenzyl)-3-(1-(6-(thiophen-2-yl)pyrimidin-4-yl)piperidin-4-yl)urea (13)

IR (KBr): ν_{max} 1622 (–C=O), 3295 (–NH) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.32–1.24 (m, 2H), 1.86 (d, 2H, *J* = 4.0 Hz), 3.15 (t, 2H, *J* = 5.0 Hz), 3.72 (s, 3H), 3.73 (brs, 1H), 4.13 (d, 2H, *J* = 2.0 Hz), 4.36 (d, 2H, *J* = 6.0 Hz), 5.95 (d, 1H, *J* = 8.0 Hz), 6.15 (t, 1H, *J* = 6.0 Hz), 6.88–6.85 (d, 2H, *J* = 6.0 Hz), 7.20–7.15 (m, 3H), 7.29 (s, 1H), 7.69 (d, 1H, *J* = 4.0 Hz), 8.03 (d, 1H, *J* = 4.0Hz), 8.42 (s, 1H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 161.55, 158.03, 158.00, 157.27, 156.94, 143.21, 132.73, 129.35, 128.31, 126.75, 113.59, 95.92, 55.00, 46.42, 42.46, 31.93. EI MS: *m/z* (rel. abund.%) 424.1 (M$^+$, 100); analy. found for C$_{22}$H$_{25}$N$_5$O$_2$S–C 62.31, H 2.01, N 16.54, O 7.57, S 7.60 (%).

1-(4-Methylbenzoate)-3-(1-(6-(2-fluoro-4-methoxyphenyl)pyrimidin-4-yl)piperidin-4-yl)urea (14)

IR (KBr): ν_{max}1582 (–C=O), 3338 (–NH) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.43–1.36 (m, 2H), 1.94 (d, 2H, J = 6.0 Hz), 3.22 (t, 2H, J = 5.0 Hz), 3.79 (s, 3H), 3.91–3.90 (m, 1H), 4.31 (d, 2H, J = 4.0 Hz), 6.41 (d, 1H, J = 8.0 Hz), 7.07–7.04 (m, 1H), 7.15 (s, 1H), 7.29 (t, 1H, J = 10 Hz), 7.43–7.41 (m, 1H), 7.52 (d, 2H, J = 4.0 Hz), 7.84 (d, 2 H, J = 4.0 Hz), 8.60 (s, 1H), 8.81 (s, 1H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 166.14, 160.36, 159.30, 158.80, 158.29, 157.79, 155.82, 155.03, 154.28, 151.79, 151.58, 148.18, 145.23, 130.47, 121.86, 119.48, 117.38, 117.29, 116.77, 115.60, 113.46, 103.39, 56.08, 51.70, 45.69, 31.68; EI MS: m/z (rel. abund.%) 480.1 (M$^+$, 100); analy. found for $C_{25}H_{26}FN_5O_4$–C 62.63, H 5.48, N 14.61, O 13.36, F 3.95 (%).

1-(3-Chlorophenyl)-3-(1-(6-(2-fluoro-4-methoxyphenyl)pyrimidin-4-yl)piperidin-4-yl) urea (15)

IR (KBr): ν_{max}1634 (–C=O), 3302 (–NH) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.43–1.35 (m, 2H), 1.93 (d, 2H, J = 6.0 Hz), 3.22 (t, 2H, J = 5.0 Hz), 3.80 (s, 3H), 3.82–3.81 (m, 1H), 4.32 (d, 2H, J = 6.0 Hz), 6.32 (d, 1H, J = 8.0 Hz), 6.94–6.91 (m, 1H), 7.09–7.05 (m, 1H), 7.30–7.16 (m, 4H), 7.42–7.27 (m, 1H), 8.61 (s, 1H), 7.66–7.65 (m, 1H), 8.57 (s,1H); ^{13}C NMR (400 MHz, DMSO-d_6): ð 161.28, 157.73, 155.92, 155.44, 154.23, 152.70, 141.95, 133.05, 130.19, 126.13, 120.58, 117.30, 116.98, 115.97, 114.47, 102.54, 102.42, 55.68, 46.27, 42.45, 31.42; EI MS: m/z (rel. abund. %) 456.1 (M$^+$, 100); analy. found for $C_{23}H_{23}ClFN_5O_2$ C 60.60, H 5.07, F 4.21, N 15.33, O 7.05, Cl 7.74 (%).

1-(4-Methoxybenzyl)-3-(1-(6-(2-fluoro-4-methoxyphenyl)pyrimidin-4-yl)piperidin-4-yl) urea (16)

IR (KBr): ν_{max}1615 (–C=O), 3319 (–NH) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.34–1.23 (m, 2H), 1.86 (d, 2H, J = 4.0 Hz), 3.17 (t, 2H, J = 5.0 Hz), 3.75 (s, 4H), 3.79 (s, 3H), 4.13 (d, 2H, J = 4.0 Hz), 4.29 (d, 2H, J = 6.0 Hz), 5.95 (d, 1H, J = 8.0 Hz), 6.15 (t, 1H, J = 6.0 Hz), 6.88 (d, 2H, J = 6.0 Hz), 7.08–7.04 (m, 1H), 7.17 (t, 3H, J = 2.5 Hz), 7.29–7.24 (dd, 1H, J = 7.0, 3.8 Hz), 7.42–7.40 (m, 1H), 8.59 (s, 1H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 161.38, 158.03, 157.29, 155.96, 155.88, 154.95, 153.51, 133.76, 128.67, 117.48, 117.24, 116.51, 116.43, 115.43, 113.59,101.62, 55.00, 53.51, 46.40, 42.28, 41.76, 31.84; EI MS: m/z (rel. abund.%) 466.1 (M$^+$, 100); analy. found for $C_{25}H_{28}FN_5O_3$–C 64.48, H 6.07, N 15.02, O 10.33, F 4.09 (%).

Brine shrimp lethality assay

Brine shrimp (*Artemia Salina*) nauplii were hatched using brine shrimp eggs in a conical shaped vessel (1 L), filled with sterile, artificial sea water of 38 g L^{-1} of sea salt and adjusted to pH 8.5 using 1 N NaOH and kept under constant aeration for 48 h. After hatching, ten nauplii were drawn through a pipette and placed in each vial containing 4.5 mL brine solution and added 300 µg mL^{-1} concentration of compounds and the final volume was made up to 5 mL using brine solution and maintained 37 °C for 24 h under the light of incandescent lamps. Assays were carried out in duplicates. The percentage lethality was determined by comparing the mean surviving larvae of test and control tubes. Podophyllotoxin was used as a positive control.

In vitro anticancer activity

The cancer cell lines, SIHA, PANC 1, MDA MB 231, IMR 32, DU 145 and A549 used in this study were procured from the American Type Culture Collection (ATCC), USA. The synthesized test compounds were evaluated for their in vitro antiproliferative activity on these six different human cancer cell lines. All the cell lines were grown in Dulbecco's modified Eagle's medium (containing 10 % FBS in a humidified atmosphere of 5 % CO_2 at 37 °C). Cells were trypsinized when sub-confluent and seeded in 96-well plates in 100 µL aliquots at plating densities depending on the doubling time of individual cell lines. A protocol of 48 h continuous drug exposure was used, and a sulforhodamine B (SRB) cell proliferation assay was used to estimate cell viability or growth. 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 and were incubated for 48 h with different doses (0.01, 0.1, 1, 10, 100 µM) of prepared derivatives. After 48 h incubation at 37 °C, cell mono layers were fixed by the addition of 10 % (wt/vol) cold trichloroacetic acid and incubated at 4 °C for 1 h and were then stained with 0.057 % SRB dissolved in 1 % acetic acid for 30 min at room temperature. Unbound SRB was washed with 1 % acetic acid. The protein bound dye was dissolved in 10 mM tris base solution for OD determination at 510 nm using a microplate reader (Enspire, Perkin Elmer, USA). 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 concentration levels. Percentage growth inhibition was calculated as:

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

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

Three dose response parameters were calculated for each experimental agent (Boyd and Paul, 1995). 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 growth 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 the drug resulting in a 50 % reduction in cell growth at the end of the drug treatment as compared to that at the beginning) indicating a net loss of cells (toxicity) following treatment was calculated from [(Ti − Tz)/Tz] × 100 = −50. Values were calculated for each of these three parameters if the level of activity is reached; however, if the effect is not reached or is exceeded, the value for that parameter was expressed as greater or less than the maximum or minimum concentration tested. Standard error (±) values were also presented in the Table 3.

Molecular docking

Molecular docking simulations were carried out using Maestro-8.5 (Schrodinger LLC) installed on RHEL-5 operating system. The structures of molecules were sketched using build module implemented in Maestro-8.5. All the sketched molecules were prepared for docking using Ligprep module. The X-ray crystal structure of EGFR-TKase (PDB Code: 2J5F) was downloaded from protein data bank (http://www.rcsb.org/pdb/explore.do?structureId=2j5f). The protein was prepared for docking using the Protein Preparation Wizard, keeping OPLS2001 Force field for minimization. The grid was constructed, keeping the reference ligand (34-JAB) as the center of the grid using GLIDE (software v5.5) Receptor Grid Generation module. The ligands were then docked with the receptor grid generated using XP-Docking implemented in Glide-5. The docked conformers were analyzed through XP-Visualizer.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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