Bioorganic & Medicinal Chemistry 22 (2014) 6876–6884

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Discovery of novel Bcr–Abl inhibitors targeting myristoyl pocket and ATP site

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ARTICLE INFO

Article history:
Received 14 October 2014
Revised 21 October 2014
Accepted 21 October 2014
Available online 27 October 2014

Keywords:
Bcr–Abl
Allosteric pocket
ATP-binding site
Myristoyl pocket
Inhibitors

ABSTRACT

Bcr–Abl plays an essential role in the pathogenesis and development of chronic myeloid leukaemia (CML). Inhibition of Bcr–Abl has great potential for therapeutic intervention in CML. In order to obtain novel and potent Bcr–Abl inhibitors, twenty seven 4,6-disubstituted pyrimidines were synthesized and evaluated herein. The biological results indicated that four compounds of them (**C4**, **C5**, **C16**, and **C23**) were potent Bcr–Abl inhibitors which were comparable to positive control. Moreover, **C4** and **C5** displayed promising antiproliferative activity against K562 cells. The results suggested that these 4, 6-disubstituted pyrimidines could serve as promising leads for further optimization of Bcr–Abl inhibitors.

1. Introduction

Chronic myeloid leukaemia (CML) is a myeloproliferative disorder characterized at the molecular level by the expression of Bcr–Abl. Bcr–Abl plays an essential role in the development of CML. Because the Bcr–Abl tyrosine kinase is active in greater than 90% of CML cases, it has becoming an attractive target for anticancer drugs discovery.[1] Identification of the disease mechanism prompted the first drug discovery efforts targeting the inhibition of Bcr–Abl as means to treat CML.[2] A number of potent Bcr–Abl inhibitors have been developed and achieved clinical success in CML patients. Imatinib (STI571) is the first drug in the family of Bcr–Abl inhibitors while Nilotinib (AMN107) and Dasatinib (BMS-345825) are second generation drugs. Ponatinib (AP24534) is an orally active Bcr–Abl inhibitor while Bosutinib (SKI-606) and Bafetinib (INNO-406) have efficacy against various point mutations in the Bcr–Abl tyrosine kinase (Fig. 1).[3] However, clinical success was soon tempered by the emergence of drug resistance.

All of these approved drugs compete with ATP for the binding site of Bcr–Abl and provide significant clinical benefit.[4] However, acquired resistance has become a major challenge for treatment of CML. An alternative strategy to discovery of new Bcr–Abl inhibitors involves the development of agents targeting Bcr–Abl sites which are different with ATP site (Fig. 2).[4] These novel small molecules may potentially be unaffected by mutations of kinase domain and retain potency against resistant Bcr–Abl variants.

Imatinib is ATP-competitive Bcr–Abl tyrosine kinase inhibitors. Alternatively, other regulatory sites on Bcr–Abl are potential targets for small-molecule inhibitors. Myristoylation at myristoyl binding pocket (allosteric site) of Bcr–Abl could stabilize kinase in its inactive conformation. It is expected that small molecules which bind to the myristoyl site could keep Bcr–Abl in inactive configuration. GNF-2 (Fig. 2) was demonstrated to bind to the myristoyl pocket which located near the carboxy terminus of ATP site.[6] It could bind to myristoyl site and display potent inhibitory activity against both wild and mutant Bcr–Abl.

Bcr–Abl mutation-induced resistance remains a major challenge for clinical management of CML. It was demonstrated that combinations of GNF-2 and Imatinib could cooperate to suppress the emergence of resistance mutations.[5] Simultaneous binding to Bcr–Abl with myristoyl site and ATP pocket could decrease the appearance of resistance-conferring mutations and result in the inhibition of both wild and mutant Bcr–Abl.[7] Encouraged by the clinical success of Imatinib as Bcr–Abl inhibitors for the treatment of CML, we made our effort to develop new small molecules to overcome resistance to ATP-competitive Bcr–Abl inhibitors.

GNF-5, the hydroxylethylamide analog of GNF-2, is a highly selective non-ATP competitive Bcr–Abl inhibitor binding to myristoyl pocket. Structural analysis of chemical features of GNF-5 and Imatinib revealed that they possessed similar core structures (indicated in blue).[8] Based on the similar core structures of Imatinib and GNF-5, we designed a series of 4,6-disubstituted

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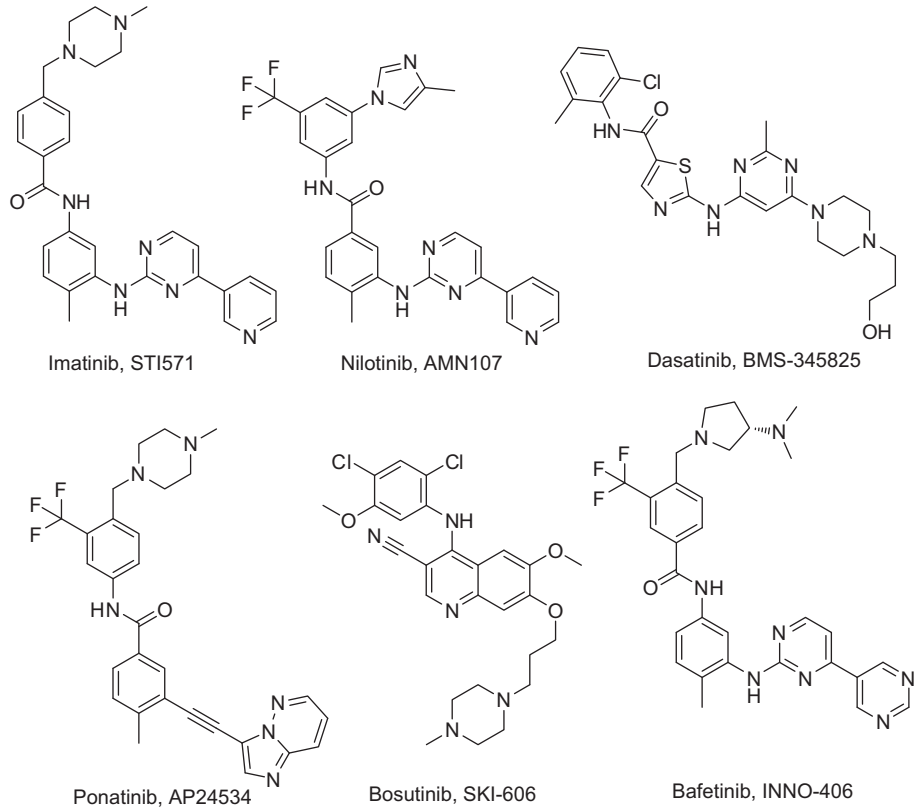


Figure 1. Structures of approved Bcr–Abl inhibitors.

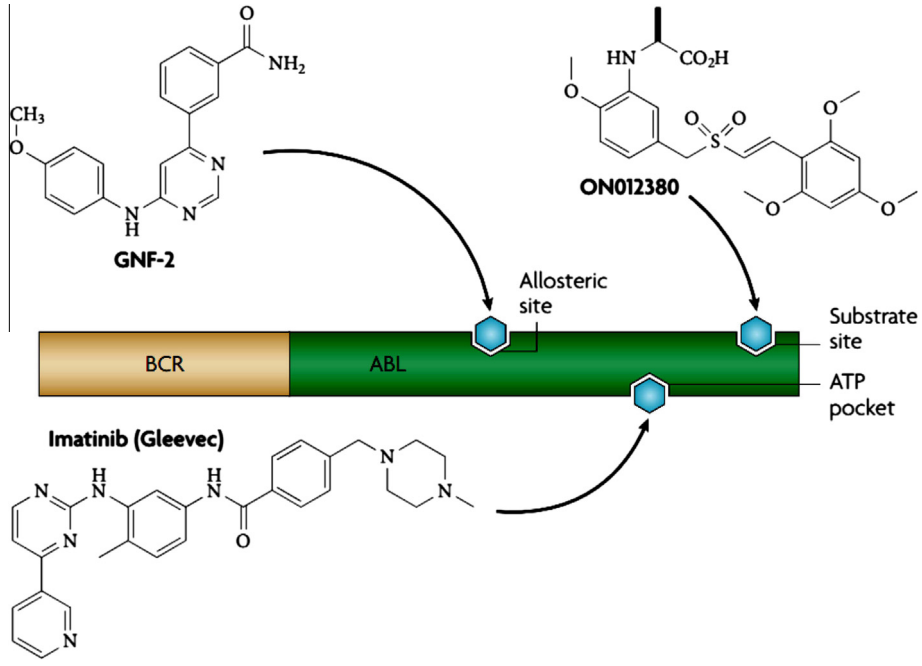


Figure 2. Different binding modes of inhibitors with Bcr–Abl.

pyrimidines to mimic the scaffold of Imatinib and GNF-5 (Fig. 3). Benzoylation piperazine was attached to the scaffold to increase the affinity with Bcr–Abl as well as the solubility.[9] Here we described a novel Bcr–Abl inhibitors design strategy, combining the structural features of two classes of Bcr–Abl inhibitors.[10] We expect that these molecules could bind to both ATP and myristoyl site of Bcr–Abl so as to overcome resistance to either agent alone.

Herein, we described the design, synthesis and biological evaluation of twenty seven 4,6-disubstituted pyrimidines as Bcr–Abl inhibitors targeting at myristoyl pocket and ATP site.

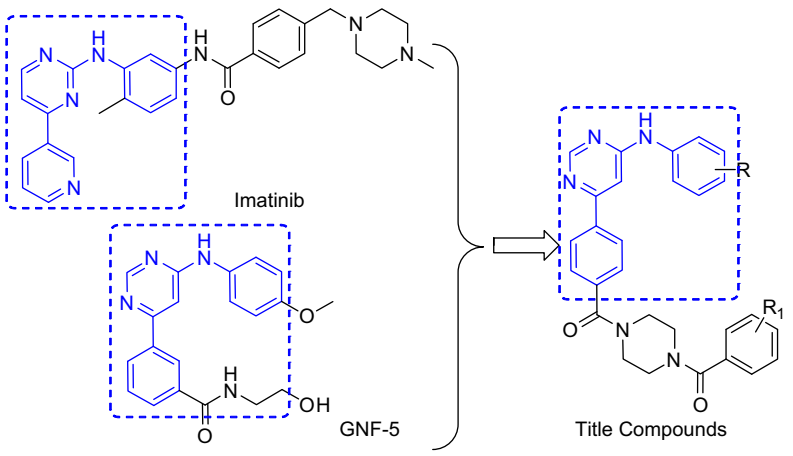


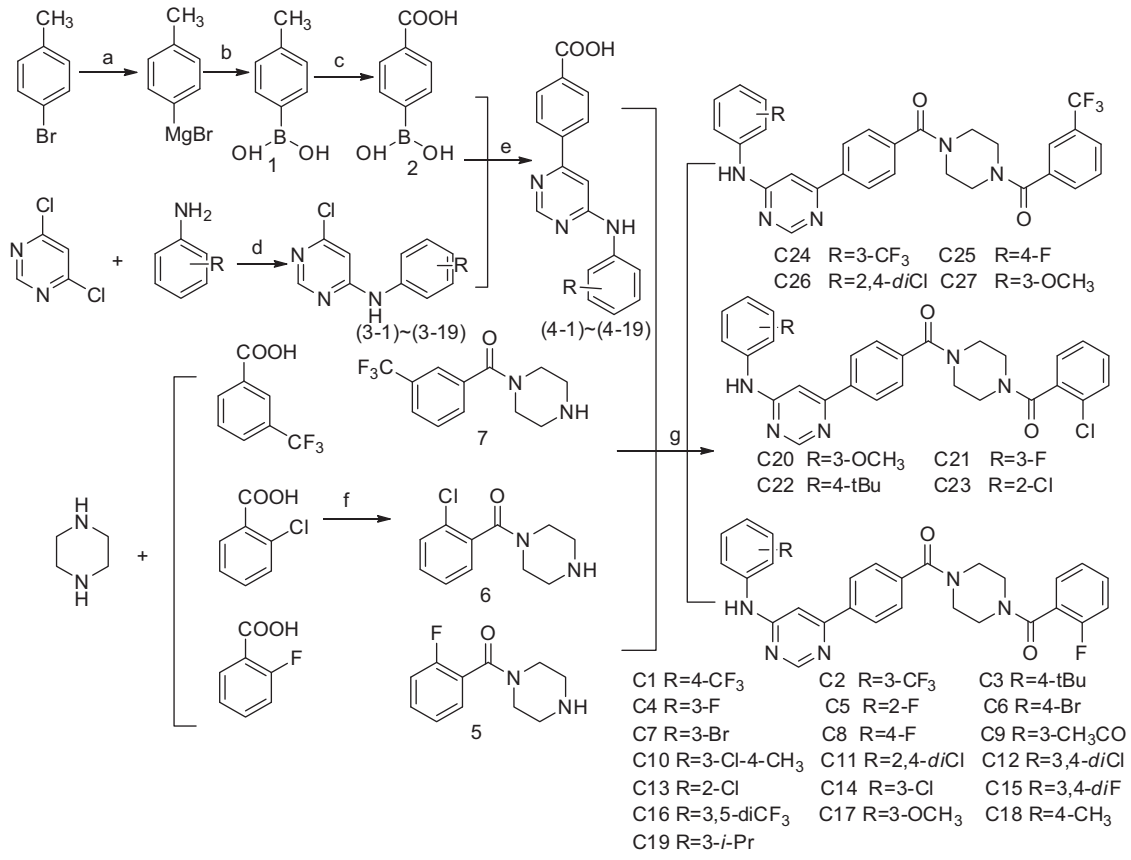
Figure 3. Design of 4,6-disubstituted pyrimidines as novel Bcr–Abl inhibitors.

Several title compounds displayed exclusive Bcr–Abl inhibitory activity and antiproliferative activity against K562 cells, with potencies similar to Imatinib. We expected to find novel lead compounds which could display Bcr–Abl inhibitory activity through binding to both ATP site and myristoyl pocket.

2. Chemistry

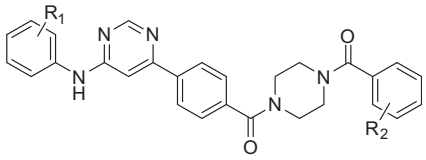
The synthetic route utilized in the preparation of the title compounds was outlined in Scheme 1. All compounds were prepared using Grignard reaction and palladium-catalyzed Suzuki cross coupling reaction as key steps.[11] 4,6-Disubstituted pyrimidines were synthesized from 4,6-dichloropyrimidine by acid-promoted amination followed by palladium-catalyzed Suzuki reactions with boronic acids to afforded key intermediates. Then condensation reaction of 4,6-disubstituted pyrimidines with various mono-benzoylation piperazines yielded title compounds.

An efficient synthesis of 4-boronobenzoic acid was developed in three steps via a slightly modified literature procedure.[12] 4-Bromotoluene was converted to corresponding Grignard reagent, followed by esterification in trimethylborate. Acidic hydrolysis provided 4-toluene boronic acid (**1**) in 60% yield after recrystallization from



Scheme 1. Reagents and conditions: (a) Mg, I_2, THF, reflux, N_2; (b) $B(OMe)_3$, THF, −20 °C; (c) NaOH (1 M), $KMnO_4$, TBAB, H_2O; (d) concd H_2SO_4, *i*-PrOH, reflux; (e) $Pd(Pph_3)_4$, Cs_2CO_3, CH_3CN/H_2O (V:V = 1:1), 90 °C; (f) CDI; THF; NaCl; piperazine/piperazine dihydrochloride (1:1); NaCl (20%), rt; (g) ClCOO-*i*Bu; THF; TEA; 0 °C → rt.

Table 1
In vitro Bcr–Abl inhibitory and antiproliferative activity against K562 cells of title compounds (IC_{50}, μM)



No	R_1	R_2	Bcr–Abl	K562	No	R_1	R_2	Bcr–Abl	K562
C1	4-CF_3	2-F	78.6	283	C2	3-CF_3	2-F	0.872	ND*
C3	4-tBu	2-F	354	>500	C4	3-F	2-F	0.017	17.8
C5	2-F	2-F	0.05	14.5	C6	4-Br	2-F	ND	306
C7	3-Br	2-F	>500	>500	C8	4-F	2-F	277	363
C9	3-CH_3CO	2-F	0.385	ND	C10	3-Cl-4-CH_3	2-F	>500	>500
C11	2,4-*di*Cl	2-F	>500	>500	C12	3,4-*di*Cl	2-F	>500	>500
C13	2-Cl	2-F	141	80.7	C14	3-Cl	2-F	91.8	ND
C15	3,4-*di*F	2-F	>500	>500	C16	3,5-*di*CF_3	2-F	0.130	60.4
C17	3-OCH_3	2-F	16.1	ND	C18	4-CH_3	2-F	ND	>500
C19	3-*i*-Pr	2-F	>500	>500	C20	3-OCH_3	2-Cl	ND	102
C21	3-F	2-Cl	6.06	ND	C22	4-tBu	2-Cl	>500	ND
C23	2-Cl	2-Cl	0.070	21.4	C24	3-CF_3	3-CF_3	0.563	25.9
C25	4-F	3-CF_3	1.44	86.9	C26	2,4-*di*Cl	3-CF_3	266	562
C27	3-OCH_3	3-CF_3	13.5	106	Imatinib			0.014	4.12

* ND = Not determined.

water. Oxidation of the benzylic carbon with $KMnO_4$ in the presence of NaOH and TBAB (tetrabutylammonium bromide) afforded 4-boronobenzoic acid (**2**) in 50% yield over three steps. Amination of 4,6-dichloropyrimidine with various aniline in the presence of concd H_2SO_4 under refluxing in 2-propanol was performed to obtain key intermediates (**3**–**1**)∼(**3**–**19**).[13] In the following step, the palladium-catalyzed Suzuki cross coupling reactions of (**3**–**1**)∼(**3**–**19**) with 4-boronobenzoic acid afforded (**4**–**1**)∼(**4**–**19**). Mono-benzoylation piperazine derivatives (**5**–**7**) were prepared from various substituted benzoic acid and piperazine using CDI as condensing agent.[14] Finally, the synthesis of title compounds (**C1**∼**C27**) was accomplished by amide bond formation through coupling reaction using the mixed anhydride method (isobutylchloroformate) successfully.[15]

3. Results and discussion

The Bcr–Abl tyrosine kinase inhibitory activity of title compounds were evaluated by using the well-established ADP-Glo assays. The antiproliferative potency of them was also identified in vitro against Bcr–Abl positive K562 cells by using MTT method.[16] Imatinib was utilized as positive control to validate the screening conditions. The in vitro enzymatic inhibitory activity and antiproliferative potency associated with the title compounds were compiled in Table 1.

It was indicated from Table 1 that some of them displayed moderate to high inhibitory activity against Bcr–Abl. Further evaluation suggested that these compounds could potent inhibit the proliferation of Bcr–Abl positive K562 cells. Four compounds (**C4**, **C5**, **C16**, **C23**) displayed potent enzymatic inhibitory activity with IC_{50} values at nanomolar ranges which were comparable to positive control. The most potent compounds (**C4** and **C5**) strongly inhibited Bcr–Abl with IC_{50} values of 0.017 and 0.050 μM, respectively. Some of them exhibited promising antiproliferative activity against K562 cells. In particularly, compounds (**C4** and **C5**) could also significantly suppress Bcr–Abl dependent K562 cells proliferation with IC_{50} values of 17.8 and 14.5 μM, respectively. In general, the antiproliferative results were consistent with the Bcr–Abl inhibitory assays. It was indicated that inhibition of Bcr–Abl might be one of the basis for anticancer activity. We found that the variety and position of halogen-substitution on aniline played important role in biological activity. It was indicated that compounds bearing substituents like fluorine, chlorine and trifluoromethyl on aniline possessed potent anticancer activity. The results indicated that the introduction of fluoro or trifluoromethyl substituent at terminal aniline might increase the enzymatic and cellar activity. We demonstrated that fluorine and trifluoromethyl were favourable structural features for achieving potent Bcr–Abl inhibition and antiproliferation effect against K562 cells.

Computational studies were carried out to investigate the potential binding modes of title compounds with ATP-binding site and myristoyl pocket of Bcr–Abl. The most potent inhibitor **C4** was selected for computational studies. Compound **C4** was docked into ATP site and myristoyl pocket of Bcr–Abl (PDB ID: 3K5V) by SYBYL-X 2.0.[17] Molecular insights based on molecular docking indicated

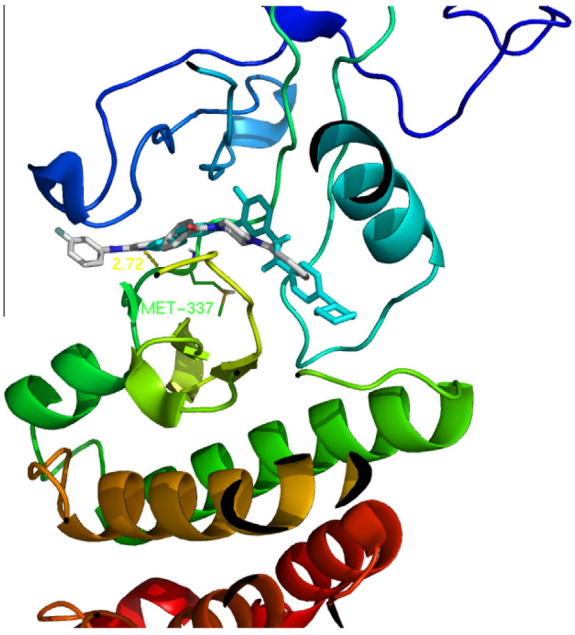


Figure 4. The predicted binding modes of C4 with ATP-binding site of Bcr–Abl.

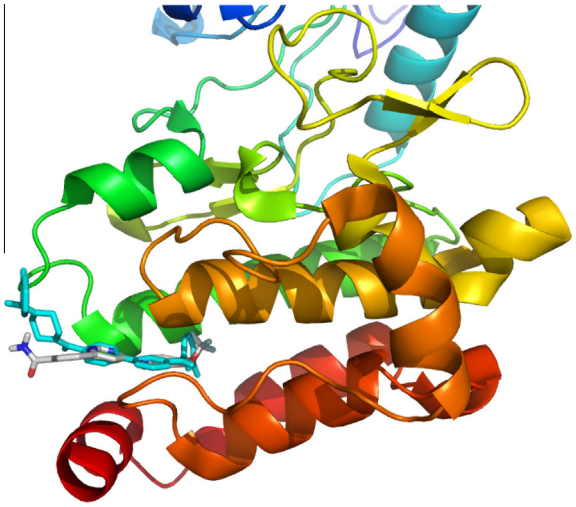


Figure 5. The predicted binding modes of C4 with myristoyl pocket of Bcr–Abl.

favorable binding mode of **C4** with both pockets. The results indicated that the representative compound **C4** could tightly bind to both ATP site and myristoyl pocket of Bcr–Abl. In the minimized complex of **C4** with ATP site of Bcr–Abl (Fig. 4), the ligand adopted the same conformation as Imatinib. In particular, there was a hydrogen bond formed between nitrogen atom of pyrimidine ring and Met 337 of ATP biding site with distance of 2.72 Å.

The myristoyl pocket is a cylindrical shaped cavity located at the C-terminus of Bcr–Abl. In the minimized complex of **C4** with myristoyl pocket of Bcr–Abl (Fig. 5), the ligand bound to receptor in a similar mode as GNF-2. Specially, the 2-fluorobenzoyl moiety might occupy a deeper position in the binding pocket than GNF-2. The proposed binding mode of C4 may account for the potential of inhibitory activity against mutants of Bcr–Abl.

These 4,6-disubstituted pyrimidines did not resemble the typical ATP-competitive Bcr–Abl inhibitors in structure.[18] Hence they are expected to target both ATP-binding site and myristoyl pocket. They could provide the potential to be unaffected by mutations in the ATP site which make resistant of CML to ATP-competitive inhibitors such as Imatinib.

4. Conclusion

In summary, a series of 4,6-disubstituted pyrimidines were developed as novel Bcr–Abl inhibitors based on Imatinib and GNF-5. Some of them displayed potent Bcr–Abl inhibition and antiproliferative potency against Bca–Abl positive leukemia cell line K562. This study demonstrated that a variety of structures can effectively target both ATP site and myristoyl pocket and provided new lead compounds for developing novel Bcr–Abl inhibitors. Further structural optimization of these promising anticancer agents will be reported in due course.

In recent studies, combinations of Imatinib and GNF-5 displayed potent Bcr–Abl inhibitory activity and antiproliferative potency against K562 cells. We proved that 4,6-disubstituted pyrimidine derivatives could be further modified as novel Bcr–Abl inhibitors targeting ATP site and myristoyl pocket. Many title compounds displayed varied degree of anticancer potency and Bcr–Abl inhibitory activity comparable to positive control. The results indicated that fluorine and trifluoromethyl were more suitable for their potency. Three compounds (**C4**, **C5**, **C23**) exhibited significant Bcr–Abl inhibitory activities, with IC_{50} values below 70 nM. Among them, **C4** exhibited potency comparable with Imatinib and might be a promising new candidate Bcr–Abl inhibitor. Moreover, some of them exhibited potent antiproliferative activities against K562 cell line. Molecular docking results indicated that these 4,6-disubstituted pyrimidines could interact more tightly with both ATP site and myristoyl pocket of Bcr–Abl.

5. Experimental

5.1. Chemistry: general procedure[18]

All solvents and reagents were obtained from commercial suppliers and purified according to standard procedure. All non-aqueous reactions were performed over dried glassware under nitrogen atmosphere. All reactions were monitored by TLC on 0.25-mm silica gel plates (fluorescence F_{254}) and visualized with UV light. Petroleum ether used refers to the fraction boiling in the range 60–90 °C. Melting points were determined on electrothermal melting point apparatus and are uncorrected. ^{1}H NMR spectra were measured on Bruker Advance (400 MHz) in $CDCl_3$ or DMSO-d_6. Coupling constants (J) are expressed in hertz (Hz) and chemical shifts (δ) of NMR are described in parts per million (ppm) units relative to internal control (TMS). Mass spectra were obtained on Shimadzu HPLC-MS-QP2010 instrument.

5.1.1. Preparation of (4-methylphenyl)boronic acid (1)[19]

A 250 mL, two-necked, round-bottomed flask containing dry magnesium (3.50 g, 150 mmol) and iodine (trace) is equipped with two rubber septum stirred under the protection of nitrogen. The solution of 4-bromotoluene (100 mmol) in 60 mL anhydrous THF was added slowly into the flask through a syringe at a rate to maintain reflux. After addition finishing, the resulting mixture was kept in refluxing for 5 h with stirring. The mixture is cooled to −30 °C, then the trimethylborate (14.1 g, 150 mmol) in 100 mL anhydrous THF was added slowly through a syringe. After addition finishing, the resulting mixture is allowed to stir at room temperature for overnight and then treated with HCl (2 mol/L, 100 mL), stirred 1 h at rt THF was removed by rotary evaporation. The aqueous layer was extracted with ethyl acetate (50 mL × 3). The combined organic layer was dried over Na_2SO_4, filtered and concentrated by rotary evaporation to give the yellow crude product. The crude product was purified by recrystallization from water and obtained a white solid (8.40 g, 62%).

5.1.2. Preparation of 4-carboxyphenylboronic acid (2)[20]

A solution of (4-methylphenyl)boronic acid (**1**) (6.80 g, 50 mmol) in 150 mL of 1 mol/L aqueous NaOH was added in one portion to a mixture of $KMnO_4$ (23.6 g, 150 mmol) and 0.50 g tetrabutylammoniumbromide in 500 mL water. The mixture was stirred at room temperature for overnight. 100 mL ethanol was added and stirred 1 h. The dark residue was filtered off and the clear filtrate was acidified with conc. HCl. The resulting precipitate was filtered out and dried under vacuum to obtain a 6.80 g white solid, yield 82%.

5.1.3. 6-Chloro-*N*-[4-(trifluoromethyl)phenyl]pyrimidin-4-amine (3–1)[21]

The 4-(trifluoromethyl)aniline (3.22 g, 20 mmol) and 4,6-dichloropyrimidine (3.88 g, 26 mmol)were dissolved in 30 mL of 2-propanol. Concd H_2SO_4 (1.50 mL) was added slowly under stirring at room temperature. The mixture was reacted under reflux and was monitored by TLC. The mixture was stored at 4 °C overnight. The precipitated product was filtered off, washed with a small amount of ice-cold 2-propanol, and dried in vacuum to afford a solid 4.90 g, yield 90%.

The intermediate compounds (**3**–**1**)∼(**3**–**19**) were prepared by using the general procedure described above.

5.1.4. 4-(6-{[4-(Trifluoromethyl)phenyl]amino}pyrimidin-4-yl) benzoic acid (4–1)[22]

Under a nitrogen atmosphere, 6-chloro-*N*-[4-(trifluoromethyl) phenyl]pyrimidin-4-amine (**3**–**1**) (4.10 g, 10 mmol), 4-carboxyphenylboronic acid (**2**) (2.00 g, 12 mmol), $Pd(PPh_3)_4$ (0.60 g, 0.50 mmol) and Cs_2CO_3 (13.0 g, 30 mmol) were suspended in a mixture of CH_3CN/H_2O (100 mL, V:V = 1:1). The mixture was heated under reflux for 48 h at 90 °C. The hot suspension was filtered and the filtrate distilled by rotary evaporation to remove acetonitrile. Water was added, and the mixture was extracted three times with EtOAc (3 × 30 ml). The aqueous layer was acidified with conc. HCl and extracted two more times with EtOAc. The combined organic layers were washed with brine and dried over Na_2SO_4. Evaporation of the solvent gave the crude product, which was purified by flash column chromatography to afford the product (2.90 g, 81%).

The key intermediate Compounds (**4**–**1**)∼(**4**–**19**) were prepared by using the general procedure described above.

5.1.5. Preparation of 1-(2-fluorobenzoyl)piperazine (5)

A solution of 2-fluorobenzoic acid (7.00 g, 0.05 mol) and CDI (8.90 g, 0.055 mol) was stirred in dry THF (30 mL) at room temperature for 30 min. In a separate round bottom flask add piperazine (10.76 g, 125 mmol) and piperazine dihydrochloride (20.0 g, 125 mmol) in 60 mL of water. Stir the reaction mixture for 5 min and add 14.0 g of NaCl. Add this brine solution to the round bottom flask containing acyl imidazole. Stir the reaction mixture for 5 hour. The mixture was filtered and the filtrate distilled by rotary evaporation to remove THF. The aqueous layer was washed with ethyl acetate (3 × 10 mL) to remove diacylated product. The PH of the aqueous layer was adjusted to about 9 using saturated solution of NaOH and washed with ethyl acetate (4 × 30 mL). The aqueous layer was discarded. The organic layer was washed with water (4 × 25 mL), dried over anhydrous Na_2SO_4 and concentrated by rotary evaporation and purified by flash chromatography to afford 1-(2-fluorobenzoyl)piperazine as colourless solid (4.90 g, 48%).

The other intermediates 1-(2-chlorobenzoyl)piperazine (**6**) and 1-[3-(trifluoromethyl)benzoyl]piperazine (**7**) were prepared by using the general procedure described above.

5.1.6. 6-(4-{[4-(2-Fluorobenzoyl)piperazin-1-yl]carbonyl} phenyl)-*N*-[4-(trifluoromethyl)phenyl]pyrimidin-4-amine (C1)

Isobutyl chloroformate (1.5 mL, 10 mmol) and triethylamine 4 mL were dissolved in 20 mL of THF and cooled to 0 °C, with vigorous stirring for 10 min. A solution of 4-(6-{[4-(trifluoromethyl)phenyl]amino}pyrimidin-4-yl)benzoic acid (**4**–**1**) (1.79 g, 5 mmol) and 2.5 mL triethylamine in 50 mL of THF was added slowly, maintaining the temperature at room temperature. The system was stirred for overnight and a solution of 1-(2-fluorobenzoyl)piperazine (**5**) in 10 mL THF was added. After 24 h, the mixture was filtered and the filtrate was concentrated by rotary evaporation to remove THF. The residue was poured into water and extracted with ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4, filtered and the ethyl acetate was removed by rotary evaporation. The production was purified by flash chromatography on silica gel using 1:5 EtOAc/petroleum ether as eluant. A single targeted compound (0.40 g) with write solid was obtained in 15% yield. Mp = 325–326 °C ;EI-MS (*m*/*z*): 549.0 (M+); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 10.2 (s, 1H), 8.9 (s, 1H), 8.05–8.19 (m, 2H), 7.98 (d, *J* = 8.0 Hz, 2H), 7.71 (d, *J* = 8.0 Hz, 2H), 7.58–7.67 (m, 2H), 7.49–7.56 (m, 1H), 7.39–7.48 (m, 1H), 7.40 (s, 1H), 7.19–7.35 (m, 2H), 3.39−3.91 (m, 8 H).

5.1.7. 6-(4-{[4-(2-Fluorobenzoyl)piperazin-1-yl]carbonyl} phenyl)-*N*-[3-(trifluoromethyl)phenyl]pyrimidin-4-amine (C2)

This compound was prepared from compounds (**4**–**2**) and (**5**) using the same procedure as described above: Yield 18%; mp = 265–267 °C; EI-MS (*m*/*z*): 548.9 (M+); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 10.11 (s, 1H), 8.83 (s, 1H), 8.29 (s, 1H), 8.06–8.17 (m, 2H), 7.95 (d, *J* = 8.0 Hz, 1H), 7.58–7.66 (m, 3H), 7.49–7.56 (m, 1H), 7.42–7.48 (m, 1H), 7.38 (d, *J* = 8.0 Hz, 1H), 7.21–7.36 (m, 3H), 3.39−3.82 (m, 8H)

5.1.8. *N*-(4-*tert*-Butylphenyl)-6-(4-{[4-(2-fluorobenzoyl) piperazin-1-yl]carbonyl}phenyl)pyrimidin-4-amine (C3)

This compound was prepared from compounds (**4**–**3**) and (**5**) using the same procedure as described above: Yield 15%; mp = 209–211 °C; EI-MS (*m*/*z*): 537.3 (M^+); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.67 (s, 1H), 8.70 (s, 1H), 7.99–8.16 (m, 2H), 7.60 (m, *J* = 8.0 Hz,4H), 7.49–7.56 (m, 1H), 7.42–7.48 (m, 1H),7.375 (d, *J* = 12.0 Hz, 2H), 7.27–7.35 (m, 2H), 7.25 (s, 1H), 3.40–3.85 (m, 8H), 1.29 (m, 9 H).

5.1.9. 6-(4-{[4-(2-Fluorobenzoyl)piperazin-1-yl]carbonyl}phenyl) -*N*-(3-fluorophenyl)pyrimidin-4-amine (C4)

This compound was prepared from compounds (**4**–**4**) and (**5**) using the same procedure as described above: Yield 16%; mp = 274–275 °C; EI-MS (*m*/*z*): 498.9 (M^+); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.98 (s, 1H), 8.80 (s, 1H), 8.05–8.16 (m, 2H),7.865 (d, *J* = 12.0 Hz, 1H), 7.61 (d, *J* = 8.0 Hz, 2H), 7.49–7.56 (m, 1H), 7.42–7.48 (m, 1H), 7.36–7.41 (m, 2H), 7.25–7.35 (m, 3H), 6.82–6.90 (t, *J* = 8.0 Hz, 1H), 3.38−3.83 (m, 8 H).

5.1.10. 6-(4-{[4-(2-Fluorobenzoyl)piperazin-1-yl]carbonyl} phenyl)-*N*-(2-fluorophenyl)pyrimidin-4-amine (C5)

This compound was prepared from compounds (**4**–**5**) and (**5**) using the same procedure as described above: Yield 18%; mp = 198–199 °C; EI-MS (*m*/*z*): 498.9 (M^+); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.475 (d, *J* = 12 Hz, 1H), 8.695 (d, *J* = 12 Hz, 1H), 8.11–8.02 (m, 2H), 7.66–7.53 (m, 6H), 7.36–7.25 (m, 3H), 7.24–7.12 (m, 2H), 3.84–3.43 (m, 8H).

5.1.11. *N*-(4-Bromophenyl)-6-(4-{[4-(2-fluorobenzoyl)piperazin-1-yl]carbonyl}phenyl)pyrimidin-4-amine (C6)

This compound was prepared from compounds (**4**–**6**) and (**5**) using the same procedure as described above: Yield 18%; mp = 288–290 °C; EI-MS (*m*/*z*): 561.1 (M^+); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.90 (s, 1H), 8.77 (s, 1H), 8.10 (d, *J* = 8.0 Hz, 1H), 7.73 (d, *J* = 8.0 Hz,2H), 7.58 (d, *J* = 8.0 Hz,2H), 7.53 (d, *J* = 8.0 Hz,2H), 7.40–7.47 (m, 1H), 7.23–7.39 (m, 4H), 6.79 (d, *J* = 8.0 Hz, 1H), 3.54–3.82 (m, 8 H).

5.1.12. *N*-(3-bromophenyl)-6-(4-{[4-(2-fluorobenzoyl)piperazin-1-yl]carbonyl}phenyl)pyrimidin-4-amine (C7)

This compound was prepared from compounds (**4**–**7**) and (**5**) using the same procedure as described above: Yield 20%; mp = 246–248 °C; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.94 (s, 1H), 8.81 (s, 1H), 8.14–8.21 (m,1H), 8.06–8.13 (m, 2H), 7.57–7.67 (m, 3H), 7.48–7.56 (m, 1H), 7.38–7.47 (m, 1H), 7.32 (t, *J* = 8.0 Hz, 4H), 7.22 (d, *J* = 8.0 Hz, 1H), 3.37–3.86 (m, 8 H).

5.1.13. 6-(4-{[4-(2-Fluorobenzoyl)piperazin-1-yl]carbonyl} phenyl)-*N*-(4-fluorophenyl)pyrimidin-4-amine (C8)

This compound was prepared from compounds (**4**–**8**) and (**5**) using the same procedure as described above: Yield 19%; mp = 285–286 °C; EI-MS (*m*/*z*): 498.9 (M^+); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.77 (d, *J* = 8.0 Hz, 1H), 8.17 (d, *J* = 8.0 Hz, 1H), 8.00–8.17 (m, 2H), 7.66–7.80 (m, 2H), 7.40–7.65 (m, 4H), 7.13–7.38 (m, 5H), 3.42–3.90 (m, 8 H).

5.1.14. 1-(3-{[6-(4-{[4-(2-Fluorobenzoyl)piperazin-1-yl]carbonyl} phenyl)pyrimidin-4-yl]amino}phenyl)ethanone (C9)

This compound was prepared from compounds (**4**–**9**) and (**5**) using the same procedure as described above: Yield 17%; mp = 244–246 °C; EI-MS (*m*/*z*): 523.1 (M^+); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.98 (s, 1H), 8.79 (s, 1H), 8.28 (s, 1H), 8.17–8.02 (m, 3H), 7.69–7.58 (m, 3H), 7.57–7.49 (m, 2H), 7.48–7.41 (m, 1H), 7.40–7.25 (m, 3H), 3.85–3.37 (m, 8 H), 2.62–2.58 (s,3H).

5.1.15. *N*-(3-Chloro-4-methylphenyl)-6-(4-{[4-(2-fluorobenzoyl) piperazin-1-yl]carbonyl}phenyl)pyrimidin-4-amine (C10)

This compound was prepared from compounds (**4**–**10**) and (**5**) using the same procedure as described above: Yield 18%; mp = 258–261 °C; EI-MS (*m*/*z*): 529.1 (M^+); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.85 (s, 1H), 8.78 (s, 1H), 8.13–8.00 (m, 3H), 7.60 (d, J = 8.0 Hz, 2H), 7.48–7.40 (m, 2H), 7.37–7.23 (m, 5 H), 3.84–3.49 (m, 8 H), 2.34–2.25 (s, 3H).

5.1.16. *N*-(2,4-Dichlorophenyl)-6-(4-{[4-(2-fluorobenzoyl) piperazin-1-yl]carbonyl}phenyl)pyrimidin-4-amine (C11)

This compound was prepared from compounds (**4**–**11**) and (**5**) using the same procedure as described above: Yield 15%; mp = 226–228 °C; EI-MS (*m*/*z*): 549.0 (M^+); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.34 (s, 1H), 8.69 (s, 1H), 8.16–8.05 (m, 2H), 7.97 (d, J = 8.0 Hz, 1H),7.72 (d, J = 2.0 Hz, 1H), 7.65 (s, 1H), 7.595 (d, J = 4.0 Hz, 2H), 7.49–7.42 (m, 2H), 7.36–7.27 (m, 1H), 7.02 (s, 2H), 3.83–3.43 (m, 8H).

5.1.17. *N*-(3,4-Dichlorophenyl)-6-(4-{[4-(2-fluorobenzoyl) piperazin-1-yl]carbonyl}phenyl)pyrimidin-4-amine (C12)

This compound was prepared from compounds (**4**–**12**) and (**5**) using the same procedure as described above: Yield 17%; mp = 288–289 °C; EI-MS (*m*/*z*): 549.1 (M^+); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 10.06 (s, 1H), 8.83 (s, 1H), 8.26 (s, 1H), 8.11 (s, 2H), 7.65–7.58 (m, 4H), 7.52 (s, 1H), 7.45 (s, 1H), 7.36–7.27 (m, 2H), 7.02 (s, 1H), 3.83–3.35 (m, 8 H).

5.1.18. *N*-(2-Chlorophenyl)-6-(4-{[4-(2-fluorobenzoyl)piperazin -1-yl]carbonyl}phenyl)pyrimidin-4-amine (C13)

This compound was prepared from compounds (**4**–**13**) and (**5**) using the same procedure as described above: Yield 20%; mp = 202–204 °C; EI-MS (*m*/*z*): 515.1 (M^+); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.30 (s, 1H), 8.67 (s, 1H), 8.03–8.14 (m, 2H), 7.89 (d, J = 8.0 Hz, 1H),7.64–7.51 (m, 4H), 7.49–7.42 (m, 1H), 7.41–7.26 (m, 4H), 7.22 (t, J = 6.0 Hz, 1H), 3.83–3.39 (m, 8 H).

5.1.19. *N*-(3-Chlorophenyl)-6-(4-{[4-(2-fluorobenzoyl)piperazin -1-yl]carbonyl}phenyl)pyrimidin-4-amine (C14)

This compound was prepared from compounds (**4**–**14**) and (**5**) using the same procedure as described above: Yield 18%; mp = 268–270 °C; EI-MS (*m*/*z*): 515.1 (M^+); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.96 (s, 1 H), 8.81 (s, 1H), 8.10 (s, 2H), 8.07 (s, 1H), 7.68–7.59 (m, 2H), 7.58–7.54 (m, 1H), 7.53–7.48 (m, 1H), 7.47–7.41 (m, 1H), 7.38 (t, J = 6.0 Hz, 1H), 7.35–7.25 (m, 3H), 7.09 (d, J = 8.0 Hz, 1H), 3.84–3.35 (m, 8H).

5.1.20. *N*-(3,4-Difluorophenyl)-6-(4-{[4-(2-fluorobenzoyl) piperazin-1-yl]carbonyl}phenyl)pyrimidin-4-amine (C15)

This compound was prepared from compounds (**4**–**15**) and (**5**) using the same procedure as described above: Yield 20%; mp = 276–278 °C; EI-MS (*m*/*z*): 517.1 (M^+); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.97 (s, 1H), 8.79 (s, 1H), 8.15–8.08 (m, 2H), 7.64 (s, 1H), 7.61 (d, J = 8.0 Hz, 2H), 7.56–7.48 (m, 1H), 7.45 (d, J = 8.0 Hz, 2H), 7.39 (s, 1H), 7.33–7.25 (m, 2H), 7.02 (s, 1H), 3.79–3.48 (m, 8H).

5.1.21. *N*-[3,5-Bis(trifluoromethyl)phenyl]-6-(4-{[4-(2-fluoro benzoyl)piperazin-1-yl]carbonyl}phenyl)pyrimidin-4-amine (C16)

This compound was prepared from compounds (**4**–**16**) and (**5**) using the same procedure as described above: Yield 24%; mp = 256–257 °C; EI-MS (*m*/*z*): 617.0 (M^+); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 10.45 (s, 1H), 8.90 (s, 1H), 8.48 (s, 2H), 8.18–8.09 (m, 2H), 7.69 (s, 1H),7.64–7.61 (m, 2H), 7.58–7.55 (m, 1H), 7.48–7.41 (m, 1H), 7.38–7.25 (m, 3H), 3.85–3.36 (m, 8H).

5.1.22. 6-(4-{[4-(2-Fluorobenzoyl)piperazin-1-yl]carbonyl} phenyl)-*N*-(3-methoxyphenyl)pyrimidin-4-amine (C17)

This compound was prepared from compounds (**4**–**17**) and (**5**) using the same procedure as described above: Yield 22%; mp = 215–216 °C; EI-MS (*m*/*z*): 511.2 (M^+); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.75 (s, 1H), 8.75 (s, 1H), 8.09 (d, J = 8.0 Hz, 2H), 7.64–7.57 (m, 2H),7.55–7.48 (m, 1H), 7.47–7.40 (m, 2H), 7.36–7.23 (m, 5H), 6.645 (d, J = 4.0 Hz, 1H), 3.77 (s, 3H), 3.75–3.39 (m, 8H).

5.1.23. 6-(4-{[4-(2-Fluorobenzoyl)piperazin-1-yl]carbonyl} phenyl)-*N*-(4-methylphenyl)pyrimidin-4-amine (C18)

This compound was prepared from compounds (**4**–**18**) and (**5**) using the same procedure as described above: Yield 19%; mp = 248–250 °C; EI-MS (*m*/*z*): 495.1 (M^+); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.645 (d, J = 12.0 Hz, 1H), 8.70 (d, J = 8.0 Hz, 1H), 8.14–8.00 (m, 2H), 7.70–7.50 (m, 5H), 7.47–7.39 (m, 1H), 7.37–7.25 (m, 3H), 7.21–7.11 (m, 2H), 3.85–3.48 (m, 8H), 2.285 (d, J = 12.0 Hz, 3H).

5.1.24. 6-(4-{[4-(2-Fluorobenzoyl)piperazin-1-yl]carbonyl} phenyl)-*N*-(3-isopropylphenyl)pyrimidin-4-amine (C19)

This compound was prepared from compounds (**4**–**19**) and (**5**) using the same procedure as described above: Yield 21%; mp = 196–197 °C; EI-MS (*m*/*z*):523.1 (M^+); ^{1}H NMR (400 MHz, DMSO) δ 9.71 (s, 1H), 8.73 (s, 1H), 8.08 (s, 2H), 7.61 (m, 2H), 7.51 (m, 2H), 7.45 (m, 1H), 7.39–7.22 (m, 5H), 6.94 (d, J = 7.6 Hz, 1H), 3.58 (m, 8H), 2.89 (h, J = 6.8 Hz, 1H), 1.23 (d, J = 6.9 Hz, 6H).

5.1.25. 6-(4-{[4-(2-Chlorobenzoyl)piperazin-1-yl]carbonyl} phenyl)-*N*-(3-methoxyphenyl)pyrimidin-4-amine (C20)

This compound was prepared from compounds (**4**–**2**) and (**6**) using the same procedure as described above: Yield 21%; mp = 227–229 °C; EI-MS (*m*/*z*): 527.3 (M^+); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.75 (s, 1H), 8.75 (s, 1H), 8.15–8.02 (m, 2H), 7.65 (s, 1H), 7.62–7.56 (m, 2H), 7.50–7.39 (m, 4H), 7.265 (d, J = 4.0 Hz, 2H), 7.02 (s, 1H), 6.68–6.60 (m, 1H), 3.77 (s, 3H), 3.75–3.37 (m, 8H).

5.1.26. 6-(4-{[4-(2-Chlorobenzoyl)piperazin-1-yl]carbonyl} phenyl)-*N*-(3-fluorophenyl)pyrimidin-4-amine (C21)

This compound was prepared from compounds (**4**–**8**) and (**6**) using the same procedure as described above: Yield 20%; mp = 252–255 °C; EI-MS (*m*/*z*): 515.3 (M^+); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.99 (s, 1H), 8.80 (s, 1H), 8.16–8.03 (m, 2H), 7.865 (d, J = 12.0 Hz, 1H), 7.68 (s, 1H), 7.63––7.54 (m, 2H), 7.48–7.38 (m, 4H), 7.33–7.28 (m, 1H), 7.04 (s, 1H), 6.92–6.81 (m, 1H), 3.83–3.39 (m, 8H).

5.1.27. *N*-(4-*tert*-Butylphenyl)-6-(4-{[4-(2-chlorobenzoyl) piperazin-1-yl]carbonyl}phenyl)pyrimidin-4-amine (C22)

This compound was prepared from compounds (**4**–**11**) and (**6**) using the same procedure as described above: Yield 20%; mp = 209–210 °C; EI-MS (*m*/*z*): 553.3 (M^+); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.67 (s, 1H), 8.70 (s, 1H), 8.16–8.00 (m, 2H), 7.65–7.53 (m, 5H), 7.52–7.41 (m, 3H), 7.375 (d, J = 12.0 Hz, 2H), 7.17 (s, 1H), 3.84–3.35 (m, 8H), 1.29 (s, 9H).

5.1.28. 6-(4-{[4-(2-Chlorobenzoyl)piperazin-1-yl]carbonyl} phenyl)-*N*-(2-chlorophenyl)pyrimidin-4-amine (C23)

This compound was prepared from compounds (**4**–**17**) and (**6**) using the same procedure as described above: Yield 21%; mp = 237–239 °C; EI-MS (*m*/*z*): 531.1 (M^+); ^{1}H NMR (400 MHz, DMSO) δ 9.96 (s, 1H), 8.81 (s, 1H), 8.17–8.02 (m, 4H), 7.64–7.53 (m, 4H), 7.52–7.40 (m, 2H), 7.38 (t, *J* = 8.1 Hz, 1H), 7.30 (s, 1H), 7.09 (d, *J* = 6.9 Hz, 1H), 3.68 (m, 8H).

5.1.29. 6-[4-({4-[3-(Trifluoromethyl)benzoyl]piperazin-1-yl} carbonyl)phenyl]-N-[3-(trifluoromethyl)phenyl]pyrimidin-4-amine (C24)

This compound was prepared from compounds (**4**–**2**) and (**7**) using the same procedure as described above: Yield 24%; mp = 220–222 °C; ESI-MS (*m*/*z*): 600.1 $(M+H)^+$; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 10.11 (s, 1H), 8.83 (s, 1H), 8.29 (s, 1H), 8.125 (d, *J* = 4.0 Hz,2H), 7.95 (d, *J* = 8.0 Hz, 1H), 7.855 (d, *J* = 4.0 Hz, 1H), 7.80 (s, 1H), 7.78–7.72 (m, 1H), 7.64–7.56 (m,4H), 7.38 (d, *J* = 8.0 Hz,1H), 7.32 (s, 1H), 3.83–3.38 (m, 8H)

5.1.30. *N*-(4-Fluorophenyl)-6-[4-({4-[3-(trifluoromethyl)benzoyl] piperazin-1-yl}carbonyl)phenyl]pyrimidin-4-amine (C25)

This compound was prepared from compounds (**4**–**8**) and (**7**) using the same procedure as described above: Yield 19%; mp = 193–195 °C; EI-MS (*m*/*z*): 549.4 (M^+); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.77 (s, 1H), 8.72 (s, 1H), 8.095 (d, *J* = 4.0 Hz, 2H), 7.91–7.83 (m, 1H), 7.80 (s, 1H), 7.76–7.70 (m, 3H), 7.59 (d, *J* = 8.0 Hz, 2H), 7.29–7.15 (m, 3H), 7.02 (s, 1H), 3.85–3.37 (m, 8H).

5.1.31. *N*-(2,4-Dichlorophenyl)-6-[4-({4-[3-(trifluoromethyl)benzoyl] piperazin-1-yl}carbonyl)phenyl]pyrimidin-4-amine (C26)

This compound was prepared from compounds (**4**–**11**) and (**7**) using the same procedure as described above: Yield 22%; mp = 152–155 °C; EI-MS (*m*/*z*): 599.3 (M^+); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.34 (s, 1H), 8.69 (s, 1H), 8.105 (d, *J* = 4.0 Hz, 2H), 7.97 (d, *J* = 8.0 Hz, 1H), 7.88–7.82 (m, 1H), 7.80 (s, 1H), 7.77–7.04 (m, 1H), 7.715 (d, *J* = 4.0 Hz, 2H), 7.63–7.55 (m, 2H), 7.50–7.41 (m,2H), 3.85–3.42 (m, 8H).

5.1.32. *N*-(3-Methoxyphenyl)-6-[4-({4-[3-(trifluoromethyl) benzoyl] piperazin-1-yl}carbonyl)phenyl]pyrimidin-4-amine (C27)

This compound was prepared from compounds (**4**–**17**) and (**7**) using the same procedure as described above: Yield 25%; mp = 84–86 °C; EI-MS (*m*/*z*): 561.3 (M^+); ^{1}H NMR (400 MHz, DMSO) δ 9.80 (s, 1H), 8.76 (s, 1H), 8.10 (d, *J* = 6.9 Hz, 2H), 7.87–7.74 (m, 3H), 7.61–7.56 (m, 2H), 7.44 (s, 1H), 7.33–7.20 (m, 4H), 6.64 (d, *J* = 6.2 Hz, 1H), 3.77 (s, 3H), 3.66–3.32 (m, 8H).

5.2. Bcr–Abl inhibitory activity assays[23]

The Bcr–Abl inhibitory activity assay was performed using ADP-Glo™ Kinase assay kit (Promega, catalog: V9101 and Abl Kinase Enzyme System (Promega, catalog: V1901) according to the manufacturer's instructions. The Abl1 reaction utilizes ATP and generates ADP. Then the ADP-Glo™ reagent is added to simultaneously terminate the kinase reaction and deplete the remaining ATP. Finally, the Kinase Detection Reagent is added to convert ADP to ATP and the newly synthesized ATP is converted to light using the luciferase reaction.

Abl was incubated with substrates, inhibitors and ATP in a final buffer of 25 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 0.01% Triton X-100, 100 μg/mL BSA, 2.5 mM DTT in 384-well plate with the total volume of 10 μL. Then the ADP-Glo™ Kinase Assay was performed in two steps once the kinase reaction is complete.[24] Subsequently, 5 μL ADP-Glo Reagent was added to stop the kinase reaction and deplete the unconsumed ATP. Only ADP and a very low background of ATP were left. Then the mixture was incubated at room temperature for 40 min and added 10 μL of Kinase Detection Reagent to convert ADP to ATP and introduced luciferase and luciferin to detect ATP. At last, the mixture was incubated at room temperature for 30–60 min and measured the luminescence with a plate-reading luminometer. The signal was correlated with the amount of ATP present in the reaction and was inversely correlated with the kinase activity.

5.3. Cell growth inhibitory activity in cancer cells[25]

The title compounds were evaluated for their antiproliferative potency against Bcr–Abl positive K562 cells using MTT method. Exponentially growing cells were harvested and plated in 96-well plates at a concentration of 1×10^4 cells/well, and then incubated for 24 h at 37 °C. The cells in the wells were treated with the title compounds respectively at various concentrations for 48 h. Then, 20 mL MTT (5 mg/mL) was added to each well and incubated for 4 h at 37 °C. Supernatant was discarded, and 150 mL DMSO was added to each well. Absorbance values were determined by a microplate reader (Bio-Rad Instruments) at 570 nm. The IC_{50} values were calculated according to inhibition ratios.

5.4. Molecular docking[26]

In order to understand the binding mode of the title compounds with both ATP site and myristoyl pocket of Bcr–Abl, we performed a molecule docking using Sybyl/Surflex-dock based on crystal structures of Bcr–Abl. The inhibitors were docked into the active site using ligand-based mode. The crystal structure of Bcr–Abl complex with Imatinib and GNF-2 was obtained from RCSB Protein Data Bank (PDB ID: 3K5V). The ligands and water molecules were removed and hydrogen was added and minimized using Tripos force field and Pullman charges. The residues in a radius 5.0 Å around ligands were selected as active site. **C4** was depicted with Sybyl/Sketch module (Tripos Inc.) and optimized applying Powell's method with Tripos force field with convergence criterion set at 0.05 kcal/(Å mol), and assigned with Gasteiger–Hückel method. Other docking parameters were kept at default.

Acknowledgment

This work was supported by the National Natural Science Foundation of China (Grant NO. 81302641 and 81302737), and the Fundamental Research Funds for the Central Universities (xjj2014140).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.10.030.

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