Discovery of Novel Bruton's Tyrosine Kinase (BTK) Inhibitors Bearing a *N*,9-Diphenyl-9*H*-purin-2-amine ScaffoldYang Ge,^{†,||} Yue Jin,^{†,||} Changyuan Wang,[†] Jianbin Zhang,[†] Zeyao Tang,[†] Jinyong Peng,[†] Kexin Liu,[†] Yanxia Li,[‡] Youwen Zhou,[§] and Xiaodong Ma^{*,†}[†]College of Pharmacy, Dalian Medical University, Dalian 116044, P. R. China[‡]Respiratory Department, The First Affiliated Hospital of Dalian Medical University, Dalian 116011, P. R. China[§]Department of Dermatology and Skin Science, University of British Columbia, Vancouver, BC V5Z 4E8, Canada

Supporting Information

ABSTRACT: Based on the pyrimidine skeleton of EGFR^{T790M} inhibitors, a series of *N*,9-diphenyl-9*H*-purin-2-amine derivatives were identified as effective BTK inhibitors. Among these compounds, inhibitors **10d**, **10i**, and **10j**, possessing IC₅₀ values of 0.5, 0.5, and 0.4 nM, displayed anti-BTK kinase activity that was as potent as the reference compounds. In particular, compound **10j** suppressed the proliferation of two typical B-cell leukemia cell lines expressing high levels of BTK with concentrations of 7.75 and 12.6 μM. The activity of the subject compound as determined by the CCK-8 method and apoptosis analysis validated that inhibitor **10j** is slightly more potent than AVL-292 and ibrutinib. The results of these experimental explorations suggested that **10j** could serve as a valuable molecule for control of leukemia pending further developments

KEYWORDS: Leukemia, BTK inhibitor, purin, synthesis, activity

Bruton's tyrosine kinase (BTK) is a member of the Tec family of nonreceptor tyrosine kinases that play a key role in the B-cell signaling pathway linking cell surface B-cell receptor (BCR) stimulation to downstream intracellular responses.^{1,2} The deregulation of BTK has been observed in numerous B-cell-derived malignancies, including acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), non-Hodgkin's lymphoma (NHL), mantle cell lymphoma (MCL), Waldenstrom's macroglobulinemia (WM), and multiple myeloma (MM).^{3,4} In 2013, ibrutinib was approved by the US Food and Drug Administration (FDA) as the first-in-class human BTK-inhibitor (**1**, Figure 1) to demonstrate clinical effectiveness and tolerability in early clinical trials for the treatment of mantle cell lymphoma.⁵ Subsequently, it was also applied to treat chronic lymphocytic leukemia (2014) and Waldenstrom's macroglobulinemia (2015), a form of non-Hodgkin's lymphoma.^{6–8}

Recently, a number of covalent and noncovalent BTK inhibitors including GS-4059 (**2**) (ClinicalTrials.gov identifier: NCT02457559),⁹ RN486 (**3**),^{10,11} AVL-292 (**4**) (ClinicalTrials.gov identifier: NCT01975610),¹² HM71224 (**5**) (ClinicalTrials.gov identifier: NCT01765478),¹³ and PLS-123 (**6**)¹⁴ have been advanced to preclinical to clinical trials (Figure 1). Notably, all currently reported covalent BTK inhibitors act primarily on the amino acid Cys481 located in the kinase hinge segment,¹⁵ which is also the preserve of all five Tec-family kinases, JAK3, EGFR, HER2, HER4, and BLK. In terms of an

epidermal growth factor receptor (EGFR), the binding for this resides at Cys797, while it is found at Cys909 in JAK3.^{16–18} Using this strategy, the novel pyrimidine scaffold, included in molecules WZ4002 (**7**),¹⁷ rociletinib (**8**),¹⁹ and osimertinib (**9**)²⁰ has been found to be a potent inhibitor that can overcome nonsmall-cell lung cancer (NSCLC) drug resistance with T790M mutation (Figure 2).

In an effort to develop more effective EGFR^{T790M} inhibitors, we previously designed and synthesized a series of *N*,9-diphenyl-9*H*-purin-2-amine analogues (**10a–m**, Figure 3) by applying the conformational constraint strategy, which is an effective method to improve ligand selectivity for a molecular target.^{21–23} Unfortunately, the assayed results showed that these synthesized compounds exhibited nearly 10-fold less potency against H1975 cells harboring the EGFR^{T790M} mutant. In addition, they also displayed weak activity against the JAK3 enzyme with IC₅₀ values of about 100 nM. Fortunately, the evaluation of these compounds for the inhibition of the BTK target provided exciting results, where several molecules appeared to inhibit BTK kinase at concentrations of 10 nM. Therefore, in this report, the potential of these molecules as BTK inhibitors is detailed by describing their synthesis, biological activity, and molecular simulation.

Received: June 11, 2016

Accepted: September 21, 2016

Published: September 21, 2016

Figure 1. Structures of exemplary BTK inhibitors.

Figure 2. Structures of novel EGFR^{T790M} inhibitors.

Figure 3. Designed strategy of title molecules.

The synthesis of title compounds **10a–m** is shown in Scheme 1.^{23,24} In this synthesis, commercially available 2,4-dichloro-5-nitropyrimidine **11** was reacted with the protected *tert*-butyl 3-aminophenylcarbamate in the presence of *N,N*-diisopropylethylamine (DIPEA) to produce 2-chloro-4-(3-*tert*-butoxycarbonylphenylamino)-5-nitropyrimidine **12** as a white solid. After coupling with various anilines, the pyrimidine template (compounds **13a–k**) was synthesized. Compounds **13a–k** were further deprotected with trifluoroacetic acid and subsequently reacted with acryloyl chloride, to produce the key intermediates **15a–k**. By reducing the nitro functionality in **15a–k** using Fe-NH₄Cl conditions, the anilines **16a–k** were prepared in excellent yields (52–68%). Finally, compounds **16a–k** were treated with triethyl orthoformate or carbon-

Scheme 1. Synthetic Route of Title Compounds **10a–m**^a

^aReagents and conditions: (a) ArNH₂, DIPEA, 1,4-dioxane, rt, 2 h, 91%; (b) ArNH₂, DIPEA, 1,4-dioxane, 60 °C, 2 h, 49–72%; (c) trifluoroacetic acid, CH₂Cl₂, 0 °C to rt, 62–85%; (d) acryloyl chloride, NaHCO₃, acetone, 10 min, 43–68%; (e) Fe-NH₄Cl, MeOH-H₂O, 2 h, 80 °C, 52–68%; (f) triethyl orthoformate, 2 h, 110 °C, 28–51%; (g) carbonyldiimidazole, CH₂Cl₂, 1 h, rt, 32–44%.

ylidiimidazole to yield the desired 2,8-dianilinopurine derivatives **10a–m**.²⁵

The newly synthesized *N*,9-diphenyl-9*H*-purin-2-amine derivatives were evaluated as BTK inhibitors in enzyme-based and cell-based assays. For comparison, AVL-292 (phase III) and the approved drug ibrutinib were also evaluated as references. All these results are reported in Table 1. Among these inhibitors, compounds **10d**, **10i**, and **10j** displayed the strongest potency to inhibit the BTK kinase, possessing IC₅₀ values of 0.5, 0.5, and 0.4 nM, respectively. Apparently, they were as potent as AVL-292 (IC₅₀ = 0.6 nM) and ibrutinib (IC₅₀ = 0.3 nM) for suppressing the BTK target. The activity tested in kinase level revealed that the sulfonamide group at the *N*-4 position of the piperazine ring was unfavorable to interact with BTK. For instance, compounds **10f** and **10h**, with IC₅₀ values of 17.1 and 13.2 nM, were 57- to 22-fold less potent for suppressing the BTK target than the reference compounds. From these tested results, it also can be seen that compounds **10c**, **10e**, and **10g**, containing a C-2' methoxy-substituted phenyl amino at C-2 position of pyrimidine core, significantly lost the capacity for inhibiting BTK (IC₅₀ values of more than 100 nM). It was speculated that the bulky methoxy group caused the steric exclusion of the BTK binding pocket, which greatly decreased the binding forces between the synthesized compounds and the BTK target. However, compound **10k**, which was produced by removing the methoxy group to the C-4 position of the phenyl ring, displayed improved suppression activity (IC₅₀ = 97.1 nM) for inhibiting the BTK kinase. Introduction of a long side chain to the phenyl ring, such as 3-morpholin-4-ylpropoxy group (compound **10j**), is quite beneficial in inhibiting the BTK (IC₅₀ = 0.4 nM) enzyme. With regard to compounds **10l** and **10m**, due to the presence

Table 1. Biological Activity of Our Synthesized Compounds^a

Compd	R	R ¹	L	Enzymatic activity (IC ₅₀ , nM)		Antiproliferation activity (IC ₅₀ , μM) ^b	
				BTK	JAK3	Ramos	Raji
10a		H		15.1	>100	11.3	11.8
10b		H		10.2	67.1	8.22	7.55
10c		MeO		>100	>100	13.2	13.7
10d		H		0.5	67.5	12.1	19.5
10e		MeO		>100	>100	>40	>40
10f		H		17.1	70.1	>40	>40
10g		MeO		>100	>100	38.6	21.9
10h		H		13.2	83.1	>40	31.7
10i		H		0.5	61.2	>40	24.2
10j		H		0.4	72.6	7.75	12.6
10k	MeO	H		97.1	67.2	>40	31.2
10l		H		>100	>100	35.2	11.9
10m		MeO		>100	>100	39.1	>40
AVL-292				0.6	--	15.1	25.30
Ibrutinib				0.3	--	8.11	15.2

^aData represent the mean of at least three separate experiments.^bDose–response curves were determined at five concentrations. The IC₅₀ values are the concentrations in micromolar needed to inhibit cell growth by 50% as determined from these curves.

of a hydroxyl group on the pyrimidine core, they exhibited very weak anti-BTK activity, with IC₅₀ values of more than 100 nM.

Figure 4. Compound **10j** induced ram0s cell apoptosis in vitro. The cells were incubated with the indicated concentrations of **10j** for 48 h, and the cells were stained with annexin V/FTIC, followed by flow cytometry analysis. One representative experiment is shown. $p < 0.05$.

In addition, all these compounds were also evaluated for their effects on the JAK3 kinase. As shown in Table 1, most of these analogues were not effective in inhibiting JAK3, with inhibition IC₅₀ values ranging from 61.2 to 83.1 nM. Interestingly, compounds **10i** (IC₅₀ = 61.2 nM) and **10j** (IC₅₀ = 72.6 nM), which have very low IC₅₀ values for inhibiting BTK kinase (0.5 and 0.4 nM, respectively), appeared to be quite potent against JAK3 kinase, suggesting that further modification of this scaffold may produce a potent JAK3 and BTK dual inhibitor.

Further testing of these molecules against B-cell leukemia cell lines (ramos and raji) using CCK-8 method are summarized in Table 1.²⁶ As shown in this table, compound **10j**, the active inhibitor against the BTK kinase (IC₅₀ = 0.4 nM), was also very potent against the B-cell leukemia cell lines with IC₅₀ values of 7.75 μM against ramos cell line and 12.6 μM against raji cell line. Although analogue **10b** had a lower IC₅₀ value (10.2 nM) than **10j** in kinase-based evaluation, it exhibited a strong suppression of these cancer cell lines (IC₅₀ values of 8.22 and 7.55 μM, respectively). Interestingly, analogue **10c**, which contains a methoxy group at C-2 position of the phenyl ring, exhibited nearly no anti-BTK activity (with an IC₅₀ value of more than 100 nM), but possessed moderate IC₅₀ value of 13.2 μM against the ramos cell line and IC₅₀ value of 13.7 μM against the raji cell line, hinting that **10c** might be employing a new mechanism to inhibit the B-cell leukemia cell lines. It found that compound **10e** with a methoxy group at the C-2

Figure 5. Effects of AVL-292, ibrutinib, and 10j on Ramos cell cycle arrest detected by flow cytometry assay. Results are representative of three separate experiments. Data are expressed as the mean $\pm$ standard deviation. $p < 0.05$.

position of the phenyl ring was not particularly sensitive to the B-cell leukemia cell lines with an IC_{50} value of more than 40 μ M. The two compounds 10l and 10m containing a special hydroxyl group on the purin core were also not very effective against the B-cell leukemia cell lines, with moderate anti-B-cell leukemia cell lines.

Additionally, the effects of the most active inhibitor 10j on apoptosis and on the cell cycle in Ramos cell line were also determined using flow cytometry analysis in parallel with AVL-292 and ibrutinib as the reference compounds. As shown in Figure 4, at a concentration of 10 μ M/L, all molecules 10j, AVL-292, and ibrutinib induced significant apoptosis (more than 41.5%). In accordance with their activity against the Ramos cell line, the novel inhibitor 10j (52.8%) displayed stronger activity to induce apoptosis than AVL-292 (41.5%) and ibrutinib (47.9%). Cell cycle analysis using flow cytometry was also performed for inhibitor 10j. As shown in Figure 5, at a concentration of 10 μ M/L, both the new synthesized molecule 10j and ibrutinib produced similar inhibition results arresting the cell cycle at the S phase. By contrast, inhibitor AVL-292 apparently arrested the cell cycle at the G2 phase.

To understand how the newly designed compounds interacted with the ATP-binding pocket of the BTK kinase domain and how this related to their binding affinity for BTK, several representative compounds, specifically the potent inhibitors 10d, 10i, and 10j and the less active inhibitor 10e, were docked using a molecular simulation into the ATP binding pocket of BTK enzyme (PDB: 3GEN).²⁷ The program

Figure 6. (a) Putative binding model of inhibitor 10d within BTK (PDB code: 3GEN). (b) Putative binding model of inhibitor 10i within BTK (PDB code: 3GEN). (c) Putative binding model of inhibitor 10j within BTK (PDB code: 3GEN). (d) Putative binding model of inhibitor 10e within BTK (PDB code: 3GEN).

AutoDock 4.2 with its default parameters was used to perform these simulations (Figure 6).^{28,29}

As indicated in the model, both active inhibitors 10d and 10j formed a strong hydrogen bond between the N-7 atom of purin core and amino acid Met477. While for compound 10i, the strong hydrogen-bond forces were produced by its N-9 atom of purin core with Leu528. It also should be noted that in the case of the active inhibitors 10d (Figure 6a) and 10i (Figure 6b), the electrophilic acrylamide was poised in a suitable position to allow the formation of a covalent bond with Cys481, while in compound 10j (Figure 6c) and 10e (Figure 6d), this enhanced interaction was not present. Instead, the polar morpholine ring in 10j and 10e pointed directly into the exterior water through a rather small channel in the protein surface, and the 4-aniline group tightly interacted with the hydrophobic pocket surrounded by Lys430, Leu408, and Leu450. Compared with 10e, inhibitor 10j also produced another hydrogen bond between the carbonyl group with amino acid Leu 408. Moreover, the more flexible morpholine side chain may generate stronger forces with Asn484 and Cys481. Therefore, compound 10j retained a high potency for inhibiting BTK kinase despite the disappearance of a covalent bond. Taken together, the docking simulation provided the possible explanations for the experimentally observed activities.

In summary, a series of novel *N*,9-diphenyl-9*H*-purin-2-amine derivatives were found to be potent BTK inhibitors. Among these compounds, inhibitors 10d, 10i, and 10j with IC_{50} values of 0.5, 0.5, and 0.4 nM, displayed a level of anti-BTK activity that was similar to the reference compounds.

Moreover, several compounds exhibited potent inhibition for the proliferation of two typical cancer cell lines expressing high levels of BTK, including the ramos and raji cell lines. In particular, compound **10j** was able to suppress these cell lines at concentrations of 7.75 and 12.6 μ M. Apoptosis analysis in the ramos cell line also indicated that **10j** was slightly more potent than AVL-292 and ibrutinib. A molecular simulation analysis showed that **10j** formed strong interactions with BTK kinase. All these results suggested that **10j** was the most active inhibitor, which will be the subject of future experimental studies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.6b00235.

Full experimental details, enzymatic-activity assay, and tumor cellular-activity assay (PDF)

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Funding

We are grateful to the National Natural Science Foundation of China (No. 81402788) and the Ph.D. Start-up Fund of Natural Science Foundation of Liaoning Province, China (No. 20141115) for the financial support of this research.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

BTK, Bruton's tyrosine kinase; EGFR^{T790M}, EGFR with T790M mutation; NSCLC, nonsmall-cell lung cancer

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(25) The spectrum data for representative compound *N*-(9-(3-aminophenyl)purin-2-((4-(1-morpholino)propoxyl)aminophenyl)) acrylamide (**10j**). Yield: 29.1%; off-white solid; ¹H NMR (DMSO-*d*₆) δ 1.82–1.87 (m, 2H), 2.36–2.41 (m, 6H), 3.51–3.62 (m, 4H), 3.91 (t, *J* = 6.4 Hz, 2H), 5.83 (d, *J* = 10.0 Hz, 1H), 6.33 (d, *J* = 16.8 Hz, 1H), 6.48–6.55 (m, 1H), 6.78 (d, *J* = 8.8 Hz, 2H), 7.51–7.66 (m, 3H), 7.68 (t, *J* = 8.8 Hz, 2H), 8.38 (s, 1H), 8.57 (s, 1H), 8.89 (s, 1H), 9.55 (s, 1H), 10.48 (s, 1H); ¹³C NMR (DMSO-*d*₆) δ 26.1, 53.5 (2C), 55.0, 66.0, 66.3 (2C), 114.4 (2C), 114.5, 118.4, 118.6, 120.5 (2C), 127.7, 128.0, 130.1, 131.7, 133.8, 135.0, 140.1, 142.2, 150.0, 151.8, 153.5, 157.1, 163.5; HRMS (ESI) for C₂₇H₂₉N₇O₃, [M+H]⁺ calcd, 500.2405; found, 500.2396.

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