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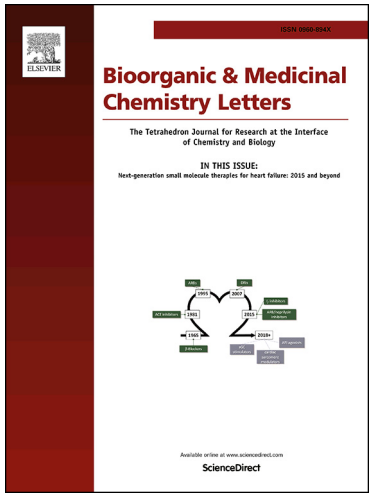

Design and synthesis of novel 1-phenyl-3-(5-(pyrimidin-4-ylthio)-1,3,4-thiadiazol- 2-yl)urea derivatives with potent anti-CML activity throughout PI3K/AKT signaling pathway

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Design and synthesis of novel 1-phenyl-3-(5-(pyrimidin-4-ylthio)-1,3,4-thiadiazol- 2-yl)urea derivatives with potent anti-CML activity throughout PI3K/AKT signaling pathway

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Abstract: In this investigation, a series of 1-phenyl-3-(5-(pyrimidin-4-ylthio)-1,3,4-thiadiazol-2-yl)urea receptor tyrosine kinase inhibitors were synthesized by a simple and efficient structure-based design. Structure-activity relationship (SAR) analysis of these compounds based on cellular assays led to the discovery of a number of compounds that showed potent activity against human chronic myeloid leukemia (CML) cell line K562, but very weak or no cellular toxicity through monitoring the growth kinetics of K562 cell during a period of 72 hours using the real-time live-cell imaging. Among these compounds, 1-(5-((6-((3-morpholinopropyl) amino)pyrimidin-4-yl)thio)-1,3,4-thiadiazol-2-yl)-3-(4-(trifluoromethyl)phenyl)urea (**7**) exhibited the least cellular toxicity and better biological activity in cellular assays (K562, IC_{50}: 0.038 μM). Compound **7** also displayed very good induced-apoptosis effect for human CML cell line K562 and exerted its effect via a significantly reduced protein phosphorylation of PI3K/Akt signal pathway by Human phospho-kinase array analysis. In vitro results indicate that 1-phenyl-3-(5-(pyrimidin-4-ylthio)-1,3,4-thiadiazol-2-yl)urea derivatives are lead molecules for further development as treatment of chronic myeloid leukemia and cancer.

Keywords: 1-phenyl-3-(5-(pyrimidin-4-ylthio)-1,3,4-thiadiazol-2-yl)urea derivatives; chronic myeloid leukemia (CML); cellular toxicity; K562 cell; PI3K/Akt signal pathway

Chronic myeloid leukemia (CML) is a malignant disease of hematopoietic stem cell caused by the t(9;22) translocation generated fusion gene BCR-ABL, a constitutively active tyrosine kinase (TK) that drives the leukemia. The t(9; 22) chromosome can occur in more than 95% of CML patients, or 5% of adult acute lymphoblastic leukemia (ALL) patients.[1-2] CML is characterised by prolonged asymptomatic period with gradual development of splenomegaly, culminating in acute leukaemia amongst untreated patients. According to the course of disease progression, clinical CML can be divided into three phases: chronic phase, acceleration phase and blast crisis phase. Most patients are in the chronic phase, and if the chronic phase is not treated timely or resistant, it will progress to an accelerated or blast phase.[3-5] Currently, specific Bcr-Abl tyrosine kinase inhibitor, Imatinib, Dasatinib or Nilotinib, is highly effective and considered standard therapy as first-line treatment for CML.[6-8] However, resistance or intolerance to these tyrosine kinase inhibitors (TKI), Heavy economic burden and reduced quality of life are often encountered that ultimately leads to disease progression.[9-10] Therefore, the development of other strategies and alterative targets for CML patients is of intense interest.

Phosphatidylinositol 3-kinase (PI3K) signaling is involved in the regulation of various cellular functions such as proliferation, differentiation, apoptosis and glucose transport. In recent years, the signaling pathway composed of PI3K and its downstream molecular Akt is closely related to the occurrence and development of human tumors.[11-13] This pathway regulates the proliferation and survival of tumor cells. Its abnormal activity can not only lead to malignant transformation of cells, but also related to tumor cell migration, adhesion, tumor angiogenesis and degradation of extracellular matrix. Currently, the tumor therapy strategy targeting the key molecules of PI3K-Akt signaling pathway is developing.[14-15] Activation of PI3K leads to activation of Akt, which activates or inhibits its downstream target proteins and then regulates cell proliferation, differentiation, apoptosis and migration.

Recently, several multi-target TKIs such as Sorafenib and Linifanib have been approved or investigated in clinical trials. Sorafenib is a potent inhibitor of several RTKs (VEGFR/PDGFR/ERK/Raf), and effectively inhibits Raf/MEK/ERK signaling

pathway.[16] Linifanib is an active multi-target RTK inhibitor of KDR and PDGFR, VEGFR.[17] The diarylurea scaffold of these compounds is thought to form multiple important hydrogen bonds with the hydrophobic pocket. Numbers of reports have recently highlighted diarylureas as potential antiproliferative agents for cancer.[18-19] Therefore, diarylurea scaffold has been identified as an effective scaffold for multikinase inhibitors, and a promising anti-cancer agents. In the present investigation, considering that the N and S atom on the thiazole ring of Dasatinib may form critical interactions with hydrophobic pocket residues, so we utilized thiadiazole ring instead of thiazole ring of Dasatinib. And then the diarylurea fragments were incorporated with the modified Dasatinib framework to synthesize a series of 1-phenyl-3-(5-(pyrimidin-4-ylthio)-1,3,4-thiadiazol-2-yl)urea derivatives, which was a potentially interesting scaffold for design of multi-target RTK inhibitors and downregulated the phosphorylation of kinases within the PI3K/Akt signaling pathway. The SAR analysis will focus on two regions: the 3/4-position substituent of phenyl ring (ring A) and 6-position substituent of pyrimidin (Figure 1).

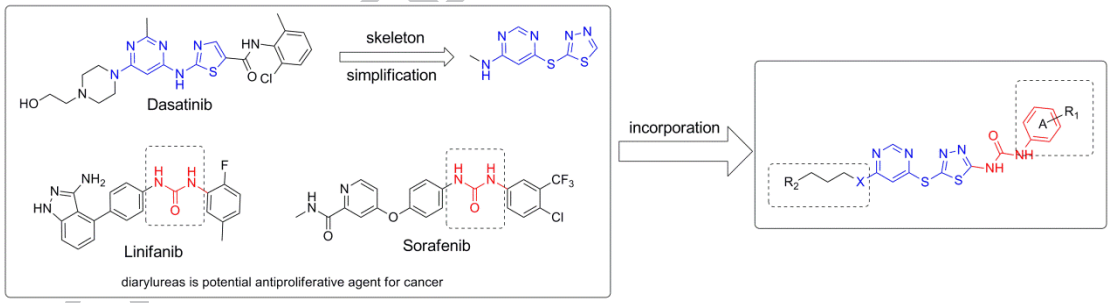
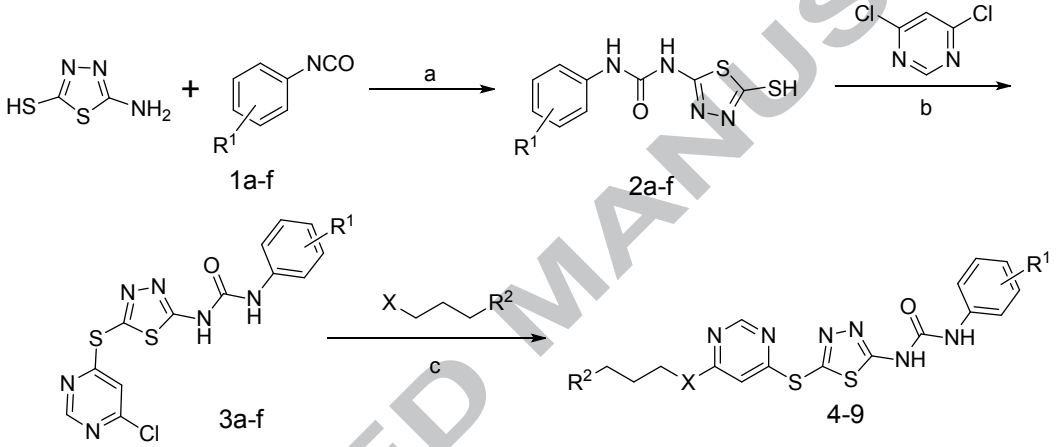


Figure 1. Design strategy based on Dasatinib and structural modifications of novel 1-phenyl-3-(5-(pyrimidin-4-ylthio)-1,3,4-thiadiazol-2-yl)urea derivatives.

The synthetic route for preparation of the title compounds was depicted in Scheme 1. Briefly, the key intermediates **2a-f** were achieved by reaction of commercially available 5-amino-1,3,4-thiadiazole-2-thiol with substituent phenyl isocyanates or diverse aromatic amines and triphosgene [20] under reflux for 3~5h using acetonitrile as a solvent in high yield (85%~95%). Then intermediates **2a-f** were reacted with commercially available 4,6-dichloropyrimidine in the presence of potassium

carbonate in acetonitrile under reflux for 8~12h to provide the 1-(5-((6-chloro-pyrimidin-4-yl)thio)-1,3,4-thiadiazol-2-yl)-3-phenylurea analogues **3a-f** in high yield (90%~96%). Finally, **3a-f** were substituted with 3-morpholinopropan-1-amine, 3-morpholinopropan-1-ol and N^1,N^1-diethylpropane-1,3-diamine in ethanol under reflux for 8-12 h to afford the 1-phenyl-3-(5-(pyrimidin-4-ylthio)-1,3,4-thiadiazol- 2-yl)urea derivatives **4-9.** A total of 10 compounds were synthesized and characterized by hydrogen nuclear magnetic resonance spectra and electrospray ionization mass spectra (see the Supporting Information).



3a:R^1=-4-CH_3
3b:R^1=-3-CF_3-4-Cl
3c:R^1=-4-Cl
3d:R^1=-4-CF_3
3e:R^1=-3-Cl-4-CH_3
3f:R^1=-3-OCH_3

4a:R^1=-4-CH_3, R^2=-morpholine, X=N
4b:R^1=-4-CH_3, R^2=-morpholine, X=O
4c:R^1=-4-CH_3, R^2=-diethylamine, X=N
5a:R^1=-3-CF_3-4-Cl, R^2=-morpholine, X=N
5b:R^1=-3-CF_3-4-Cl, R^2=-morpholine, X=O
5c:R^1=-3-CF_3-4-Cl, R^2=-diethylamine, X=N
6:R^1=-4-Cl, R^2=-morpholine, X=N
7:R^1=-4-CF_3, R^2=-morpholine, X=N
8:R^1=-3-Cl-4-CH_3, R^2=-morpholine, X=N
9:R^1=-3-OCH_3, R^2=-morpholine, X=N

Scheme 1. Synthesis of 1-phenyl-3-(5-(pyrimidin-4-ylthio)-1,3,4-thiadiazol-2-yl)urea derivatives. Reagents and conditions: (a) acetonitrile, reflux, 3~5h, 85%~95%; (b) potassium carbonate, acetonitrile, reflux, 8~12h, 90%~96%; (c) ethanol, reflux, 8~12h, 35%~60%.

The results of the antiproliferative activities of these compounds and Imatinib (a standard drug approved by FDA) were summarized in Table 1. Figure 2 showed cellular toxicity comparison between standard drug Imatinib and compounds **4-9** on

K562 cells. Cell proliferation and cell killing experiments were tested with the method of the real-time live-cell imaging, and kinetics of cell growth was monitored and recorded by an InCucyteZOOM device during a period of 72 hours.

Firstly, we changed the substituent of 6-position pyrimidin with different groups and fixed the substituent of phenyl ring (**4a** vs **4b** vs **4c, 5a** vs **5b** vs **5c**). Obviously, there is no significant difference in the biological activities of compounds **4a-c** or **5a-c**. However, the cellular toxicity of compounds **4b** and **5b** was significantly higher than that of **4a** or **4c** and **5a** or **5c**, respectively (shown in Figure 2A and 2B). The cellular toxicity and antiproliferative activities of compounds **4a** and **5a** are similar to those of **4c** and **5c**, respectively. These results imply that the substituent of 6-position pyrimidin does not affect the bioactivities but change the cellular toxicity of compounds to K562 cells.

We next fixed 6-(3-morpholinopropyl)amino substituent of pyrimidin and varied the 3/4-position substituent of phenyl ring (**4a, 5a, 6, 7, 8, 9**). Among these compounds, **4a** (4-methyl phenyl, IC_{50} = 0.056 μM), **7** (4-trifluoromethyl phenyl, IC_{50} = 0.038 μM), **8** (3-chloro-4-methyl phenyl, IC_{50} = 0.035 μM) showed excellent activities compared to standard drug Imatinib (IC_{50} = 0.14 μM). The most potent compound corresponds to **7** displayed a very weak or no cellular toxicity against K562 cells compared to **8** (shown in Figure 2C). The results indicate that the antiproliferative activity of compound **4a** and **7** is superior to that of Imatinib, and the cellular toxicity is also slightly superior or similar to Imatinib, which indicating that the anti-viability activities of compounds **4a** and **7** against K562 cells were not due to cellular toxicity (shown in Figure 2D). Considering that compound **7** has the least cellular toxicity and better biological in cellular assays, further studies were performed just on compound **7.**

Table 1. Anti-proliferation activities of 1-phenyl-3-(5-(pyrimidin-4-ylthio)-1,3,4-thiadiazol-2-yl)urea derivatives **4-9** against K562 cells

Comp.	K562 (IC_{50}, μM)	**Comp.**	K562 (IC_{50}, μM)

4a	0.056±0.0028	**6**	0.11±0.013
4b	0.078±0.0044	**7**	0.038±0.0013
4c	0.014±0.001	**8**	0.035±0.0025
5a	0.12±0.025	**9**	0.69±0.081
5b	0.15±0.026	**Imatinib**	0.14±0.010
5c	0.28±0.064		

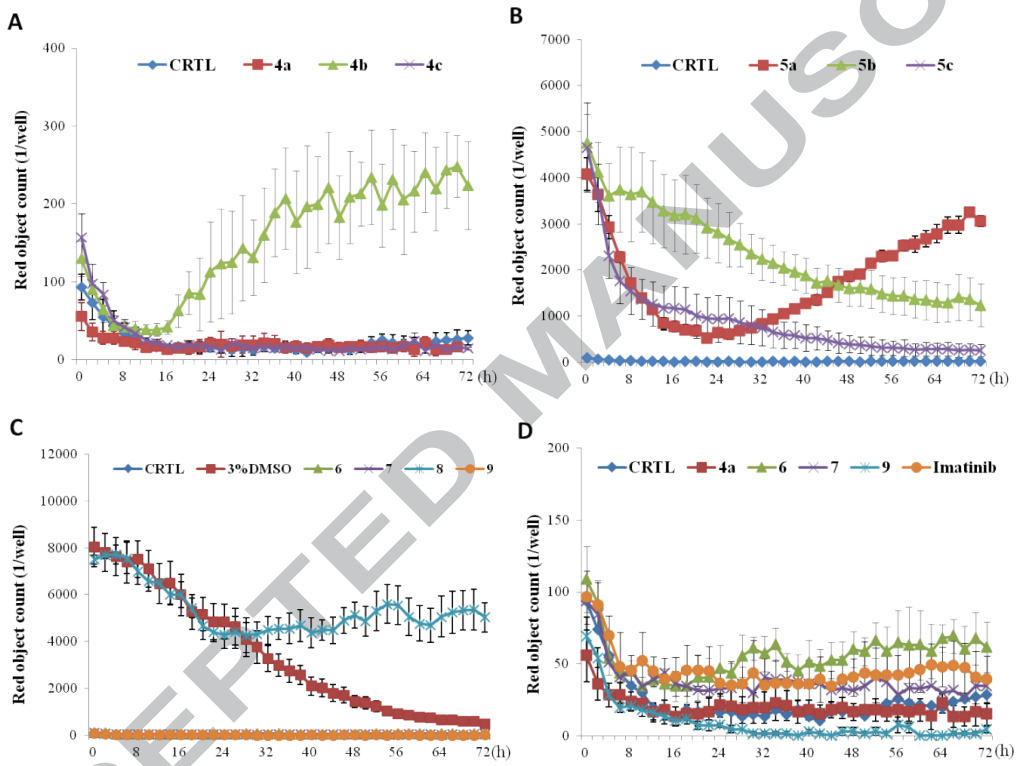


Figure 2. Cellular toxicity of compounds **4-9** on K562 cells (see the Supporting Information for the experimental operation). In this experiment, kinetics of growth was monitored and recorded by an InCucyteZOOM device during a period of 72 hours. All the conditions were in triplicates and the assay was repeated twice. (A) Cellular toxicity of compounds **4a, 4b** and **4c** against K562 cells. (B) Cellular toxicity of compounds **5a, 5b** and **5c** against K562 cells. (C) Cellular toxicity of compounds **6, 7, 8, 9** against K562 cells. (D) Cellular toxicity of compounds **4a, 6, 7, 9** and Imatinib against K562 cells.

To confirm compound **7** could induce apoptosis, we further performed cell apoptosis

assays using the CellEvent® Caspase-3/7 Green ReadyProbes® reagent. The total apoptotic cell count was monitored and recorded by an InCucyteZOOM device during a period of 48 hours. As shown in Figure 3, compound **7** at 10 μM significantly induced apoptosis. Apoptotic cell count significantly increased from 24 h to 48 h after administration, and was different from the vehicle control ($P < 0.0001$). Both compound **7** and Imatinib inhibited K562 cell proliferation, but Imatinib had stronger apoptosis effect on K562 cells than compound **7** at the same concentration of 10 μM. These results revealed that compound **7** inhibited proliferation and induced apoptosis in K562 cells, which was the same to most of the clinical anticancer chemotherapy agents.

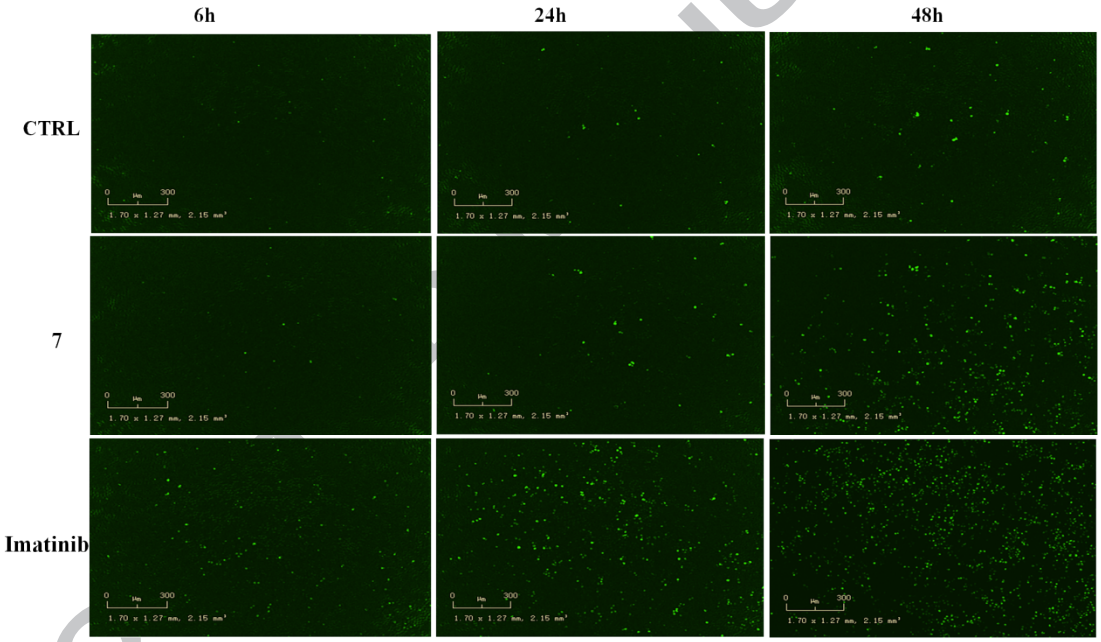


Figure 3. K562 cells were loaded with CellEvent® Caspase-3/7 Green ReadyProbes® reagent, then treated with 10 μM compound **7** and Imatinib, respectively, compared to the vehicle control for 48 hours. Untreated cells (CTRL) have minimal fluorescence, while treated cells (**7** and Imatinib) have a significant increase in fluorescence (shown in green) indicating apoptosis (see the Supporting Information for the experimental operation).

We next performed a human phospho-kinase array, which simultaneously determine relative phosphorylation levels in over 40 different kinases, and disclosed the effects of the treatment with compound **7** on activation of the AKT downstream signaling

pathways in K562 cells (Figure 4A). In the human phospho-kinase array, treatment with compound **7** clearly downregulated the phosphorylation of kinases within the PI3K/Akt signaling pathway, AKT1/2/3 (S473, T308) and its downstream kinases p70S6K (T421/S424), TOR (S2448) and GSK-3α/β(S21/S9). Phosphorylation of the kinases RSK1/2/3 (S380/S386/S377) and ERK1/2 (T202/Y204, T185/Y187) involved in the Ras/Raf/MEK/ERK pathway was decreased after addition of compound **7** in K562 cells. Whereas the phosphorylation of Src kinases (Src, Lck, PYK2) signaling pathways was slightly reduced upon treatment with compound **7**. We slao detected significant changes in the phosphorylation of P53 (S392, S46, S15), p27 (T198). Phosphorylation of kinases that belong to the MAPK pathway (p38, HSP27, JNK) and STAT pathway was differentially effected upon compound **7**. Together, these data suggest that a major mechanism by which compound **7** mediates K562 cells is through down-regulation of AKT signaling pathway, all resulting in lower cell survival, proliferation and apoptosis. In addition, the array results further revealed that the compound **7** could inhibit the effect of the phosphorylation of WNK1 (T60) and PRAS40 (T246) kinases.[21] The down-regulation of WNK1 activation may indicate that other AKT-independent pathways could also play roles in the observed effects of cell growth inhibition and cell apoptosis. [22-23]

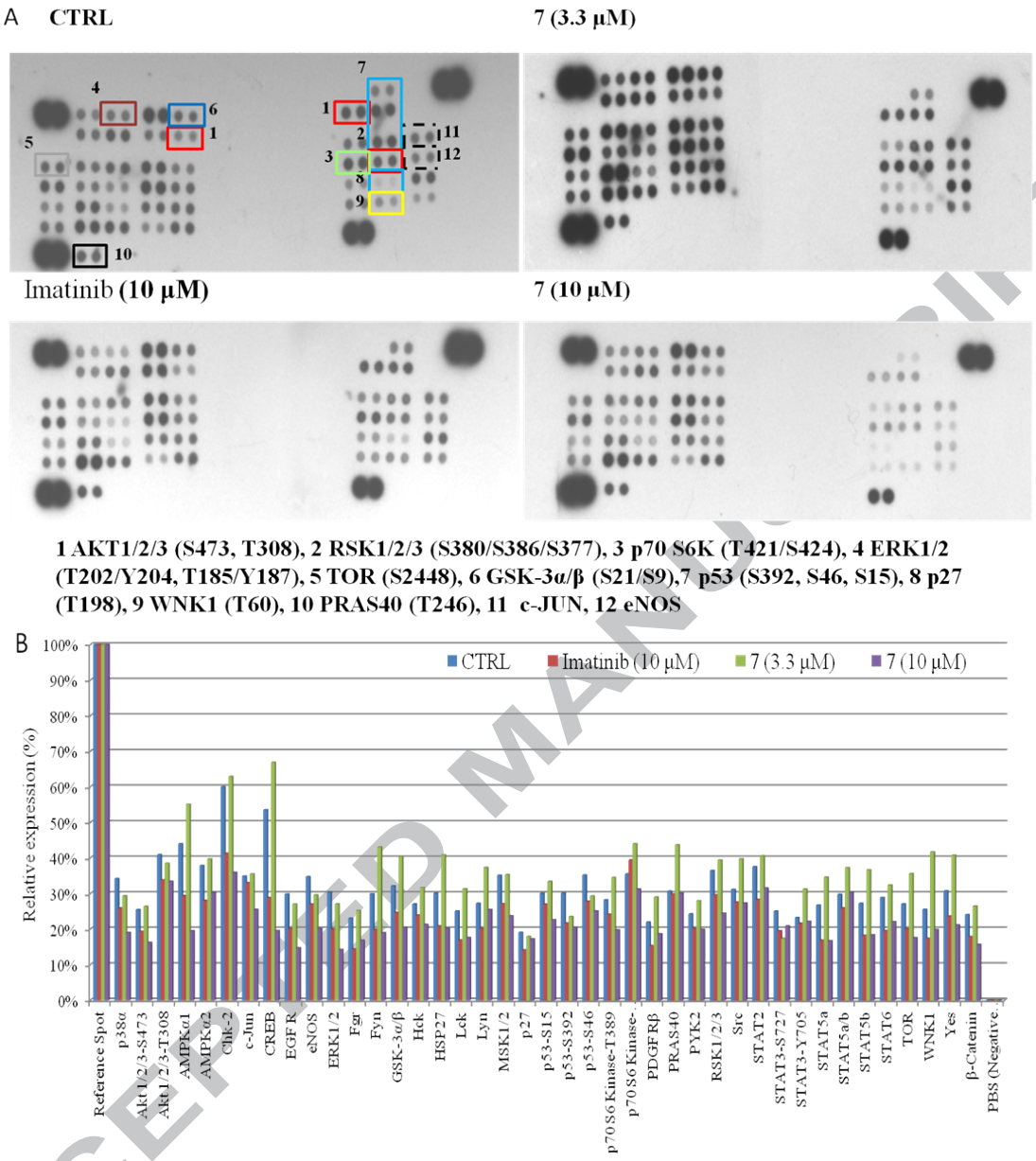


Figure 4. Compound **7** activate PI3K/Akt signaling pathway (see the Supporting Information for the experimental operation). (A) Representative images showing the changes of kinase phosphorylation in the Human Phospho-kinase Array on K562 cells treated with compound **7** and Imatinib. Select phosphorylated proteins on the kinase array membrane are indicated. (B) Relative expression of indicated protein in K562 cells treated for 48 hours.

In this study, a series of 1-phenyl-3-(5-(pyrimidin-4-ylthio)-1,3,4-thiadiazol-2-yl)urea derivatives were synthesized and evaluated for antiproliferative activity against human chronic myeloid leukemia cell line K562. As expected, these compounds

exhibited remarkable effects on human chronic myeloid leukemia cell line K562. Especially, compounds **4a** (IC_{50} = 0.056 μM) and **7** (IC_{50} = 0.038 μM) showed much better inhibitory activity than standard drug Imatinib (IC_{50} = 0.14 μM) against human chronic myeloid leukemia cell line K562, and the cellular toxicities of compounds **4a** and **7** were also slightly superior or similar to Imatinib. In conclusion, SAR studies of 1-phenyl-3-(5-(pyrimidin-4-ylthio)-1,3,4-thiadiazol-2-yl)urea derivatives based on structure design combined with cellular assays led to the discovery of a number of potent TKI inhibitors. Compound **7** was the most active one and lower cellular toxicity in cellular levels. It also displayed very good induced-apoptosis effect for human CML cell line K562. Human phospho-kinase array analysis showed that compound **7** exerted its effect via a significantly reduced phosphorylation of downstream signal proteins, mainly via the PI3K/Akt kinase. Overall, compound **7** could be taken as a good lead compound for further lead optimization for the treatment of CML. The further structure expansion of these new series of compounds and evaluation of their pharmacological profile, as well as improvement of inhibition ability are still on-going.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found online version, at

References and Notes

Design and synthesis of novel 1-phenyl-3-(5-(pyrimidin-4-ylthio)-1,3,4-thiadiazol-2-yl) urea derivatives with potent anti-CML activity throughout PI3K/AKT signaling pathway

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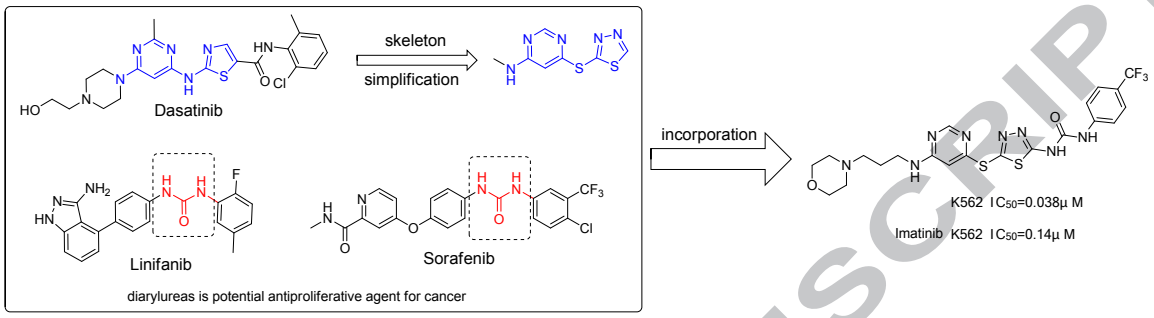

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