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# Targeting Cysteine Located Outside the Active Site: An Effective Strategy for Covalent ALKi Design

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**ABSTRACT:** Potent inhibitors of ALK are highly desired because of the occurrence of drug resistance. We herein firstly report the development of a rationally designed inhibitor, **Con B-1**, which can covalently bind to Cys1259, a cysteine located outside the ALK active site by linking a warhead with Ceritinib through a 2,2'-Oxybis(ethylamine) linker. The *in vitro* and *in vivo* assays showed **ConB-1** is a potent selective ALKi with low toxicity to normal cells. In addition, the molecule showed significant improvement of anticancer activities and potential antidrug resistant activity compared with Ceritinib, demonstrating the covalent inhibitor of ALK can be a promising drug candidate for the treatment of NSCLC. This work may provide a novel perspective on the design of covalent inhibitors.

## INTRODUCTION

ALK is a member of the family of insulin-like tyrosine kinase receptors.<sup>1</sup> It was proved that ALK is a strong carcinogenic gene that enhances the development of a variety of cancers.<sup>2</sup> The incidence of ALK gene rearrangement in NSCLC is 3–7%.<sup>3</sup> Patients diagnosed with ALK-rearranged lung cancer can benefit from treatment with ALK tyrosine kinase inhibitors.<sup>4</sup> FDA-approved drugs that target the abnormal ALK protein are Crizotinib,<sup>5</sup> Ceritinib,<sup>6</sup> Alectinib,<sup>7</sup> Brigatinib,<sup>8</sup> and Lorlatinib.<sup>9</sup> Nevertheless, acquired resistance to these drugs usually emerges after one year of treatment, and it is still an urgent need to develop novel and potent ALKis.<sup>10</sup>

Covalent inhibitors have attracted more and more attention in dealing with the insufficient activity and drug resistance in drug research because of the improved potency and prolonged duration of action.<sup>11</sup> Meanwhile, covalent inhibitors especially with acrylamide as the warhead, such as Osimertinib,<sup>12</sup> Ibrutinib,<sup>13</sup> Neratinib<sup>14</sup> and Afatinib,<sup>15</sup> which have all been approved by the FDA in recent years, can also be safe.<sup>16</sup>

With the development of structural biology and computeraided drug-design technology, the rational design of covalent inhibitors has made great progress by targeting the cysteine residue in the active pocket.<sup>17</sup> However, more than half of kinase targets bear no such cysteine residue.<sup>11a,18</sup> In this work, we describe the design of covalent inhibitors targeting the Cys1259 located outside the active pocket of ALK by incorporating a warhead to Ceritinib utilizing a linker. Through exploring the linker spacing and component, **ConB-1** was identified, which showed potent, highly selective ALK inhibition activity and superior anticancer activity. This work showcases a strategy of covalent inhibitor design by targeting the cysteine outside the active site, which might be applicable to other therapeutic targets (Figure 1).

## RESULTS AND DISCUSSION

**Structure-Guided Design of ALK Covalent Inhibitors.** To develop a covalent inhibitor for ALK, Ceritinib, a potent

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Figure 1. (a) Schematic illustration of molecular design in this work; (b) Molecular structure components of Con B-1.

ALKi approved by FDA for treatment of NSCLC, was used as the template (Figure 2a). Through observing the cocrystal structure of ALK and Ceritinib (pdb: 4MKC),<sup>19</sup> we noticed that Cys1259, although located outside of the Ceritinib binding site, can still be a promising residue for covalent targeting by linking a warhead with Ceritinib through a linker (Figure 2b). As for the warhead, acrylamide was selected as the electrophile because of its convenient synthesis and inertness toward glutathione.<sup>20</sup> The length and composition of the linker are crucial for the activity, because without a proper linker, the warhead with linker may not only fail to form a covalent bond with Cys1259 but also impede the noncovalent binding of Ceritinib part with ALK. So, in this study, two kind of linkers with different lengths were examined (the structures were depicted in Figure 2c)

**Chemistry.** The synthetic approach for the target compounds were outlined in Schemes 1 and 2. The compounds in the two series shared similar synthetic methods. For **Con B-1** as an example, firstly, installation of the acrylamide group on **a1** under basic conditions afforded **b1** in overall moderate yield; secondly, *tert* butoxycarbonyl moiety of intermediate **b1** was removed by stirring in trifluoroacetic acid for 30 min to obtain compound **c1**; finally, amide

condensation reaction was in progress between intermediate d1 and c1 by stirring with HATU and DIPEA in DMF to afford target molecule Con B-1.

In Vitro Bioassays and SAR. The biochemical potency of the compounds to ALK were examined at two concentrations (200 nM and 20 nM). Among all the molecules, four compounds in Con A series (Con A-1, 2, 3, 4) and three in Con B series (Con B-1, 2, 3) showed potent inhibition of ALK (Figure 3a, Table S1). It is worth noticing that the molecues with  $n^2 = 1$  showed low inhibitory activity against ALK compared with other molecules, which might be ascribed to the lower flexibility of the methylene group that could not allow the warhead to adopt the suitable direction to form a covalent bond with the terminal Cys1259.

To investigate the functional consequence of inhibiting ALK in cells, five cancer cell lines (H3122, H2228, H1299, A549, and Hela) were treated with the compounds at different concentrations, and the growth was monitored through the MTT assay (Table S2). Among the cell lines, H3122 and H2228 were reported as ALK-positive human NSCLC cell lines.<sup>19,21</sup> As despicted in Figure 3b, five of the compounds displayed potent antiproliferative activities (Con A-1, Con A-3, Con A-4, Con B-1, and Con B-3), and four of them showed advantaged bioactivity compared with Ceritinib (Figure 3c).

On the other hand, the toxicity of covalent inhibitors should not be underestimated. The biologically active molecules were tested against normal human liver cell line LO2 to learn their toxicities. As a result, at a concentration of 5  $\mu$ M, all the compounds showed a similar inhibition rate to the cells (Figure 3d, Table S3), indicating that compared with Ceritinib, the covalent inhibition of ALK do not increase the toxicity to normal cells. Because of the highest potency in *in vitro* bioactivity and low toxicity to normal cells, **Con B-1** was selected for further biological and mechanism investigation.

High selectivity may be the most important feature in the design of a safe and effective covalent inhibitor.<sup>11a,22</sup> In this study, to learn the selectivity of **Con B-1**, several targets (IGF1R, INSR, FLT3, and FGFR2) were selected because they were the main targets of Ceritinib.<sup>6</sup> As a result, **Con B-1** showed high selectivity, with IC <sub>50</sub> value of 0.5 nM to ALK comparing with 7.1, 12.6, 875, and >1000 nM to IGF1R, INSR, FLT3, and FGFR2, respectively (Figure 4a, Table S4).



Figure 2. (a) Chemical structure of Ceritinib; (b) Binding mode of Ceritinib in ALK (PDB ID:4MKC); (c) Chemical structures of designed molecules.

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## Scheme 1. Synthesis of Compounds in Con B Series<sup>4</sup>



"Reagents and conditions: (i) acryloyl chloride, DIPEA, THF, ice bath; (ii) TFA, 50 °C; (iii) for n2 = 1: Ceritinib, *tert*-Butyl bromoacetate,  $K_2CO_3$ , Acetonitrile, reflux, TFA; for n2 = 2: Ceritinib, Methyl acrylate, Et<sub>3</sub>N, Methanol, rt, NaOH, Methanol/H<sub>2</sub>O, 60 °C; (iv) HATU, DIPEA, DMF, rt.





"Reagents and conditions: (i) acryloyl chloride, DIPEA, THF, ice bath; (ii) TFA, 50 °C; (iii) for n2 = 1: Ceritinib, *tert*-Butyl bromoacetate,  $K_2CO_3$ , Acetonitrile, reflux, TFA; for n2 = 2: Ceritinib, Methyl acrylate,  $Et_3N$ , Methanol, rt, NaOH, Methanol/H<sub>2</sub>O, 60 °C; (iv) HATU, DIPEA, DMF, rt.

**Determination of Covalent Inhibition of ConB-1.** First, to prove the ability of acrylamide in binding with the Cys1259, a new molecule (**Re-ConB-1**) with the double bond of acrylamide group changed to a single bond was synthesized, and it showed a dramatic decrease by 25-fold (0.5 nM vs 13.2 nM) in binding activity against ALK when compared with **ConB-1** (Figure 4b,c), demonstrating the great contribution of covalent binding to the bioactivity. Next, to further investigate the covalent binding ability, **ConB-1** was incubated with L-cysteine (the molar ratio of L-cysteine and **Con B-1** = 1:5), and LC-MS analysis revealed a peak in the LC trace with a mass of

891.3649 Da, corresponding to addition of one molecule of Lcysteine to **ConB-1** (Figure.S1). Furthermore, to confirm that **ConB-1** can covalently bind to ALK, an excess amount of **ConB-1** was preincubated with ALK for 4 h, and a peak at m/z20276.81 corresponding to the complex of ALK with **ConB-1** was observed, demonstrating that **Con B-1** could efficiently form a single covalent bond with ALK (Figure 5).

Long Time Inhibitory Effect and ATP Competitive Experiment of Con B-1 on ALK. To monitor the influence of the remote covalent bond to the inhibitory activity with time, ALK and Con B-1 were incubated for 30 min at room

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Figure 3. (a) Inhibition rate (%) of compounds against ALK at two concentrations; (b) Antiproliferative activities of molecules targeting human cancer cell line H3122; (c) Chemical structures of molecules with advantaged anticancer bioactivities compared with Ceritinib; (d) Antiproliferative activities of molecules targeting human normal cell line LO2.



Figure 4. (a) Selectivity of Con B-1 against ALK, IGF1R, INSR, FLT3, and FGFR2; (b) Chemical structure of Con B-1 and Re-Con B-1; (c) Inhibition rates of the molecules (Con B-1, Re-Con B-1, and Ceritinib) against ALK at different concentrations.

temperature. The free compounds were removed through the column, and the substrate was added to start the reaction. The reaction rate was monitored for 2 h. In this experiment, normal enzyme activity group was set as the positive control group, and the nonenzyme group was the negative control group (Figure 6a). As a result, during the 2 h incubation period, the conversion rate was low compared with positive control, demonstrating the continuous potent inhibitory effect of **Con B-1** to ALK. It should be pointed out that the conversion rate was slowly growing during the incubation period, suggesting that Ceritinib as kinase-recognition scaffold in this molecule can leave the binding pocket through thermodynamic motion. Because of the restriction of the terminal covalent bond, the free Ceritinib group might enter the binding pocket again,

which might indicate the phenomenon that the kinase inhibitory activity of **Con B-1** is higher than Ceritinib.

Apart from that, to identify whether **Con B-1** is competitive for ATP, the inhibitory effect of **Con B-1** to ALK at a high concentration of ATP (1 mM) was conducted. After a 10 min incubation, as depicted in Figure 6b, **Con B-1** still holds an advantage compared with Ceritinib at a high ATP concentration; meanwhile, the  $IC_{50}$  value (1.4 nM) of **Con B-1** to ALK at a high concentration of ATP was upregulated (0.5 nM at ATP Km). The above experiments indicated that the binding of kinase-recognition scaffold of **Con B-1** to ALK is reversible, and the covalent bond outside the binding pocket can improve the binding affinity to the target protein.



Figure 5. ConB-1 covalently modified ALK in MS assay. Mass spectra of of (a) ALK alone, (b) ALK preincubated with ConB-1 (MW:769.3).

**Bioactivity of Con B-1 to Mutant ALK and Cell Lines.** ALK-L1196M, ALK-G1202R and the corresponding cell lines BaF3-ALK-L1196M, BaF3-ALK-G1202R were employed to identify the possible antidrug resistant activity of **ConB-1**. As depicted in Figure 7a, **Con B-1** was more active than Ceritinib in ALK-L1196M but less active in ALK-G1202R. In BaF3 models of ALK mutant cell lines, **Con B-1** showed similar activity (Figure 7b). It was assumed that in ALK-G1202R, the binding mode of **Con B-1** was changed and unable to form an effective covalent bond with Cys1259, which led to the decrease of the binding affinity of **ConB-1**.

**Binding Mode Analysis of Con B-1.** To further investigate the binding mode of **Con B-1** with ALK, molecular modeling studies were conducted. It was shown that in the ATP binding pocket, **Con B-1** adopts a similar binding mode with Ceritinib. Apart from that, with the optimized linker, the acrylamide group succeeded in binding with the Cys1259 residue, which endowed **con B-1** with higher bioactivity compared with Ceritinib (Figure 8a,b).

Western Blot Analysis of Con B-1. To examine the effects of Con B-1 on ALK signaling, Western blot analysis was performed. After 24 h treatment of H3122 cells with Con B-1 at different concentrations, the expression levels of p-ALK, AKT, p-AKT, STAT3, and p-STAT3 were evaluated. As shown

in Figure 9, the expression of p-ALK, p-AKT, and STAT3 were decreased in response to **Con B-1** treatment. Meanwhile, the expression of AKT and p-STAT3 was slightly downregulated after the treatment of increasing concentration of **Con B-1**. Collectively, these data indicated that ALK signal pathway can be effectively inhibited by treatment with **Con B-1** in H3122 cell line.

**Pharmacokinetic Studies of Con B-1.** The pharmacokinetic properties of **Con B-1** were evaluated in Sprague– Dawley (SD) rats. As shown in Table 1, **Con B-1** showed a plasma clearance of 16.98 mL·kg<sup>-1</sup>·min<sup>-1</sup> administered intravenously in rats, whereas oral administration in rats gave half-time ( $T_{1/2}$ ) of 2.89 h. Oral bioavailability (F) of 12.1% was achieved. Apparently, the oral bioavailability of the molecule was not ideal compared with Ceritinib,<sup>6</sup> which can be ascribed to the large molecular weight and the amide bonds in the molecule. Further optimizations could be conducted in the future, and we will report it on due course.

*In Vivo* Antiproliferative Activity of Compound Con B-1. The *in vivo* antitumor activity of Con B-1 was conducted based on the novel covalent binding mode and promising cellular activity. As depicted in Figure 10, the growth of xenograft tumors was inhibited by Con B-1 in a dose-dependent manner. TGIs of 62%, 49.7% and 39.7% were observed in the H3122 xenograft model at dose of 50, 20, and 10 mg/kg, respectively. Ceritinib as positive control in this assay was less active than Con B-1 with TGI of 34% at dose of 20 mg/kg. In addition, Con B-1 did not cause significant weight loss during the treatment period demonstrating its low toxicity.

### CONCLUSIONS

In this work, by fine-tuning of the type and length of linker attached to Ceritinib, a first-in-class ALK covalent inhibitor targeted the Cys1259, which located outside the ATP binding pocket was discovered.

Compared with Ceritinib, as a covalent molecule, **Con B-1** has several advantages, including the improvement of ALK inhibition activity, the enhancement of antiproliferative activity, and higher inhibitory activity on drug-resistant cell lines.

It should also be pointed out that, different with the traditional covalent compound, due to the restriction of the covalent bond located outside of ALK active pocket, the Ceritinib moiety might leave the ATP pocket through the



Figure 6. Inhibitory effect of Con B-1 on (a) ALK in 2 h incubation and (b) higher ATP concentration.

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Figure 7. Bioactivity of Con B-1 to mutant ALK and cell lines. (a) Bioactivity of Con B-1 to ALK mutations; (b) Bioactivity of Con B-1 to BaF3 models of ALK mutations.



**Figure 8.** (a) Binding mode of **Con B-1** in ALK; (b) Overlap of the binding modes of Ceritinib (green carbon atoms) and **Con B-1** (gray carbon atoms). In molecular modeling study, the X-ray crystal structure of ALK (PDB ID: 4MKC) was obtained as starting point.<sup>19</sup>



Figure 9. Western blot analysis of key ALK signaling proteins.

molecular dynamics, which is consistent with the ATP competitive experiments and long time inhibitory effect.

It has been estimated that only 39% of kinases (200 of 518) contain a cysteine in the ATP-binding pocket, which restricted the design of covalent inhibitors.<sup>11a,18</sup> The strategy of targeting cysteine outside the active site in this work may expand the range of proteins that can be targeted for covalent inhibition.

## EXPERIMENTAL SECTION

Human Cell Lines. H3122 (Human nonsmall cell lung cancer), H2228 (Human nonsmall cell lung cancer), H1299 (Human nonsmall cell lung cancer), A549 (Human lung cancer cells), HeLa (Human cervical cancer), and LO2 (human normal hepatocyte line) were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.). BaF3-ALK-G1202R and BaF3-ALK-L1196 M were from Sundia MediTech Company, Ltd. All cell lines were maintained at 37 °C under a humidified 5% CO<sub>2</sub> atmosphere according to supplier recommendations.

Chemical Synthesis. All solvents and reagents obtained from commercial sources were used without further purification. Flash column chromatography was performed using silica gel from Qingdao Haiyang. <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were recorded on a Bruker AMX 400 spectrometer and were calibrated using TMS or residual deuterated solvent as an internal reference (CDCl<sub>3</sub>: <sup>1</sup>H,  $\delta$  = 7.26 ppm;

## Table 1. Pharmacokinetic Parameters for Con B-1 in Rats

IV $(1 \text{ mg/kg})^{a}$			PO (10 mg/kg) <sup>b</sup>			
CL (mL/min/kg)	Vss (ml/kg)	T <sub>max</sub> (h)	C <sub>max</sub> (ng/mL)	AUCinf (ng·h/ml)	T <sub>1/2</sub> (h)	F(%)
16.98	2806.62	2.67	157	859	2.89	12.1

<sup>a</sup>Vehicle: 5% DMA/10% Solutol HS 15/85% saline. <sup>b</sup>Vehicle: 5% DMA/10% Solutol HS 15/85% saline.



Figure 10. Pharmacodynamic profile of Con B-1 in vivo. (a) Growth inhibitory effect of Con B-1 on established H3122 xenografts in female BALB/c nude mice (N = 6 per group); (b) Body weight of the mices during the dosage period.

<sup>13</sup>*C*, *δ* = 77.16 ppm, DMSO-d<sub>6</sub> 2.50 ppm, CD<sub>3</sub>OD 3.31 ppm). All of the target compounds were examined by HPLC, and the purity of the biologically tested compounds was ≥95%

Synthesis of tert-Butyl (2-(2-acrylamidoethoxy)ethyl)carbamate (**b1**). To a solution of acryloyl chloride (181 mg, 2 mmol) in THF (15 mL) at 0 °C, was added a solution of *tert*-butyl (2-(2-aminoethoxy)-ethyl)carbamate (**a1**, 408 mg, 2 mmol) and DIPEA (417 mg, 3 mmol) in THF. The reaction mixture was stirred for 1 h under the ice bath. Upon completion, the reaction was quenched with water and extracted with EtOAc. The organic layer was washed twice with water, dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The crude material was purified by column chromatography to afford target molecule as a colorless oil (392 mg, yield 76%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  = 6.22 (dd, *J* = 17.0, 2.0, 1H), 6.13 (dd, *J* = 17.0, 9.9, 1H), 5.55 (dd, *J* = 9.9, 2.0, 1H), 3.53–3.47 (m, 2H), 3.46 (s, 4H), 3.27–3.18 (m, 2H), 1.37 (s, 9H). HRMS (DART-TOF) calculated for C<sub>12</sub>H<sub>23</sub>N<sub>2</sub>O<sub>4</sub> [M + H]<sup>+</sup> m/z 259.1658, found 259.1645.

Synthesis of tert-Butyl (2-(2-(2-acrylamidoethoxy)ethoxy)ethyl)carbamate (**b2**). The title compound was obtained from *tert*-butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate following a similar synthesis procedure to that of **b1** (colorless oil, yield 69%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  = 6.30 (dd, *J* = 17.0, 1.3, 1H), 6.23–6.06 (m, 1H), 5.64 (d, *J* = 10.1, 1H), 3.70–3.51 (m, 9H), 3.40–3.24 (m, 2H), 1.46 (d, *J* = 6.6, 9H). HRMS (DART-TOF) calculated for C<sub>14</sub>H<sub>27</sub>N<sub>2</sub>O<sub>5</sub> [M + H]<sup>+</sup> m/z 303.1920, found 303.1893.

Synthesis of 2-(4-(4-((5-Chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methylphenyl)piperidin-1-yl)acetic Acid (d1). To a solution of Ceritinib (560 mg, 1 mmol) in acetonitrile (15 mL) was added tert-butyl bromoacetate (195 mg, 1 mmol) and K<sub>2</sub>CO<sub>3</sub> (414 mg, 3 mmol), and the resulting solution was stirred under reflux conditions for 5 h. Subsequently, the reaction was quenched with water and extracted with EtOAc, and the organic layer was dried over anhydrous sodium sulfate, concentrated, and purified by flash chromatography on the silica gel column to afford the intermediate as a yellow solid (511 mg, yield 76%). The intermediate was dissolved in 20 mL of TFA and stirred for 5 h. After that, 10 mL of DCM was added into the reaction solution, and the mixture was concentrated under reduced pressure to obtain a brown oil. The mixture was purified by flash chromatography on a silica gel column to afford the title compound as a white solid (421 mg, yield 90%). <sup>1</sup>H NMR (400 MHz, MeOD)  $\delta$  = 8.29 (d, J = 7.9, 1H), 8.23 (s, 1H), 7.99 (d, J = 7.9, 1H), 7.70 (t, J = 7.7, 1H), 7.51 (t, J = 7.6, 1H), 7.40 (s, 1H), 6.92 (s, 1H), 4.72-4.59 (m, 1H), 4.13 (s, 2H), 3.82 (t, J

= 18.3, 2H), 3.35 (s, 4H), 3.13 (t, J = 11.7, 1H), 2.18 (d, J = 12.2, 3H), 2.13–1.92 (m, 4H), 1.37–1.16 (m, 13H). HRMS (DART-TOF) calculated for C<sub>30</sub>H<sub>39</sub>ClN<sub>5</sub>O<sub>5</sub>S [M + H]<sup>+</sup> m/z 616.2360, found 616.2347.

Synthesis of 3-(4-(4-((5-Chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methylphenyl)piperidin-1-yl)propanoic Acid (d2). To a solution of Ceritinib (330 mg, 0.6 mmol) in methanol (50 mL) was added methyl acrylate (78 mg, 0.9 mmol) and  $Et_3N$  (240 mg, 2.4 mmol). The solution was stirred for 8 h. After that, the methanol was removed through reduced pressure, and then the mixture was dissolved with EtOAc and washed with water for 3 times. The organic layer was dried over anhydrous sodium sulfate, concentrated, and purified by flash chromatography on a silica gel column to afford the intermediate as a white solid (348 mg, yield 90%). The intermediate was dissolved in 10 mL of methanol/H<sub>2</sub>O (1:1), and to this solution NaOH (200 mg, 5 mmol) was added and stirred at 60 °C for 10 h. After that, the methanol was removed through reduced pressure, and the pH of water layer was adjusted to 2 with dilute hydrochloric acid. Then the mixture was dissolved with EtOAc and washed with water for 3 times. The organic layer was dried over anhydrous sodium sulfate, concentrated, and purified by flash chromatography on silica gel column to afford the intermediate as a white solid (323 mg, yield 95%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  = 9.51 (s, 1H), 8.58 (d, J = 8.4, 1H), 8.16 (d, J = 1.2, 1H), 8.03 (d, J = 3.2, 1H), 7.93 (d, J = 7.9, 1H), 7.62 (dd, J = 11.3, 4.4, 1H), 7.58–7.51 (m, 1H), 7.27 (d, J = 6.5, 1H), 6.75 (d, J = 37.6, 1H), 4.56 (dt, J = 11.1, 5.6, 1H), 3.51–3.14 (m, 3H), 2.81 (ddd, J = 47.2, 24.3, 12.3, 3H), 2.17 (d, J = 8.1, 3H), 1.34 (dd, J = 14.8, 6.5, 13H). HRMS (DART-TOF) calculated for C<sub>31</sub>H<sub>41</sub>ClN<sub>5</sub>O<sub>5</sub>S [M +  $H^{+}_{-}$  m/z 630.2517, found 630.2508.

Synthesis of N-(2-(2-(3-(4-(4-((5-Chloro-4-((2-(isopropylsulfonyl)phényl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2 methylphenyl)piperidin-1-yl)propanamido)ethoxy)ethyl)acrylamide (Con B-1). b1 (46 mg, 0.2 mmol) was dissolved in 5 mL of trifluoroacetic acid and stirred at 50 °C for 1 h. Subsequently, DCM (10 mL) was added into the reaction solution, and the mixture was concentrated under reduced pressure to obtain brown oil (c1). The resulting crude intermediate was used in the next step without further purification. To a solution of the above intermediate, d2 (126 mg, 0.2 mmol), and HATU (45.6 mg, 0.12 mmol) in DMF (10 mL) was added DIPEA (38.7 mg, 0.3 mmol). After being stirred for 15 h, the reaction was quenched with water, separated, dried over anhydrous sodium sulfate, concentrated, and purified by flash chromatography on silica gel column to afford the title compound as a white solid (65 mg, yield 42%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  = 9.51 (s, 1H), 8.58 (d, J = 8.4, 1H), 8.16 (s, 1H), 8.03 (s, 1H), 7.98-7.90 (m, 1H), 7.62 (t, J = 7.3, 1H), 7.54 (d, J = 10.5, 1H), 7.25 (d, J = 8.8, 2H), 6.76 (s, 1H), 6.28 (dd, J = 17.0, 1.3, 1H), 6.15 (dd, J = 17.0, 10.1, 1H), 5.62 (dd, J = 10.2, 1.3, 1H), 4.62–4.47 (m, 1H), 3.68– 3.41 (m, 8H), 3.24 (ddd, J = 37.5, 22.1, 8.9, 3H), 2.86–2.66 (m, 3H), 2.49 (t, J = 6.2, 2H), 2.26 (t, J = 11.3, 3H), 2.16 (s, 3H), 1.76 (dt, J = 21.8, 11.9, 5H), 1.34 (dd, J = 19.4, 6.5, 13H). <sup>13</sup>C NMR (101 MHz,  $CDCl_3$ )  $\delta = 172.78, 165.78, 157.47, 155.35, 144.69, 138.50, 134.63,$ 

131.29, 130.80, 128.02, 127.38, 126.45, 124.93, 123.63, 123.14, 120.82, 111.15, 105.90, 72.02, 69.99, 69.48, 55.48, 54.26, 53.94, 39.35, 38.79, 37.79, 32.58, 22.28, 18.93, 15.37. HRMS (DART-TOF) calculated for  $C_{38}H_{53}ClN_7O_6S$  [M + H]<sup>+</sup> m/z 770.3467, found 770.3418.

Synthesis of N-(2-(2-(2-(3-(4-((5-Chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methylphenyl)piperidin-1-yl)propanamido)ethoxy)ethoxy)ethyl)acrylamide (Con B-2). The title compound was obtained from b2 and d2 following a similar synthesis procedure to that of Con B-1 (white solid, yield 39%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  = 9.51 (s, 1H), 8.58 (d, J = 8.3, 1H), 8.16 (s, 1H), 8.02 (s, 1H), 7.93 (dd, J = 8.0, 1.5, 1H), 7.67-7.59 (m, 1H), 7.53 (s, 1H), 7.31-7.26 (m, 1H), 6.78 (s, 1H), 6.28 (dd, J = 17.0, 1.6, 2H), 6.12 (dd, J = 17.0, 10.2, 1H), 5.68–5.56 (m, 1H), 4.54 (dt, J = 12.1, 6.1, 1H), 3.69–3.41 (m, 11H), 3.25 (dq, J = 13.0, 6.5, 1H), 3.14 (d, J = 10.9, 2H), 2.69 (dd, J = 26.2, 14.6, 3H), 2.46 (t, J = 6.2, 2H), 2.27-2.12 (m, 5H), 1.78 (dd, J = 33.0, 11.0, 5H), 1.44–1.29 (m, 12H). <sup>13</sup>C NMR (101 MHz,  $CDCl_{2}$ )  $\delta = 172.45, 165.61, 157.48, 155.36, 144.69, 138.51, 134.62, 138.51, 138.51, 134.62, 138.51, 138.50, 138.50, 138.50, 138.50, 138.50, 138.50, 138.50, 138.$ 131.29, 130.86, 127.90, 127.28, 126.38, 124.94, 123.64, 123.12, 120.77, 111.20, 71.87, 70.43, 70.12, 69.86, 55.48, 54.25, 53.97, 39.27, 38.99, 32.62, 29.70, 22.27, 18.94, 15.37. HRMS (DART-TOF) calculated for  $C_{40}H_{57}CIN_7O_7S$  [M + H]<sup>+</sup> m/z 814.3729, found 814.3760.

Synthesis of N-(15-(4-(4-((5-Chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl) amino)-5-isopropoxy-2-methylphenyl)piperidin-1-yl)-13-oxo-3,6,9-trioxa-12azapentadecyl)acrylamide (Con B-3). The title compound was obtained from b3 and d2 following similar synthesis procedure to that of **Con B-1** (white solid, yield 42%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$ = 9.51 (s, 1H), 8.59 (d, J = 8.3, 1H), 8.20-8.14 (m, 1H), 8.06-7.97 (m, 2H), 7.93 (dd, J = 8.0, 1.5, 1H), 7.68–7.59 (m, 1H), 7.53 (s, 1H), 7.28–7.22 (m, 1H), 6.78 (s, 1H), 6.28 (dd, J = 17.0, 1.6, 1H), 6.14 (dd, J = 17.0, 10.2, 1H), 5.62 (dd, J = 10.1, 1.6, 1H), 4.63-4.48 (m, 1H), 3.72-3.56 (m, 12H), 3.55-3.43 (m, 4H), 3.27 (dq, J =13.7, 6.9, 1H), 3.12 (d, J = 11.4, 2H), 2.78–2.64 (m, 3H), 2.44 (t, J = 6.4, 2H), 2.16 (s, 4H), 1.86–1.65 (m, 5H), 1.42–1.28 (m, 14H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  = 172.69, 165.72, 157.49, 155.35, 144.69, 138.51, 137.29, 134.64, 131.28, 130.92, 127.80, 127.23, 126.33, 124.90, 123.62, 123.12, 120.75, 111.11, 105.84, 71.77, 70.43, 70.38, 70.24, 70.10, 69.96, 55.48, 54.31, 54.00, 39.30, 39.01, 38.03, 32.73, 29.69, 22.28, 18.94, 15.37. HRMS (DART-TOF) calculated for  $C_{30}H_{38}Cl_2N_5O_4S [M + H]^+ m/z 634.2022$ , found 634.2013.

Synthesis of N-(2-(2-(2-(4-(4-((5-Chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2methylphenyl)piperidin-1-yl)acetamido)ethoxy)ethyl)acrylamide (Con B-4). The title compound was obtained from b1 and d1 following a similar synthesis procedure to that of Con B-1 (white solid, yield 33%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  = 9.51 (s, 1H), 8.58 (t, J = 6.3, 1H), 8.16 (d, J = 2.6, 1H), 8.03 (d, J = 6.3, 1H), 7.93 (dd, J = 8.0, 1.4, 1H), 7.66–7.59 (m, 1H), 7.54 (s, 1H), 7.46 (t, J = 5.9, 1H), 7.25 (d, J = 8.9, 2H), 6.77 (s, 1H), 6.34–6.26 (m, 1H), 6.23– 6.11 (m, 1H), 5.66-5.60 (m, 1H), 4.65-4.49 (m, 1H), 3.69-3.45 (m, 9H), 3.26 (dt, J = 13.7, 6.8, 1H), 3.07 (s, 2H), 2.98 (d, J = 11.4, 2H), 2.32 (dd, J = 11.7, 9.6, 2H), 2.17 (d, J = 13.0, 3H), 1.84–1.62 (m, 4H), 1.43–1.28 (m, 12H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  = 171.16, 165.63, 157.48, 155.35, 144.69, 138.51, 137.18, 134.64, 131.29, 130.81, 127.86, 127.28, 126.42, 124.92, 123.64, 123.13, 120.80, 111.01, 105.84, 71.81, 70.16, 69.43, 62.11, 55.48, 55.01, 39.36, 38.52, 37.64, 32.94, 22.29, 18.93, 15.37. HRMS (DART-TOF) calculated for  $C_{37}H_{51}CIN_7O_6S [M + H]^+ m/z$  756.3310, found 756.3319.

Synthesis of N-(2-(2-(2-(2-(4-(4-((5-Chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methylphenyl)piperidin-1-yl)acetamido)ethoxy)ethoxy)-ethyl)acrylamide (**Con B-5**). The title compound was obtained from **b2** and **d1** following a similar synthesis procedure to that of **Con B-1** (white solid, yield 37%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  = 9.51 (s, 1H), 8.58 (t, *J* = 6.1, 1H), 8.16 (d, *J* = 3.0, 1H), 8.02 (s, 1H), 7.93 (dd, *J* = 8.0, 1.5, 1H), 7.67–7.59 (m, 1H), 7.54 (d, *J* = 6.3, 1H), 7.30–7.26 (m, 1H), 6.79 (s, 1H), 6.35–6.23 (m, 2H), 6.13 (dd, *J* =

17.0, 10.2, 1H), 5.61 (td, J = 9.8, 3.4, 1H), 4.67–4.52 (m, 1H), 3.66– 3.48 (m, 12H), 3.32–3.21 (m, 1H), 3.06 (s, 2H), 2.98 (d, J = 11.4, 2H), 2.74–2.61 (m, 1H), 2.31 (dd, J = 11.5, 9.2, 2H), 2.16 (s, 3H), 1.82–1.67 (m, 4H), 1.41–1.30 (m, 12H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta = 170.89$ , 165.58, 157.48, 155.36, 144.69, 138.50, 137.32, 134.63, 131.29, 130.85, 127.83, 127.29, 126.41, 124.92, 123.63, 123.13, 120.79, 111.10, 105.85, 71.82, 70.43, 70.13, 70.09, 69.84, 62.10, 55.48, 54.99, 39.26, 38.80, 37.60, 32.94, 22.30, 18.94, 15.37. HRMS (DART-TOF) calculated for C<sub>37</sub>H<sub>51</sub>ClN<sub>7</sub>O<sub>6</sub>S [M + H]<sup>+</sup> m/z 756.3310, found 756.3319.

Synthesis of N-(1-(4-(4-((5-Chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2methylphenyl)piperidin-1-yl)-2-oxo-6,9,12-trioxa-3-azatetradecan-14-yl)acrylamide (Con B-6). The title compound was obtained from b3 and d1 following a similar synthesis procedure to that of Con B-1 (white solid, yield 21%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  = 9.52 (s, 1H), 8.58 (t, J = 7.2, 1H), 8.16 (d, J = 2.9, 1H), 8.02 (s, 1H), 7.93 (dd, J = 8.0, 1.5, 1H), 7.67-7.60 (m, 1H), 7.57-7.47 (m, 2H), 7.27-7.23 (m, 1H), 6.79 (s, 1H), 6.28 (dt, J = 7.7, 2.7, 1H), 6.19-6.08 (m, 1H), 5.67-5.58 (m, 1H), 4.69-4.52 (m, 1H), 3.68-3.49 (m, 16H), 3.32-3.21 (m, 1H), 3.08 (d, J = 17.3, 2H), 2.98 (d, J = 11.4, 2H), 2.30 (dt, J = 11.3, 5.7, 2H), 2.21–2.12 (m, 3H), 1.82–1.65 (m, 4H), 1.42–1.29 (m, 12H). <sup>13</sup>C NMR (101 MHz,  $CDCl_3$ )  $\delta$  = 170.82, 165.57, 157.48, 155.35, 144.70, 138.50, 137.36, 134.64, 131.29, 130.93, 127.78, 127.23, 126.28, 124.91, 123.63, 123.13, 120.78, 111.07, 105.84, 71.74, 70.56, 70.50, 70.24, 70.19, 69.83, 62.09, 55.48, 54.99, 39.29, 38.82, 37.62, 32.93, 29.69, 22.30, 18.94, 15.37. HRMS (DART-TOF) calculated for  $C_{41}H_{59}ClN_7O_8S [M + H]^+ m/z$ 844.3834, found 844.3829.

Synthesis of tert-Butyl (3-Acrylamidopropyl)carbamate (f1). The title compound was obtained from *tert*-butyl (3-aminopropyl)carbamate following a similar synthesis procedure to that of **b1** (white solid, yield 71%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  = 6.25–6.15 (m, 1H), 6.09 (dd, *J* = 17.0, 9.9, 1H), 5.54 (dd, *J* = 9.9, 1.8, 1H), 3.28 (td, *J* = 12.0, 4.9, 2H), 3.10 (dd, *J* = 11.8, 5.8, 2H), 1.65–1.52 (m, 2H), 1.36 (s, 9H). HRMS (DART-TOF) calculated for C<sub>11</sub>H<sub>21</sub>N<sub>2</sub>O<sub>3</sub> [M + H]<sup>+</sup> *m*/z 229.1552, found 229.1545.

Synthesis of tert-Butyl (4-Acrylamidobutyl)carbamate (**f2**). The title compound was obtained from *tert*-butyl (4-aminobutyl)-carbamate following similar a synthesis procedure to that of **b1** (white solid, yield 76%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  = 6.27 (dd, *J* = 17.0, 1.6, 2H), 6.13 (dd, *J* = 17.0, 10.2, 1H), 5.62 (dd, *J* = 10.2, 1.6, 1H), 4.72 (s, 1H), 3.35 (q, *J* = 6.4, 2H), 3.13 (s, 2H), 1.62–1.49 (m, 4H), 1.44 (s, 9H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  = 165.74, 156.23, 130.99, 126.13, 79.27, 39.20, 28.41, 27.71, 26.50. HRMS (DARTTOF) calculated for C<sub>12</sub>H<sub>23</sub>N<sub>2</sub>O<sub>3</sub> [M + H]<sup>+</sup> *m*/*z* 243.1709, found 243.1701.

Synthesis of tert-Butyl (6-Acrylamidohexyl)carbamate (**f3**). The title compound was obtained from *tert*-butyl (6-aminohexyl)-carbamate following a similar synthesis procedure to that of **b1** (colorless oil, yield 68%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  = 6.28 (dd, J = 17.0, 1.4, 1H), 6.12 (dd, J = 17.0, 10.2, 1H), 5.62 (dd, J = 10.2, 1.3, 1H), 3.32 (dd, J = 13.1, 6.7, 2H), 3.11 (dd, J = 12.6, 6.2, 2H), 1.60–1.41 (m, 13H), 1.34 (dd, J = 8.8, 5.6, 4H). HRMS (DARTTOF) calculated for C<sub>14</sub>H<sub>26</sub>N<sub>2</sub>O<sub>3</sub>Na [M + Na]<sup>+</sup> m/z 293.1841, found 293.1856.

Synthesis of tert-Butyl (8-Acrylamidooctyl)carbamate (**f4**). The title compound was obtained from *tert*-butyl (8-aminooctyl)carbamate following a similar synthesis procedure to that of **b1** (colorless oil, yield 67%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  = 6.27 (dd, J = 17.0, 1.2, 1H), 6.11 (dd, J = 17.0, 10.2, 1H), 5.62 (dd, J = 10.2, 1.1, 1H), 4.56 (s, 1H), 3.09 (dd, J = 12.6, 6.2, 2H), 1.59–1.39 (m, 13H), 1.28 (d, J = 17.7, 9H). HRMS (DART-TOF) calculated for C<sub>16</sub>H<sub>30</sub>N<sub>2</sub>O<sub>3</sub>Na [M + Na]<sup>+</sup> m/z 321.2154, found 321.2149.

Synthesis of N-(3-(3-(4-(4-((5-Chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2methylphenyl)piperidin-1-yl)propanamido)propyl)acrylamide (**Con A-1**). The title compound was obtained from f1 and d2 following a similar synthesis procedure to that of **Con B-1** (white solid, yield 52%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  = 9.50 (s, 1H), 8.58 (d, *J* = 8.4, 1H), 8.14 (d, *J* = 8.9, 2H), 7.93 (d, *J* = 7.9, 1H), 7.62 (t, *J* = 7.8, 1H), 7.54 (s, 1H), 7.27–7.22 (m, 1H), 6.77 (s, 1H), 6.35–6.22 (m, 1H), 6.14 (dd, *J* = 17.0, 10.2, 1H), 5.63 (d, *J* = 10.2, 1H), 4.61–4.48 (m, 1H), 3.47–3.21 (m, 6H), 3.12 (d, *J* = 11.3, 2H), 2.78–2.64 (m, 3H), 2.45 (t, *J* = 6.2, 2H), 2.23–2.08 (m, 5H), 1.83 (d, *J* = 13.3, 2H), 1.77–1.60 (m, 4H), 1.34 (dd, *J* = 19.1, 6.5, 13H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  = 173.40, 165.87, 157.49, 155.38, 155.34, 144.69, 138.52, 137.19, 134.64, 131.25, 127.79, 127.17, 126.01, 124.90, 123.66, 123.11, 120.74, 111.00, 105.82, 71.74, 55.47, 54.38, 54.01, 38.10, 35.77, 35.60, 32.85, 32.69, 29.89, 22.26, 18.93, 15.37. HRMS (DART-TOF) calculated for C<sub>37</sub>H<sub>51</sub>ClN<sub>7</sub>O<sub>5</sub>S [M + H]<sup>+</sup> *m*/*z* 740.3361, found 740.3400.

Synthesis of N-(4-(3-(4-(4-((5-Chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2methylphenyl)piperidin-1-yl)propanamido)butyl)acrylamide (Con A-2). The title compound was obtained from f2 and d2 following a similar synthesis procedure to that of Con B-1 (white solid, yield 67%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  = 9.51 (s, 1H), 8.58 (d, J = 8.3, 1H), 8.16 (s, 1H), 8.03 (s, 1H), 7.93 (dd, J = 8.0, 1.4, 1H), 7.62 (dd, J = 11.4, 4.3, 1H), 7.54 (s, 1H), 7.31-7.23 (m, 2H), 6.75 (s, 1H), 6.75 (s, 1H), 6.33–6.22 (m, 1H), 6.17–6.06 (m, 1H), 4.55 (dt, J = 12.1, 6.1, 1H), 3.46-3.11 (m, 7H), 2.90-2.68 (m, 3H), 2.49 (t, J = 5.9, 2H), 2.31 (t, J = 11.4, 2H), 2.16 (s, 3H), 1.88 (d, J = 12.7, 2H), 1.76-1.67 (m, 2H), 1.65–1.53 (m, 4H), 1.35 (dd, J = 20.1, 6.5, 12H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  = 172.69, 165.83, 157.46, 155.36, 155.33, 144.70, 138.49, 136.78, 134.66, 131.27, 130.98, 127.93, 127.27, 126.19, 124.88, 123.63, 123.15, 120.80, 110.97, 105.86, 71.87, 55.49, 54.31, 53.88, 39.13, 38.53, 37.77, 32.57, 32.00, 27.18, 26.48, 22.26, 18.93, 15.37. HRMS (DART-TOF) calculated for C38H53ClN7O5S  $[M + H]^+ m/z$  754.3517, found 754.3531.

Synthesis of N-(6-(3-(4-(4-((5-Chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2 methylphenyl)piperidin-1-yl)propanamido)hexyl)acrylamide (Con A-3). The title compound was obtained from f3 and d2 following a similar synthesis procedure to that of Con B-1 (white solid, yield 62%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  = 9.52 (s, 1H), 8.58 (d, J = 8.4, 1H), 8.16 (s, 1H), 8.04 (d, J = 10.3, 2H), 7.93 (dd, J = 7.9, 1.3, 1H), 7.67-7.58 (m, 1H), 7.52 (s, 1H), 7.25 (d, J = 9.7, 1H), 6.78 (d, J = 16.4, 1H), 6.31-6.20 (m, 1H), 6.09 (dd, J = 17.0, 10.2, 1H), 5.90 (s, 1H), 5.64–5.54 (m, 1H), 4.58–4.46 (m, 1H), 3.27 (tq, J = 13.9, 6.8, 5H), 3.13 (d, J = 11.3, 2H), 2.72 (dd, J = 12.2, 6.9, 3H), 2.43 (t, J = 6.1, 2H), 2.27-2.13 (m, 5H), 1.85 (d, J = 12.6, 2H), 1.67 (dd, J = 22.0, 12.1, 2H), 1.60–1.47 (m, 4H), 1.42–1.29 (m, 16H). <sup>13</sup>C NMR  $(101 \text{ MHz}, \text{ CDCl}_3) \delta = 172.49, 165.61, 157.47, 155.37, 144.65,$ 138.50, 137.10, 134.63, 131.29, 131.01, 127.95, 127.44, 126.09, 124.91, 123.62, 123.13, 120.84, 111.20, 105.90, 72.02, 55.49, 54.36, 53.87, 39.11, 38.58, 37.94, 32.84, 32.37, 29.43, 29.39, 26.22, 26.10, 22.29, 18.93, 15.37. HRMS (DART-TOF) calculated for  $C_{40}H_{57}ClN_7O_5S [M + H]^+ m/z$  782.3830, found 782.3863.

Synthesis of N-(8-(3-(4-(4-((5-Chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2methylphenyl)piperidin-1-yl)propanamido)octyl)acrylamide (Con A-4). The title compound was obtained from f4 and d2 following a similar synthesis procedure to that of Con B-1 (white solid, yield 57%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  = 9.52 (s, 1H), 8.58 (d, J = 8.3, 1H), 8.16 (s, 1H), 8.02 (s, 2H), 7.93 (dd, J = 8.0, 1.4, 1H), 7.67-7.58 (m, 1H), 7.53 (s, 1H), 7.27 (d, J = 6.0, 1H), 6.75 (s, 1H), 6.26 (ddd, J = 17.0, 6.5, 1.4, 1H), 6.10 (td, J = 17.4, 9.1, 1H), 5.66-5.53 (m, 1H), 4.61-4.45 (m, 1H), 3.39-3.09 (m, 8H), 2.73 (dd, J = 14.2, 5.1, 3H), 2.46 (t, J = 6.0, 2H), 2.24 (t, J = 11.1, 2H), 2.17 (s, 3H), 1.86 (d, J = 12.1, 2H), 1.71 (dd, J = 22.6, 11.9, 2H), 1.60–1.43 (m, 5H), 1.41– 1.28 (m, 21H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  = 172.20, 165.51, 157.47, 155.36, 144.70, 138.50, 134.64, 131.29, 131.00, 127.90, 127.29, 126.09, 124.91, 123.61, 123.14, 120.82, 110.98, 105.92, 71.84, 55.49, 54.33, 53.85, 39.51, 39.03, 37.80, 32.67, 29.47, 29.40, 29.09, 29.05, 26.85, 26.75, 22.28, 18.93, 15.37. HRMS (DART-TOF) calculated for  $C_{42}H_{61}CIN_7O_5S [M + H]^+ m/z 810.4143$ , found 810.4150.

Synthesis of N-(3-(2-(4-(4-((5-Chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2methylphenyl)piperidin-1-yl)acetamido)propyl)acrylamide (**Con A**- **5**). The title compound was obtained from **f1** and **d1** following a similar synthesis procedure to that of **Con B-1** (white solid, yield 35%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  = 9.51 (s, 1H), 8.58 (d, *J* = 8.2, 1H), 8.15 (s, 1H), 8.01 (s, 1H), 7.93 (dd, *J* = 8.0, 1.5, 1H), 7.66–7.60 (m, 2H), 7.57 (s, 1H), 7.27–7.21 (m, 1H), 6.81 (s, 1H), 6.72 (t, *J* = 6.0, 1H), 6.28 (dd, *J* = 17.0, 1.5, 1H), 6.15 (dd, *J* = 17.0, 10.2, 1H), 5.64 (dd, *J* = 10.2, 1.5, 1H), 4.66–4.54 (m, 1H), 3.38 (td, *J* = 12.4, 6.4, 4H), 3.27 (dq, *J* = 13.7, 6.8, 1H), 3.10 (s, 2H), 2.99 (t, *J* = 10.9, 2H), 2.39 (d, *J* = 14.2, 2H), 2.16 (s, 3H), 1.85–1.69 (m, 6H), 1.35 (dd, *J* = 23.4, 6.5, 12H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  = 165.90, 157.48, 155.36, 155.32, 144.74, 138.50, 137.20, 134.64, 131.28, 131.22, 127.78, 127.13, 126.01, 124.92, 123.66, 123.13, 120.78, 111.05, 105.80, 71.69, 61.86, 55.48, 55.08, 37.53, 35.88, 35.70, 32.80, 29.80, 29.69, 22.28, 18.94, 15.37. HRMS (DART-TOF) calculated for C<sub>36</sub>H<sub>49</sub>ClN<sub>7</sub>O<sub>5</sub>S [M + H]<sup>+</sup> *m/z* 726.3204, found 726.3223.

Synthesis of N-(4-(2-(4-(4-((5-Chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxymethylphenyl)piperidin-1-yl)acetamido)butyl)acrylamide (Con A-6). The title compound was obtained from  $f_2$  and  $d_1$  following a similar synthesis procedure to that of Con B-1 (white solid, yield 33%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  = 9.51 (s, 1H), 8.58 (d, J = 8.2, 1H), 8.15 (s, 1H), 8.06 (d, J = 5.2, 1H), 8.02 (s, 1H), 7.93 (dd, J = 8.0, 1.5, 1H), 7.68-7.59 (m, 1H), 7.54 (s, 1H), 7.30-7.22 (m, 1H), 6.75 (s, 1H), 6.33 (t, J = 5.5, 1H), 6.25 (dd, J = 17.0, 1.6, 1H), 6.12 (dd, J = 17.0, 10.1, 1H), 5.60 (dd, J = 10.1, 1.6, 1H), 4.64-4.48 (m, J)1H), 3.44-3.15 (m, 7H), 2.86-2.68 (m, 3H), 2.47 (t, J = 6.2, 2H), 2.28 (t, J = 11.3, 2H), 2.16 (s, 3H), 1.87 (d, J = 12.5, 2H), 1.78-1.55 (m, 7H), 1.34 (dd, J = 20.3, 6.5, 12H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta = 165.75, 157.48, 155.35, 144.71, 138.50, 134.64, 131.28, 130.98,$ 127.85, 127.21, 126.20, 124.91, 123.65, 123.13, 120.76, 111.04, 105.83, 71.78, 61.99, 55.48, 55.06, 39.22, 38.52, 37.57, 32.79, 29.69, 27.60, 26.45, 22.29, 18.94, 15.37. HRMS (DART-TOF) calculated for  $C_{37}H_{51}CIN_7O_5S [M + H]^+ m/z$  740.3361, found 740.3348

Synthesis of N-(6-(2-(4-(4-((5-Chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2methylphenyl)piperidin-1-yl)acetamido)hexyl)acrylamide (Con A-7). The title compound was obtained from f3 and d1 following a similar synthesis procedure to that of Con B-1 (white solid, yield 36%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  = 9.51 (s, 1H), 8.57 (t, J = 7.1, 1H), 8.16 (d, J = 3.0, 1H), 8.02 (s, 1H), 7.93 (d, J = 7.8, 1H), 7.62 (t, J = 7.6, 1H), 7.56 (d, J = 8.2, 1H), 7.25 (d, J = 8.7, 1H), 6.79 (s, 1H), 6.27 (d, J = 17.0, 1H), 6.11 (dd, J = 17.0, 10.3, 1H), 5.82 (s, 1H), 5.62 (d, I = 10.2, 1H), 4.58 (dt, I = 12.3, 6.1, 1H), 3.29 (dg, I = 20.8, 6.7, I)5H), 3.01 (dd, *J* = 30.0, 19.2, 3H), 2.67 (dd, *J* = 23.3, 12.7, 1H), 2.35 (d, J = 9.6, 2H), 2.22–2.11 (m, 3H), 2.05 (d, J = 8.8, 1H), 1.77 (dd, J = 25.4, 11.2, 4H, 1.65-1.48 (m, 5H), 1.46-1.29 (m, 15H).  $^{13}C$ NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  = 165.60, 155.36, 144.72, 138.50, 134.63, 131.29, 131.00, 127.27, 126.17, 124.94, 123.64, 123.13, 120.81, 111.09, 105.85, 71.83, 55.48, 55.00, 39.12, 38.54, 37.55, 29.70, 29.33, 26.11, 26.07, 22.29, 18.94, 15.37. HRMS (DART-TOF) calculated for  $C_{39}H_{55}CIN_7O_5S [M + H]^+ m/z$  768.3674, found 768.3659.

Synthesis of N-(8-(2-(4-(4-((5-Chloro-4-((2-(isopropylsulfonyl)phényl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-. methylphenyl)piperidin-1-yl)acetamido)octyl)acrylamide (Con A-8). The title compound was obtained from f4 and d1 following a similar synthesis procedure to that of Con B-1 (white solid, yield 29%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  = 9.51 (s, 1H), 8.58 (d, J = 8.2, 1H), 8.16 (d, J = 3.2, 1H), 8.04 (d, J = 18.4, 1H), 7.93 (dd, J = 8.0, 1.6, 1H), 7.66–7.59 (m, 1H), 7.56 (d, J = 11.3, 1H), 7.24 (s, 1H), 6.80 (d, J = 13.0, 1H), 6.30-6.23 (m, 1H), 6.07 (dd, J = 17.0, 10.3, 1H), 5.68-5.58 (m, 2H), 4.64-4.52 (m, 1H), 3.39-3.24 (m, 5H), 3.00 (dd, *J* = 26.5, 15.3, 3H), 2.34 (t, *J* = 10.7, 2H), 1.75 (dd, *J* = 32.2, 10.2, 4H), 1.53 (d, J = 6.6, 5H), 1.40–1.30 (m, 19H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  = 165.52, 157.48, 155.36, 144.71, 138.50, 134.63, 131.29, 130.96, 127.86, 127.32, 126.18, 124.93, 123.64, 123.14, 120.83, 111.12, 100.00, 71.85, 62.05, 55.49, 55.00, 39.54, 38.90, 37.60, 32.88, 29.70, 29.49, 29.02, 26.75, 22.30, 18.93, 15.37. HRMS (DART-TOF) calculated for  $C_{41}H_{59}ClN_7O_5S [M + H]^+ m/z$  796.3987, found 796.3981.

**ALK Inhibition Assays.** The ALK activity assay was conducted through Mobility shift assay. Briefly, the compound, wild-type ALK

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(Carna, 08-518), ALK-L1196 M (Carna, 08-529), ALK-G1202R (Carna, 08-544), Kinase substrate22 and ATP (Km, Sigma) were diluted in kinase buffer to the indicated concentrations. The assay plate was covered and incubated at room temperature for 10 min. To learn the inhibitory activity of **Con B-1** at higher ATP concentration, a concentration of 1 mM was applied in this study. The data were collected on Caliper EZ Reader and presented in Excel. The curves were fitted by Graphpad Prism 5.0.

**Cell Viability Assay.** Cells were cultured at 37 °C with 5% CO<sub>2</sub> in RPMI 1640 or DMEM, supplemented with 10% (v/v) fetal bovine serum (Gibco), and 1% (v/v) penicillin– streptomycin (HyClone). Cells in logarithmic phase were seeded in a 96-well plate at  $2-5 \times 10^3$  cells per well for 24 h (37 °C, 5% CO<sub>2</sub>). Then, an equal volume of medium containing various concentrations of test compounds was added to each well. After 72 h, MTT was added, and the cells were incubated for an additional 1–4 h. The absorbance values (OD) of the 96-well plate was measured at 450 nm using a Spectra MAX M5 microplate spectrophotometer (Molecular Devices). The IC<sub>50</sub> values were the means of at least three independent experiments and calculated by GraphPad Prism 5 software.

**Molecular Modeling.** To predict the possible binding mode of **Con B-1** with ALK, Molecular modeling was performed by using autodock 4.0. The X-ray crystal structure of ALK (PDB ID: 4MKC) was obtained as starting point.

Mass Spectrometry Analysis for Identifying Covalent Binding Nature of ConB-1. L-Cysteine was incubated with DMSO or a 5-fold molar excess of ConB-1 for 8 h at 4 °C in water. Reactions were then analyzed by LC-MS using a Shimadzu LC and autosampler system (Shimadzu) interfaced to an LTQ ion trap mass spectrometer.

ALK samples were incubated with 20 molar excess of the **ConB-1** for 4 h at rt before being quenched by adding 0.1% trifluoroacetic acid (aq.). The resulting samples were separated over an HPLC column with 5-95% acetonitrile in water as an eluent. The MS data were analyzed using an Agilent LC/MSD Time-of-Flight Mass Spectrometer equipped with an electrospray ionization source.

**Inhibitory Effect of Con B-1 on ALK in 2 h Incubation.** In this experiment, 70 fold the final concentration of ALK (87.5 nM) and 200-fold the  $IC_{50}$  (100 nM) of **Con B-1** were incubated for 30 min at room temperature. The free compounds were removed through the column, and the substrate was added to start the reaction. Then the reaction rate was monitored for 2 h to determine whether the combination of the compound and the enzyme is reversible or irreversible. The normal enzyme activity group was set as the positive control group, and the nonenzyme group was the negative control group.

Western Blotting. H3122 cells were treated with Con B-1 at the concentrations of 0, 10, 50, 200, and 500 nM for 24 h at 37 °C, then the cells were harvested, washed in ice-cold PBS, and lysed with RIPA buffer, protease inhibitors, phosphatase cocktails A and B, and PMSF (1 mM). Protein concentration was determined by the BCA Protein Assay Kit (Beyotime#p0012s). The samples were subjected to SDS-PAGE and then transferred onto PVDF membranes (Millpore). The membranes were incubated overnight at 4 °C with the primary antibody in 5% BSA/TBST buffer with gentle shaking, then washed with 1× TBS/T 3 times, followed by incubation for 1 h with a 1/5000 dilution of secondary HRP antibody in 5% nonfat milk/TBST. Antibody information: p-ALK (Cell Signaling Technology; catalogue no. 3341s), AKT (wanleibio; catalogue no. WL0003b), p-AKT (wanleibio; catalogue no. WL02908), STAT3 (abcam; catalogue no. ab68153), p-STAT3 (Cell Signaling Technology; catalogue no. 9145T), GAPDH (Cell Signaling Technology; catalogue no. 2118). The target blots were detected with chemiluminescence system.

**Pharmacokinetic Studies.** Male SD rats (200–220 g, N = 3 per group) were dosed intravenously with 1 mg/kg of **Con B-1** prepared in 5% DMA/10% Solutol HS 15/85% saline, and orally with 10 mg/kg in 0.5% CMC-Na. Blood samples were taken at 0 (prior to dosing), 0.25, 0.5, 1, 2, 4, 6, 12, and 24 h following oral dosing and at 0 (prior to dosing), 0.083, 0.25, 0.5, 1, 2, 4, 8, and 24 h following intravenous dosing. The samples were collected from the jugular vein and stored

in ice  $(0-4 \, ^\circ C)$ . Plasma was obtained from the blood samples by centrifugation (8000 rpm for 6 min at 2–8  $^\circ C$ ) and stored at –80  $^\circ C$ . All samples of the compound were determined by LC–MS/MS (Shimadzu; API 4000). The assay lower limit of quantitation (LLOQ) was 1 ng/mL for **Con B-1** in plasma. The pharmacokinetic parameters were analyzed by noncompartmental methods using WinNonlin 5.2 (accomplished by Sichuan XPiscoric Inc.).

In Vivo Xenograft Studies. The female BALB/c nude mice were purchased (Beijing HFK Bioscience Co. ltd., Beijing, China). H3122 cells were harvested during the exponential-growth phase and washed twice with serum-free medium. Mice (6-7) weeks old and weighed 18–22 g) were injected subcutaneously with  $5 \times 10^6$  H3122 cells, which were suspended in 0.1 mL of serum and antibiotic-free growth medium. The tumors were allowed to grow to 150-200 mm<sup>3</sup>, at which point the mice were divided randomly (6 mice for each group). The mice were dosed orally with Con B-1 (10, 20, 50 mg/kg/d, dissolved in 10% NMP/90% PEG300, vehicle (10% NMP/90% PEG300), and Ceritinib (positive control, 20 mg/kg/d, dissolved in 10% NMP/90% PEG300). The body weight and tumor volume were measured every 3 days. The tumor volume was determined with Vernier calipers and calculated as follows: tumor volume =  $a \times b^2/2$ (a, long diameter; b, short diameter). Percentage of tumor growth inhibition (TGI) was determined after 15 days of treatment as the reduction in size of tumors in treated groups vs vehicle controls. All experimental protocols and animal handling procedures were approved by the Institutional Animal Care and Use Committee of Sichuan University.

## ASSOCIATED CONTENT

#### **1** Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.0c01707.

Inhibition rate (%) of compounds against ALK; antiproliferative activities of various cancer cell lines; antiproliferative activities of active compounds against human normal cell LO2; selectivity of Con B-1; covalent adduct nature of ConB-1; HPLC traces for Con A-3, Con A-4, Con B-1, and Con B-3 (PDF) Molecular formula strings and some data (CSV)

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## **Author Contributions**

<sup>¶</sup>G.Y., X.Z., and C.P. contributed equally to this work. G.Y.Y. performed the chemical synthesis and wrote the manuscript. X.X.Z., L.Y., C.L.P., H.F.S., and J.Y. assisted in chemical synthesis and characterization. S.K.L., X.Y.H., B.N., and M.Z. generated cell lines and designed and supervised biological studies. R.L. supervised the project and provided computational docking support and advice on compound design. D.Y.L. provided experimental support. R.L. conceived of and led this study.

#### Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS USED

ALK, anaplastic lymphoma kinase; ALKi, anaplastic lymphoma kinase inhibitors; NSCLC, nonsmall cell lung cancer; HATU, hexafluorophosphate azabenzotriazole tetramethyl uranium; DIPEA, *N*,*N*-diisopropylethylamine; DMF, dimethylformamide; MTT, methyl thiazolyl tetrazolium; SAR, structure activity relationship; LC-MS, liquid chromatography-mass spectroscopy; SD rats, Sprague-Dawley rats; TGIs, tumor growth inhibitions; TFA, Trifluoroacetic acid; DCM, dichloromethane

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