ABSTRACT: Targeted inhibition of anaplastic lymphoma kinase (ALK) dramatically improved therapeutic outcomes in the treatment of ALK-positive cancers, but unfortunately patients invariably progressed due to acquired resistance mutations in ALK. Currently available drugs are all type-I inhibitors bound to the ATP-binding pocket and are most likely to be resistant in patients harboring genetic mutations surrounding the ATP pocket. To overcome drug resistance, we rationally designed a novel kind of “bridge” inhibitor, which specially bind into an extended hydrophobic back pocket adjacent to the ATP-binding site of ALK. The novel type-I 1/2 inhibitors display excellent antiproliferation activity against ALK-positive cancer cells and appear superior to two clinically used drugs, crizotinib and ceritinib. Structural and molecular modeling analyses indicate that the inhibitor induces dramatic conformational transition and stabilizes unique DFG-shifted loop conformation, enabling persistent sensitivity to different genetic mutations in ALK. These data highlight a rationale for further development of next-generation ALK inhibitors to combat drug resistance.
mutations but is also inactive against other mutations, such as G1202R, I1171, and F1174V. \(^{14,15}\) Similarly, other ALK inhibitors also exhibit inconstant potency against various resistant mutants of ALK. \(^{16}\) On April 28, 2017, FDA granted accelerated approval to brigatinib, \(^{17}\) which is also classified as a second-generation inhibitor and cannot cover the broad range of drug-resistant mutations either. The results from phase II trial enrolling 222 crizotinib-resistant patients showed that the overall response rate (ORR) of two different dose strategies was 45% and 55%, respectively. \(^{18}\) Brigatinib is now in both first-line setting and phase II study with patients resistant to ceritinib and alectinib. Besides, several other ALK inhibitors are currently under clinical assessment, such as lorlatinib (Phase I/II, Pfizer), \(^{19}\) entrectinib (Phase II, Ignitya), \(^{20}\) and ensartinib (Phase II, Xcovery). \(^{21}\) Lorlatinib is a highly active ALK inhibitor with IC\(_{50}\) lower than 0.07 nM and can block most mutations resistant to crizotinib, ceritinib, and alectinib. In a phase I/II trial on 43 patients, the ORR of lorlatinib was 46% with or without treatment of prior ALK inhibitors. \(^{22}\) Entrectinib can target both ALK and NTRK1–3 fusion protein. In a phase I study enrolling 24 patients without treatment with other ALK inhibitors, the ORR of entrectinib was 57%, 100%, and 85% for ALK\(^+\), NRTK1\(^−3\), and ROS1\(^+\) patients, respectively. \(^{23}\) Remarkably, the ORR of ensartinib (X-396) in a phase I study enrolling 52 resistant patients was 88% by serial plasma sequencing that helps to select specific patients. \(^{24}\)

Currently available drugs and candidates in ongoing clinical trials are all type-I inhibitors bound to the ATP-binding pocket of ALK (Figure 1a) in its active DFG (Asp-Phe-Gly)-in state, and thus are most likely to be ineffective in harboring genetic mutations surrounding the ATP pocket (e.g., L1196M, G1202R, G1269A, and S1206Y), which are the main cause of drug resistance observed in clinic. In contrast, there exists another kind of inhibitor, so-called type-II or type-I\(^{1/2}\) inhibitors, occupying an extended hydrophobic tunnel (see in Figure 1a) adjacent to the ATP-binding site, which could induce a distinct DFG-out or DFG-shifted loop conformation. These compounds show several advantages over the type-I inhibitors, such as improved kinase selectivity, slower off-rates with extended residence time, \(^{25}\) and coverage of broad ALK mutations. So far, only a few type-II or type-I\(^{1/2}\) inhibitors of ALK have been identified, and the potency of these molecules is generally week, \(^{26,27}\) thus hindering further development.

We hypothesized that designing a new scaffold that bridges both the ATP-binding pocket and the hydrophobic back pocket might be an effective way to identify a potent type I\(^{1/2}\) inhibitor with improved ligand binding affinity toward both wild-type and drug-resistant mutants of ALK, as well as kinase selectivity to avoid potential off-target effects. In the present work, a series of novel type-I\(^{1/2}\) inhibitors were rationally designed and synthesized, and the experimental results demonstrate that these compounds show potent inhibitory activity in in vitro ALK enzyme-based assay and superior antiproliferation activity in three ALK-positive cancer cell lines (NCI-H2228, NCI-H3122, and Karpass-299), when compared to the two clinically used drugs, crizotinib and ceritinib. Furthermore, the sensitivity of compound 001-017 against four resistant ALK mutants was also found to be better than those of both crizotinib and ceritinib. Structural and molecular modeling analyses indicate that the inhibitor induces dramatic conformational transition and stabilizes unique DFG-shifted loop conformation enabling persistent sensitivity to different resistant mutations in ALK.

Figure 1. Rational design of possible “bridge molecules” that could target both ATP-binding site and back pocket. (a) Representation of the binding pocket of ALK. Protein structure is shown in gray ribbon. The ATP-binding pocket is colored in green, and the hydrophobic back pocket is colored in orange. The structure of the shifted DFG motif is highlighted and shown in cyan. (b) Schematic illustration of the potential pharmacophore representing the interactions in the hinge region and hydrophobic back pocket. Part of the piperidine carboxamide structure that locates in the linker region and the deep back pocket is displayed and highlighted. Hydrogen bond donor and acceptor are shown by the green circles labeled D and A, respectively. The circle labeled HYD represents the hydrophobic moiety that occupies the adenine ring region. (c) Rational design of type-I\(^{1/2}\) inhibitors. Various building structures substituted in different regions are also listed.
RESULTS AND DISCUSSION

Rational Design of Type-I\textsuperscript{1/2} Inhibitors of ALK Targeting Both ATP-Binding Site and Additional Hydrophobic Back Pocket. Type-I\textsuperscript{1/2} inhibitors are a novel class of kinase inhibitors that target the back pocket of ATP in either catalytically active or inactive DFG-in loop state, and can be recognized as a hybrid of type-I and type-II inhibitors.\textsuperscript{28} The back pocket is typically hydrophobic with its size and shape regulated by the type of gatekeeper residue, and the cavity cannot be occupied by ATP. Piperidine carboxamide 1 (see in Figure 3e), which binds into the extended hydrophobic back pocket, was first reported as an ALK inhibitor by Bryan and co-workers in 2012,\textsuperscript{26} and is at present the only type-I\textsuperscript{1/2} inhibitor of ALK. However, the binding affinity of piperidine carboxamide 1 is relatively weak (inhibitory IC\textsubscript{50} = 0.174 \(\mu\)M in ALK enzyme-based assay), and there is no relevant cellular data reported so far. In our previous study, the binding features of piperidine carboxamides were comprehensively analyzed by using combined molecular modeling strategies.\textsuperscript{29} We observed that piperidine carboxamides can form strong interactions with the residues surrounding the hydrophobic back pocket, but lack favorable contacts with the residues in the ATP-binding site. Thus, in the present work, part of the piperidine carboxamide structure which binds to the linker region and the hydrophobic back pocket of ALK (see in Figure 1b) is harnessed as a chemical probe to build "bridge molecules" that could occupy both the ATP-binding site and the hydrophobic back pocket.

Type-I inhibitors bind to the ATP-binding pocket and typically recognize the active DFG-in loop conformation of the target. Generally they mimic the adenine moiety of ATP and fit into the ATP pocket by forming hydrogen bond interactions with the backbone atoms of the corresponding residues in the hinge region as well as establishing hydrophobic contacts in the adenine-binding region. Therefore, in order to construct a similar pharmacophore containing hydrogen bond donors and/or acceptors and hydrophobic moieties, pyrrole pyrimidine, with a rigid conjugate structure, was proposed and tethered to the starting piperidine carboxamide structure (Figure 1c).

Molecular modeling study indicates that two hydrogen bonds can be formed between the residue Met1199 and the nitrogen atoms of pyrrole pyrimidine, which can well mimic the hydrogen bonds with the adenine moiety. The R\textsuperscript{1} region also

<table>
<thead>
<tr>
<th>Compound</th>
<th>R\textsuperscript{1}</th>
<th>IC\textsubscript{50}, nM (± SD)</th>
<th>Compound</th>
<th>R\textsuperscript{1}</th>
<th>IC\textsubscript{50}, nM (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crizotinib</td>
<td>—</td>
<td>4.66 (± 0.53)</td>
<td>001-007</td>
<td>—</td>
<td>148.15 (± 9.88)</td>
</tr>
<tr>
<td>LDK-378 (Certinib)</td>
<td>—</td>
<td>3.94 (± 0.21)</td>
<td>001-008</td>
<td></td>
<td>11.48 (± 1.01)</td>
</tr>
<tr>
<td>001-001</td>
<td>H</td>
<td>52.47 (± 3.78)</td>
<td>001-009</td>
<td>R</td>
<td>6.01 (± 0.53)</td>
</tr>
<tr>
<td>001-002</td>
<td>H</td>
<td>5.34 (± 0.27)</td>
<td>001-010</td>
<td>R</td>
<td>18.85 (± 0.79)</td>
</tr>
<tr>
<td>001-003</td>
<td>O</td>
<td>81.97 (± 5.18)</td>
<td>001-011</td>
<td>H</td>
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<td>001-005</td>
<td>&gt; 2000</td>
<td></td>
<td>001-013</td>
<td>R</td>
<td>37.88 (± 4.33)</td>
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<tr>
<td>001-006</td>
<td>H</td>
<td>11.88 (± 0.94)</td>
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</table>

\textsuperscript{a}All values are the average of \( n \geq 2 \) ± standard deviation.

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Table 1. ALK Kinase Inhibitory Activity (IC\textsubscript{50}) for Compound 001-001–001-13 with R\textsuperscript{1} Substitutions

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resides in a relatively hydrophobic environment between the adenine-binding region and the solvent accessible region. According to previous studies of type-I inhibitors, a phenyl ring which typically forms hydrophobic interactions with the glycine-rich loop residue Leu1122 is a choice of high priority and therefore was introduced to pyrrole pyrimidine at position 1 to optimally induce a $\alpha = 90^\circ$–$110^\circ$ angle change (Figure 1c).

In addition, compounds with different substitutions on the pyridine ring and various heterocyclic groups were also designed and synthesized. The R$^2$ moiety at the para-position of R$^3$ protrudes into the solvent accessible region and was replaced by several basic amine-containing solubilizing groups. With respect to the hydrophobic R$^3$ region, various functional groups, such as trifluoromethoxy, trifluoromethyl, methoxy, cyano and chloro groups, were designed and replaced at the ortho-, meta-, and para-position of the phenyl ring for better binding.

**Inhibitory Potency in Biochemical Assay and Structure–Activity Relationship (SAR) Studies.** To evaluate the inhibitory activity against ALK in in vitro models, time-resolved fluorescence resonance energy transfer (TR-FRET) assays were carried out for all the synthesized compounds as well as two positive control drugs, crizotinib and ceritinib. The IC$_{50}$ value of each compound was determined from the fitted dose–response curve, and the data are summarized in Table 1 to Table 3. Initially, we replaced the R$^1$ moiety with a substituted phenyl ring and R$^3$ with a para-trifluoromethylphenyl group, leading to analogue 001-001. The inhibitory IC$_{50}$ value of this compound is 52.47 nM, which is about 3-fold more potent than both marketed drugs.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R$^2$</th>
<th>R$^3$</th>
<th>IC$_{50}$, nM ($\pm$SD) $^a$</th>
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</thead>
<tbody>
<tr>
<td>001-014</td>
<td>$^O$N$^+$</td>
<td>-H</td>
<td>11.2 ($\pm$ 2.31)</td>
</tr>
<tr>
<td>001-015</td>
<td>$^O$N$^+$</td>
<td>-H</td>
<td>0.6 ($\pm$ 0.04)</td>
</tr>
<tr>
<td>001-016</td>
<td>$^O$N$^+$</td>
<td>-methyl</td>
<td>20.25 ($\pm$ 0.97)</td>
</tr>
<tr>
<td>001-017</td>
<td>$^O$N$^+$</td>
<td>-methyl</td>
<td>0.27 ($\pm$ 0.03)</td>
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</tbody>
</table>

$^a$ All values are the average of $n = 2 \pm$ standard deviation.

A fluorine or trifluoromethyl group significantly worsens IC$_{50}$. In sharp contrast, compound 001-002 with a more hydrophilic amino group substituted at the para-position exhibits noticeably improved activity (IC$_{50}$ = 5.34 nM), a ~10-fold increase relative to that of 001-001. By replacing the phenyl ring with a more hydrophilic pyridine ring at R$^3$, both compounds 001-006 and 001-008 show improved activity. The tethering position of the pyridine has little influence on the activity. Compound 001-007 with a methoxyl group substituted at the para-position of the pyridine ring shows a 10-fold decrease in activity compared with 001-006. Besides pyridine, five-membered heterocycles were also examined. We are pleased to find that the IC$_{50}$ values of the responding analogues (001-009, 001-0011, and 001-012) are all less than 10 nM.

During the first round of optimization, the para-position (R$^3$) of the left phenyl group seems very sensitive to structural changes. We decided to fine-tune R$^2$ in the next phase (Table 2), which directly protrudes into the solvent accessible region. Several hydrophilic nitrogen-containing solubilizing groups were examined. Compounds 001-015 and 001-017 show excellent inhibitory potency with IC$_{50}$ values in the picomolar range, 0.6 nM and 0.27 nM, respectively. Under the same assay conditions, the IC$_{50}$ readouts of crizotinib and ceritinib are 4.66 nM and 3.94 nM, respectively. In comparison, 001-017 is substantially more potent than both marketed drugs.

Optimization was also carried out for the moiety that sits in the deep hydrophobic back pocket (phenyl ring on the right, R$^3$) (see in Table 3). This phenyl group was substituted with various functionalities. Compound 002-004 without any substituent on the phenyl ring exhibits ~100-fold decreased activity relative to 001-017, demonstrating the importance of having a trifluoromethoxy moiety at the para-position. Substitution of the trifluoromethoxy group at the ortho-position of the R$^3$ phenyl (002-003) has a negative effect with IC$_{50}$ = 61.12 nM. Trifluoromethylation at the meta-position results in a potent analogue 002-008 (IC$_{50}$ = 0.64 nM). Replacing the trifluoromethoxy group by other groups, including trifluoromethyl, methylsulfonfyl, sulfur trifluoride, methoxy, cyano and.
chloro groups, all lead to weaker inhibitors. For example, analogue 002-006 with a hydrophilic methylsulfonyl group has an IC_{50} value of 347.52 nM. This observation validates the fact that the back pocket is primarily surrounded by hydrophobic amino acids, so the interactions between inhibitors and protein in this region should be predominantly hydrophobic. The importance of the R^3 phenyl was further confirmed by replacing it with other rings, such as pyridine, cyclohexane, or smaller saturated heterocyclic groups (002-012, 002-013, 002-016, and 002-017). Weak inhibition was generally obtained, with the IC_{50} values ranging from 291.38 nM to 1921.69 nM.

Superior Cellular Activity against NSCLC and ALCL Cell Lines. The analogues were assessed for their bioactivities to inhibit the proliferation of ALK-positive cancer cells, including two NSCLC cell lines expressing EML4-ALK fusion (NCI-H2228 and NCI-H3122) and an ALCL cell line Karpas-299 with NPM-ALK fusion. Serial dilutions from 5000 ng/mL to 1.6 ng/mL were tested for each inhibitor, and the cell viability was determined by the MTT assay. The experimental results for several representative inhibitors are summarized in Figure 2, and the full cellular data for all the compounds can be found in Supplementary Table S1. As we can see in Figure 2b, at high concentrations all compounds show good inhibitory activities against NCI-H2228, while at low concentrations significant divergence is observed. The inflection point of the activity appears at the concentration of 40 ng/mL, and the differences become greater at 8 ng/mL where the inhibition ratios of cirizotinib and ceritinib drop sharply to 20.59% and 36.57%, respectively. At this concentration, compounds 001-017 and 002-008 give 71.67% and 63.08% inhibition of cell

<table>
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<th>Compound</th>
<th>R^3</th>
<th>IC_{50} nM (± SD)</th>
<th>Compound</th>
<th>R^3</th>
<th>IC_{50} nM (± SD)</th>
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<td>002-001</td>
<td></td>
<td>3.15 (± 0.45)</td>
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<td>1.65 (± 0.79)</td>
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<td>61.12 (± 3.22)</td>
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<td>39.87 (± 2.23)</td>
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<td>347.52 (± 7.64)</td>
<td>002-015</td>
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<td>159.5 (± 7.33)</td>
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<td>0.86 (± 0.13)</td>
<td>002-016</td>
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<td>1921.69 (± 14.59)</td>
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<td>002-008</td>
<td></td>
<td>0.64 (± 0.07)</td>
<td>002-017</td>
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<td>1205.98 (± 9.12)</td>
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<td>002-009</td>
<td></td>
<td>2.18 (± 0.18)</td>
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</tbody>
</table>

*All values are the average of n ≥ 2 ± standard deviation.
viability. When tested at the concentration of 1.6 ng/mL, cirizotinib and ceritinib show no inhibition, while 001-017 and 002-008 deliver remarkable results, 69.09% and 56.16% inhibition. These results indicate that 001-017 and 002-008 are excellent antiproliferation inhibitors against NCI-H2228, which are superior to both cirizotinib and ceritinib.

We further examined the inhibitory activities of all the synthesized compounds against NCI-H2228 at the lowest concentration of 1.6 ng/mL (shown in Figure 2c). Ten compounds show higher inhibition ratios than cirizotinib and ceritinib, among which four compounds (001-017, 002-007, 002-008, and 002-011) display >50% inhibitory potency. Meanwhile, the antiproliferation activities against Karpas-299 and H3122 cell lines were also examined. As shown in Figure 2d,e, results similar to those of NCI-H2228 are observed. Compound 001-017 exhibits higher potency than 002-008 in both cells. It is not surprising that the cellular activity of 001-017 is the best among all the tested compounds since the enzyme-based IC_{50} value of 001-017 (0.27 nM) is the lowest. As a result, 001-017 emerges as a potential candidate with potent enzymatic activity against ALK and excellent antiproliferation activity in different ALK-positive cancer cell lines.

Moreover, the influence of compound 001-017 on downstream signaling pathways was also examined, and the results are illustrated in Figure 2f,g, which confirms the reduction of phospho-ALK, phospho-AKT, and phospho-STAT3 levels following 001-017 treatment. According to our results, both 001-017 and ceritinib can inhibit autophosphorylation of ALK in NCI-H2228 and Karpas-299 cell lines, and can cause substantial prevention of phosphorylation of AKT and STAT3 in a dose-dependent manner. It was also observed that compound 001-017 is more potent than ceritinib. At 10 nM, 001-017 completely suppresses ALK phosphorylation, and inhibition is observed even at 1 nM, demonstrating the unparalleled activity of 001-017 in suppressing relevant signaling pathways.

**Structural Basis and Conformational Transition upon Type-I1/2 Inhibitor Binding.**

The binding geometries of all the compounds were first predicted by the Glide docking simulations, followed by 5 ns MD simulations, and the binding free energies were subsequently calculated by the MM/GBSA methodology. Different solute dielectric constants (ε_{sol} = 1, 2, and 4) were applied to get insights into the influence on the overall accuracy, which is represented by the linear correlation.
coefficient (R) between the predicted binding free energies and the experimental -pIC\textsubscript{50} values from six models. In order to find the best prediction model, two crystal structures of ALK (PDB entries: 4FNZ and 4DCE) were applied with the solute dielectric constants set to 1, 2, or 4 (4FNZ-1, 4FNZ-2, 4FNZ-4, 4DCE-1, 4DCE-2, and 4DCE-4). The binding free energies were calculated based on the MM/GBSA methodology with the solute dielectric constant set of 1. (c) Schematic representation of the conformational shifts of the residues surrounding the hydrophobic back pocket upon the type-I\textsuperscript{1/2} inhibitor binding. The original unbound DFG-in protein structure is shown in cyan and the DFG-shifted structure is colored in yellow. (d) 2-D schematic diagram of the binding patterns for compound 001-017. (e, f) Protein-inhibitor interaction spectra on a per-residue basis for piperidine carboxamide 1 and compound 001-017. The data were generated by the MM/GBSA binding free energy decomposition analysis. (g) Differences of the binding free energies (\(\Delta G_{\text{compound-017}} - \Delta G_{\text{piperidine carboxamide 1}}\)) between piperidine carboxamide 1 and 001-017 on a per-residue basis with the important residues highlighted.

Figure 3. Molecular modeling and insights into the binding mechanism. (a) Heat map of the correlation coefficients between experimental -pIC\textsubscript{50} values and predicted binding free energies from six models. In order to find the best prediction model, two crystal structures of ALK (PDB entries: 4FNZ and 4DCE) were applied with the solute dielectric constants set to 1, 2, or 4 (4FNZ-1, 4FNZ-2, 4FNZ-4, 4DCE-1, 4DCE-2, and 4DCE-4). (b) Linear correlation between the predicted binding free energies and the experimental -pIC\textsubscript{50} values using the crystal structure of 4FNZ. The binding free energies were calculated based on the MM/GBSA methodology with the solute dielectric constant set of 1. (c) Schematic representation of the conformational shifts of the residues surrounding the hydrophobic back pocket upon the type-I\textsuperscript{1/2} inhibitor binding. The original unbound DFG-in protein structure is shown in cyan and the DFG-shifted structure is colored in yellow. (d) 2-D schematic diagram of the binding patterns for compound 001-017. (e, f) Protein-inhibitor interaction spectra on a per-residue basis for piperidine carboxamide 1 and compound 001-017. The data were generated by the MM/GBSA binding free energy decomposition analysis. (g) Differences of the binding free energies (\(\Delta G_{\text{compound-017}} - \Delta G_{\text{piperidine carboxamide 1}}\)) between piperidine carboxamide 1 and 001-017 on a per-residue basis with the important residues highlighted.
behaviors, but the contribution of each residue varies. The key residues that assist the preferential binding of 001-017 to piperidine carboxamide 1 are highlighted in Figure 3g. The contributions from the residues Leu1122, Met1199, and Phe1271 are significantly higher in the binding of 001-017 to ALK. Both the hydrophobic interaction with Leu1122 and the hydrogen bonding with Met1199 in the ATP-binding region are enhanced. The dominating π-π interaction with Phe1271 in the back pocket is also strengthened. These results suggest that the binding of 001-017 to both the ATP-binding site and hydrophobic back pocket is significantly stronger than that of piperidine carboxamide 1, leading to much higher overall inhibitory activity toward ALK.

**Sensitivity against Drug-Resistant Mutants of ALK.** The emergence of drug resistant mutants of ALK in the clinic has become a great challenge for cancer therapy. In this study, the sensitivity of compound 001-017 against four clinically resistant mutants of ALK (L1196M, C1156Y, R1275Q, and F1174L) was also evaluated, and the results are summarized in Figure 4a. L1196 M is a gatekeeper mutation, analogues to the mutation in the EGFR-T790M kinase domain, and is believed to cause resistance to the first-line drug, crizotinib. The second-generation inhibitor, ceritinib, was found to be more sensitive to the L1196M mutant. In our assays, the inhibitory IC<sub>50</sub> values of crizotinib and ceritinib against L1196M ALK are 80.21 nM and 9.36 nM, respectively, consistent with the reported data. The activity of compound 001-017 (IC<sub>50</sub> = 7.51 nM) against the L1196 M ALK is similar to that of ceritinib. For the C1156Y mutant, both crizotinib and ceritinib show comparable inhibitory activities (IC<sub>50</sub> = 15.77 nM and 12.95 nM, respectively), but compound 001-017 is more than 3-fold more potent, with IC<sub>50</sub> = 4.7 nM. In addition, compound 001-017 against the R1275Q mutant is about 7-fold more potent than crizotinib and 3-fold more potent than ceritinib. For the F1174L mutant, the sensitivities of ceritinib and 001-017 (IC<sub>50</sub> = 10.39 nM and 9.81 nM, respectively) are similar, which are about 2-fold more active than that of crizotinib. In summary, compound 001-017 exhibits superior sensitivity against different genetic mutations in ALK compared with both crizotinib and ceritinib.

**Kinase Selectivity.** Compound 001-017 was designed as a type-I<sup>1/2</sup> inhibitor that binds into an extended back pocket that does not exist in most kinases. So it was expected that type-I<sup>1/2</sup> inhibitors would have improved kinase selectivity. To explore the kinase selectivity profile of 001-017, the inhibitory activity on a panel of 35 kinases from different families was tested. The results are summarized in Figure 4b, and the detailed IC<sub>50</sub> values against specific kinases are listed in Supplementary Table S2. Compound 001-017 shows very weak or no inhibition against most kinases. The IC<sub>50</sub> values of only two kinases, LTK (0.42 nM) and ROS1 (7.88 nM), are lower than 10 nM. It is not surprising that 001-017 shows high inhibitory activity against LTK since the sequence identity between LTK and ALK is very high (~78% in the kinase domain). Besides, ROS1 also shows relatively high sequence identity (~52% in the kinase domain) to ALK<sup>31</sup>. Apart from LTK and ROS1, none of the other kinases are found to be highly sensitive to 001-017 (>1000-fold decrease in activity), indicating that 001-017 is a highly selective ALK inhibitor.

Numerous point mutations of ALK have been identified clinically that are resistant to available therapeutic drugs. There is an urgent need to develop new inhibitors with novel chemical structures to combat drug resistance. To our knowledge, drugs currently on the market and those in clinical trials are all ATP-competitive type-I inhibitors of ALK. In this work, we designed and synthesized a series of type-I<sup>1/2</sup> inhibitors with very potent inhibitory activity against ALK in enzyme-based assay, with five compounds (001-015, 001-017, 002-007, 002-008, and 002-011) showing picomolar IC<sub>50</sub>. The most potent analogue, 001-017, is >500-fold (IC<sub>50</sub> = 0.27 nM) more active than the reference compound, piperidine carboxamide 1 (IC<sub>50</sub> = 174 nM), and significantly more potent than crizotinib (IC<sub>50</sub> = 4.66 nM) and ceritinib (IC<sub>50</sub> = 3.94 nM). On the basis of the results of structural analysis, strong hydrophobic interactions with the residues Ile1171 and Phe1271, and two hydrogen bonds with Lys1115 and Gly1269 are formed in the extended back pocket, accounting for the excellent binding affinity of 001-017. 001-017 also shows an improved efficacy compared to both crizotinib and ceritinib against ALK-addicted cancer cells, such as NSCLC expressing EML4-ALK (NCI-H2228 and NCI-H3122) and ALCL expressing NPM-ALK (Karpas-299). We examined the influence of 001-017 on the downstream signal transduction in three cancer cell lines and confirmed the reduction of phospho-ALK, phospho-STAT3 and phospho-AKT level following the 001-017 treatment. Although the full downstream targets of the ALK signaling remain unclear, the expression of these targets can be useful markers for clinical assessment. Further investigations would be needed to clarify the exact signaling pathways of ALK and help to explore possibilities for combination therapy clinically.

To determine the potency of 001-017 to drug-resistant mutants of ALK, one of the most frequently gatekeeper mutation, L1196M, was tested first. Compound 001-017 shows high activity against the L1196M mutant. Despite a slightly
weaker binding affinity of 001-017 to the L1196M mutant compared with the native ALK, the IC<sub>50</sub> value of 001-017 against this cell line is >10-fold lower than that of crizotinib. The efficacy of 001-017 against other three major resistant mutants of ALK (C1156Y, R1275Q, and F1174L) was also evaluated, and the results showed 001-017 displays superior sensitivity compared to both crizotinib and ceritinib in general.

Type-II and/or type-I<sup>1/2</sup> inhibitors bind into an extended hydrophobic back pocket, available to only a few kinases. Thus, the kinase selectivity of type-II and/or type-I<sup>1/2</sup> inhibitors is always higher than that of the ATP-competitive type-I inhibitors. In this study, the inhibitory activities of 001-017 against 35 kinases from different families were examined. Results confirm that 001-017 is a highly selective ALK inhibitor, with ~94% kinases found immune to 001-017 (>1000-fold decrease in activity).

■ CONCLUSION

In conclusion, we designed a new molecular scaffold that binds to not only the ATP-binding pocket but also an extended hydrophobic back pocket, leading to very potent inhibitors against both wild-type and resistant mutants of ALK. An added benefit of this design is that the extended back pocket cannot be accessed in most kinases, therefore offering improved kinase selectivity. We expect that the inhibitors presented in this work will inspire the next generation therapy for ALK-positive cancers and provide a new strategy for developing novel ALK inhibitors to combat drug resistance.

■ EXPERIMENTAL SECTION

Compound Synthesis. All solvents were distilled according to general practice prior to use. All reagents were purchased and used without further purification unless specified otherwise. Solvents for flash column chromatography were technical grade and distilled prior to use. Analytical thin-layer chromatography (TLC) was performed using Huanghai silica gel plates with HSGF 254. Visualization of the developed chromatogram was performed by UV absorbance (254 nm) and appropriate stains. Flash column chromatography was performed using Qingdao Haiyang Chemical HG/T2354-92 silica gel (200−300 mesh) with the indicated solvent system according to standard techniques. 1H NMR and 13C NMR data were recorded on Bruker 400 MHz (100 MHz for 13C, 376 MHz for 19F) nuclear resonance spectrometers unless otherwise specified, respectively. Chemical shifts (δ) in ppm are reported as quoted relative to the residual signals of chloroform (1H 7.26 ppm and 13C 77.16 ppm). Multiplicities are described as s (singlet), bs (broad singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and coupling constants (J) are reported in Hertz (Hz). 13C NMR spectra were recorded with total proton decoupling.

The synthesis of compound 2 (Figure 5a): In a flamed-dried flask under Ar, NaH (3.91 g, 60% in mineral oil, 97.7 mmol) was weighed, followed by addition of DMF (150 mL). The resulting mixture was cooled to 0 °C. A solution of compound 1 (10 g, 65 mmol) in 50 mL of DMF was added slowly over a 20 min period, and the reaction solution was stirred for 30 min.

Figure 5. (a−c) General synthetic routes of 7H-pyrrolo[2,3-d]pyrimidine derivatives.
Benzensulfonyl chloride (8.3 mL, 65 mmol) was added, and the mixture was warmed to r.t. and stirred for 1 h. Water was carefully added and the resulting precipitate was collected by filtration and dried in a vacuum to obtain compound 2 (17.3 g, yield: 90%) as a crystalline solid.

The synthesis of compound 3: In a flame-dried flask under Ar, LDA (6 mL, 2 M in THF) was added. The solution was cooled to −78 °C, and compound 2 (2.3 g, 7.83 mmol) in THF (25 mL) was added dropwise. The mixture was stirred for 1.5 h at −78 °C, and a solution of Li 2 (2.3 g, 9 mmol) in THF was added dropwise. The reaction mixture was stirred for 3 h. Water was carefully added and the mixture was extracted with three times of DCM. The organic phase was washed with brine, dried over Na2SO4, filtered, and concentrated to give crude product 3 (3.2 g, yield: 98%) as a yellow solid.

The synthesis of compound 4: To a stirred solution of compound 3 (1.2 g, 2.86 mmol) in THF (15 mL) was added 5 M sodium hydroxidemethanolic solution. After 30 min, the solvent was removed under reduced pressure, and sat. NH4Cl was added. The resulting precipitate was filtered, washed with water, and dried to afford compound 4 (783 mg, yield: 98%) as a yellow solid.

The synthesis of compound 5: To a mixture of compound 4 (600 mg, 2.15 mmol) and ethyl (35)-piperidine-3-carboxylate (406 mg, 2.58 mmol) in MeCN (30 mL) was added Dipea (1 mL, 6.45 mmol), and the mixture was stirred at 120 °C under Ar for 48 h. The reaction mixture was cooled to r.t. and filtered to give compound 5 (629 mg, yield: 68%) as a white solid.

The synthesis of compound 6: LiOH (1 g, 2.5 mmol) was added to a mixture of compound 5 (1 g, 2.5 mmol) in THF/ H2O = 30 mL/10 mL. The reaction solution was stirred at r.t. for 4 h. The mixture was acidified to pH = 2–3 and concentrated. Sat. NaHCO3 was added until a large amount of solid precipitate appeared. The solid was collected by filtration and dried in a vacuum to obtain compound 6 (860 mg, yield: 93%).

Suzuki reaction (Figure 5b): The aryl iodide compound (100 mg, 1 equiv), 3 equiv corresponding boronic acid, 0.2 equiv of Pd(dppf)Cl2, 5 equiv of K2PO4 were added sequentially to a solution of dioxane/H2O = 3 mL/1 mL. The reaction mixture was stirred under Ar at 90 °C for 15 h. The solvent was removed under a vacuum, and the residue was purified by silica gel flash column chromatography and recrystallization to afford the product.

The synthesis of the amide compound (Figure 5c): acido (100 mg, 1 equiv), 1.5 equiv of EDCI, 1.5 equiv of HOAT were added to 3 mL of DCM. The corresponding amine (1.5 equiv) and 4 equiv of Dipea were added. The reaction mixture was stirred at r.t. overnight. Water was added and the mixture was extracted with DCM three times. The organic phase was washed with brine, dried over Na2SO4, filtered, and purified by silica gel flash column chromatography and recrystallization to afford product.

After the modifications of the chemical structure, 34 compounds were obtained in total, and the spectroscopic data of all the compounds can be found in the Supporting Information.

Cell-Based Antiproliferation Assay. Initially, cells were collected and seeded on 96-well plates in RPMI-1640 growth media +10% fetal bovine serum (FBS) at ~17,000 cells/well and incubated overnight at 37 °C in 5% CO2. The next day, serial compound dilutions were added into the assigned wells, and the plates were further cultured at 37 °C for 72 h. Cell proliferation was assessed using thiazolyl blue tetrazolium bromide (MTT) assay by adding 5 mg/mL MTT into each well, and the plates were then cultured for another 4 h. The converted dye was dissolved by MTT buffer overnight, and the absorbance of each well was finally detected at 570 nm with the control wavelength of 650 nm.

Immunoblotting Analysis. Cells were collected and lysed in cell lysis buffer (Beyotime Biotechnology, P0013) containing 1 mM PMSF and inhibitor cocktail. Cells were sonicated for 15 s to complete cell lysis and shear DNA, and the samples were then heat to 95–100 °C for 5 min. Subsequently, 20 μL samples were loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were then electrotransferred to polyvinylidene fluoride (PVDF) membrane (Millipore). After transfer, the membrane was washed with TBS for 5 min and incubated with blocking buffer (5% BSA) for 1 h at room temperature. Later, the membrane was incubated with diluted primary antibody (anti-ALK (Cell Signaling Technology, #3333), anti-Phospho-ALK (Cell Signaling Technology, #3341), anti-AKT (Cell Signaling Technology, #4691), anti-Phospho-AKT (Cell Signaling Technology, #4058), anti-STAT3 (Cell Signaling Technology, #4904), anti-Phospho-STAT3 (Cell Signaling Technology, #9145), and anti-GAPDH (Beyotime Biotechnology, AG019). After further incubation with anti-rabbit or anti-mouse IgG, HRP-linked secondary antibody (Beyotime Biotechnology), the bands bound with HRP were detected using BeyoECL Plus (Beyotime Biotechnology, P0018).

Kinase Selectivity Assay. The experiments were conducted by ChemPartner Co., Ltd. CHK2 and MARK1 assays were conducted using ADP-Glo luminescent assay with ATP concentration at Kon. PI3Kr assays were also conducted by Kinase-Glo luminescent assay with ATP concentration at 25 μM. mTOR kinase assays were carried out using Lancette Ultra assay with ATP concentration at Kon. The experiments of the other 31 kinases were carried out by Mobility shift assay with ATP concentration at Kon. Staurosporine or PI 103 or IKK-16 were set as the reference compound. The compounds were tested from 5 μg/mL to 0.00025 μg/mL for total of 10 concentrations (3-fold dilution). The data were fitted in XLFit to obtain IC50 values. Equation used is Y = bottom + (top-bottom)/(1 + (IC50/X)^HillSlope).

Simulation Modeling. All docking simulations were performed using the Glide module in Schrodinger 9.0.32 Preparations of the crystal structures of ALK/inhibitor complexes (PDB codes: 4FNZ and 4DCE) were carried out with the Protein Preparation wizard module. All crystallographic waters were removed from the original structures, and the missing hydrogen atoms and partial charges were added to the proteins. A restrained partial minimization was then performed with the maximum root-mean-square deviation (RMSD) value set to 0.3 Å. The receptor grid boxes were produced and centered on the original crystallographic inhibitors by the Receptor grid generation module. The scaling factors for van der Waals radii and partial atomic charge cutoff were set to 0.8 and 0.15, respectively. Preparations of the inhibitors were all accomplished by the LigPrep module with protonated states generated at pH = 7.0 ± 2.0. All the other parameters were set to the default values. Finally, all the inhibitors were docked into the binding pocket of ALK using the extra precision (XP) scoring mode.

The predicted binding structures from the docking simulations served as the starting structures for molecular
The PME method was applied to handle long-range electrostatics extended 8 Å from any solute atom. The Particle Mesh Ewald (PME) method was applied to inhibitors and proteins, respectively. Each complex was immersed into a periodic TIP3P water box which was extended 8 Å from any solute atom. The Particle Mesh Ewald (PME) method was applied to handle long-range electrostatics. A three-step minimization was performed before each MD simulation: (1) 1000 cycles of minimizations with 50 kcal/mol/Å² restraint on the backbone carbons, including 500 cycles of steepest descent and 500 cycles of conjugate gradient minimizations; (2) the same 1000 cycles of minimizations without restraint, including 1000 cycles of steepest descent and 4000 cycles of conjugate gradient minimizations. Afterward, the temperature of each system was gradually elevated from 0 to 1 atm and P = 300 K) MD simulations were performed with the time step set to 2 fs. The SHAKE algorithm was used to handle all hydrogen bonded atoms, and the resulting MD trajectories were saved every 10 ps.

The binding free energy (ΔGbind) of each inhibitor was predicted by the Molecular Mechanics/Generalized Born Solvent Area (MM/GBSA) method implemented in Amber. The modified Generalized Born (GB) model, (gb = 2), which has advantages over other models, was utilized to predict the polar part of desolvation energy (ΔGpol), and the nonpolar part of desolvation (ΔGnonpol) was calculated based on the solvent accessible surface area (SASA) computed by the LCP algorithm. The exterior dielectric constant was set to 80, and different solute dielectric constant (εs = 1, 2, or 4) was applied to get insights into the influence on the overall accuracy. The conformational entropic contribution (−TΔS) was not considered in the calculations due to high computational cost and low prediction accuracy.44 The MM/GBSA decomposition analysis was applied to calculate the contribution of each residue to inhibitor binding.

ASSOCIATED CONTENT
5 Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.7b00419.

Antiproliferation activity of all the synthesized compounds against NCI-H2222, Karpas-299 and NCI-H3122, kinase selectivity of compound 001-017 toward 35 kinases from different families, and the NMR spectroscopic data for all the compounds (PDF)

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Notes
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