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Structure-based design, synthesis and characterization of the first irreversible inhibitor of Focal Adhesion Kinase

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ABSTRACT: Focal Adhesion Kinase signaling pathway and its functions have been involved in the development and aggressiveness of tumor malignancy, it then presents a promising cancer therapeutic target. Several reversible FAK inhibitors have been developed and are being conducted in clinical trials. On the other hand, irreversible covalent inhibitors would bring many desirable pharmacological features including high potency and increased duration of action. Herein we report the structure-guided development of the first highly potent and irreversible inhibitor of the FAK kinase. This inhibitor showed a very potent decrease of autophosphorylation of FAK in squamous cell carcinoma. A cocrystal structure of the FAK kinase domain in complex with this compound revealed the inhibitor binding mode within the ATP binding site and confirmed the covalent linkage between the targeted Cys427 of the protein and the inhibitor.

Focal adhesion kinase (FAK) is an ubiquitously expressed non-receptor protein tyrosine kinase and a scaffold protein localized to focal adhesions, which plays important roles in many biological process including survival, proliferation, angiogenesis, adhesion, motility, metastasis, tumor microenvironment, epithelial to mesenchymal transition (EMT) as well as cancer stem cell renewal.¹⁻³ FAK is also over-expressed in a variety of solid and non-solid tumors, which make it a promising therapeutic target.³⁻⁴ In recent years, numerous clinical trials have been developed with small FAK inhibitor molecules.⁵⁻⁶ The results of such

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3 inhibitors are interesting since the decrease of tumor growth and metastasis development was
4 observed in some preclinical models and in cancer patients, even if some adverse events have
5 been revealed.⁷ In order to enhance the efficacy of anti-FAK cancer treatments the trials are
6 conducted either individually or in combination. Recent advances in the evaluation of FAK
7 inhibitors in preclinical and clinical models are very promising.⁷⁻⁸ Furthermore, FAK has
8 recently been shown to be localized in the nucleus and could promote survival and cell
9 proliferation by interacting directly with p53.⁹ FAK inhibition could also trigger immune-
10 mediated tumor regression through the control of regulatory T-cell (Treg) in the tumor
11 environment.¹⁰

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20 The development of covalent irreversible inhibitors to target kinases have recently
21 garnered significant interest.¹¹⁻¹² From a therapeutic point of view, the main advantages of
22 irreversible inhibitors are i) an increase of the target residence time to obtain durable
23 inhibition without maintaining continuous drug exposure; ii) a better ability to avoid drug
24 resistance by reducing the development of resistance mutations.¹³ Although there is only very
25 few of irreversible tyrosine kinase inhibitors used as therapeutic agents in the fields of
26 oncology nowadays, the number of these new inhibitors is steadily increasing to enter clinical
27 trials.

32 33 34 **RESULTS AND DISCUSSION**

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37 **Design of Irreversible Covalent Inhibitors.** In order to assess the feasibility of
38 designing irreversible inhibitors of FAK, our strategy entailed identifying a suitable cysteine
39 residue within or next to the ATP binding pocket. This approach would be amenable by using
40 a kinome-wide sequence analysis in connection with 3D structure of the ATP site. In
41 particular, we noticed that FAK contains a cysteine residue in the Glycine-rich loop region
42 closer to the ATP site, a feature which is not observed in most of other human kinases (Figure
43 1A). The distance between Cys427 and a reversible inhibitor, such as TAE226 (PDB ID:
44 2JKK)¹⁴ or PHM16 (PDB ID: 4BRX)¹⁵ in the ATP binding site is less than 10 Å (Figure 1B).

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52 Based on the two-step process for kinase inhibition, we designed our inhibitors, which
53 first bind reversibly to the enzyme, forming a non-covalent complex, and then form a covalent
54 binding with the residue Cys427 of the enzyme to provide an irreversible complex (Figure S1,
55 equation 1). Pyrimidine derivatives, especially 2,4-dianilinopyrimidine derivatives, are one of
56 the important scaffolds found in many compounds described by pharmaceutical companies
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and academic laboratories.^{6,16–18} These pyrimidine-containing inhibitors efficiently target the FAK scaffolding function and suppress tumor growth through inhibition of Akt signaling or activation of p53 signaling and its downstream targets.¹⁰ Our design strategy required the synthesis of 2,4-pyrimidine derivatives as a scaffold to maintain affinity similar to reversible inhibitors (compound **2**, Figure 1C), and inclusion of a linker with Michael acceptor which could react with the nucleophilic Cys427 residue. A number of diverse linkers were evaluated, we quickly focused upon two linkers, a squarate (3,4-bisaminocyclobutane-1,2-dione) (**5** and **6**) and a 1,2,3-triazole (**7** and **8**) motif which are able to sustain a good orientation to target Cys427 in the Glycine-rich loop region of the FAK kinase. Concerning the electrophile motif, an acrylate group was chosen to maintain reactivity (**5**, **7** and **8** in Figure 1C).

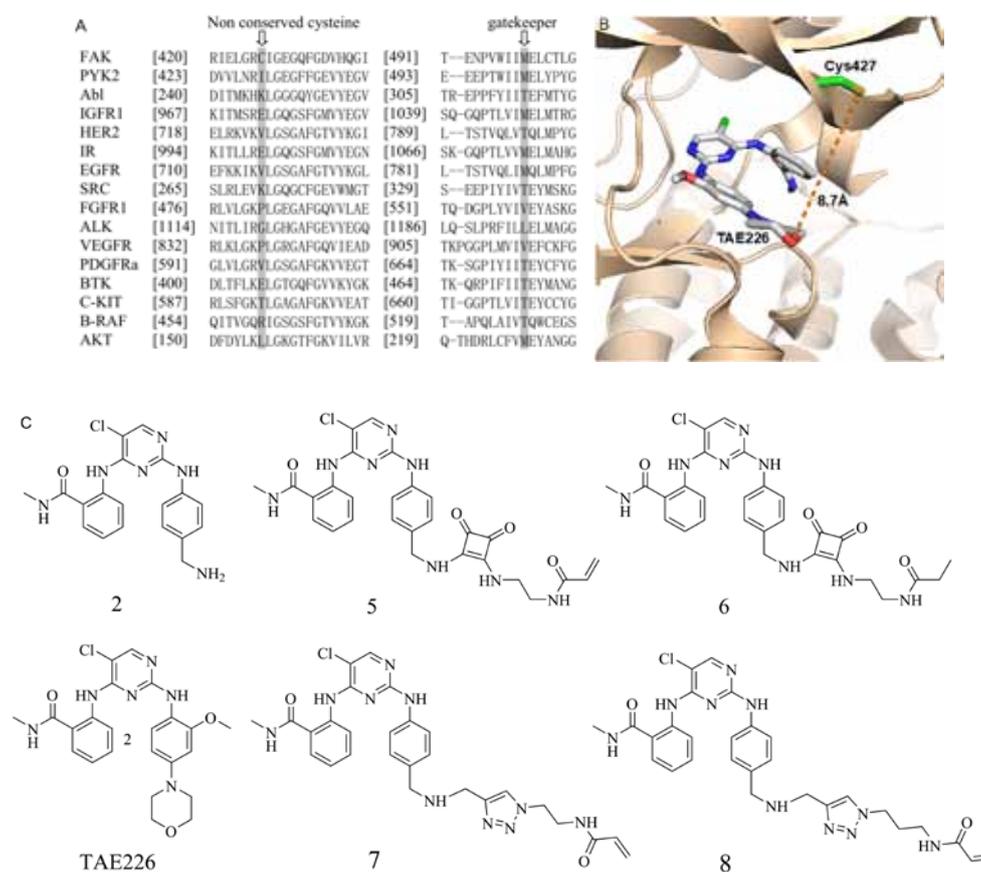
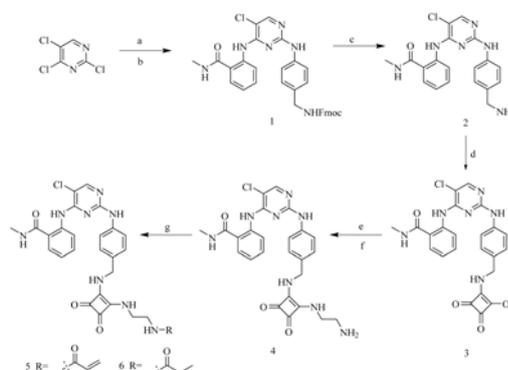


Figure 1. Design of a potent and electrophilic inhibitor of FAK guided by structural bioinformatics. (A) Partial sequence alignment of FAK with 15 other protein kinases, (B) Structural assessment of cysteine residue 427 for covalent binding to FAK. The structure shows the FAK kinase (tan ribbon) bound to TAE226 (white sticks) (PDB ID: 2JKK). The Cys427 side chain is colored in green and its distance to TAE226 indicated. (C) Reversible and irreversible inhibitors of FAK.

Synthesis of FAK Inhibitors. The synthetic route of new 2,4-pyrimidine compounds is outlined in Scheme 1 (for compounds **5** and **6**) and Scheme S1 (for compounds **7** and **8**). Starting from commercially available 2,4,5-trichloropyrimidine, the first chlorine atom in position 4 of 2,4,5-trichloropyrimidine is displaced by 2-amino-N-methylbenzamide in the presence of NaHCO₃ in EtOH at reflux to afford monosubstituted intermediate in a yield of 93%. This was further substituted to compound **1** in the presence of the corresponding (9H-fluoren-9-yl)methyl(4-aminobenzyl)carbamate and HCl in n-BuOH in a yield of 53%. The amino protecting Fmoc group of **1** was removed using piperidine to provide the key intermediate **2**. Then, the electrophilic 1,4-addition reactions occurred between compound **1** and dimethyl squarate in the presence of DIPEA at room temperature to afford compound **3** in a yield of 81%. This was further condensed with tert-butyl (2-aminoethyl)carbamate in the presence of DIPEA, followed by the deprotection of Boc group to give compound **4**. Finally, acylation of the primary amine with acryloyl chloride or propionyl chloride gave the expected, targeted covalent inhibitor **5** and the corresponding reversible inhibitor **6**, without the electrophilic warhead. Another potential irreversible inhibitors **7** and **8** bearing a 1,3,5-triazole linker were also synthesized in a similar manner (Scheme S1).

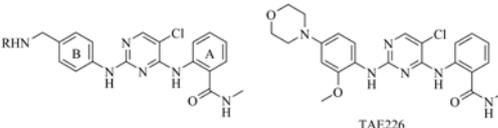


Scheme 1. Synthesis of FAK targeted covalent inhibitor **5** and reversible inhibitor matched-pair **6**. Reagents and conditions: a) 2-amino-N-methylbenzamide, NaHCO₃, EtOH, reflux; b) (9H-fluoren-9-yl)methyl (4-aminobenzyl)carbamate, HCl, n-BuOH, 120 °C; c) piperidine, DMF, RT; d) dimethyl squarate, DIPEA, DMF, RT; e) tert-butyl (2-aminoethyl)carbamate, DIPEA, DMF, 70 °C; f) TFA, DCM, RT; g) Acryloyl chloride or propionyl chloride, NEt₃, DMF, RT.

Biochemical Characterization of FAK Inhibitors. To establish whether our rationally designed targeted covalent inhibitors **5**, **7** and **8** could form irreversible complexes, their

inhibitory potency were first assessed using an ADP-Glo™ kinase assay. One previously reported inhibitor of FAK, TAE226, was used as a control. In this assay, TAE226 showed IC₅₀ value of 5.8 nM against the kinase activity of FAK (Table 1), which was similar to previously reported data.¹⁹

Table 1. In Vitro Enzymatic Activities of Novel 2,4-diarylamino-pyrimidines compared with TAE226



| N° | R | IC ₅₀ (nM) |
|----------|---|-----------------------|
| 2 | H | 26 ± 2.2 |
| 5 | Sq(CH ₂) ₂ NHCOCH=CH ₂ | 0.6 ± 0.04 |
| 6 | Sq(CH ₂) ₂ NHCOCH ₂ CH ₃ | 4.7 ± 0.5 |
| 7 | CH ₂ Tr(CH ₂) ₂ NH COCH=CH ₂ | 2.3 ± 0.3 |
| 8 | CH ₂ Tr(CH ₂) ₃ NH COCH=CH ₂ | 1.2 ± 0.1 |
| TAE226 | | 5.8 ± 0.6 |

Sq: squaramide group; Tr: 1,2,3-triazole group

As expected, our selected 2,4-dianilinopyrimidine scaffold (compound **2**) showed a potent inhibitory activity to FAK (IC₅₀ = 26 nM), as shown in Table 1. Interestingly, the introduction of different groups on the amine function of the benzylamino moiety (ring B) resulted in a very large increase in inhibitory potency on FAK kinase activity (compounds **5**, **6**, **7** and **8**), showing specific contributions of these groups. Compounds bearing a 1,2,3-triazole group (**7** and **8**) gave an inhibitory potency of 2.3 and 1.2 nM, respectively, which are more potent than TAE226. Interestingly, compound **5** bearing a squarate group demonstrated the best inhibitory activity (IC₅₀ = 0.6 nM), which was largely better than that of the matched pair **6**, without a reactive acryloyl group (IC₅₀ = 4.7 nM).

As irreversible inhibitors always increase inhibitory potency over time, we then investigated time-dependence inhibition of FAK kinase by the putative irreversible inhibitors **5**, **7** and **8**, as compared with the reversible matched pair **6**. As shown in Figure 2, only compound **5** displayed an increase in its inhibitory potency over 2 h, which was consistent with the formation of a covalent bond. As expected, the reversible matched pair **6** displayed

no time-dependence. Unfortunately, the putative irreversible inhibitors of FAK **7** and **8** displayed no time-dependence in this assay.

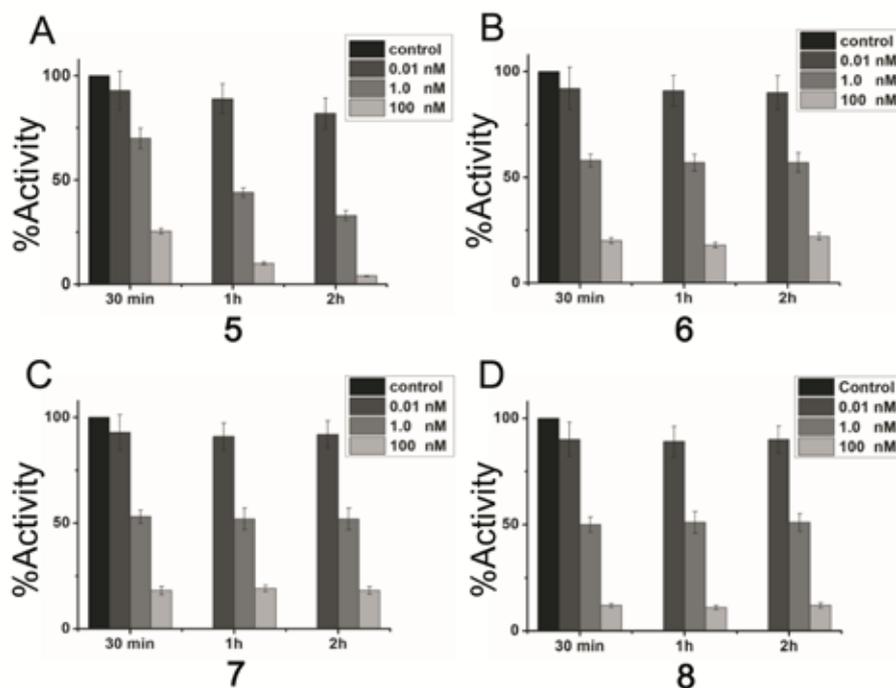


Figure 2. (A) Targeted covalent inhibitor **5** shows time-dependent inhibition of FAK kinase, (C-D) No time-dependent inhibition of FAK kinase with targeted reversible inhibitors **6**, **7**, **8**.

Since high intracellular ATP concentrations under physiological conditions influence the cellular potency of ATP-competitive inhibitors of protein kinases, non-ATP competitive inhibitors provide distinct advantages to conventional ATP-competitive binders. Thus, developing irreversible inhibitors is an attractive and alternative strategy to achieve the non-ATP competitive inhibition of kinase-mediated signaling. Therefore, we studied non-competitive character versus ATP of the putative irreversible inhibitor **5**, compared with the reversible matched pair **6**. Indeed, as shown in Figure 3, inhibitor **5** showed the same inhibition at the different concentrations of ATP. On the contrary, the inhibitory potency of the matched pair **6** on FAK kinase activity decreased with increasing concentration of ATP.

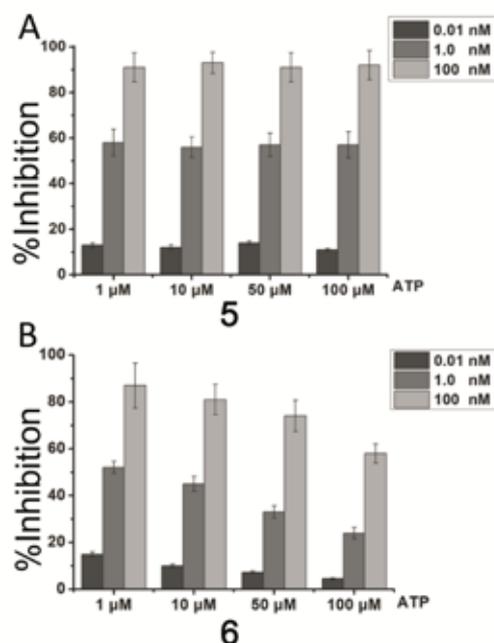


Figure 3. (A) Targeted covalent inhibitor **5** shows non-ATP dependent inhibition of FAK kinase, (B) ATP-dependent inhibition of FAK kinase with targeted reversible inhibitor **6**.

Crystal Structure of FAK Bound to Inhibitors. To confirm the formation of a covalent bond and to verify that the binding mode of the bis-anilinopyrimidine part is similar to the reversible counterparts, we resolved the cocrystal structures of FAK kinase domain successively with compounds **5** (PDB ID: 6GCX), **6** (PDB ID: 6GCW) and **7** (PDB ID: 6GCR). As shown in Figures 4, the mode of interaction of these compounds with the FAK kinase domain is very similar to that observed in complex with TAE226 or PHM16. Indeed, our inhibitors stabilize an unusual helical conformation of the DFG motif (D564-F565-G566) in which the phi torsion angle of Asp564 is rotated by 113° , compared to the active kinase domain. Compounds **5**, **6** and **7** contain a common 5-chloro-2-anilino-4-(2-methylcarbamoyl)anilinopyrimidine scaffold, which binds well to the hinge region of the FAK kinase. Three hydrogen bonds are observed in the crystal structure of FAK bound to inhibitors. Among them, two are formed between the nitrogen in the pyrimidine and 2-aniline moieties with the backbone nitrogen and the carbonyl group of Cys502 in the kinase hinge (Figure 4A-C). Another one is formed between the CO of carboxamide group of our inhibitor and the backbone nitrogen of Asp564 of the DFG motif. Some of hydrophobic interactions are observed between the carbon atoms of the 2-aniline ring and Ile428 and Gly505, but also between carbons in the pyrimidine ring and Ala452 and Leu553. As already observed in the

crystallographic structure of a complex between FAK kinase domain and TAE226, the chlorine atom on the pyrimidine ring is located into the ATP binding pocket, near the gatekeeper residue Met499 (Figure S2).

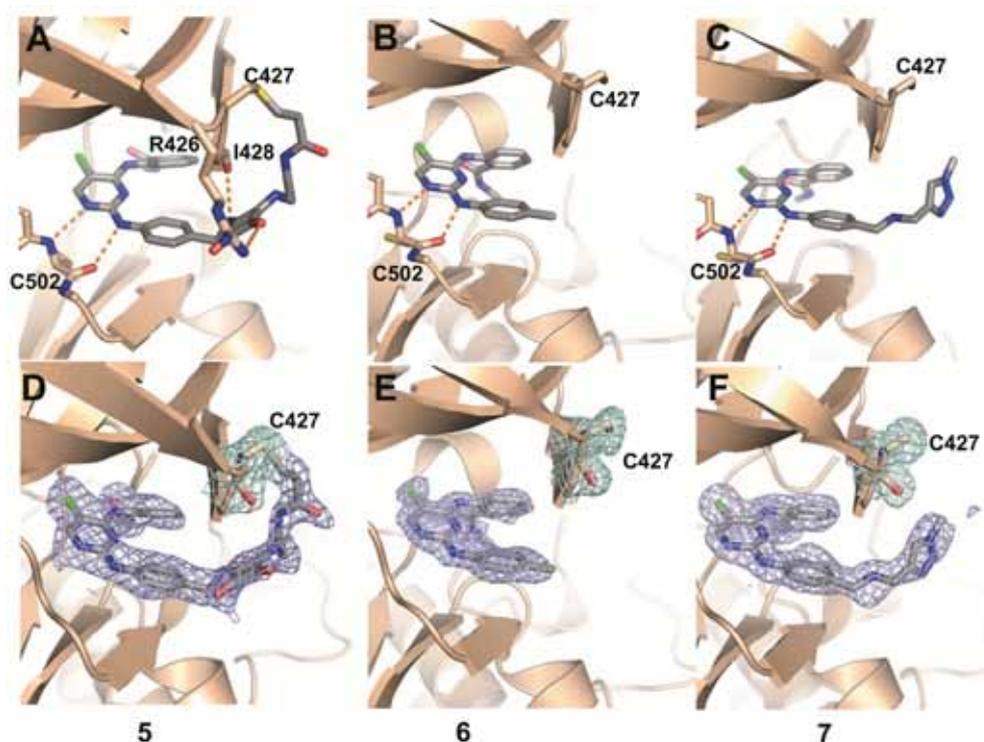


Figure 4. Crystal structures of the FAK kinase domain in complex with inhibitors **5** (PDB ID: 6GCX), **6** (PDB ID: 6GCW) and **7** (PDB ID: 6GCR). (A-C) The active site of the FAK kinase domain bound **5**, **6** and **7** are shown. Inhibitors are shown in grey for ordered regions and white for disordered regions. Although the white regions are modeled, they are not supported by electron density as can be seen in panels A-C. Key hydrogen bonds between the compounds and the FAK kinase are shown as orange dashed lines. (D-F) 2Fo-Fc electron density countered a 1σ is shown for the compounds (blue mesh) and Cys427 (cyan mesh), which are shown in stick representation. The FAK kinase is shown as ribbon in tan. Note that only compound **5** exhibits continuous density connecting to Cys427.

Interestingly, for **5**, it is observed that two hydrogen bonds are formed between the side chain nitrogen of Arg426 and the carbonyl of the squarate, and the CO of Ile428 forms a hydrogen bond with the NH of the squaramide attached to the benzylamino moiety (Figure 5). On the contrary, 1,2,3-triazole group can not make this type of the interaction (Figure 5), supporting that the binding of the squarate group to the FAK active site is crucial to guide the electrophilic group of **5** for reaction with the nucleophilic thiol group of Cys427 (Figure 4A).

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Furthermore, only the cocrystal structure with **5** revealed a strong and continuous electron density between C427 and the electrophilic carbon in **5** (Figure 4D) and thus clearly confirmed a covalent bond formation.

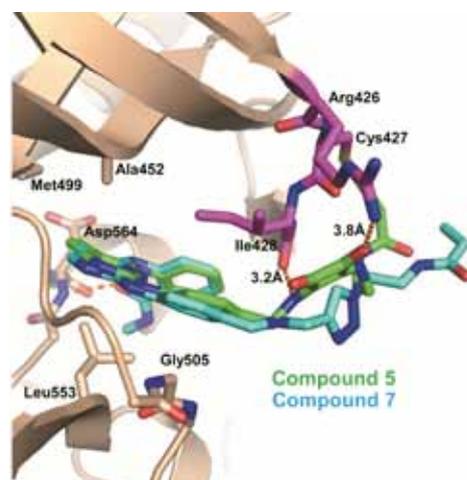


Figure 5. Superposition of the two structures of compounds **5** and **7** bound to FAK. The compounds **5** (green), **7** (blue) and side chains in FAK interacting with the compound are shown in stick representation. Hydrogen bonds are shown as orange dashed lines.

Anti-proliferation activity of compound 5 in SCC cells. Having established the potency and irreversibility of inhibitor **5** binding to the FAK active site in vitro, we investigated the inhibition of the full-length FAK kinase by **5** in squamous cell carcinoma (SCC) cells, which were induced in K14CreER FAK^{lox/lox} mice using a two stage chemical skin carcinogenesis assay as previously described.²⁰ In a previous study, we have demonstrated that PF-562,271, a reversible inhibitor of FAK kinase was able to block the 3D proliferation of the SCC cells, cause squamous cell carcinoma regression and inhibit tumor growth in vivo.²¹ The anti-proliferation of the irreversible inhibitor **5** against SCC cells was first assessed using cell nuclei counts, and VS-4718, a highly potent reversible inhibitor of FAK which is under clinical development,²² was used as a control. Treatment of SCC FAK-WT cells with **5** resulted in a dose-dependent inhibition of proliferation with similar ED₅₀ ($1.73 \pm 0.1 \mu\text{M}$) as that of VS-4718 ($1.49 \pm 0.2 \mu\text{M}$) (Figure S3).

Covalent inhibition of FAK by compound 5 in SCC cells. We tested the inhibition of FAK autophosphorylation in SCC cell line with the irreversible inhibitor **5**. As shown in Figure 6a, compound **5** blocked Tyr397 phosphorylation in a dose-dependent manner and FAK autophosphorylation was significantly inhibited at low concentrations in these cancer

cell lines, which is comparable to VS-4718. This is also consistent with its inhibitory activity against the FAK kinase, suggesting that this compound is able to effectively inhibit cellular FAK autophosphorylation and phosphorylation of kinase targets at low concentrations. Then, we examined the reversibility of the autophosphorylation of Tyr397 of FAK by performing washout experiments. Upon washout, VS-4718 lost its inhibition capacity against FAK, which is coherent with its reversible property. In contrast, **5** retained potent inhibition of FAK after washing out the inhibitor, and demonstrated covalent binding (Figure 6d).

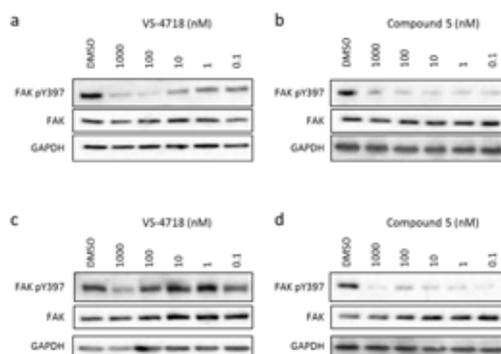


Figure 6. Inhibition of FAK autophosphorylation in SCC cells. (a-b) **5** and VS-4718 inhibited the autophosphorylation of FAK in a dose-dependent manner; (c-d) Washout experiments showed that the inhibition of FAK autophosphorylation could be reversed for the reversible inhibitor VS-4718, but not for the irreversible inhibitor **5**. The experiments were repeated three times and were consistent.

Kinase Selectivity Profile of Covalent Inhibitor 5. In order to investigate the selectivity of the irreversible inhibitor **5**, we tested this compound in vitro against a panel of ten kinases (EGFR, HER2/ERBB2, IGF1R, c-Kit, PDGFR, Pyk2, c-Src, IRR, and IR) determined by Eurofins Pharma Discovery Services, Poitiers, France to investigate its selectivity (Table S1, Supporting Information). A TR-FRET based kinase protocol was used with human recombinant protein in the presence of 1 μ M of compound **5**. Interestingly, this compound showed only 21% inhibition on the IR (insulin receptor) kinase even at the high concentration of 1 μ M. This is in contrast to TAE226, which exhibited high potency towards IR ($IC_{50} = 26$ nM).¹⁹ Even if TAE226 has displayed a potent antitumor effect both in vitro and in vivo in a large variety of cancers such as brain tumors,²³ esophageal cancer,²⁴ breast cancer,²⁵ ovarian cancer,²⁶ and gastrointestinal stroma tumor,²⁷ TAE226 never entered in clinic studies, because this compound inhibited the insulin receptor with a high potency and showed important side effects in animal studies.¹⁹

CONCLUSIONS

In summary, we described the first irreversible sub-nanomolar inhibitor of FAK containing a pyridimine scaffold and a Michael acceptor as electrophile up to date through structure-based and medicinal chemistry optimization. The crystal structure of the FAK kinase domain in complex with this inhibitor was resolved to display the formation of a covalent bond. Beyond generating and characterizing the covalent FAK inhibitor, our approach can direct the design of new electrophilic inhibitors which target a noncatalytic cysteine in proteins. The irreversible inhibitor **5** showed a highly potent decrease of autophosphorylation of FAK in SCC cells, suggesting that this compound could efficiently block a key event of the intracellular FAK signaling pathway. It is worth noting that **5** exhibited an anti-proliferative effect in SCC cells similar to that of VS-4718, which is currently under phase I studies. Moreover, this compound displayed low inhibition against the IR (insulin receptor) kinase, which might provide a new mode of action and overcome the side effects observed with TAE226. Further studies into molecular mechanisms of this type of compounds against cancer including anti-proliferation of human cancer cells and the regulation of tumorigenesis and metastatic progression is underway in the laboratory to demonstrate the advantages for the development of novel cancer therapies.

METHODS

Chemistry. Methods for the synthesis of compounds, characterization, and spectra are described in detail in the Supporting Information.

Enzyme Inhibition Studies. Inhibitory potency in a FAK enzymatic assay was determined using an ADP-Glo™ kinase assay. Briefly, 4 μL of assay mixture containing 0.5 μg FAK substrate, 5 ng FAK, 8 μM ATP and 1 μL of compounds at desired concentrations in Kinase reaction buffer (40 mM Tris, 20 mM MgCl_2 , 0.1 mg/mL BSA, 2 mM MnCl_2 , 2 μM DTT) was added into a 384-well plate. After incubation at 37 °C for 1 h, the kinase reaction was stopped by the addition of 5 μL of ADP-Glo™ Reagent to deplete the unconsumed ATP and leave only ADP. The plate was incubated at room temperature for 40 min, and added by 10 μL of Kinase Detection Reagent to convert ADP to ATP and introduce luciferase and luciferin to detect ATP. After incubation at room temperature for 15–20 minutes, the luminescence signal was detected with an Enspire plate reader. The IC_{50} value was determined for each compound, from a sigmoid dose–response curve using Graph-Pad Prism (GraphPad Software, San Diego, CA, USA).

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3 **Inhibition of FAK Autophosphorylation in SCC cells.** SCC FAK-WT cells were
4 cultured in Glasgow Minimum Essential Medium (10% foetal calf serum, 2 mM l-glutamine,
5 NEAA, sodium pyruvate and MEM vitamins). Cells were seeded in 6 well plates at 100,000
6 cells per well. Compounds were made up in DMSO to 1000x final concentration and 24 hours
7 after cell seeding were added to the culture media resulting in a final DMSO concentration of
8 0.1%. Compounds were incubated on cells for 48h before cell lysis. For wash out
9 experiments, cells were washed with PBS twice and fresh media was added for 3 hours.

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16 For cell lysis, briefly, wells were washed in ice-cold PBS, drained and cells lysed in MD
17 Anderson buffer (1% Triton X 100, 50 mM Hepes pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂
18 and 1 mM EGTA) supplemented with a protease and phosphatase tablet (Sigma-Aldrich). Cell
19 lysates were clarified by centrifugation at 12,000x g for 10 minutes and the protein
20 concentration determined by Bradford assay (Thermo Fisher Scientific). 20 µg protein of each
21 lysate was resolved using 4–15% polyacrylamide gels (Bio-Rad) and transferred to PVDF
22 membranes, blocked and probed with primary antibodies overnight. Blots were washed in
23 TBS/0.1% Tween and incubated with anti-rabbit conjugated horseradish peroxidase. Blots
24 were washed in TBS/0.1% Tween and visualised with BM Chemiluminescence Western
25 Blotting Substrate (Sigma-Aldrich) on a Bio-Rad ChemiDoc imager. Blot densitometry was
26 determined using Image Lab software (Bio-Rad).

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36 **Crystallization and Structure Determination.** The kinase domain of the avian FAK
37 protein (FAK411–686) was expressed and purified following a previously described assay.²⁸
38 The inhibitor-FAK complex was formed by incubation of the compound (final concentration
39 10 mM) with the protein (8 mg/mL) at 4 °C overnight. Crystals of the complex were grown,
40 mixing an equal volume of the complex with the crystallization condition (100 mM Tris, pH
41 8.5, 100 mM Li₂SO₄, 20–24% PEG4000, 10 mM TCEP). Crystals were cryoprotected by a
42 quick soak into the following cryosolution 100 mM Tris pH 8.5, 100 mM Li₂SO₄, 24%
43 PEG4000, 10 mM TCEP, 35% ethylene glycol and then flash-frozen. Diffraction data were
44 collected at synchrotron facilities in beamline ID30-1 at ESRF (Grenoble, France)
45 (compounds **5**, **7**) or the XALOC beamline at ALBA (compound **6**) and processed with
46 XDS.²⁹ To provide an initial set of phases, the molecular replacement protocol in Phaser was
47 used using the FAK kinase model from PDB 2JKK.³⁰ Refinement was carried out using the
48 program Refmac and manual rebuilding was performed with Coot.^{31–32} The Dundee
49 PRODRG2 Server was used to create the initial model for inhibitors **5**, **7** and **10**.³³ Final R-
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3 factors are 22.3/26.2 (Rwork/Rfree) for FAK/**5**, 19.5/22.3 for FAK/**6** and 21.7/27.4 for
4 FAK/**7**.
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6 7 **ASSOCIATED CONTENT**

8 9 Supporting Information

10 The Supporting Information is available free of charge on the ACS Publications website at
11 DOI:
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13 Additional figures, experimental methods and analyses (PDF)
14

15 16 17 **Accession Codes**

18 The structures of FAK domain bound to compounds **5**, **6** and **7** have been deposited in
19 the Protein Data Bank with PDB accession codes 6GCX, 6GCW and 6GCR, respectively.
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22 23 24 **AUTHOR INFORMATION**

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29 30 Author Contributions

31 ‡These authors contributed equally.
32

33 34 **Notes**

35 The authors declare no competing financial interest.
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37

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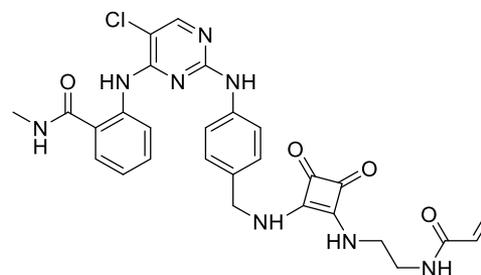
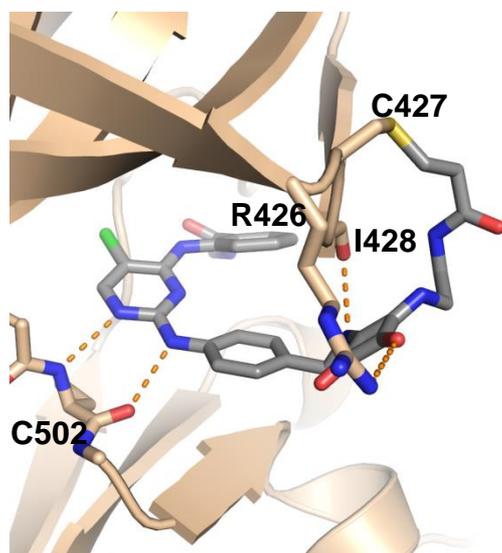
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3 **Structure-based design, synthesis and characterization of the first**
4 **irreversible inhibitor of Focal Adhesion Kinase**
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7 Exp ́dite Yen-Pon, Bo Li, Marta Acebr ́n-Garcia de Eulate, C ́dine Tomkiewicz-
8 Raullet, John Dawson, Daniel Lietha, Margaret C. Frame, Xavier Coumoul,
9 Christiane Garbay, Melanie Etheve-Quelquejeu, Huixiong Chen
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FAK: IC₅₀ = 0.6 ± 0.04 nM