Discovery of Indoline-Based, Natural-Product-like Compounds as Probes of Focal Adhesion Kinase Signaling Pathways

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With the goal of identifying small molecule modulators of protein−protein interactions, we developed a solid-phase synthesis method, which was then successfully utilized in a library generation of 164 aminoindoline-derived, natural-product-like compounds. This library and several other related intermediates synthesized during this project were then subjected to different biological assays in search of small molecule modulators of focal adhesion kinase (FAK)-mediated signaling pathways. This study included (i) an in vitro, full length FAK inhibition assay, (ii) a cell proliferation assay, and (iii) a wound healing assay. In FAK inhibition assay, eight library members (**5**−**12**) and three aminoindoline derivatives (**13**, **14**, and **2**) were identified as promising candidates. Compounds **13** and **2** inhibited the FAK activity by 25−45% at 10 μM. These two lead compounds also showed activity in a wound healing assay. To our knowledge, these aminoindoline-derived small molecules belong to a new family of FAK inhibitors.

Introduction

Because of the growing desire to dissect signaling pathways using small molecules, the need for the chemical probes that achieve these ends is also emerging rapidly.[1−5] Inspired by bioactive natural products that already have an established track record in this arena, the quest for developing solution- and solid-phase methods leading to high-throughput generation of natural product-inspired chemical entities has also grown.[6−8]

With the objective of populating this chemical space, we launched a program few years ago that aims to develop solution- and solid-phase high-throughput synthesis methods leading to the library generation of alkaloid natural product-inspired compounds. In particular, we focused our attention on developing this program around two scaffolds[9−12] (i.e., indoline and tetrahydroquinoline) that are commonly found in a wide variety of bioactive alkaloid natural products. A wide abundance of the indoline substructure in alkaloid natural product family was the driving force for us to initiate this library generation program and to explore the chemical space around this moiety. Using the aminoindoline scaffold (see **1**, Scheme 1), we report herein our study that led to the library generation and the identification of several library members as well as other related compounds as modulators of focal adhesion kinase (FAK) signaling pathways.

Results and Discussion

To develop the solid-phase synthesis and the subsequent library generation plans on scaffold **4**, compound **2** was needed as the starting material. As shown in **4**, there are three potential diversity sites that would allow us to explore the chemical space around the aminoindoline scaffold. The synthesis of aminoindoline derivative **1** was reported by us earlier,[9] and this could easily be converted to compound **2** in a few simple transformations. The sequence for producing **2** from compound **1** included (i) −OBz hydrolysis, (ii) oxidation, (iii) allylation (to prodcue 1:1 diastereomeric mixure), (iv) −OMEM removal, (v) alkylation/protection, (vi) *N*-Teoc removal, (vii) *N*-Fmoc, and (viii) −OTHP removal. With the use of the Broad Institute's alkylsilyl polystyrene macrobeads (loading 1.0125 mmol/g) and the solid-phase synthesis loading protocol developed earlier, compound **2** could be successfully loaded onto the resin (89% loading determined after cleavage from the solid support), producing derivative **3**.

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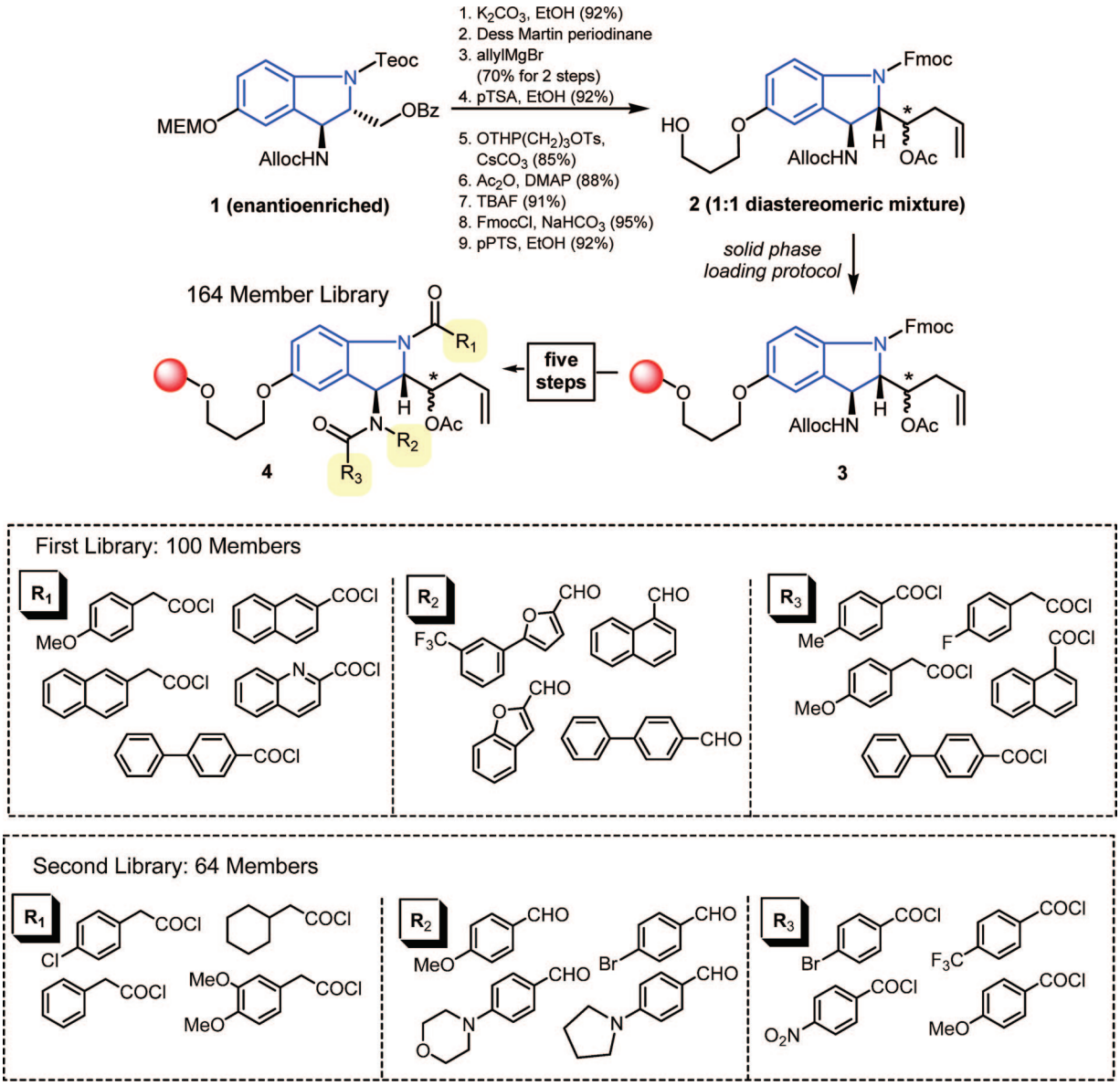
Scheme 1. Solid-Phase Synthesis Method for the Library Generation[a]



[a] The details are provided in the Experimental Section.

In a five-step sequence on solid phase, the final product **4** could be easily obtained from **3**. These steps included (i) *N*-Fmoc removal, (ii) *N*H-amidation (first diversity, R_1), (iii) *N*-Alloc removal, (iv) *N*-alkylation by reductive amination (second diversity, R_2), and (v) *N*-(R_2)-amidation (third diversity, R_3). Following the successful solid-phase synthesis of **4**, the use of the IRORI split-and-mix-type technology was then applied for generating two libraries with a total of 164 compounds. The diversity element in these libraries was selected to explore the identification of small molecule modulators of several protein−protein interactions-based signaling pathways. For example, with the objective of mapping the extended, shallow hydrophobic domains of protein surfaces, a wide variety of aromatic hydrophobic groups were chosen in the library planning for the choice of the diversity groups.

The complete analytical information on the library members (i.e., HPLC-MS) is provided in the Supporting Information. (Note: The HPLC yield of the products ranges from approx 50−96% and in most cases it is higher than >65−70%.) In one study, small molecules obtained from these libraries and several aminoindoline-derived intermediates (180 compounds in total) were then subjected to biological assays in search of chemical modulators of focal adhesion kinase (FAK) signaling. FAK belongs to a family of nonreceptor tyrosine kinase, and it plays a role in signal transduction pathways associated with integrin-mediated cell adhesions.[13,14] FAK plays an important role in cancer progression through the involvement in cellular processes including cell survival, cell migration, and cell invasion.[14−17] A wide variety of cancer cells and tissues have an overexpression of FAK, supporting the role of FAK in human tumorigenesis and metastasis.[16] Because of these biological implications, there is a growing interest in finding small molecule modulators of FAK that could further be utilized as chemical tools to enhance our current understanding in this complex area.[18−21] Another advantage in finding these functional tools is that they could also pave the way in developing new therapeutic agents to function as anticancer compounds.

In this study, human recombinant full-length FAK was incubated in kinase buffer containing ATP and the substrate with or without the presence of the indoline-derived compounds at 10 μM final concentration. The detailed information on all the biological assays

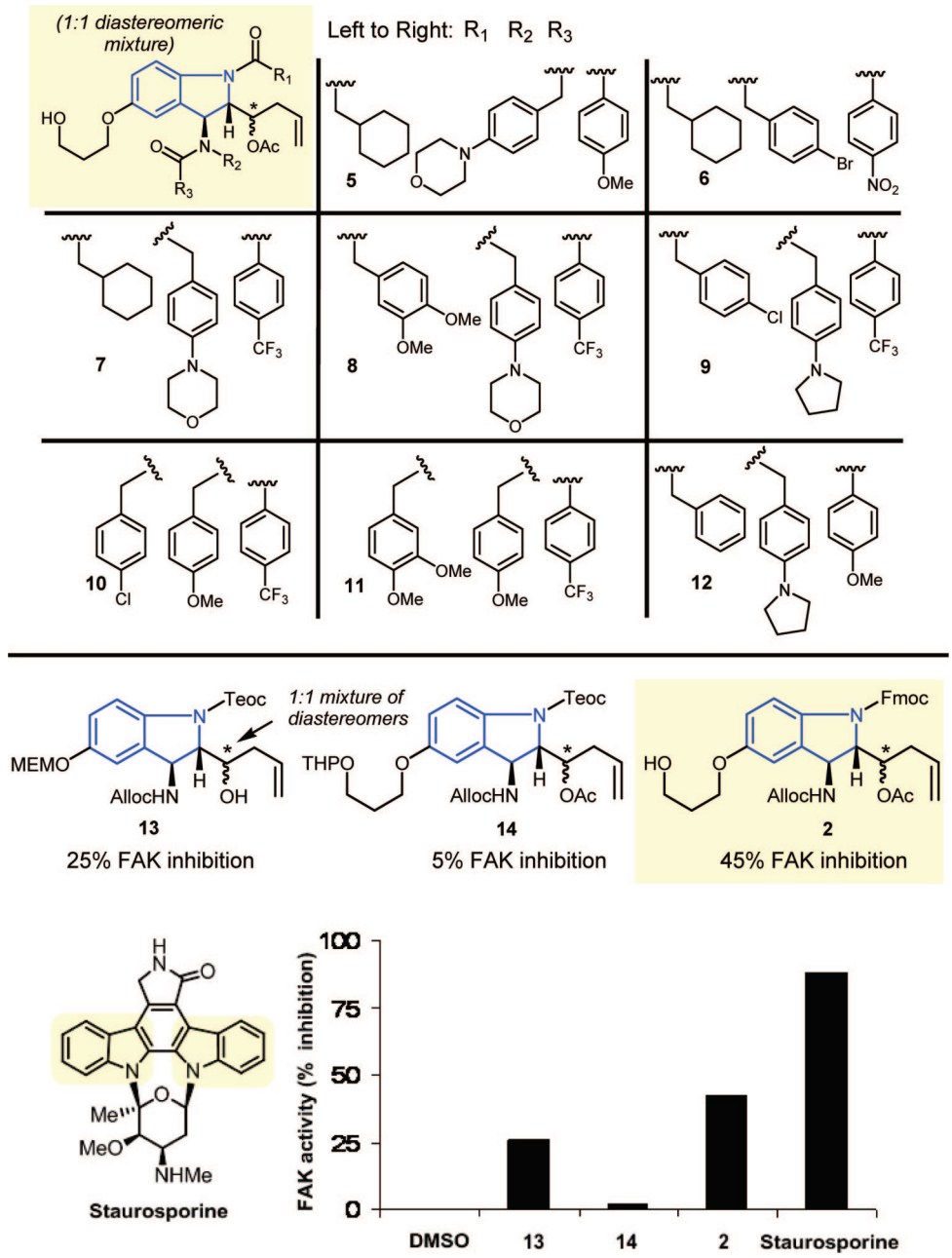


Figure 1. Eight library members (**5**−**12**) and three aminoindoline derivatives (**13**, **14**, and **2**) were identified as FAK inhibitors. The library members **5**−**12** inhibited the FAK activity by 10−25% at 10 μM. The aminoindoline derivative, **2**, showed the FAK inhibition by 45% at 10 μM.

is provided in the supporting material. This study led to the discovery of several library members (Figure 1, **5**−**12**) and three aminoindoline derivatives (**13**, **14**, and **2**) (these three compounds were synthesized as ~1:1 mixture of diastereomers that was not possible to separate by flash chromatography) as novel inhibitors of the FAK activity. The library members **5**−**12** were identified as weak inhibitors (i.e., 10−25% inhibition of the FAK activity at 10 μM). It was interesting to discover that compounds **13** and **2** inhibited the FAK activity by 25% and 45% at 10 μM, respectively (see Figure 1; natural product staurosporine, a known kinase inhibitor, was used as a control).

Indoline derivatives **13**, **14**, and **2** were also tested in cell proliferation assay and showed the inhibitory activity (IC_{50}), that is, 24 μM (**13**), >100 μM (**14**), and 16 μM (**2**) (see Figure 2A). MDA231-M cells were pretreated with these compounds at 25 μM for 1 h, and that led to a significant decrease in phosphorylated tyrosine 397-FAK as compared to control (see Figure 2B). Finally, these compounds were also tested to explore their properties in cell scratch motility assay as described earlier[22] and were found to be active at 25 μM (see Figure 3). The cause of this effect is not clear at this stage. Further work would be needed to provide a better explanation if this is caused by the involvement of FAK in cell migration or compound **2** is working through a different mechanism.

To obtain a better understanding of the interaction of **2**, both isomers (*R*)-**2** and (*S*)-**2** were docked to the crystal structure of human FAK cocrystallized with a sub-micromolar pyrrolopyrimidine inhibitor (PDB code 2ETM). Ligprep (Schrödinger, NY) was first used to generate both isomers of compound **2**. Glide (Schrödinger, NY) was then used to dock the two isomers and to identify the best docking pose, obtained with (*R*)-**2** (see Figure 4).[23] Since the allyl

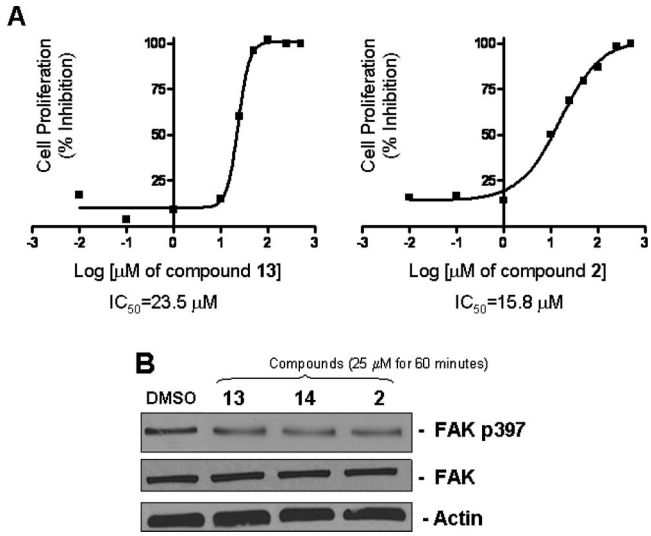


Figure 2. Effect of compounds **2**, **13**, and **14** on cell proliferation (A) and FAK kinase activity (B). (A) MTT assay of MDA231-M cells treated with increasing concentration of compound **13** (IC_{50} = 23.5 μM) and compound **2** (IC_{50} = 15.8 μM). (B) Expression level of pY397-FAK (P-FAK), FAK, and Actin in MDA231-M cells pretreated with or without compounds **2**, **13**, and **14** (25 μM, 1 h) examined by Western blot.

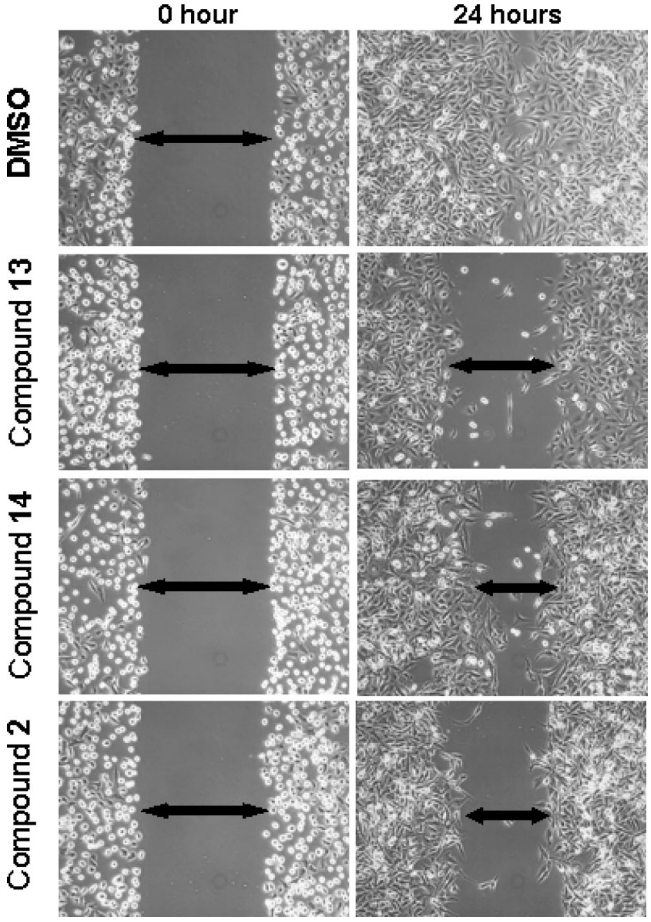


Figure 3. Wound healing assay using MDA231-M cells on treatment with or without the aminoindoline derivatives **13**, **14**, and **2** at 25 μM.

group was predicted to point toward the outside of the binding pocket, both isomers appear to fit into the pocket according to the docking results. The energy of the system was further minimized with flexible receptor side-chains with ICM (Molsoft LLC, CA).[24] Because all FAK ligands cocrystallized to date form a hydrogen bond with the backbone nitrogen of Cys502, this interaction was imposed during docking. A pose was identified where (*R*)-**2** makes a hydrogen bond with the backbone nitrogen of Cys502 and hydrogen bonds with the side-chains of Arg550 and Ser568. The fluorene moiety of (*R*)-**2** is buried in a hydrophobic side-cavity of FAK next to the activation loop.

Summary

To summarize, we report a library generation of 164 compounds that were obtained from an aminoindoline scaffold and the discovery of three compounds with a promising response to FAK inhibition. These aminoindoline derivatives belong to a novel class of FAK inhibitors and could serve as useful chemical probes to enhance our current understanding of FAK-mediated signaling pathways. Further, work is warranted to probe their mechanism of action in this area and to explore their applications in developing a new family of anticancer agents.

Experimental Section

All reactions were carried out in flame-dried glassware under an atmosphere of nitrogen with magnetic stirring. Thin-layer chromatography (TLC) was done on EMD (art. 5715-7) precoated silica gel 60 F_{254} glass plates (layer thickness 0.25 mm). Visualization was affected with a UV lamp (254 nm) or by staining with ammonium molybdate/ceric sulfate solution. Flash column chromatography was performed using silica gel 60 (40−63 μm, Silicycle) or Biotage Horizon Flash Chromatography System. Solvents were purified as follows: Trace amounts of water and oxygen from THF, DMF, and dichloromethane were removed using columns containing activated alumina and copper under N_2. Triethylamine, pyridine, ethyl ether, and toluene were obtained from commercial suppliers (EMD and Aldrich) and used without further purification. NMR spectra were recorded on a Bruker DRX 400 MHz spectrometer. All chemical shifts are reported in parts per million (δ). ^{1}H NMR (400 MHz) spectra were recorded at room temperature in $CDCl_3$ or C_6D_6 solutions and referenced to residual $CHCl_3$ (7.27 ppm) or C_6H_6 (7.16 ppm). Fully decoupled ^{13}C NMR (100 MHz) spectra were recorded in $CDCl_3$ or C_6D_6 solutions. The center peaks of $CDCl_3$ (77.0 ppm) and C_6D_6 (128.7 ppm) were used as the internal reference. Mass spectra were carried out on a VG Quattro I (Micromass) mass spectrometer equipped with a pneumatically assisted electrospray ionization source, operating in positive mode. HPLC were performed using a Hewlett-Packard (Agilent) 1100 Series equipped with a diode array detector and a NovaPack C18 (3.9 × 300 mm) column. The enantiomeric excess was determined by chiral HPLC, using a Hewlett-Packard (Agilent) 1090 Series II Liquid Chromatograph equipped with a diode array detector and a CHIRACEL-OD column. HPLC/MS were performed using Waters equipment: Waters micromass ZQ ESCI Multi-Mode ionization, Waters 996 Photodiode Array Detector (254 nm), and Waters 2795 Separation Module with Phenomenex Spherisorb 3 ODS-2 column. Small-scale solid phase reactions (1−50 mg resin) were performed in 2 mL fritted polypropylene Bio-Spin chromatography columns. Medium-scale solid phase reactions (20−200

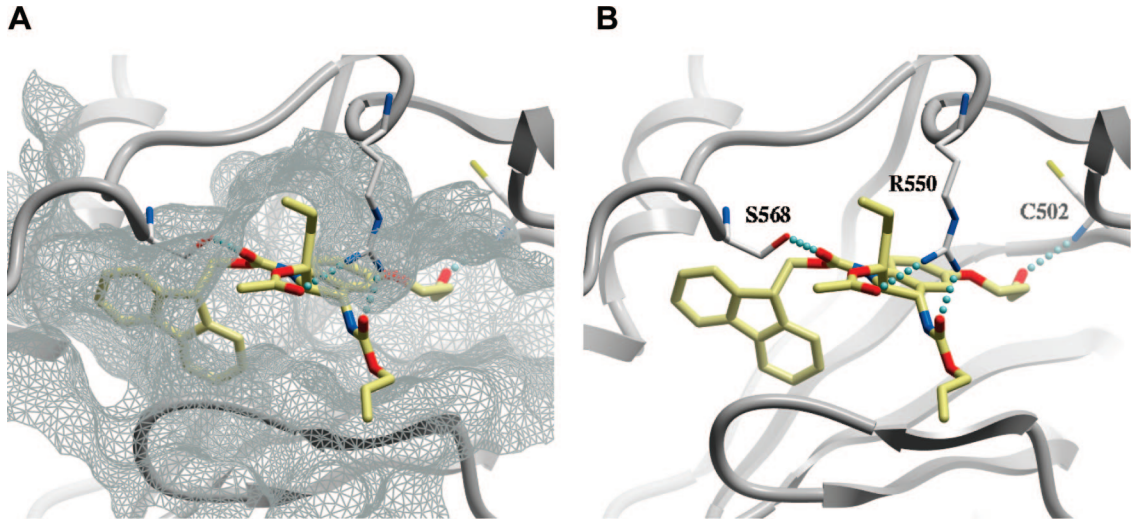
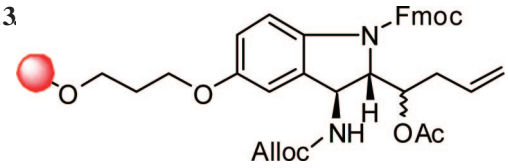


Figure 4. Docked conformation of (*R*)-**2** complexed to the ATP site of FAK. (*R*)-**2** is deeply buried in the pocket at the interface of the FAK N- and C-terminal lobes (A) and makes hydrogen bonds with the backbone of Cys502 as well as the side-chains of Arg550 and Ser568, while the fluorene ring system makes extensive hydrophobic interactions (B).

mg) were performed in 10 mL polypropylene PD-10 columns. Agitation of solid phase reactions was performed using a Barnstead-Thermolyne Labquake shaker. The linker cleavage reactions (<50 mg of beads) were carried out in 1.5 mL eppendorf tubes. Vacuum removal of solvents for the linker cleavage reactions was accomplished using Genevac HT-4 Atlas Evaporator.

The detailed synthesis procedure and the analytical information on compounds, **1a**, **13**, **13a**, **1c**, **1d**, **14**, **1e**, **1f**, and **2** are provided in the earlier published research article (see, ref 10).

Compound 3



3-[Diisopropyl (*p*-methoxyphenyl) silyl] propyl functionalized resin (750 mg, 1.0125 mmol/g of silane, ICCB batch MX-19) were swollen in CH_2Cl_2 (9.0 mL) under N_2 for 30 min. The solvent was then drained under positive N_2 pressure. A solution of trifluoromethanesulfonic acid in CH_2Cl_2 (0.45M, 13.5 mL, 6.06 mmol) was added by syringe. The resin was then left to sit for 20 min under N_2. The acid solvent was drained under positive N_2 pressure, and the resin was washed with CH_2Cl_2 (9.0 mL). The activated resin was treated with 2,6-lutidine (0.924 mL, 8.08 mmol) for 30 min, followed by addition of a solution of compound **2** (1.267 g, 2.025 mmol) in CH_2Cl_2 (4.0 mL). The resin was gently shaken overnight. The resin was washed with CH_2Cl_2 (3×), THF (3×), and CH_2Cl_2 (3×). The beads were then dried on the lyophilizer overnight to give the loaded beads **3** (1.132 g, 89% loading). (Loading was performed in 3 different batches using 250 mg of resin).

Cleavage. The loaded resin (20 mg) in Eppendorf tube was swelled in THF (0.5 mL) for 30 min and treated with HF/pyridine solution (15.0 μL). The reaction tube was shaken for 2 h. Methoxytrimethylsilane (150 μL) was added, and the tube was shaken for another 30 min. The solution was removed and the resin was washed with THF. All solvents were combined and concentrated. The crude sample was purified by column chromatography (1:1 ethyl acetate/hexanes) to give the product **2** (8.5 mg, 89% loading).

Library Generation (100-Member Library). Fmoc Removal. The beads (1.62 g, 1.232 mmol) were swelled in DMF (150 mL) for 30 min. The solvent was then drained. A solution of 20% piperidine in DMF (200 mL) was added to the beads. The beads were shaken for 2.5 h. The resin was washed with CH_2Cl_2 (3×), THF (3×), CH_2Cl_2 (3×), and then they were dried on the lyophilizer overnight.

Acid Chloride Coupling (1st Diversity). The beads from above were split into 5 parts (each part contains 20 MicroKans, 0.3 g, 0.246 mmol), each of which was mixed with dry CH_2Cl_2 (30 mL), collidine (200 μL, 1.476 mmol), and 5 different acid chlorides (0.739 mmol). The mixtures were shaken independently at room temperature for 36 h. Each reaction mixture was washed with CH_2Cl_2 (3×), THF (3×) CH_2Cl_2 (3×), and the beads were then dried on the lyophilizer overnight.

Alloc Removal. The 100 MicroKans were mixed with *N*-methylmorpholine (20 mL) and acetic acid (9.6 mL) in CH_2Cl_2 (150 mL). Tetrakis(triphenylphosphine) pallidum(0) (3.77 g, 3.27 mmol), followed by triphenylphosphine (4.20 g, 15.68 mmol), was added, and the mixtures were wrapped with aluminum foil and were allowed to stir gently for 24 h. After filtration, the MicroKans were washed with CH_2Cl_2 (3×), THF (3×), CH_2Cl_2 (3×) and then dried under high vacuum overnight.

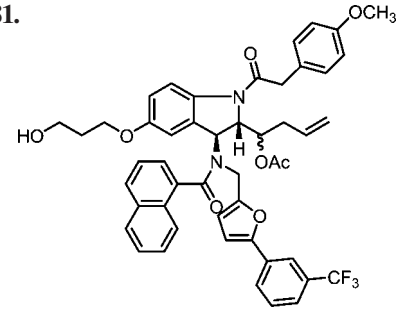
Reductive Amination (2nd Diversity). The beads resulted from the above were split into 4 parts (each part contains 25 MicroKans, 0.405 g, 0.308 mmol) Each of these 4 parts were swelled in trimethylorthoformate (30 mL), mixed with $NaCNBH_4$ (50 mg, 0.77 mmol), a stock solution (trimethylorthoformate/MeOH/AcOH, 5.0 mL/1.0 mL/90 μL), and one of the 4 corresponding aldehyde (0.77 mmol). The mixtures were shaken independently at room temperature for 24 h. Each reaction mixture was washed with THF (3×), CH_2Cl_2 (3×), and THF (3×), and the beads were then dried on the lyophilizer overnight.

Acid Chloride Coupling (3rd Diversity). The beads from above were split into 5 parts (each part contains 20 MicroKans, 0.3 g, 0.246 mmol), each of which was mixed with dry CH_2Cl_2 (35 mL), collidine (325 μL, 1.476 mmol), and 5 different acid chlorides (1.230 mmol). The mixtures were shaken independently at room temperature for 40 h. Each reaction mixture was washed with CH_2Cl_2 (3×), THF (3×), and CH_2Cl_2 (3×), and the beads were then dried on the lyophilizer overnight.

Cleavage. The beads in each MicroKan were transferred into Eppendorf tubes, were swelled in THF (0.5 mL) for 30 min, and were treated with HF/pyridine solution (15.0 μL). The reaction tube was shaken for 2 h. Methoxytrimethylsilane (150 μL) was added, and the tube was shaken for another 30 min. The solution was removed, and the resin was washed with THF. All solvents were combined and concentrated. All the library members were analyzed by HPLC and MS.

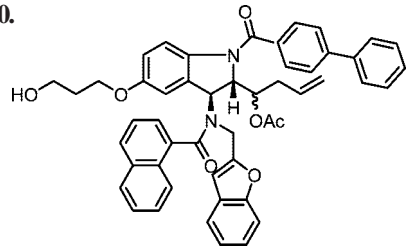
Second 64-Member Library. The second 64-member Library generation was undertaken using the same procedure as described above.

Compound RD-380-81.



^{1}H NMR (400 MHz, DMSO-d_6, 120 °C): δ 8.07−7.93 (m, 2H), 7.93−7.81 (m, 1H), 7.81−7.68 (m, 2H), 7.67−7.45 (m, 6H), 7.28−7.20 (m, 2H), 7.20−7.10 (m, 3H), 6.94−6.73 (m, 4H), 6.73−6.59 (m, 1H), 6.03−5.48 (m, 2H), 5.48−5.18 (m, 1H), 5.18−4.65 (m, 3H), 4.35−4.03 (m, 1H), 4.01−3.81 (m, 3H), 3.81−3.75 (m, 1H), 3.70 (s, 2H), 3.67 (s, 1H), 3.57−3.44 (m, 2H), 2.32 (s, 3H), 2.25−2.09 (m, 1H), 2.00−1.84 (m, 1H), 1.84−1.68 (m, 2H). ^{13}C NMR (100 Mz, DMSO-d_6, 100 °C): δ 170.0, 169.8, 169.2, 159.2, 156.7, 156.5, 138.2, 134.1, 131.1, 131.0, 130.6, 130.1, 129.6, 129.3, 128.9, 127.9, 127.7, 127.6, 127.2, 126.0, 125.8, 124.4, 114.9, 114.8, 109.0, 73.0, 67.7, 67.1, 66.6, 66.5, 58.4, 56.0, 33.1, 21.7, 21.1, 20.5. MS (ES$^+$): *m*/*z* 847.4 (M + 1).

Compound RD-380-90.



^{1}H NMR (400 MHz, DMSO-d_6, 120 °C): δ 8.04−7.95 (m, 2H), 7.94−7.89 (m, 1H), 7.72−7.62 (m, 5H), 7.60−7.54 (m, 2H), 7.51−7.48 (m, 2H), 7.47−7.34 (m, 4H), 7.28−7.22 (m, 3H), 7.20−7.11 (m, 3H), 6.99−6.83 (m, 1H), 6.73−6.66 (m, 1H), 6.50−6.28 (m, 1H), 6.05−5.71 (m, 1H), 5.65−5.24 (m, 2H), 5.05−4.80 (m, 2H), 4.73−4.44 (m, 2H), 3.91 (t, J = 6.28 Hz, 2H), 3.57 (t, J = 6.33 Hz, 2H), 2.53−2.48 (m, 2H, merged in DMSO peak, evidence from Tocsy), 2.32 (s, 3H), 1.87−1.77 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6, 100 °C): δ 169.5, 166.4, 155.2, 153.5, 141.3, 138.8, 134.9, 132.9, 132.7, 128.7, 128.4, 128.2, 127.8, 127.5, 127.3, 126.7, 126.2, 125.9, 124.6, 124.5, 124.0, 123.7, 123.6, 122.2, 120.3, 117.0, 115.5, 110.6, 110.3, 104.2, 72.3, 66.7, 65.2, 57.1, 31.7, 20.3, 19.1. MS (ES$^+$): *m*/*z* 785.4 (M + 1).

In Vitro Kinase Assay. Aminoindoline-derived library and several related compounds were tested in a search for small molecule inhibitors of FAK. In a typical study, human recombinant full-length FAK was incubated in kinase buffer containing ATP and the substrate for 4 h at room temperature with or without the presence of the aminoindoline-derived compounds at 10 μM final concentration. The remaining ATP in solution was then quantified utilizing the Kinase-Glo-luminescence kit (Promega). This study led to the discovery several aminoindoline-derived compounds.

Cell Proliferation Assay. The exponentially growing cells (1×10^3 cells) were seeded in 96-well plates, and 18 h later, they were then continuously treated with compounds **13**, **14**, and **2** dissolved in DMSO. The final concentration of DMSO was less than 0.05%. Following this, after 96 h, the cell survival was evaluated using 3-(4,5-dimethylthiazo-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) metabolic assay as described in the literature.[25] The IC_{50} for the cell proliferation assay was then determined: 23.5 μM (**13**), >100 μM (**14**), and 15.8 μM (**2**).

Cell Motility Assay. The cell motility was investigated using the scratch motility assay as described earlier.[22] In this study, cells were grown on sterile coverslips for 24 h and were then wounded by cell scraping using a micropipette tip. Following the culture washing, the cells were incubated at 37 °C in the presence of compounds **13**, **14**, and **2** (25 μM) for the reported time period to allow the migration toward the gaps, and the wound healing areas were examined under the microscope.

In Silico Procedure and Tools. Glide (Schrödinger, NY) was first used to identify the best docking pose for compound **2** in the crystal structure of the active conformation of chicken FAK (PDB code 2J0L).[23] This docking pose was then used as a starting point for flexible docking of compound **2** to the crystal structure of human FAK cocrystallized with a sub-micromolar pyrrolopyrimidine inhibitor (PDB code 2ETM). The energy of the system was minimized with flexible receptor side-chains using ICM (Molsoft LLC, CA).[24] Because all FAK ligands cocrystallized to date form a hydrogen bond with the backbone nitrogen of Cys502, this interaction was imposed during docking.

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Supporting Information Available. Full characterization of compounds and additional information. This material is available free of charge via the Internet at http://pubs.acs.org.

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