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Building skeletally diverse architectures on the Indoline Scaffold: The discovery of a chemical probe of focal adhesion kinase signaling networks

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A B S T R A C T

Inspired by bioactive indoline alkaloid natural products, here, we report a divergent synthesis approach that led to skeletally diverse indoline alkaloid-inspired compounds. The natural product-inspired compounds obtained were then subjected to a series of in vitro and cellular assays to examine their properties as modulators of focal adhesion kinase (FAK) activity. This study resulted in the identification of a promising lead inhibitor of FAK (**42**), which also showed activity in a wound healing and cell invasion assay. The in silico study of the lead compound (**42**) was also undertaken.

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1. Introduction

The post-genomics chemical biology age has brought challenges to the biomedical research community trying to develop a better understanding of protein–protein interaction-based signaling networks.[1–7] This has resulted in a growing use of small molecules as chemical probes for understanding these networks.[8–10] There are several advantages in the use of small molecules. These advantages include the ability of the small molecules (i) to interact with the biological targets in a reversible manner, (ii) to selectively modulate only one of the multiple interactions being made by the biological target, and (iii) to interfere with the cellular machinery in a non-destructive manner. Another advantage is that, in addition to developing useful chemical probes for understanding cellular machinery, the study of these probes also has the potential, in certain cases, to develop into new therapeutic approaches in drug discovery and the probes could function as starting candidates for drug design.

2. Results and discussion

2.1. Synthesis of a diverse set of indoline-derived compounds

Several indole and indoline alkaloid natural products are known to interfere with protein surfaces involved in protein–protein interactions.[11] Some of the bioactive alkaloid natural products (e.g., staurosporine, **1**, vindoline, **2**, and vinblastine, **3**) are shown in Figure 1. As a result, we were interested in synthesizing alkaloid-inspired compounds having the indoline scaffold containing an additional medium size cyclic ring to generate skeletally diverse compounds.[12,13] Indoline-based compounds are quite abundant in nature and are considered highly privileged building blocks in medicinal chemistry as well.[14] Since natural products containing

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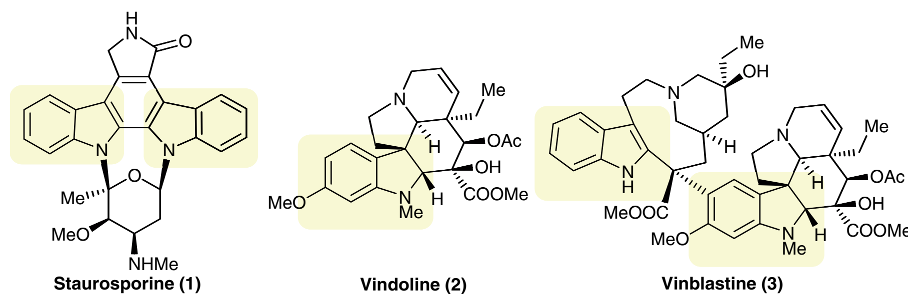


Figure 1. Indole and indoline alkaloid natural products as modulators of protein–protein interactions.

this scaffold have led to interesting biological properties, this warranted further structural modifications to its backbone.

In our synthetic planning to generate skeletally diverse indoline-based tricyclic compounds, the objective was to synthesize generic compounds **5**–**9** from a common functionalized intermediate such as **4** as shown in Figure 2. Compound **4** as a bicyclic scaffold possesses the phenolic hydroxyl group and an amino alcohol functionality. The primary hydroxyl group on **4** could be converted to the corresponding aldehyde and homoaldehyde derivatives and be further subjected to allylation and vinylation reactions, producing allylic and vinylic derivatives. Through developing the ring closing metathesis approaches, the indoline scaffold could then lead to the formation of tricyclic compounds **5–9**. The RCM reaction[15–17] has been extensively utilized in formation of enone/enamide moieties in solution phase and several examples in the literature further validated our approach. Alternatively, **9** could be derived from the regioselective opening of an epoxide in **8** to form the enamide moiety. As a result, an attractive feature to this strategy is that indoline-based tricyclic derivatives **5, 6, 7**, and **9** all contain the enamide moiety and could be further subjected to stereocontrolled diversity-based reactions. In addition to the amino alcohol moiety, the phenolic hydroxyl group on indoline scaffold, **4**, could act as an anchoring site onto solid support in solid phase reactions to generate indoline-based library members. In a skeletal diversity-based synthesis approach, the formation of indoline-based tricyclic derivatives would provide a novel entry to a wide variety of complex, natural product-inspired polycyclic compounds on solid support.

Central to this idea is the development of an efficient solution method to obtain an indolinol derivative (**12**, Scheme 1). This derivative is a versatile building block and can be utilized in the synthesis of indoline-based, natural product-inspired polycyclic derivatives. This building block has three orthogonal functional groups including a phenolic hydroxyl moiety that is protected as –OMEM. Upon deprotection, this functional group could serve as an anchoring site in solid phase synthesis. These derivatives are easy to synthesize on a large scale (i.e., 10–20 g) and the synthesis was carried out as follows. The -OMEM ether of 3-(*C*-allyl)-4-nitrophenol **10c** (see the Supporting Material) was synthesized from 2-nitro-5-hydroxybenzaldehyde in four steps. The allyl derivative was then subjected to Sharpless dihydroxylation reaction giving the dihydroxy derivative, **11**. Surprisingly, the ee of this reaction was low and the reason is not clear at this stage. The primary-OH group was then protected as –OBz, followed by tosylation of the secondary –OH group. The tosyl derivative was then hydrogenated over 10% Pd/C (THF) for the nitro group reduction. The corresponding amine was then treated with K_2CO_3 in DMF at 40 °C giving the indoline derivative, **12** (after the *N*-Alloc protection) in high yield. In another study, the corresponding vinyl and allyl derivatives, **19** and **21** (see **22** and **23** to explain the stereocontrol outcome) were easily obtained using (*S*)-(+)-indolinemethanol (**17**) as the starting material.

Using the ring closing metathesis as the key step, the generation of tricyclic architectures, **25**, **27**, **29**, **31**, and **33** is shown in Scheme 2. Indolinol **12** was converted to the corresponding aldehyde **13** (80%). Following this, compound **13** was then subjected to allylation reaction. Thus, treatment of **13** with allylMgBr in the presence of $ZnCl_2$, followed by the protection of the hydroxyl groups, gave **14** and **15** as a separable mixture of diastereomers. (*Note.* the stereochemistry was assigned in the cyclic products obtained from the RCM.) At this point, the stage was set to explore the olefin ring closing metathesis reaction with different substrates. For our model studies to explore the RCM reaction, pure diastereomers, **14** and **15** were independently converted to *N*-acryloyl derivatives (**24** and **26**). With the 1st generation Grubbs' 10 mol% catalyst, it was a delight to observe the cyclic enamide formation (**25** and **27**) in high yields within 2–5 min at the room temperature. For other three systems, compounds **29**, **31**, and **33** were independently obtained from **16**, **19**, and **21**. All of them successfully generated the tricyclic products, **29**, **31**, and **33** that utilized the 2nd generation Grubbs' catalyst in the ring closing metathesis.

The synthesis plans leading to tricyclic architectures, **38** and **41** are shown in Scheme 3. To achieve these goals, compound **13** was converted into an olefin through a Wittig reaction (96%). The *N*-al-

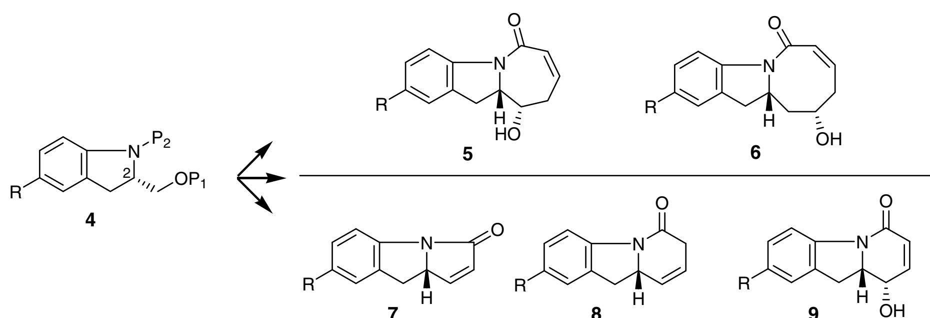
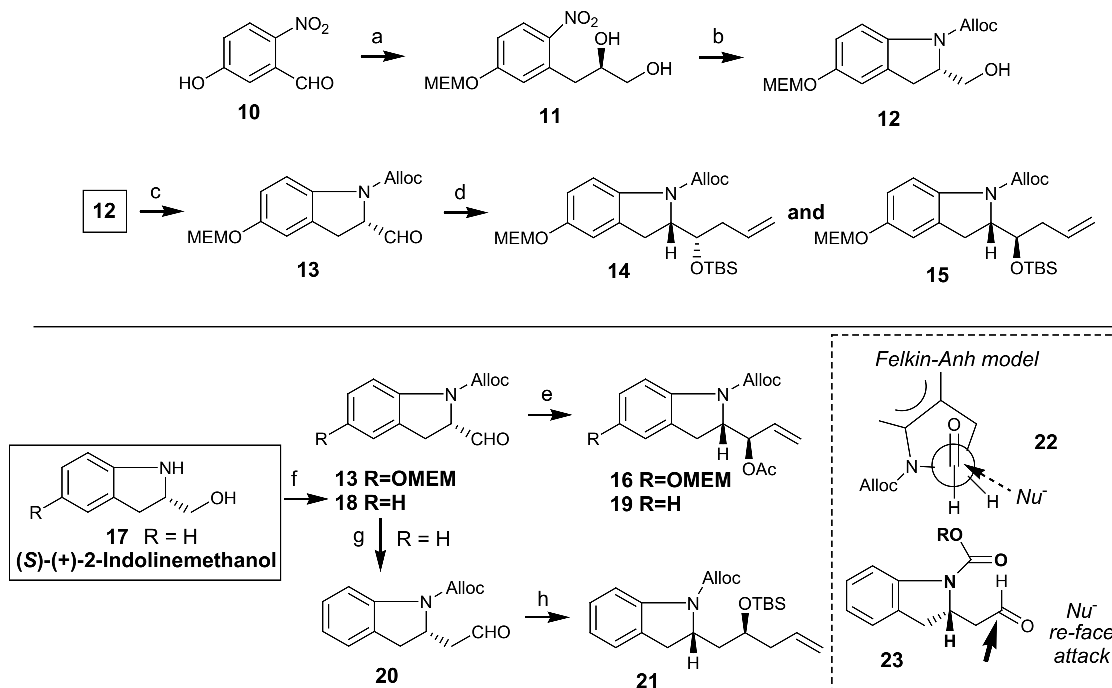
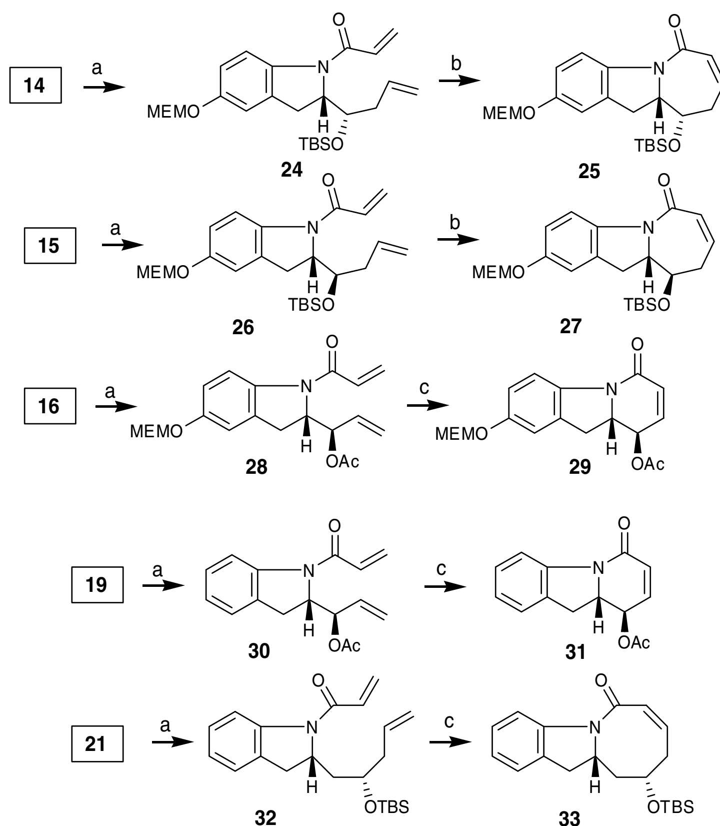


Figure 2. Skeletally diverse tricyclic architectures derived from an indolinol scaffold.



Scheme 1. Synthesis of the indoline skeleton. Reagents and conditions: (a) i—$Ph_3PHC(OMe)Cl$, *t*-BuOK, 0 °C, 96%; ii—1.5 HCl, 60 °C, 98%; iii—NaHMDS, Ph_3PMeBr, THF, 0 °C, 86%; iv—MEMCl, DIPEA, rt, 89%; v—sharpless dihydroxylation reaction-AD mix-β, 99%; (b) i—BzCl, pyridine, −5 °C, 24h, 89%; ii—*p*-TsCl, DMAP, 35 °C, 96%; iii—H_2, 10% Pd/C, K_2CO_3, rt; iv—K_2CO_3, DMF, 40 °C, 80% for 2 steps; v—K_2CO_3, MeOH, rt, 83%; vi—AllocCl, DIPEA, −78 °C, 94%; (c) SO_3 pyridine, 80%; (d) i—AllylMgBr, $ZnCl_2$, −78 °C to rt, 41% for **13a** (*syn*) and 47% for **13b** (*anti*); ii—TBDMSCl, imidazole, 83% for **14** (*syn*) and 88% for **15** (*anti*); (e) i—vinylMgBr, −78 °C to rt; ii—Ac_2O, DMAP, **16**: 37% and **19**: 70% for 2 steps; (f) i—AllocCl, DIPEA, −78 °C; ii—SO_3 pyridine, 91%; (g) i—$Ph_3PHC(OMe)Cl$, *t*-BuOK, 0 °C, 96%; ii—*p*TSA, 40 °C, 94%; (h) i—AllylMgBr, −78 to −15 °C (ii) TBDMSCl, imidazole, 65% for 2 steps.



Scheme 2. Synthesis of tricyclic architectures by RCM. Reagents and conditions: (a) i—$Pd(PPh_3)_4$, acetic acid, 4-methylmorpholine, rt; ii—acryloyl chloride, DIPEA, rt, 74–95% for 2 steps; (b) 10 mol% 1st generation Grubbs' catalyst, rt, 90–97%; (c) 10 mol% 2nd generation Grubbs' catalyst, rt, 85–92%.

loc group was then removed and substituted by coupling with vinyl acetic acid on the cyclic amine to afford **34**. Upon treatment with Grubbs' 1st generation catalyst, **34** was converted into **35** in a RCM reaction. Compound **35** was then subjected to epoxidation conditions using DMDO and **36** was the only product observed by ^{1}H NMR. Treatment with LiHMDS followed by coupling with phenylacetic acid using DIC produced **37**. Reaction of **37** with benzenethiol afforded **38** in a stereocontrolled manner containing two diversity sites. In a similar manner, the series of reactions that included (i) Wittig reaction, (ii) *N*-alloc removal, and (iii) *N*-acryloylation (77%), on **13** yielded the product **39**. Indoline-based tricyclic derivative **40** was obtained from **39** in 75% yield by RCM reaction with the 1st generation Grubbs' catalyst. As a test study, **40** on reaction with PhSH/Et_3N gave the Michael-type product, **41**, as the major isomer (ratio 5.5:1). Compounds shown in Figure 3 (see, **42** and **43)** were also synthesized as a part of the study leading to the development of the solid phase synthesis program (not shown in this paper). The details of the synthesis procedure are provided in the Supporting Material.

2.2. Testing of indoline-derived, chemical modulators of FAK

With the goal of finding small molecule modulators of focal adhesion kinase (FAK), the diverse collection of indoline-derived compounds was then subjected to several biological assays. Belonging to the family of non-receptor tyrosine kinase, FAK plays a crucial role in signal transduction pathways that are initiated by integrin-mediated cell adhesions and growth factor receptors.[18–24] As an adapter protein,[25–27] FAK is involved in regulating several cellular processes including cell survival, cell proliferation, and cell migration and invasion in the development and progression of cancer.[24,28–31] It is well documented that an over expression of FAK is commonly seen in a wide variety of human cancers.[32] This further confirms the role of FAK in tumorigenesis, metastasis, and survival signaling.[23,28,32] Due to these reasons, there is a growing interest in identifying small molecules that could serve as chemical probes to enhance our current understanding of signaling networks involving FAK.[33–39] In addition to this, some of these probes could also become an excellent starting point in developing new therapeutic agents for cancer.

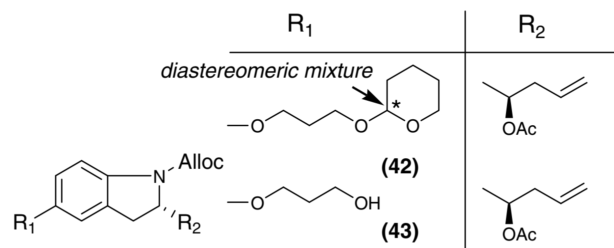


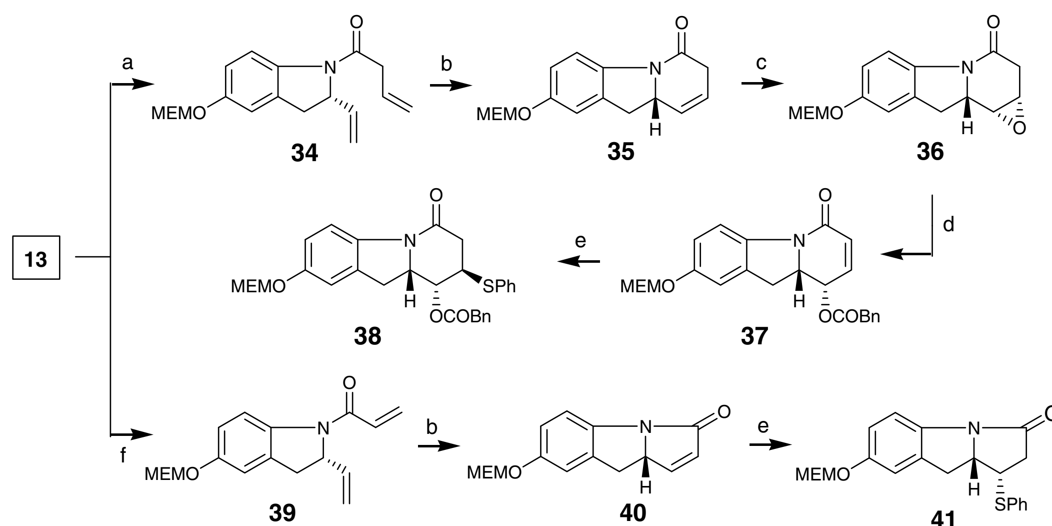
Figure 3. Two other indoline derivatives (see the Supporting Material for the synthesis details).

2.3. In vitro kinase assay

All the indoline-derived compounds were tested in a search for small molecule inhibitors of FAK. In a typical study, human recombinant full-length FAK (60 ng) was incubated in kinase buffer containing ATP (2 μM) and substrate (20 μg/ml) for 4 h at room temperature with or without the presence of the indoline-derived compounds at 30 μM final concentration. Remaining ATP in solution was then quantified utilizing the Kinase-Glo-luminescence kit (Promega). This study led to the discovery of a novel indoline-derived compound, compound **42**, which successfully inhibited FAK kinase activity (30% inhibition at 30 μM) (Fig. 4A). Additional studies examining the structure–activity relationship of compound **42** and FAK will be necessary in order determine if this compound is functioning as an ATP competitor or allosteric inhibitor.

2.4. Cell proliferation assay

Exponentially growing cells (1×10^3 cells) were seeded in 96-well plates. After 18 h cells were continuously treated with compound **42** dissolved in DMSO. The final concentration of DMSO was less than 0.05%. Following this, after 96 h, cell survival was evaluated using the 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) metabolic assay as previously described.[40] The inhibitory activity (IC_{50}) of compound **42** was determined to be 38.7 μM (Fig. 4B).



Scheme 3. Exploration of functional group diversity. Reagents and conditions: (a) i—NaHMDS, Ph_3PMeBr, THF, 0 °C, 96%; ii—$Pd(PPh_3)_4$, acetic acid, 4-methylmorpholine, rt, 90%; iii—vinyl acetic acid, DIC, HOBt, DMF, rt, 61%; (b) 25 mol% 1st generation Grubbs' catalyst, dichloromethane, for **35**: rt, 1 h, 77%, for **40**: reflux, 48 h, 75%; (c) DMDO, acetone; (d) i—LiHMDS, −78 °C, 95%; ii—phenylacetic acid, DMAP, DIC, 96%; (e) benzenethiol, triethylamine, 0 °C, 97% for **38** and 95% for **41**; (f) repeat step (a) (i) and (ii), (iii) acryloyl chloride, pyridine, 0 °C, 77%.

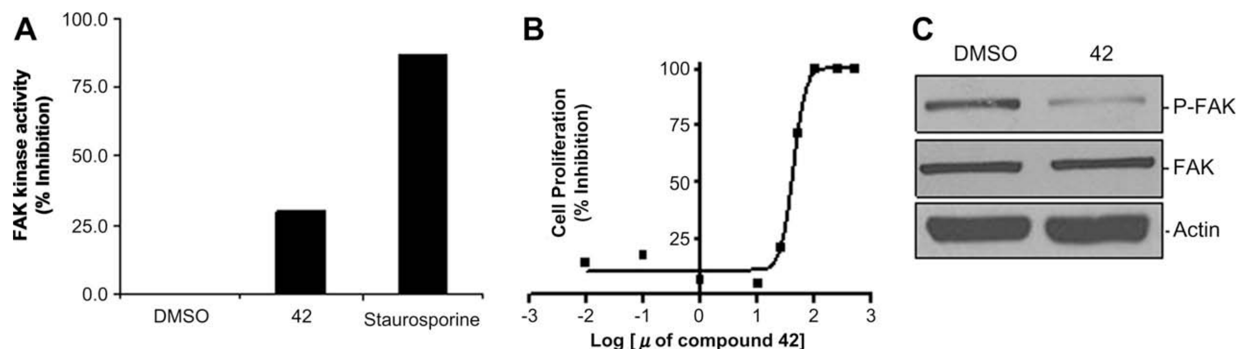


Figure 4. Effect of compound **42** on FAK kinase activity (A and C) and cell proliferation (B). (A) In vitro kinase assay of full-length FAK with 30 μM of indicated compounds. (B) MTT assay of MDA231-M cells treated with increasing concentrations of compound **42**. IC_{50} = 38.7 μM. (C) Expression level of pY397-FAK (P-FAK), FAK, and actin in MDA231-M cells pretreated with or without compound **42** (25 μM, 1 h) examined by Western blot. Compound **42** pretreatment led to a significant decrease in phosphorylated tyrosine 397-FAK as compared to control (DMSO).

2.5. Cellular FAK activity

The level of phosphorylated tyrosine 397 (the major autophosphorylation site of FAK) in MDA231-M cells was evaluated in cells pretreated with or without compound **42** (Fig. 4C). Compound **42** treated MDA231-M cells demonstrated reduced levels of phosphorylated tyrosine 397, indicative of the reduced FAK activity.

2.6. Docking simulation

Compound **42** was docked with Glide (Schrödinger, NY)[41] to a grid representation of the catalytic site of human FAK co-crystallized with a sub-micromolar pyrrolopyrimidine inhibitor (PDB code 2ETM, http://www.pdb.org). A constraint was used during docking that forced hydrogen bonding of compound **42** to the backbone nitrogen of Cys502, since a similar interaction was observed in all FAK co-crystal structures available. The resulting complex was then locally minimized with flexible receptor side chains in the internal coordinates space with ICM (Molsoft LLC, CA).[42] Two hydrogen bonds are observed between **42** and FAK, the first one between the acyl carbonyl of Alloc and the backbone nitrogen of Cys502, the other between the piperidine oxygen of compound **42** and the backbone nitrogen of Asp564 (Fig. 5).

This first generation hit confirms that our indoline-based chemical series is suitable for FAK inhibition. Docking studies suggest opportunities to optimize **42**. For instance, the side chains of E506 and K454 are less than 5 Å away from docked **42**, but are not making polar interaction. Additionally, another structure of FAK (PDB code 2J0L)[26] shows an alternate conformation of the activation loop with a larger catalytic site that may accommodate more potent derivatives.

2.7. Cell motility assay (Fig. 6)

The effect of compound **42** on cell motility was investigated using the wound healing assay as described earlier.[43] In this study, cells were grown on sterile coverslips for 24 h and were then wounded by cell scraping using a micropipette tip. Cultures were washed and then incubated with fresh culture media at 37 °C with or without the presence of compound **42** (25 μM) for the indicated time periods. Cells were allowed to migrate and heal the wound. Photomicrographs were taken at each time point in order to examine the wound healing areas (Fig. 6).

2.8. Cell invasion assay (Fig. 7)

Cell invasion experiments were performed with 8 μm porous chambers coated with Matrigel (Becton–Dickinson) as described earlier.[43] Briefly, serum starved cells were placed into the upper compartment (30,000 cells) of a 24-well Boyden chamber with or without compound **42** (25 μM) and the chambers were then placed into 24-well culture dishes containing 400 μl of DMEM 0.2% BSA with 10% serum (lower compartment) as chemoattractant. Invasive cells are able to invade through the Matrigel, infiltrating through the pores and attaching themselves to the underside of the membrane. The invasive cells underneath the membrane were fixed and stained after 48 h. Filters were viewed under bright-field 40× objective and the counting was performed for three fields in each sample. As shown in Figure 7, reduced invasive activity of MDA231-M cells was observed in the presence of compound **42**, as compared to DMSO treated control.

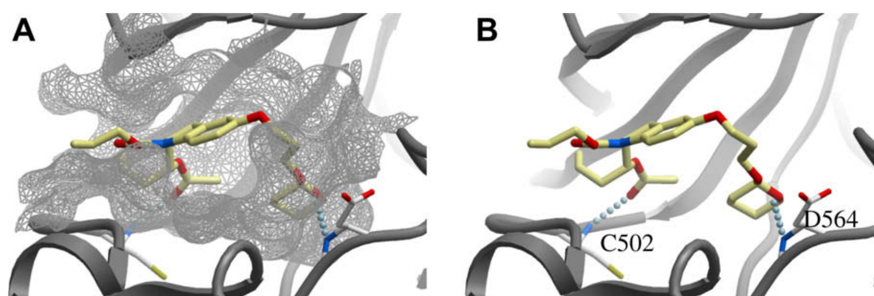


Figure 5. Docked conformation of compound **42** complexed to the ATP site of FAK. Compound **42** is deeply buried in the pocket at the interface of the FAK N- and C-terminal lobes (A) and makes hydrogen bonds with the backbone amide groups of Cys502 and Asp564 (B).

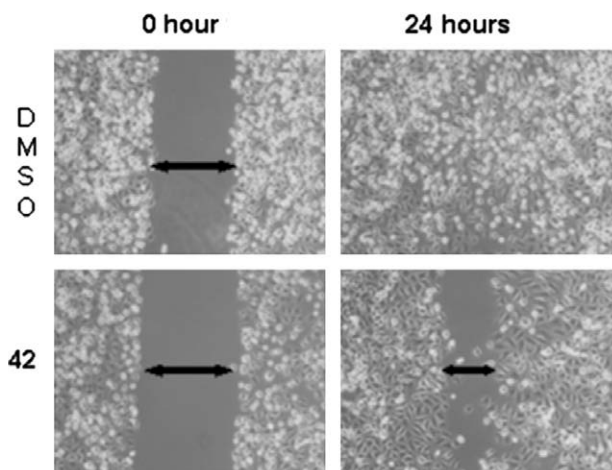


Figure 6. The effect of compound **42** on the MDA231-M cell motility. Wound healing assay of MDA231-M cells treated with or without the presence of **42** (25 μM). Arrows indicative of remaining wound area.

3. Summary

With the goal of obtaining diverse architectures from the indoline scaffold, several modular approaches were developed. This allowed us to generate a wide variety of indoline-based bicyclic and tricyclic compounds. To extend the scope of these methods on solid phase for high-throughput generation of indoline-based libraries, further work is in progress. The highly diverse collection obtained in this program (through the solution phase synthesis) was then examined in a search of chemical modulators of FAK activity. Toward this objective, the collection was subjected to full-length FAK inhibition assay and this led to the discovery of a novel class of moderate small molecule inhibitor of FAK (compound **42**). To explore the scope of **42** further, it was also investigated as a modulator of cell migration through the wound healing assay and showed a significant inhibitory activity of cell migration. Moreover, compound **42** was also able to demonstrate its inhibitory activity in the Boyden chamber invasion assay, indicative of its ability to inhibit cell movement on a three-dimensional scale and/or by reducing the cells' ability to produce extracellular matrix degrading enzymes, such as matrix metalloproteinases. Although the precise mechanism of action of **42** is not clear at this stage, it certainly serves as a good starting point in developing next generation derivatives and warrants further investigation.

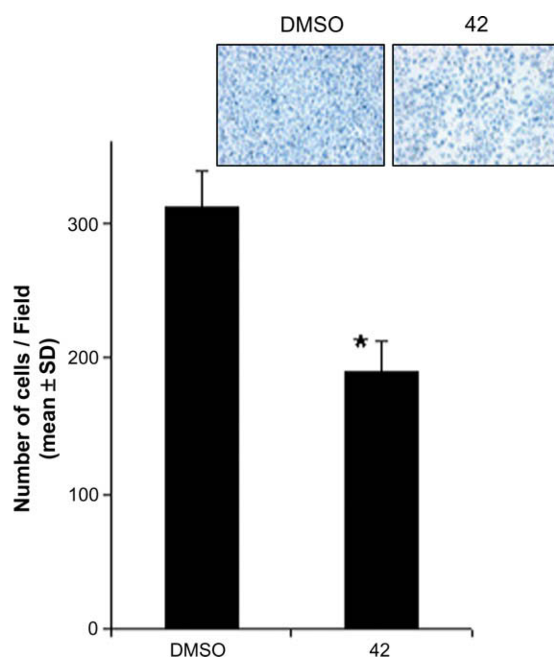


Figure 7. Effect of compound **42** on MDA231-M cell invasion. Boyden chamber invasion assay of MDA231-M cells treated with or without the presence of compound **42** (25 μM). Cells treated with compound **42** demonstrated a significant reduction in their ability to invade. ($^{*}P < 0.05$ compound **42** vs DMSO, $N = 6$).

Acknowledgments

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Supplementary data

Experimental details and full characterization data for all new compounds are provided. This material is available free of charge via the internet. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.09.025.

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