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Protein kinase affinity reagents based on a 5-aminoindazole scaffold

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ABSTRACT

Affinity reagents that target protein kinases are powerful tools for signal transduction research. Here, we describe a general set of kinase ligands based on a 5-aminoindazole scaffold. This scaffold can readily be derivatized with diverse binding elements and immobilized analogs allow selective enrichment of protein kinases from complex mixtures.

Protein kinases are a key family of enzymes that regulate reversible protein phosphorylation cascades in cells. Protein kinases catalyze the transfer of a phosphate group from ATP to a target protein, serving as regulatory elements in signal transduction pathways.[1] Proper control of protein kinase activity is essential for normal cellular behavior and aberrant kinase function has been correlated with a number of diseases.[2] Currently there are 11 small molecule protein kinase inhibitors approved for clinical use, with dozens of others undergoing clinical evaluation.[3,4]

Due to the promise of protein kinases as drug targets, significant efforts have been made to investigate this large enzyme family. However, only a small percentage of the kinase superfamily (>500 members in humans) has adequately been functionally analyzed. Characterization of protein kinase expression and function in diverse cellular contexts provides important insight into these enzymes but global profiling remains challenging due to their generally low cellular abundance compared to other proteins. Studies performed on the yeast proteome of *S. cerevisiae* found that 63% of protein kinases were expressed at concentrations ranging from 1 nM to 50 nM, which is significantly lower than the average protein concentration in cells.[5,6] A valuable technique that has aided the global analysis of protein kinases is the use of immobilized kinase inhibitors that selectively enrich these low abundance enzymes. These affinity reagents have been used to identify the cellular targets of small molecule inhibitors, provide exhaustive analysis of kinase inhibitor selectivity, and delineate phosphorylation profiles of protein kinases and their substrates.[7–19] While currently existing affinity reagents have proven to be very useful, there still remains a need for new and diverse chemical tools for studying kinase function. Affinity reagents based on inhibitors that are tuned to conserved binding elements in the ATP-binding sites of protein kinases allow for unbiased profiling of these enzymes and increase the sensitivity of currently existing analytical techniques. Furthermore, a general scaffold that that can be readily modified should allow subsets of kinases to be enriched and analyzed. Here we describe the synthesis and biochemical characterization of an indazole pharmacophore, that is, a general protein kinase ligand. In addition to possessing selectivity for protein kinases over other cellular enzymes, this indazole core can be easily derivatized for proteomics applications using a modular synthetic approach.

An inhibitor based on the 5-aminoindazole scaffold was previously identified as a promiscuous inhibitor of protein kinases.[20] We selected the indazole scaffold as a general protein kinase ligand due to its ability to exploit conserved binding elements in the ATP-binding site. Furthermore, the modular nature of this scaffold allows for diverse functionalities to be introduced at multiple positions. Figure 1 shows how the 5-aminoindazole core interacts with the ATP-binding sites of protein kinases. The indazole pharmacophore lies in the narrow ATP-binding pocket, forming similar hydrophobic interactions as the adenine ring of ATP.[21] Furthermore, the indazole scaffold is able to make three hydrogen bonds with the conserved hinge region, which is one more than the adenine ring of ATP. This binding orientation projects substituents at the *C*-5 amine of the indazole core into a deep hydrophobic pocket and the amine at the *C*-3 position out of the binding pocket into solvent. The solvent accessibility of the *C*-3 position makes this site an ideal position for linker or reporter attachment.

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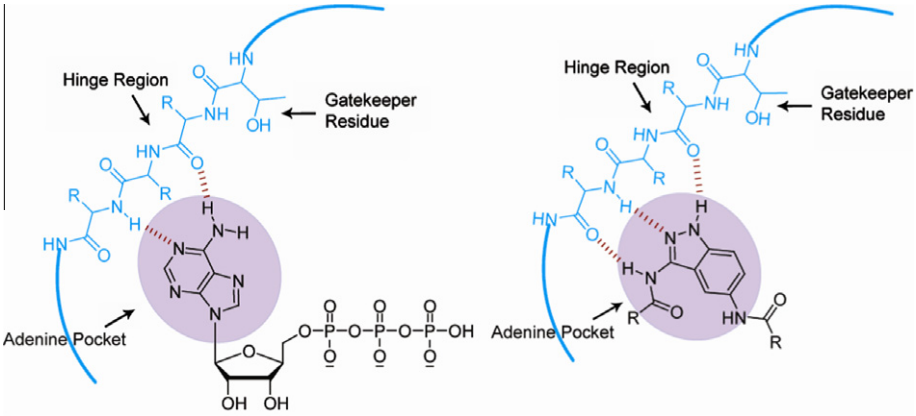
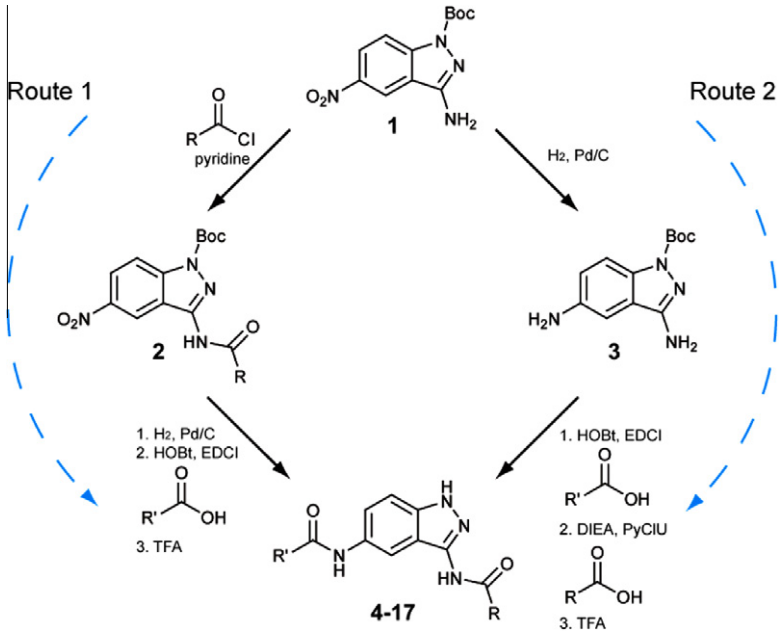


Figure 1. A schematic representation of ATP (left) and the 5-aminoindazole scaffold (right) bound to the ATP-binding pocket of a protein kinase. The indazole scaffold occupies the adenine binding region of the pocket and makes three hydrogen bonds (dashed lines) to the amide backbone of the protein kinase hinge region.

To probe the generality of the indazole scaffold and the potential of this core to serve as a versatile affinity reagent, two flexible synthetic schemes were developed (Scheme 1). Both synthetic routes were used to generate a small panel of compounds based on the indazole core (Fig. 2). The key in synthesizing this panel is the ability to selectively modify the *C*-3 and *C*-5 amines of the indazole core. Indazole scaffold **1**, which was used in both synthetic routes, was generated in two-steps. 2-Fluoro-5-nitrobenzonitrile and hydrazine monohydrate were refluxed in ethanol to generate the heterocyclic indazole core. Selective Boc-protection of the N-1 position yielded the starting scaffold for derivatization. In the first synthetic route (Route 1), the *C*-3 amine of nitroindazole **1** was acylated with an acid chloride of interest to give **2**. Subsequent reduction of the nitro group via palladium-catalyzed hydrogenation yielded the reduced indazole to which various carboxylic acids were coupled using standard amide bond-forming conditions. Final compounds **4–15** were generated by deprotection with TFA. Synthetic route 2 allows increased diversity to be introduced at the 3-position. First, nitroindazole **1** was reduced via palladium-catalyzed hydrogenation to generate aniline **3**. Next, the *C*-5 amine was selectively acylated with carboxylic acids that had been activated with carbodiimide coupling reagents. Subsequent acylation of the *C*-3 position with acid chlorides containing linkers for immobilization or tag attachment yielded fully derivatized indazole intermediates. The coupling reagent chlorodipyrroli dinocarbenium hexafluorophosphate (PyClU) was found to be an extremely mild and efficient means for generating acid chloride coupling partners for this reaction. Final compounds **16** and **17** were generated by deprotection with TFA.

To determine which substituents are readily accommodated in the ATP-binding sites of protein kinases, indazole-based inhibitors **4–15** were tested for their ability to inhibit three diverse protein kinases: SRC (a tyrosine kinase), PKA (an AGC kinase), and CLK1 (a CMGC kinase). The percent inhibition of each kinase at a 1 μM concentration of inhibitor is shown in Supplementary Table 1. Most compounds based on the indazole core were found to be sub-micromolar inhibitors of these three kinases. We observed that compounds containing a phenylacetic acid group at the 5-position, particularly with substitution at the *meta*-position, are potent inhibitors of the protein kinases tested. Additionally,



Scheme 1. Functionalization of the indazole scaffold.

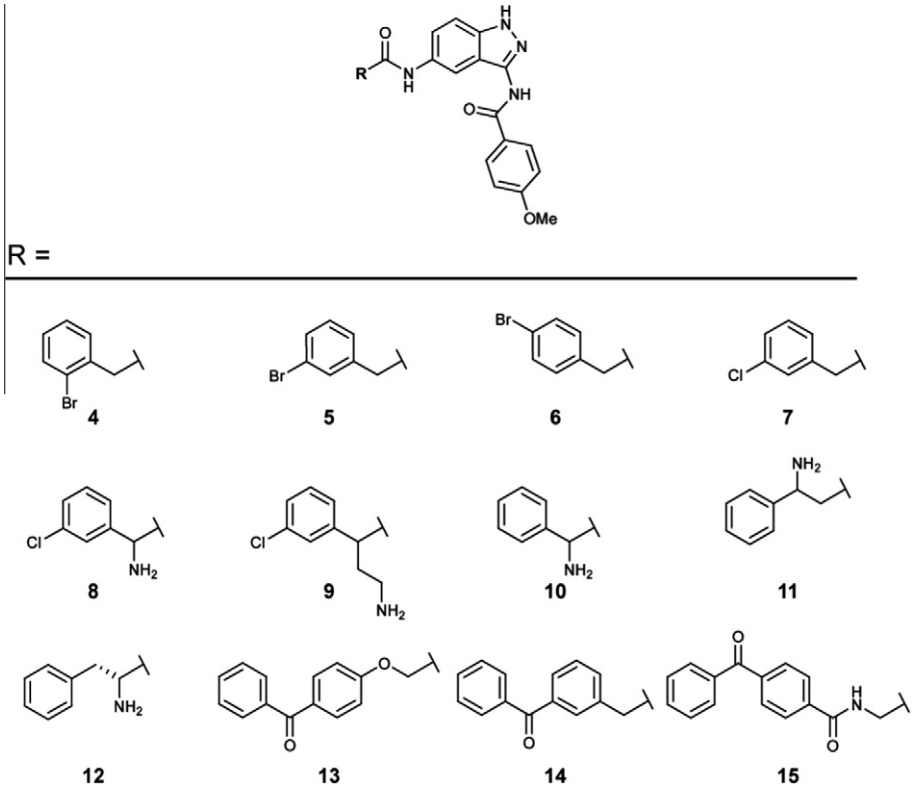


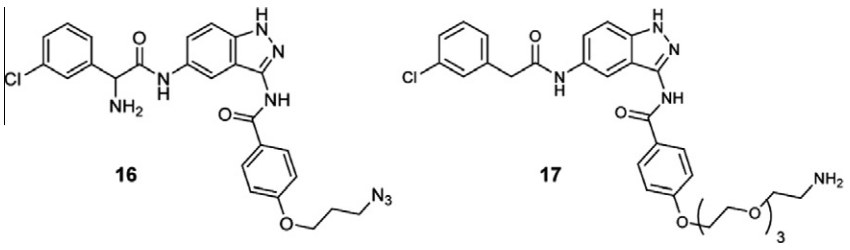
Figure 2. Indazoles that were generated and tested for their ability to inhibit the protein kinases SRC, PKA, and CLK1.

substituents at the α-position of the phenylacetic moiety improve inhibitor potency across the protein kinase panel.

In order to further confirm that the indazole scaffold is a general kinase binder, a smaller panel of five compounds was assayed against an expanded set of protein kinases (SRC, CLK1, CDK2 (CMGC kinase), Aurora A (CAM kinase), and PAK4 (STE kinase)). Promiscuous inhibitors **7**, **8**, and **9** were selected from the original panel. In addition, a modified version of **7**, which contains an amino-polyethylene glycol (PEG) linker (analog **17**), and a modified version of **8**, which contains an azidopropyl linker (analog **16**), were also tested. Gratifyingly, each of these compounds was found to be a fairly potent inhibitor of the kinases tested, with several compounds displaying IC_{50}s in the low nanomolar range (Table 1). Compound **9**, which contains an ethylamino group at the α-position of the phenylacetic moiety, displayed the broadest inhibitory profile. Most importantly, linker-containing compounds **16** and **17** are similarly effective inhibitors as their parent compounds (**7** and **8**) against all of the kinases tested. These results confirm that the 3-position of the indazole scaffold can be used as a site for immobilization and that modification with a long tether will not disrupt the interaction of this pharmacophore with most kinases.

To demonstrate the utility of indazole-based affinity reagents, a series of pull-down experiments were performed. Compound **17** was selected for immobilization on solid support. ECH Sepharose resin, which contains free carboxylic acid groups, was coupled to the free amine of **17** (Fig. 3A). The efficiency of inhibitor

Table 1
IC_{50} values (nM) of compounds **7**, **8**, **9**, **16** and **17** against the kinases SRC, CLK1, CDK2, Aurora A, and PAK4. Values shown are the average of three assays ± SEM



Compound	SRC	CLK1	CDK2	Aurora A	PAK4
7	530 ± 70	27 ± 3	150 ± 20	17 ± 1	140 ± 8
8	140 ± 10	<10	1800 ± 300	30 ± 4	64 ± 6
9	45 ± 6	<10	360 ± 30	8 ± 1	24 ± 1
16	150 ± 20	Not tested	2700 ± 600	18 ± 1	22 ± 2
17	420 ± 40	21 ± 3	230 ± 40	19 ± 1	410 ± 10

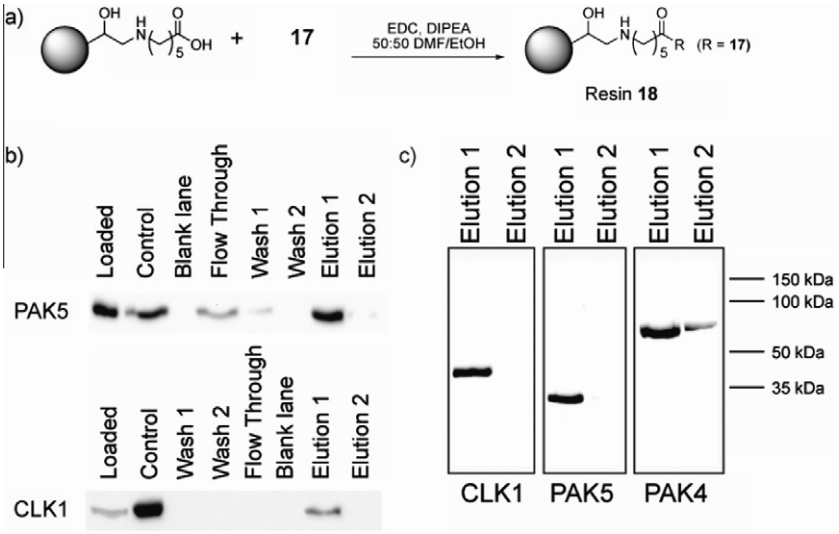


Figure 3. Pull-down assays. (a) Procedure for generating affinity resin **18**. (b) Enrichment of protein kinases from *E. coli* lysate using affinity resin **18**. His6-PAK5 and His6-CLK1 were added to bacterial lysate and enriched using a standard pull-down protocol. Collected fractions were subjected to SDS–PAGE, transferred to nitrocellulose and analyzed using HisProbe-HRP (Pierce). Lane 2 contains a purified standard for each kinase tested. (c) Eluted fractions from CLK1, PAK5, and PAK4 pull-down experiments were subjected to SDS–PAGE and analyzed by silver staining (SilverXpress, Invitrogen).

immobilization was monitored by analytical HPLC in the presence of an internal standard. Optimized coupling conditions resulted in 63% of inhibitor **17** being immobilized. Final affinity resin **18** was generated by quenching any unreacted sites with ethanolamine.

To characterize resin **18** in a complex protein mixture, affinity pull-down assays were performed with purified protein kinases that had been added to *Escherichia coli* (*E. coli*) lysate. This is an attractive model for enrichment studies because *E. coli* lack endogenous eukaryotic protein kinases and only exogenously added kinases should be retained. The protein kinases PAK4, PAK5 and CLK1 were each added to *E. coli* lysate and the resultant kinase/lysate mixtures were incubated with resin **18**. After 4 h of incubation, the resin was washed multiple times, and bound protein was eluted with an excess of free inhibitor **7**. Collected fractions were subjected to SDS–PAGE and analyzed by Western Blot Analysis or silver staining. Figure 3B shows Western Blot Analysis (HisProbe-HRP) of the pull-down experiments performed with PAK5 and CLK1. In both kinases, a majority of the kinase is captured by resin **18** and very little protein is detected in the washes. Furthermore, an ATP-competitive ligand is able to efficiently elute both kinases from the resin. Silver staining of the eluted fractions from each enrichment experiment show that only the exogenously added kinase is present (Fig. 3C). This demonstrates that probe **18** is able to selectively enrich protein kinases over other highly abundant ATP-binding proteins and enzymes present in *E. coli*, including metabolic enzymes and heat shock proteins.

In conclusion, compounds based on the 5-aminoindazole scaffold appear to be general ligands for protein kinases. This scaffold can readily be modified with a wide array of binding elements that mediate inhibitor potency and selectivity. The ability to rapidly generate diverse affinity reagents based on a general protein kinase ligand should allow extensive coverage of the kinome. In addition, specific subsets of kinases can be targeted by using more selective indazole reagents. Importantly, the 5-aminoindazole scaffold can be derivatized with flexible linkers that allow immobilization on a solid support or reporter attachment. In vitro activity assays demonstrate that linker-derivatized versions of 5-aminoindazoles do not have reduced potencies against most kinases. Furthermore, an immobilized version of these inhibitors serves as an effective affinity reagent in kinase enrichment experiments. The use of affinity reagents based on the 5-aminoindazole scaffold to study kinase function will be reported in due course.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.10.069.

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