

Discovery of a Potent Allosteric Kinase Modulator by Combining Computational and Synthetic Methods

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Dedicated to Robert Huber

Abstract: The rational design of allosteric kinase modulators is challenging but rewarding. The protein kinase PDK1, which lies at the center of the growth-factor signaling pathway, possesses an allosteric regulatory site previously validated both *in vitro* and *in cells*. ANCHOR.QUERY software was used to discover a potent allosteric PDK1 kinase modulator. Using a recently published PDK1 compound as a template, several new scaffolds that bind to the allosteric target site were generated and one example was validated. The inhibitor can be synthesized in one step by multicomponent reaction (MCR) chemistry when using the ANCHOR.QUERY approach. Our results are significant because the outlined approach allows rapid and efficient scaffold hopping from known molecules into new easily accessible and biologically active ones. Based on increasing interest in allosteric-site drug discovery, we foresee many potential applications for this approach.

Compounds with an allosteric mode of action enable modulation of the protein function that cannot be accomplished by active-site inhibitors.^[1] Additionally, allosteric receptor sites are often less conserved than active sites in a protein family. Hence, more selective receptor modulation is possible and often new therapeutic qualities can be accomplished. For example, most ATP-competitive kinase inhibitors show limited selectivity, and therefore clean and selective regulation of a cellular pathway is often prohibited. While nonselective kinase inhibitors were considered advantageous for targeting more than one signaling pathway in cancer, some clinical kinase inhibitors show considerable toxicity owing to polypharmacology. More-selective kinase inhibitors are potentially better suited for combination with other selective targeted drugs in future personalized treatments. As a subgroup of the human kinome, the AGC kinases

provide a unique opportunity to discover allosteric modulators since a regulatory allosteric site, the so called PIF-pocket, is well established.^[2] Amongst these kinases, 3-phosphoinositide-dependent protein kinase-1 (PDK1) is of particular interest owing to its potential usefulness for treating diabetes or cancer. Recently, we introduced ANCHOR.QUERY as a specialized pharmacophore search technology that brings interactive virtual screening of novel protein–protein inhibitors to the desktop.^[3] Unlike other software, ANCHOR.QUERY leverages more than 31 million compounds with an approximately 2 billion preformed conformer space of low-molecular-weight compounds that are easily accessible by multicomponent reaction (MCR) chemistry. MCR enables the diverse assembly of many bioactive scaffolds in a convergent one-pot manner.^[4] So far, we have applied the ANCHOR.QUERY software to the design of antagonists of the protein–protein interaction between p53 and MDM2 by using a deeply buried tryptophan as an anchor and template.^[5] Multiple active scaffolds were discovered and validated by solving the cocrystal structures.^[6] Herein, we report for the first time that ANCHOR.QUERY can also be successfully applied to query fragments derived from a small-molecule/protein cocrystal structure.

The starting point for our discovery is the cocrystal structure of the known small molecule **1** in the PIF-pocket of PDK1 (Figure 1).^[7] ANCHOR.QUERY automatically proposed the deeply buried phenyl group of compound **1** as being the highest-energy contributing fragment for the binding of the molecule. This fragment was thus used as the template for drug design. Further analysis reveals that the carboxylic acid of **1** forms multiple hydrogen bonds and charge–charge interactions to a Lys and Arg of the receptor. Finally, the second phenyl group of **1** resides on a hydrophobic but solvent-exposed patch of amino acids that forms a rather flat pocket. Based on this analysis, a three-point pharmacophore model was queried against an approximately 2 billion conformer library based on 27 MCRs. Compound **2** was proposed amongst the top results and can be synthesized by the one-pot reaction of benzaldehyde **3**, primary amine **4**, and glutaric anhydride **5**. The corresponding MCR is called the Castagnoli reaction and is very suitable for the synthesis of libraries of compounds.^[8] The MCR diastereoselectively yields only the *trans* product **2**, however, this is produced as a racemate (Scheme 1).

PDK1 binds to the C-terminal hydrophobic motif of substrates and this docking interaction with the PIF-pocket of

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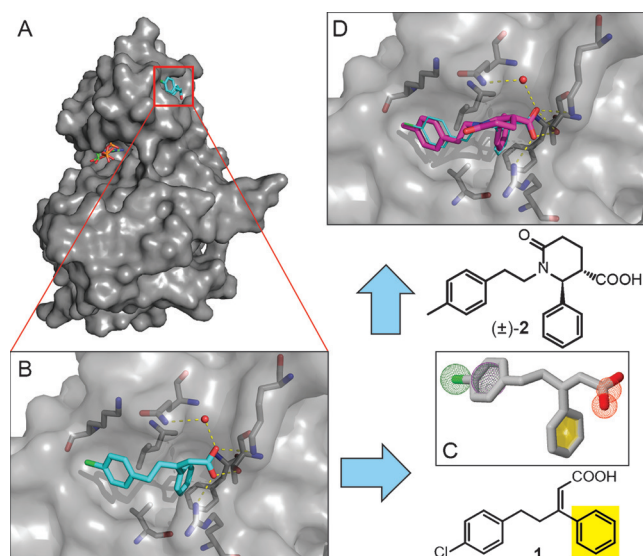
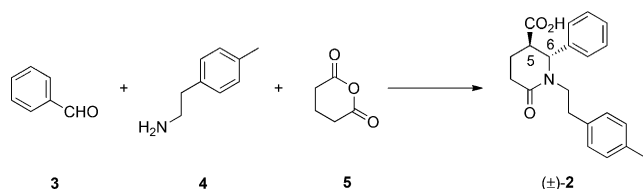


Figure 1. Evolution of an allosteric PIF-pocket-targeting PDK1 inhibitor by using ANCHOR.QUERY software. A) PDK1 (PDB ID: 3HRF) showing the active site with ATP (green/orange stick model) and the remote PIF-pocket filled with ligand **1** (cyan stick model). B) Zoomed view of the PIF-pocket showing key interactions of the carboxyl group with Arg131, Thr148, and Lys76, and a water-mediated hydrogen bond to Gln150, as well as halogen bonding with the Lys115 backbone carbonyl. C) The ANCHOR.QUERY-generated pharmacophore model based on ligand **1**, including the phenyl anchor (yellow), the negative charge (red), a second aromatic region (magenta), and a hydrophobic region (green). D) The top resulting molecule **2** (cyan stick model) of the query, shown aligned with molecule **1** (magenta lines) in PDK1 after energy optimization.



Scheme 1. Castagnoli-3CR to give PDK1 inhibitor **2**.

PDK1 is necessary for the phosphorylation of substrates such as S6K, SGK, PRK, and PKC isoforms. The C-terminal sequence of PRK2 (PIFtide: REPRILSEEEQEMFRDF-DYIADWC, hydrophobic motif underlined) has vastly higher affinity to the PIF-pocket of PDK1 than the hydrophobic motif sequences derived from other substrates.^[9] Similar to compound **1** and PIFtide,^[7,10] low micromolar concentrations of the original compound **2** proposed by ANCHOR.QUERY stimulated the *in vitro* activity of PDK1 when using the peptide T308tide as a substrate. The structure–activity relationship (SAR) was investigated and an alpha-screen interaction–displacement assay revealed that the majority of the Castagnoli compounds indeed are able to fully disrupt the high-affinity interaction between PDK1 and PIFtide (Figure 2 and Table S1 in the Supporting Information). There is an indication of halogen bonding in the R¹ position, with 4-chloro (11 μM) and 4-bromo (8.1 μM) com-

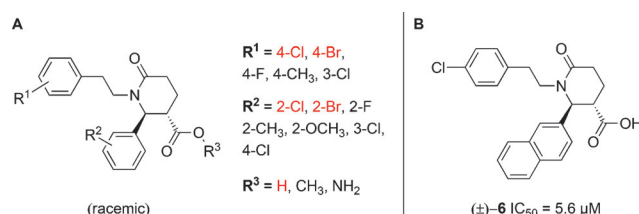


Figure 2. Structure–activity relationship. A) General structure indicating the best substituents (highlighted in red). B) The most potent compound.

pounds being more potent than the 4-fluoro counterpart (26 μM) that is not capable of halogen bonding.^[11] Shortening of the linker at the δ-lactam nitrogen atom from phenethyl to benzyl, or replacing it with an aliphatic *tert*-butyl group eliminates the ability to displace PIFtide. In the R² position, an *ortho*-substituted phenyl ring is preferred over *meta* and *para* substitution and the most potent substituents are *ortho*-chloro (11 μM) and *ortho*-bromo (8.5 μM), which both display around 3-fold better potency than the *ortho*-fluoro (28 μM) or *ortho*-methyl (9.4 μM) compounds.

Additionally, employing 2-naphthaldehyde results in the most potent compound (±)-**6** (5.6 μM, Figure 2), whereas an aliphatic group reduces the ability to displace PIFtide entirely. The R³ position favors a carboxylic acid (11 μM), with the analogous ester derivative being less potent (40 μM) and the primary amide not able to displace PIFtide at all. Finally, racemic **7** (R¹ = 4-Cl; R² = 2-Cl; R³ = H, Figure 3) showed good potency (11 μM), and separation of the two enantiomers by chiral SFC illustrates a difference between the two enantiomers: enantiomer **7A** (7.0 μM) is 2-fold more potent than enantiomer **7B** (15 μM) for disrupting the PDK1–PIFtide interaction (Figure S2). These results show that the Castagnoli scaffold displaces PIFtide with higher potency

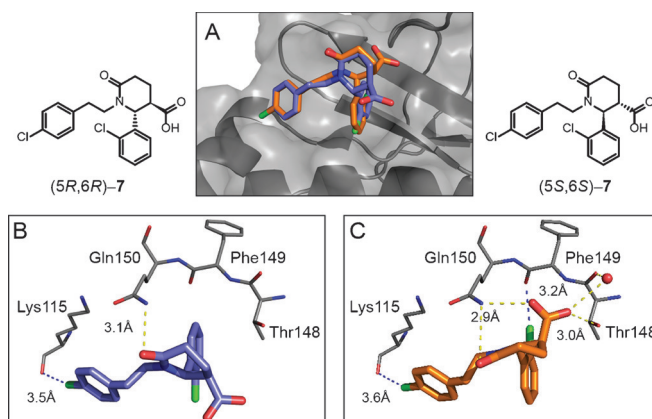


Figure 3. Cocystal structure of the enantiomers of **7** in the PIF-pocket of PDK1. A) Overall crystal structure showing (5R,6R)-**7** (blue stick model) and (5S,6S)-**7** (orange stick model). B) Top view (towards the phenyl anchor) of (5R,6R)-**7** in the PIF-pocket. C) Top view (towards the phenyl anchor) of (5S,6S)-**7** in the PIF-pocket. In (B) and (C), hydrogen and halogen bonds are shown as yellow and blue dotted lines, respectively.

than, for example, the current best compound PS210^[12] (a dicarboxylate derivative of **1**, 20 μM) and is amongst the most potent compounds reported. To assess the important issue of selectivity of the Castagnoli scaffold against kinases other than PDK1, a kinase profiling study was carried out. Racemic compound **7** did not alter the activity of any of the 50 kinases tested in this panel (Table S2), thus indicating a high degree of selectivity. In the selectivity screen, the tested compound also did not affect the activity of PDK1 measured using PDKtide as a substrate, an assay that is not sensitive to small compounds binding to the PIF-pocket.^[12]

In order to elucidate the structural mode of action of **7**, we obtained its cocrystal structure with PDK1. The crystal structure was solved to 1.24 Å resolution. In contrast to previous crystal structures of complexes of PDK1 with small-molecule compounds, Lys76 is invisible owing to side chain movement. Interestingly, both enantiomers of **7** cocrystallized in the structure in a ratio of approximately 1:1. The receptor–ligand interactions are dominated by hydrophobic contacts, electrostatic interactions, and halogen bonding (Figure 3).^[13] In both molecules, the 2-chlorophenyl substituent is the anchor and is deeply buried in the pocket, as with **2**. The orientation, however, of the 2-chlorophenyl substituent is different in the two enantiomers. Whereas in the structure of the (5*S*,6*S*)-**7** isomer, the 2-chloro substituent forms a short contact (3.2 Å) to the backbone carbonyl oxygen of Phe149, it is turned by approximately 180° in the other enantiomer. The carboxyl moiety of the (5*R*,6*R*)-**7** isomer appears to displace the usual conformation of the Arg131 head group in the crystal structure, nonetheless, a charge–charge interaction would be feasible with different conformations of Arg131. The different orientation of the other enantiomer precludes this interaction. Instead, the (5*S*,6*S*)-**7** isomer carboxyl forms hydrogen bonds with the Gln150 side-chain amide and the Thr148 hydroxy group, and a water-mediated hydrogen bond to Phe149. The δ -lactam carbonyl oxygen from (5*R*,6*R*)-**7** forms an additional hydrogen bond with Gln150. In contrast to their differential ability to disrupt the interaction of PDK1 with PIFTide, the two enantiomers activated PDK1 with undistinguishable AC_{50} values (AC_{50} = concentrations that gives 50% of maximum activation). Such difference may be due to off-rate by the off rate of enantiomer A, a feature that is more relevant for the displacement of PIFTide. In both enantiomers, the 4-chlorophenylethyl side chain displays hydrophobic interactions with Leu155, Ile118, Ile119, Lys115, and Val124, and additionally, a halogen bond to the backbone carbonyl oxygen of Lys115. The alignment of the energy-minimized ANCHOR.QUERY-derived structure of **2** and the observed crystal structure of **7** is very good (Figure 4). The phenyl group is placed in the deep pocket of the anchor and there is a good overlap between the δ -lactam and second aromatic ring region.

Recently, modulators of PDK1 directed at the PIF-pocket have been described by several groups. Disulfide trapping by using a library of low-molecular-weight disulfides was applied and several hits resulted.^[14] Disulfide trapping, however, is a highly specialized technology based on cysteine-mutated proteins and difficult-to-access screening libraries of disulfides.^[15] Moreover, hits derived from disulfide trapping have

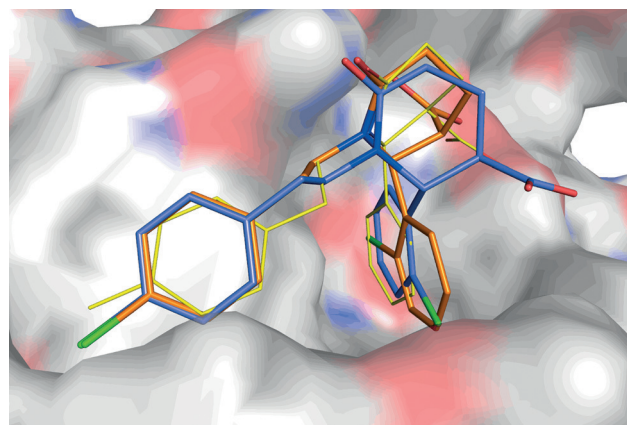


Figure 4. Alignment of the ANCHOR.QUERY energy-minimized binding pose of **2** (yellow lines), and observed the cocrystal structure of (5*R*,6*R*)-**7** (purple stick model) and (5*S*,6*S*)-**7** (orange stick model).

to be further processed into drug-like compounds by removing the sulfhydryl trap in a nontrivial process. Allosteric PDK1 inhibitors can also be discovered through high-throughput screening, an expensive technology accessible mostly to pharmaceutical companies. Another group identified allosteric ligands for the PIF-pocket by using NMR-based screening of a fragment library.^[16] Compounds resulting from fragment screening are often very weak binders and have to be optimized in a nontrivial step-wise process. In addition, this approach is highly specialized and requires expensive instrumentation, which is not generally accessible to researchers. Finally, allosteric modulators directed to the PIF-pocket of PDK1 have been designed based on high-resolution crystal structures taking into account the knowledge of the protein–protein interaction and based on previous crystal structures.^[7,12,17] Despite the structural simplicity of the PIF-pocket binders, complex multistep sequential synthesis is often required. For example, **1** is the product of a 4-step sequential synthesis and the required starting materials are available in only limited diversity, thus making detailed SAR studies challenging. Taken together, all currently described allosteric PDK1 inhibitors are weaker binders than compound **7**. The method we propose herein is complementary to the previously used approaches. The hits based on different scaffolds can be efficiently accessed in one chemical step from commercially available starting materials by using MCR chemistry. The binding of the compounds is verified by biochemical methods and one cocrystal structure could be solved. The discovered scaffold shows very good selectivity for PDK1 over all other screened kinases. The ANCHOR.-QUERY platform derived compound and the cocrystal structure align well. MCR chemistry allows fast and efficient synthesis of these compounds and more detailed biochemical studies will be reported in the near future. The simple approach described herein is based on freeware and uses straightforward chemistry, which can be performed by any skilled personnel. No specific instrumentation that is not present in standard laboratories is needed. We therefore predict a wide use of this method for many different drug-discovery applications.

Experimental Section

One-pot synthesis of compound **2**: A solution of benzaldehyde (102 μ L, 1.0 mmol, 1.0 equiv) and 2-(*p*-tolyl)ethylamine (145 μ L, 1.0 mmol, 1.0 equiv) in acetonitrile (1 mL, 1.0 M) was heated in the microwave at 110 °C for 15 min. The reaction was concentrated under vacuum and *p*-xylene (2 mL) and glutaric anhydride (114 mg, 1.0 mmol, 1.0 equiv) were added to the residue. The resulting reaction mixture was heated under reflux for 6 h. Upon cooling to room temperature, the product precipitated from solution. The solid was collected by filtration, washed with ice-cold ether (3 $\times$ 5 mL) and dried. The product **2** was obtained as a white solid (170 mg, 0.50 mmol, 50%). Melting point: 194 °C; ^1H NMR (500 MHz, CDCl_3) δ = 7.37 (t, J = 7.5 Hz, 2H), 7.30 (t, J = 7.3 Hz, 1H), 7.20 (d, J = 7.4 Hz, 2H), 7.02 (s, 4H), 5.08 (d, J = 3.3 Hz, 1H), 4.24–4.05 (m, 1H), 2.90 (dd, J = 8.8 Hz, J = 4.2 Hz, 1H), 2.87–2.78 (m, 2H), 2.75–2.63 (m, 2H), 2.63–2.52 (m, 1H), 2.27 (s, 3H), 2.12–2.02 (m, 1H), 2.02–1.88 ppm (m, 1H); MS (ESI): m/z (%): 360.2 (100) [M^+ + Na], 338.2 (55) [M^+ + H].

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