# Virtual Docking Approaches to Protein Kinase B Inhibition

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**Abstract:** We examined some in silico approaches to identify Akt (protein kinase B) inhibitors. Experimental validation of selected compounds was achieved using a fluorescence-based enzymatic assay and a substrate phosphorylation assay involving the protein GSK-3. We report on success and failure obtained by using several strategies including FlexX, GOLD, and CSCORE, where the 100−200 top-scoring compounds from a 50000-compound library were experimentally tested. This study led to the identification of low micromolar Akt1 inhibitors.

Protein phosphorylation plays a central role in many cellular events such as proliferation, differentiation, survival, and angiogenesis.[1] Consequently, unregulated kinase activity can result in uncontrolled cellular growth and inappropriate regulation of apoptosis, which is a key mechanism in oncogenesis suppression.[2]

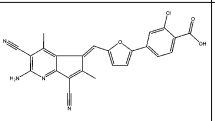
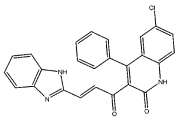
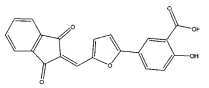
Within this scenario Akt, also known as protein kinase B (PKB), has recently caught scientists' attention, since its aberrant activation has been recognized to be responsible for a wide range of proliferative and antiapoptotic processes in many human tumors.[3] Akt is a subfamily consisting of three different cellular isoforms, namely, Akt1 (PKBα), Akt2 (PKB$\beta$), and Akt3 (PKB$\gamma$ ). Akt1 is mostly involved in breast cancer and in gastric adenocarcinomas; Akt2 is amplified in ovarian, pancreatic, and breast cancers; and Akt3 is amplified in breast cancer and prostate cell lines.[4]

Akt1 is composed of a kinase domain, a N-terminal pleckstrin homology (PH) domain, and a short carboxy-terminal tail region. This protein is activated when Thr308 and Ser473 are phosphorylated.[5] Once activated, Akt1 inhibits apoptosis and stimulates cell cycle progression by phosphorylating numerous targets in various cell types, including cancer cells. Consequently, the development of molecules capable of blocking protein kinase B activity is a valuable route for anticancer drug discovery.[6−9]

Nowadays, high-throughput screening of large chemical databases is a common approach for lead identification. However, given the 3D structure of the protein target, it should be possible to restrict the number of compounds to be tested by using computational docking studies.

In this paper, we describe a number of approaches based on the reported crystal structure of Akt1 kinase. This methodology allowed us to select several potential inhibitors on the basis of their predicted ability of docking into the ATP binding site.

**Table 1.** Structures of Compounds Showing Inhibitory Activity against Akt1

| Molecule | ID | Structure | $IC_{50}$ (μM) | Ki (μM) |
|---|---|---|---|---|
| **1** | 6025233 |  | 2.6 | 1.1 |
| **2** | 5809365 |  | 4.5 | 3.9 |
| **3** | 5378650 |  | 25.1 | 20.8 |

A target binding site was derived from the crystal structure of the ternary complex involving Akt1, nonhydrolyzable form of ATP (AMP-PNP pdb id: 1O6K), and the peptide substrate derived from GSK-3$\beta$.[10] The protein active site was defined including those residues within 6.5 Å from the ATP mimic. Hydrogen atoms were calculated using SYBYL[11] (Tripos, St. Louis, MO), and water molecules, peptide substrate, and the ATP mimic were eliminated. A total of 50000 compounds (Chembridge, San Diego, CA) were subsequently docked and ranked according to the software FlexX (BioSolveIT, Sankt Augustin, Germany).[12,13] In an initial attempt, we selected the top 2000 compounds and ranked them with other scoring functions using CSCORE[14] (SYBYL). Subsequently, we experimentally tested at 10 $\mu$M the top 100 compounds according to Drugscore,[15] the top 200 compounds according to Goldscore,[16] and another top 200 compounds according to Chemscore.[17] Disappointingly, only one inhibitor (**2**, 5809365) common in Goldscore and Chemscore selection was found through the Akt1 assay (Table 1), while no inhibitor was found among compounds selected by Drugscore. In addition, we also docked the FlexX top 4000 compounds using GOLD[18] and subsequently selected and tested the top 200 compounds. Once again, **2** resulted as the only inhibitor (Figure 1A).

On the basis of these results, we relied on another strategy described in Figure 1B. Here, the top 4000 compounds out of 50000 docked compounds were selected using FlexX and Drugscore (BioSolvIT). The top 4000 docked structures were further evaluated and ranked according to Goldscore and Chemscore functions (CSCORE). A list of common 200 compounds was then selected among ranked top 700 compounds according to both scoring functions (Figure 1B). Visual analysis of the 200 docked structures resulted in the elimination of 100 compounds with improbable docking geometry. The remaining 100 compounds were experimentally tested up to 30 $\mu$M against Akt1. The inhibitory activity was evaluated for the selected compounds by using Z'-LYTE kit assay provided by Invitrogen Corporation.[19] Among the experimentally tested compounds at least three emerged as interesting inhibitors, two of which

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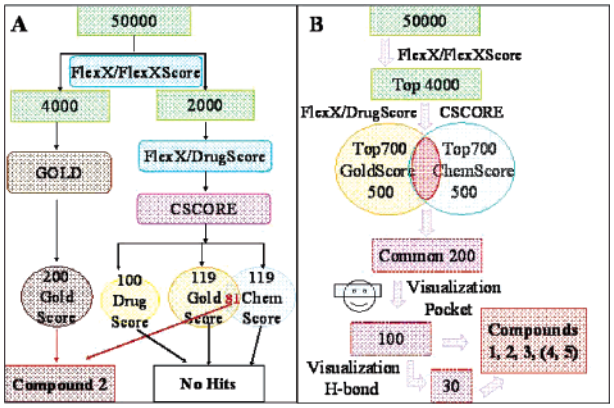



**Figure 1.** Schematic representations of the virtual docking approaches adopted.

showing $IC_{50}$ values in the low-micromolar range. Particularly, **1** and **2** (Table 1) inhibited Akt1 in a concentration range comparable to that of H-89, the only known commercially available Akt inhibitor,[20] yielding $IC_{50}$ of 2.3 and 4.5 $\mu$M, respectively (Figure 2A,B). Compound **3** showed an $IC_{50}$ of 25.1 $\mu$M. Remaining selected compounds did not show any inhibitory activity up to 30 $\mu$M.

A second assay was carried out to further evaluate the inhibitory activity for **1** and **2** by using an immunoblotting assay with anti-phospho-GSK-3$\alpha/\beta$ and GSK-3 as a substrate (Figure 2D−E). In agreement with the Z'-LYTE assay, both compounds inhibited GS3K phosphorylation in the low micromolar range.

To confirm and extend these findings, we measured the $K_i$ value and the type of inhibition of Akt1 by compounds **1**−**3** (Figure 2C). For these purposes, we initially determined the $K_m$ and the $V_{max}$ of the enzymatic reaction involving the peptide provided by the Z'-LYTE kit assay and Akt1 by varying the concentration of ATP. The above parameters appeared to be 7.9 $\mu$M and 0.0205 $\mu$mol min$^{-1}$ mg$^{-1}$, respectively. We then used a 10 $\mu$M concentration of **1**, 20 $\mu$M of **2**, and 50 $\mu$M of **3** to identify the inhibitors' $K_i$ values (Table 1). Because all our inhibitors affected the $K_m$ rather than the $V_{max}$ of the reaction (Figure 2C), they can be truly considered ATP-competitive inhibitors of Akt1.

To rule out the possibility of eventual nonspecific interactions, we also verified that no substantial changes in the $IC_{50}$ values for **1** were detected when increasing 10-fold the protein concentration and when preincubating the compounds with Akt1 for 30 min prior measuring its $IC_{50}$ value. These tests have been shown to give dramatically different $IC_{50}$ values in the presence of nonspecific ligand−protein interactions.[21]

In addition, we tested our compounds against a nonrelated protein kinase, Abl1,[22] which is a tyrosine kinase under investigation in our laboratory. Our compounds did not inhibit this kinase up to 100 $\mu$M. We currently do not have data on the selectivity of our compounds for the different Akt isoforms. Therefore, using our structure-based approach, we were able to identify three inhibitors of Akt1 (Table 1), two of which show an inhibitory activity comparable to that of H-89 (Figure 2). On the basis of the docked geometry and in agreement with our experimental data, it appears that all three inhibitors place themselves nicely into the catalytic site of the ATP, resembling the binding of the

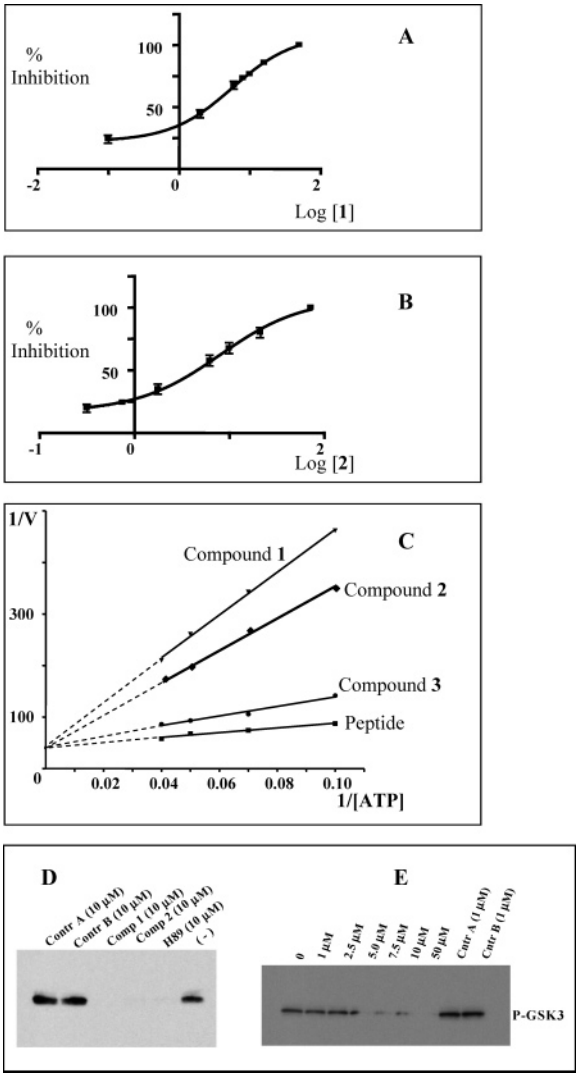



**Figure 2.** (A) $IC_{50}$ evaluation for **1** (2.6 $\mu$M). The Hill slope for this curve is 1.1. (B) $IC_{50}$ evaluation for **2** (4.5 $\mu$M). Corning 384-well low-volume plates (20 $\mu$L) were used. The fluorescent enzymatic assay has been performed following the protocol provided by Invitrogen Corporation, using a fluorescent plate reader (Victor2, Perkin-Elmer). $IC_{50}$ values were determined by fitting the data to the sigmoidal dose response equation and plotting the observed percentage of inhibition versus the logarithm of inhibitor concentration using GraphPad Prism. (C) Lineweaver−Burk $K_m$ and $K_m$(app) evaluation for Akt1. Each measurement was performed in triplicate. The $K_m$ and $V_{max}$ values of the enzymatic reaction were determined at 25 °C by using increasing ATP concentrations (5, 10, 15, 20 and 25 $\mu$M). The $K_i$ and the $K_{m(app)}$ were calculated at fixed inhibitor concentration, as reported in the text. All constant values were evaluated by fitting the data to the Lineweaver−Burk plot. (D) Akt1 inhibition assay using GSK-3 as a substrate, showing a comparison of **1** and **2** (Table 1) with H89 at 10 $\mu$M. (E) Dose response for **1**. Akt (10 ng of recombinant enzyme) in 25 $\mu$L 1× kinase buffer (25 mM Tris, pH 7.5; 5 mM $\beta$-glycerol phosphate; 2 mM dithiothreitol; 0.1 mM $Na_3VO_4$; 10 mM $MgCl_2$) was mixed with 2.5 $\mu$L of DMSO(1% stock) or MPA-D (100 $\mu$M in 1% DMSO). Samples were incubated on ice for 1.5 h, at which time 1 $\mu$g of GSK-3 fusion protein (cell signaling), which served as the substrate, was added followed by ATP (200 $\mu$M) to each reaction mixture. After the suspensions were incubated at 30 °C for 20 min, the reaction was terminated by the addition of 3× SDS sample buffer (187.5 mM Tris-HCl, pH 6.8; 6% SDS; 30% glycerol; 150 mM dithiothreitol; 0.03% bromophenol blue). The samples were boiled for 5 min, and the proteins were separated on a 12% SDS−polyacrylamide gel and subsequently transferred to a nitrocellulose membrane. Membranes were incubated with rabbit polyclonal anti-phospho-GSK-3$\alpha/\beta$ (Ser21/9) (cell signaling).

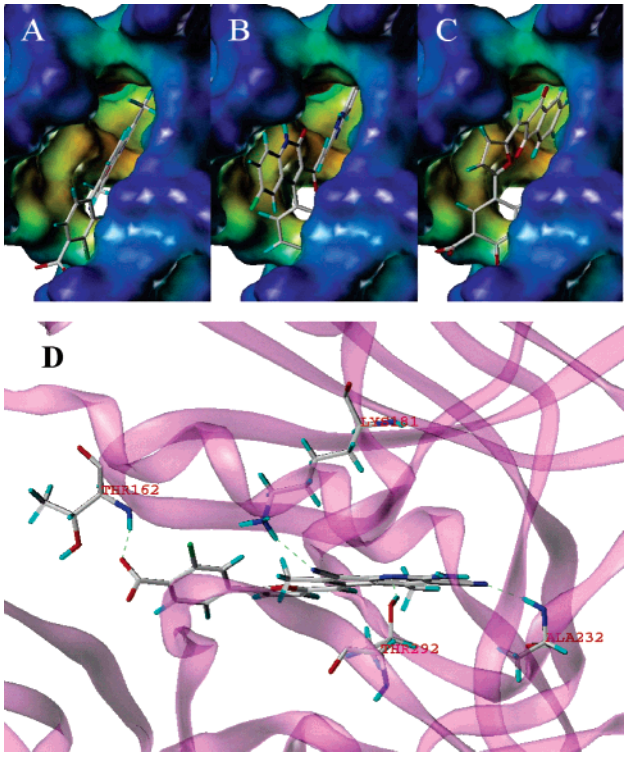
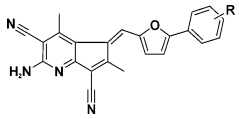



**Figure 3.** (A−C) Docked structures of **1**−**3** into the ATP binding site of Akt1. The 2D structures of 50 000 compounds were converted to 3D structures using CONCORD[25] or CORINA.[26] Two docking programs were used to screen compounds against Akt1 kinase. The FlexX program applied Drugscore to determine docked conformers. The GOLD package docked ligands using the Goldscore fitness function. Consensus scoring was obtained by using CSCORE (SYBYL). (D) Hydrogen bonds between **1** and residues present in the Akt1 catalytic pocket.

**Table 2.** Derivatives of **1** and Their Inhibitory Activity against Akt1




| compd | R | $IC_{50}$ ($\mu$M) |
|---|---|---|
| **1** | *p*-COOH, *m*-Cl | 2.6 |
| **4** | *p*-COOH | 126 |
| **5** | *m*-COOH | 60.2 |

adenosine moiety of the cofactor (Figure 3A−C). Indeed, each compound is involved in H-bonding interactions with residues Lys181, Ala232, Thr292, and Thr162 (Figure 3D) similar to interactions observed in the crystal structure of Akt1 in complex with AMP-PNP.

Accordingly, measurement of inhibitory properties of 13 additional analogues of **1** revealed that only **4** and **5**, both capable of forming H-bonds with the above-mentioned residues, showed appreciable inhibition in the micromolar range (Table 2). Therefore, the ability of a given compound to be involved in H-bond interaction appears to be essential in all inhibitor−Akt1 complexes as previously reported for other protein kinases[23] and in other docking studies.[27] In fact, when the ability to form H-bonds is taken into account for the selection of candidate inhibitors, only 30 compounds would be selected. As described in Figure 1B, our selected 30 compounds contain all three hits, thus yielding a hit rate of 10%.

Despite the availability of many reliable in silico approaches and robust in vitro commercially available assays, discovering Akt inhibitors still remains a challenging task. Although several attempts have been performed in this field, there are presently no marketable inhibitors against Akt1 besides H-89. In fact, a very recent study based on high-throughput screening led to the characterization of only two Akt1 low-micromolar inhibitors out of 270 000 tested compounds.[23] Moreover, during our ongoing efforts to identify molecules capable of blocking Akt-kinases activity, we tested a library of 2000 natural products (Microsource) at concentrations up to 30 $\mu$M against Akt1, but no compound emerged as an effective low-micromolar inhibitor (data not shown).

In conclusion, we describe two different structure-based strategies we adopted in order to discover compounds inhibiting Akt1. When we applied the strategy described in Figure 1A, simply relying on results provided by the scoring functions, the hit rate appeared to be only slightly superior to the one expected from a random approach (0.01−0.5%).[24] However, when our docking methodology outcome was analyzed by taking into account a consensus between scoring functions and H-bonding patterns similar to those observed in the crystal structure of Akt1 in complex with AMP-PNP, a remarkable 10% hit rate was achieved (Figure 1B).

We believe that the two low-micromolar inhibitors described here may represent a starting point for finding potent and selective molecules capable of preventing Akt1 activity in human tumoral cells.

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