

An ATP-Site On-Off Switch That Restricts Phosphatase Accessibility of Akt

Kui Lin,^{1*} Jie Lin,¹ Wen-I Wu,² Joshua Ballard,² Brian B. Lee,¹
Susan L. Gloor,^{2†} Guy P. A. Vigers,² Tony H. Morales,² Lori S. Friedman,¹
Nicholas Skelton,¹ Barbara J. Brandhuber^{2*}

The protein serine-threonine kinase Akt undergoes a substantial conformational change upon activation, which is induced by the phosphorylation of two critical regulatory residues, threonine 308 and serine 473. Paradoxically, treating cells with adenosine 5'-triphosphate (ATP)-competitive inhibitors of Akt results in increased phosphorylation of both residues. We show that binding of ATP-competitive inhibitors stabilized a conformation in which both phosphorylated sites were inaccessible to phosphatases. ATP binding also produced this protection of the phosphorylated sites, whereas interaction with its hydrolysis product adenosine 5'-diphosphate (ADP) or allosteric Akt inhibitors resulted in increased accessibility of these phosphorylated residues. ATP-competitive inhibitors mimicked ATP by targeting active Akt. Forms of Akt activated by an oncogenic mutation or myristoylation were more potently inhibited by the ATP-competitive inhibitors than was wild-type Akt. These data support a new model of kinase regulation, wherein nucleotides modulate an on-off switch in Akt through conformational changes, which is disrupted by ATP-competitive inhibitors.

INTRODUCTION

The serine-threonine protein kinase Akt (also known as protein kinase B or PKB) plays a central role in propagating growth signals, metabolism, and cell survival, making it an attractive cancer therapeutic target (1–3). The three human Akt protein isoforms (Akt1, 2, and 3) have multiple domains: an N-terminal pleckstrin homology (PH) domain, an interdomain linker, a kinase domain, and a C-terminal hydrophobic motif (4). Akt is activated by a multistep process that results in phosphorylation of two critical residues, Thr³⁰⁸ in the activation loop and Ser⁴⁷³ in the hydrophobic motif, which induces a substantial conformational change that leads to a greater than 1000-fold increase in its kinase activity (5–7). An initiating step in the activation of Akt is its recruitment to the plasma membrane. The PH domain directs the translocation of Akt from the cytosol to the plasma membrane by binding to the products of phosphatidylinositol 3-kinase (PI3K) (4). Before Akt's membrane translocation and phosphorylation, the protein assumes a PH-in conformation in which the PH domain interacts extensively with the kinase domain and crucial adenosine 5'-triphosphate (ATP) binding site residues are involved in interdomain interactions with residues in the PH domain (8, 9). Upon membrane translocation and subsequent phosphorylation, Akt assumes a catalytically active PH-out conformation characterized by movement of the PH and kinase domains relative to each other, allowing formation of a catalytically competent kinase (8). In addition to the movement of the regulatory PH domain, during the activation of Akt, the conserved DFG motif in the kinase domain shifts from the inactive DFG-out conformer, which is stabilized in the PH-in conformation by interactions with the PH domain, to the active DFG-in conformer in which the phenylalanine residue has rotated out of the ATP binding site and the aspartic acid residue is positioned to coordinate ATP-associated metal ions (6, 9, 10). The pivotal position of

Akt in the PI3K-Akt-mTOR (mammalian target of rapamycin) pathway, which promotes many cellular activities critical for oncogenesis such as cellular proliferation, and its frequent activation in human cancers have led to the pursuit of Akt inhibitors for cancer therapy (11). Both active site-directed ATP-competitive inhibitors and non-ATP-competitive allosteric compounds have been reported. Although allosteric inhibitors block Akt phosphorylation (12), ATP-competitive inhibitors paradoxically increase phosphorylation of Akt in cells (13–15).

Here, we investigated the molecular mechanism by which ATP-competitive inhibitors induce increased phosphorylation of Akt and demonstrate that ATP site occupation causes stabilization of a conformation in which the phosphorylated sites are protected from dephosphorylation by phosphatases. These findings suggest a mechanism for regulating kinase activity through nucleotide binding, with important therapeutic implications for the different classes of Akt inhibitors.

RESULTS

ATP-competitive inhibitors protect Akt from dephosphorylation

GDC-0068 is a highly selective ATP-competitive pan-Akt inhibitor (fig. S1) (16). Similar to other ATP-competitive inhibitors, GDC-0068 induces a dose-dependent increase in phosphorylation of Akt at both Thr³⁰⁸ and Ser⁴⁷³ residues in multiple cell lines, despite inhibiting Akt signaling as evidenced by reduced phosphorylation of multiple Akt downstream targets, such as proline-rich Akt substrate of 40 kD (PRAS40), the transcription factors FoxO1 and FoxO3a, and the ribosomal protein S6 (Fig. 1A). This increased phosphorylation of Akt is observed at concentrations that do not suppress phosphorylation of S6, consistent with a mechanism that is independent of the feedback regulation involving p70S6 kinase downstream of Akt (17). Although this effect occurs through a kinase-intrinsic mechanism (15), the precise mechanism has not been elucidated and could occur through increased phosphorylation of Akt by its upstream kinases or reduced dephosphorylation of Akt by phosphatases.

¹Genentech Inc., South San Francisco, CA 94080, USA. ²Array BioPharma Inc., Boulder, CO 80301, USA.

*To whom correspondence should be addressed. E-mail: klin@gene.com (K.L.); bbrandhuber@arraybiopharma.com (B.J.B.)

†Present address: Luca Technologies Inc., Golden, CO 80401, USA.

We first inquired whether GDC-0068 caused increased phosphorylation of Thr³⁰⁸ by phosphoinositide-dependent protein kinase 1 (PDK1), the physiological kinase for this site (5). GDC-0068 did not significantly affect the ability of PDK1 to phosphorylate Thr³⁰⁸ in unactivated recombinant Akt (Fig. 1B). We then examined the effect of GDC-0068 on the ability of phosphatases to dephosphorylate active Akt. As previously reported (18), protein phosphatase 2A (PP2A) caused a dose-dependent de-

phosphorylation of Akt1 on both Thr³⁰⁸ and Ser⁴⁷³ residues (Fig. 1C), an effect that was blocked when Akt was preincubated with GDC-0068. This protective effect was reduced if GDC-0068 and PP2A were added simultaneously to Akt1 (Fig. 1D), consistent with the protection requiring binding of GDC-0068 to Akt1. GDC-0068 did not affect the ability of PP2A to dephosphorylate activated MEK2 (mitogen-activated or extracellular signal-regulated protein kinase kinase 2) (Fig. 1E), or a peptide

substrate in a phosphatase assay (fig. S2A), demonstrating that GDC-0068 does not directly inhibit PP2A. Analogous protective effects from PP2A were observed for active Akt2 and Akt3 incubated with GDC-0068 or other ATP-competitive Akt inhibitors either structurally related to GDC-0068, such as GNE-692 (19) and GNE-376 (20), or structurally unrelated, such as GSK690693 (14) (figs. S1 and S3, A to E). Similarly, the ATP-competitive compounds also protected Akt from dephosphorylation by PP1 (fig. S4) with no direct inhibitory effect on PP1 activity (fig. S2B). We also tested the protein phosphatase PHLPP1, which dephosphorylates Akt on Ser⁴⁷³ but not Thr³⁰⁸ (21), and GDC-0068 also protected activated Akt2 from dephosphorylation at Ser⁴⁷⁴ (the equivalent of Ser⁴⁷³ in Akt1) by this enzyme (fig. S3F). In contrast, preincubation of active Akt1 with an allosteric inhibitor of Akt1 and Akt2, Akti-1/2 (22), did not protect the phosphorylation sites but resulted in more efficient dephosphorylation of Ser⁴⁷³ by PP2A, with no significant effect on the dephosphorylation of Thr³⁰⁸ (Fig. 1F).

If the increase in Akt phosphorylation observed in cells is primarily due to protection from dephosphorylation, pretreatment of cells with an ATP-competitive compound should lock Akt in the phosphorylated state, and Akt should remain phosphorylated when its upstream kinases PDK1 and mTOR complex 2 (mTORC2) (23) are subsequently inhibited (fig. S5, scenario A). Conversely, if the increased phosphorylation is due to increased phosphorylation by upstream kinases, pretreatment with an ATP-competitive inhibitor followed by treatment with inhibitors of the upstream kinases should lead to dephosphorylation of Akt as a result of persistent phosphatase activity (fig. S5, scenario B). Treatment of NIH 3T3 cells expressing human Akt1 with ATP-competitive Akt inhibitors resulted in increased phosphorylation of Akt on both Thr³⁰⁸ and Ser⁴⁷³ (Fig. 2). Addition of the dual PI3K and mTOR kinase inhibitor, GNE-493 (24), did not block the increased phosphorylation of Thr³⁰⁸ or Ser⁴⁷³ when added to cells after pretreatment with the Akt inhibitors (Fig. 2A). Likewise, the

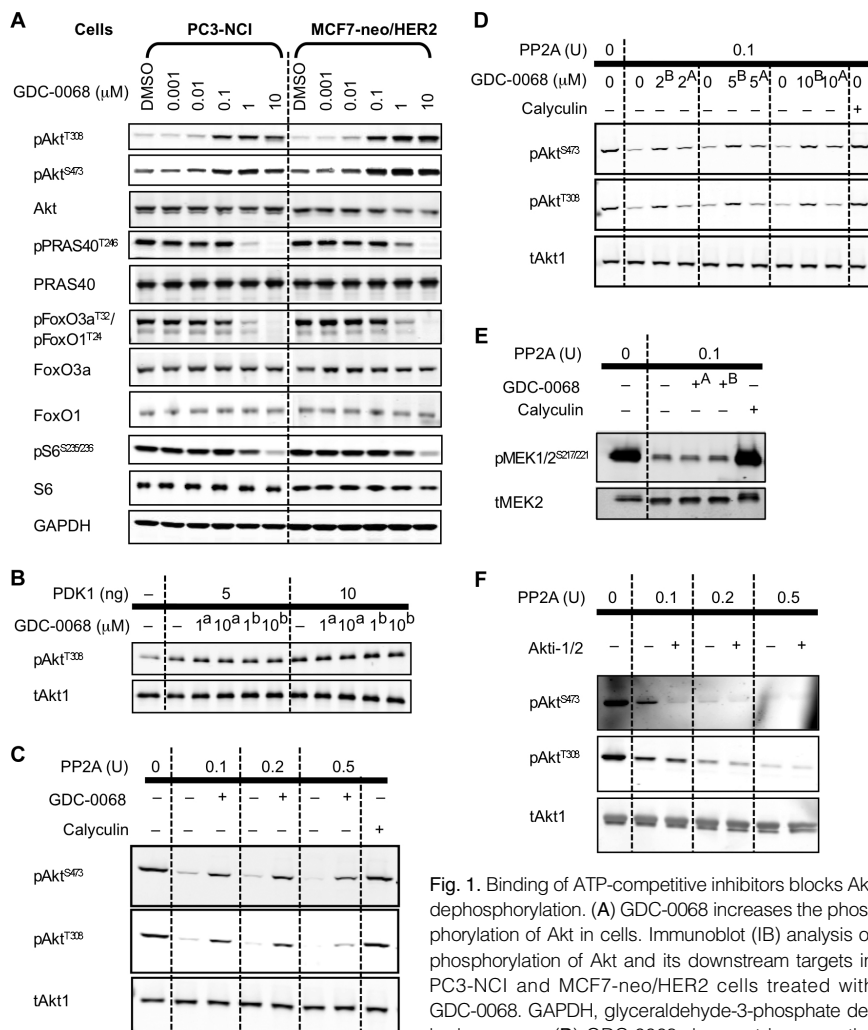


Fig. 1. Binding of ATP-competitive inhibitors blocks Akt dephosphorylation. (A) GDC-0068 increases the phosphorylation of Akt in cells. Immunoblot (IB) analysis of phosphorylation of Akt and its downstream targets in PC3-NCI and MCF7-neo/HER2 cells treated with GDC-0068. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (B) GDC-0068 does not increase the phosphorylation of inactive Akt1 by PDK1. GDC-0068

was either added simultaneously (^a) with PDK1 or preincubated with Akt (^b) before PDK1 was added. (C) Preincubation of GDC-0068 with activated His-Akt1 blocks dephosphorylation by PP2A. (D) Protection from dephosphorylation requires binding of GDC-0068 to Akt1. GDC-0068 was either preincubated with Akt1 (^b) or added simultaneously (^a) with PP2A. (E) GDC-0068 does not block dephosphorylation of phosphorylated MEK by PP2A. GDC-0068 was either preincubated with MEK2 (^b) or added simultaneously (^a) with PP2A. (F) The allosteric Akt inhibitor Akti-1/2 increased dephosphorylation of Ser⁴⁷³. Activated His-Akt1 was preincubated with Akti-1/2 before addition of PP2A. Representative data from at least three experiments are shown. Quantification and statistical analysis are provided in table S4.

PDK1 inhibitor BX-795 (25) caused only a slight reduction in the phosphorylation of Thr³⁰⁸ when added after pretreatment with the Akt inhibitors (Fig. 2B). As expected for both scenarios, blocking both PI3K and mTOR kinase activities with GNE-493 before incubation with the Akt inhibitors effectively blocked the increased phosphorylation on both Thr³⁰⁸ and Ser⁴⁷³ residues, whereas pretreatment with the PDK1 inhibitor BX-

795 blocked increased phosphorylation of the PDK1 site Thr³⁰⁸. These results are consistent with scenario A (fig. S5), in which the increase in phosphorylation of Akt is predominantly due to protection from dephosphorylation rather than increased phosphorylation by upstream kinases.

ATP-competitive inhibitors block the accessibility of phosphorylated Thr³⁰⁸ and Ser⁴⁷³ sites

Akt is a structurally plastic enzyme, with large conformational differences observed between the inactive and the active kinase domain structures as demonstrated for Akt2 (6), including disorder-to-order changes in the N-terminal lobe (N lobe), activation loop, and hydrophobic motif. Analysis of Akt crystal structures provides a basis for understanding the mechanism by which inhibitor binding stabilizes the phosphorylated state of the enzyme. The crystal structure of GDC-0068-bound activated Akt1 kinase was solved to 2.0 Å (table S1) and revealed that GDC-0068 does not directly interact with either phosphorylated residue (Fig. 3A) because the inhibitor binds about 20 Å from both phosphorylation sites. However, GDC-0068 binds between the N lobe and the C-terminal lobe (C lobe) of the kinase and appears to restrict intra- and interdomain movement through its extensive contacts with residues in both lobes (table S2). When Akt is in an inhibited, conformationally restricted state, the phosphate group on Thr³⁰⁸ has intramolecular interactions with Arg²⁷³ and Lys²⁹⁷ (Fig. 3B). In addition, both phosphorylated Thr³⁰⁸ and the phosphomimetic residue, Ser⁴⁷³→Asp (S473D), are positioned close to the main surface of the kinase and do not protrude from the surface in an extended conformation (Fig. 3C), thus limiting the accessibility to phosphatases. Similarly, the activated structures of Akt1 (Fig. 3D and table S1) and Akt2 (6) in complex with adenylyl-imidodiphosphate (AMP-PNP) and a glycogen synthase kinase-3β (GSK3β) substrate peptide reveal limited accessibility to the phosphorylation sites.

To test whether the interactions of the phosphate group of Thr³⁰⁸ with the basic residues in the activation loop and Ser⁴⁷³ with the hydrophobic motif contribute to the protection against dephosphorylation, we mutated Arg²⁷³ and Ser⁴⁷³ into alanines in myristoylated Akt1 (MyrAkt1), a membrane-attached form of Akt that is constitutively phosphorylated and activated in cells. When expressed in MEF3T3 cells, the MyrAkt1 Arg²⁷³→Ala (R273A) mutant exhibited reduced phosphorylation at both Thr³⁰⁸ and Ser⁴⁷³ residues compared to wild-type MyrAkt1, and the Ser⁴⁷³→Ala (S473A) mutant showed diminished phosphorylation at Thr³⁰⁸ (Fig. 3E). These effects could be partially rescued by okadaic acid, an inhibitor of protein serine-threonine phosphatases, suggesting that these mutations promote dephosphorylation of Akt and that the intramolecular interactions of the phosphorylated residues provide some degree of protection for activated Akt against phosphatases even in the absence of the ATP-competitive inhibitors. Treatment with GDC-0068 increased the phosphorylation of the R273A mutant at both Ser⁴⁷³ and Thr³⁰⁸, although with reduced efficiency at the Thr³⁰⁸ site, consistent with the hypothesis that interaction with Arg²⁷³ is required for the optimal protection of Thr³⁰⁸ phosphorylation. GDC-0068 also rescued phosphorylation of the S473A mutant at Thr³⁰⁸ to a small extent, suggesting that interaction of phosphorylated Ser⁴⁷³ with the hydrophobic motif can further increase GDC-0068-mediated stabilization of the Thr³⁰⁸ site or increase the affinity of GDC-0068 to the activated conformation of Akt (or both).

We hypothesized that the ATP-competitive inhibitor-bound conformation of phosphorylated Thr³⁰⁸ in the activation loop and phosphorylated Ser⁴⁷³ in the hydrophobic motif would make these sites inaccessible not only to phosphatases but also to phosphorylation site-specific antibodies under native, nondenaturing conditions. The ATP-competitive inhibitors

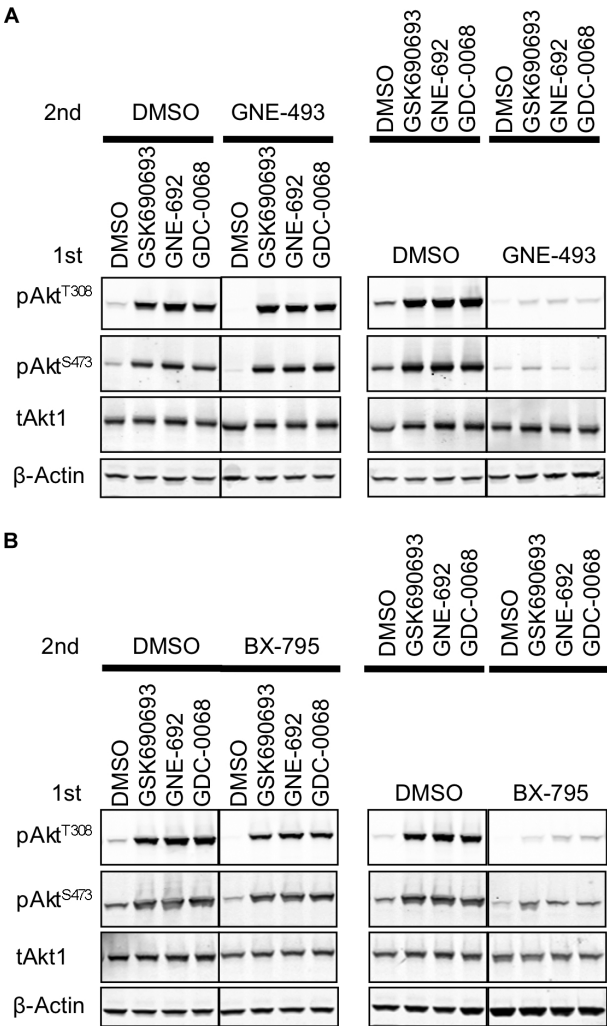


Fig. 2. ATP-competitive inhibitors block dephosphorylation of already phosphorylated Akt in cells rather than causing de novo phosphorylation. (A) NIH 3T3 cells expressing FLAG-Akt1 were pretreated with cycloheximide and MG-132 to block protein synthesis and degradation. Cells were then first treated with DMSO or 1 μM of the indicated ATP-competitive Akt inhibitors for 45 min, followed by DMSO or 1 μM of the dual PI3K/mTOR inhibitor GNE-493 for another 45 min, or vice versa. (B) NIH 3T3 cells expressing FLAG-Akt1 were treated and analyzed as in (A), except GNE-493 was replaced with a PDK1 inhibitor, BX-795. Representative data of three experiments are shown. Quantification and statistical analysis are provided in table S4.

GSK690693 and GDC-0068 both induced increased phosphorylation of Thr³⁰⁸ and Ser⁴⁷³ of FLAG-tagged Akt1 as detected by immunoblotting of denatured whole-cell lysates with anti-phosphorylated Thr³⁰⁸ and Ser⁴⁷³ antibodies (Fig. 4A). Okadaic acid also caused increased phosphorylation at Ser⁴⁷³ and, to a less extent, Thr³⁰⁸, consistent with previous reports (8). An anti-FLAG antibody immunoprecipitated more FLAG-Akt1 phosphorylated at Thr³⁰⁸ and Ser⁴⁷³ from cells treated with GSK690693, GDC-0068, or okadaic acid compared to that pulled down from dimethyl sulfoxide (DMSO)-treated cells. Furthermore, the relative ratios were similar to those seen in the denatured whole-cell lysates. The phosphospecific antibodies immunoprecipitated most of the Akt phosphorylated at Thr³⁰⁸ and Ser⁴⁷³ in okadaic acid-treated cells. However, the phosphospecific antibodies failed to pull down most of Akt phosphorylated at these sites in cells treated with the two Akt inhibitors. These observations are consistent with the decreased accessibility of phosphorylated Thr³⁰⁸ and Ser⁴⁷³ in the ATP-competitive inhibitor-bound forms of Akt. In comparison, a nonphosphospecific antibody that recognizes epitopes in the N- and C-terminal regions of Akt could proportionally pull down phosphorylated Akt in cells with the different treatments (Fig. 4A), suggesting that these epitopes are less affected by the shift in conformational equilibrium induced by the ATP-competitive inhibitors.

Similar protection from the antibodies was also observed in vitro with purified recombinant His-tagged, catalytically active Akt1 phosphorylated

on both Thr³⁰⁸ and Ser⁴⁷³ (Fig. 4B). Incubation of active His-Akt1 with an ATP-competitive inhibitor reduced the ability of phosphospecific Ser⁴⁷³ and Thr³⁰⁸ antibodies, but not that of anti-His antibody, to immunoprecipitate Akt. Immunoprecipitation of active His-Akt1 incubated with the allosteric inhibitor Akti-1/2 was equivalent for the anti-phosphorylated Thr³⁰⁸ antibody but enhanced for the anti-phosphorylated Ser⁴⁷³ antibody compared to DMSO-treated control, consistent with the dephosphorylation assay results (Fig. 1F). These observations are in accordance with the allosterically inhibited structure of Akt1, which shows that several residues in the activation loop and hydrophobic motif binding groove are displaced by the PH domain relative to the activated structure, thereby increasing the accessibility of Thr³⁰⁸ and Ser⁴⁷³ (9), which most likely contributes to the rapid dephosphorylation of Akt in cells treated with the allosteric inhibitors (12).

The reduced accessibility to phosphorylation site-specific antibodies suggests that detection methods dependent on recognition of phosphorylated Akt under nondenaturing conditions may underestimate the amount of phosphorylated Akt in cells treated with the ATP-competitive inhibitors. For example, the Meso Scale Discovery (MSD) phosphorylated Akt/total Akt kits use an immobilized capture antibody for total Akt and a SULFO-TAG-labeled detection antibody for phosphorylated Akt or a different epitope in Akt. We found that under nondenaturing conditions, incubation of activated recombinant Akt with the ATP-competitive inhibitors tested resulted in reduced detection of phosphorylated Thr³⁰⁸ and Ser⁴⁷³ compared to vehicle treated control (Fig. 5A). Fluorescence resonance energy transfer (FRET)-based homogeneous time-resolved fluorescence (HTRF) sandwich immunoassays can be used to detect the abundance of phosphorylated epitopes by use of two monoclonal antibodies coupled to HTRF donor and acceptor dyes, one specific for the phosphorylated residue to be detected, the second to another epitope in the kinase. We used an anti-phosphorylated Thr³⁰⁸ antibody labeled with a cryptate donor and an anti-His antibody labeled with a d2 acceptor to assess the availability of phosphorylated Thr³⁰⁸ epitope. We found that this assay was also sensitive to the presence of ATP-competitive inhibitors. As low a concentration as 0.08 μ M of GDC-0068 caused ~50% reduction in the FRET signal (Fig. 5B).

ATP binding protects Akt phosphorylation sites, whereas adenosine 5'-diphosphate has the opposite effect

Because the crystal structures of Akt1 bound to GDC-0068 and to AMP-PNP were similar, we hypothesized that ATP binding to activated Akt1 could also protect the phosphorylation sites. Indeed, incubation of active His-Akt1 with ATP or AMP-PNP resulted in reduced accessibility to the phosphorylated Thr³⁰⁸ antibodies (Fig. 5, A, C, and D). The effect on accessibility to phosphorylated Ser⁴⁷³ was less

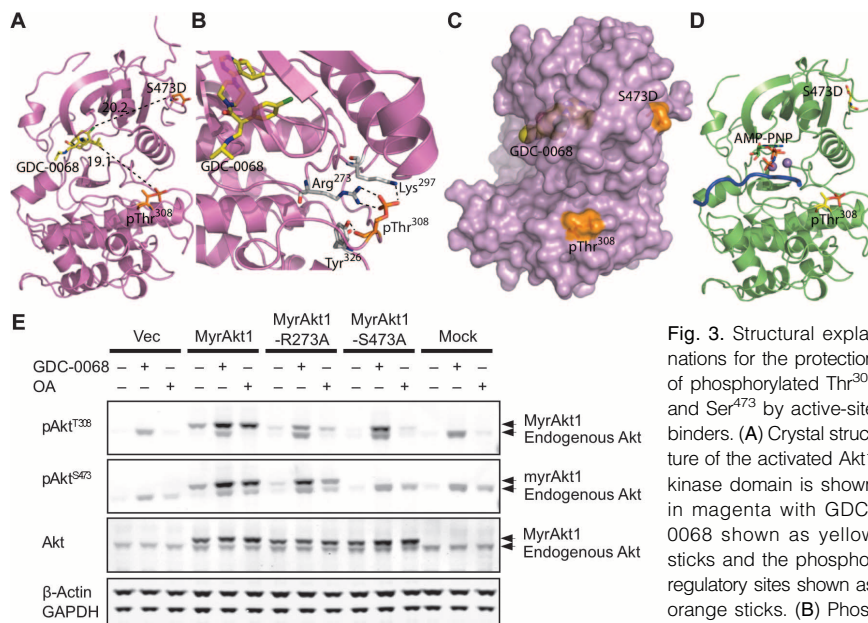


Fig. 3. Structural explanations for the protection of phosphorylated Thr³⁰⁸ and Ser⁴⁷³ by active-site binders. (A) Crystal structure of the activated Akt1 kinase domain is shown in magenta with GDC-0068 shown as yellow sticks and the phosphoregulatory sites shown as orange sticks. (B) Phosphorylated Thr³⁰⁸ (orange sticks) interacts with Arg²⁷³, Lys²⁹⁷, and Tyr³²⁶ in the structure of Akt1 complexed to GDC-0068 (yellow sticks). (C) Surface representation of Akt1 complexed to GDC-0068 (yellow) illustrating the surface locations of phosphorylated Thr³⁰⁸ (orange) and S473D (orange). (D) Crystal structure of the activated Akt1 kinase domain is shown as a cartoon (green) complexed to AMP-PNP (orange sticks), Mn²⁺ (purple spheres), and GSK3 β peptide (blue ribbon) illustrated in the same orientation as Akt1 in (A). (E) Effects of the R273A and S473A mutations on phosphorylated Akt. MEF3T3 cells transfected with myristoylated wild-type Akt1, R273A, or S473A mutants were serum-starved and treated with or without GDC-0068 or okadaic acid (OA) for 1 hour. Cell lysates were analyzed by Western blots. Representative data of three experiments are shown. Quantification and statistical analysis are provided in table S4.

on both Thr³⁰⁸ and Ser⁴⁷³ (Fig. 4B). Incubation of active His-Akt1 with an ATP-competitive inhibitor reduced the ability of phosphospecific Ser⁴⁷³ and Thr³⁰⁸ antibodies, but not that of anti-His antibody, to immunoprecipitate Akt. Immunoprecipitation of active His-Akt1 incubated with the allosteric inhibitor Akti-1/2 was equivalent for the anti-phosphorylated Thr³⁰⁸ antibody but enhanced for the anti-phosphorylated Ser⁴⁷³ antibody compared to DMSO-treated control, consistent with the dephosphorylation assay results (Fig. 1F). These observations are in accordance with the allosterically inhibited structure of Akt1, which shows that several residues in the activation loop and hydrophobic motif binding groove are displaced by the PH domain relative to the activated structure, thereby increasing the accessibility of Thr³⁰⁸ and Ser⁴⁷³ (9), which most likely contributes to the rapid dephosphorylation of Akt in cells treated with the allosteric inhibitors (12).

pronounced (Fig. 5, A and D), suggesting that ATP may be less effective than ATP-competitive inhibitors at stabilizing the interaction between phosphorylated Thr⁴⁷³ and the hydrophobic groove. ATP also reduced recognition by non-phosphosite-specific Akt antibodies, either with an Akt1-specific antibody that recognizes an epitope within the PH domain (Fig. 5D; this effect was also seen with AMP-PNP) or with the anti-total Akt antibodies included in the MSD kit (Fig. 5A).

Extensive enzymatic characterization of another AGC kinase, the cAMP (cyclic adenosine monophosphate)-dependent protein kinase (PKA), indicates that adenosine 5'-diphosphate (ADP) release is a rate-limiting component of enzyme turnover (26, 27) suggestive of structural changes in the protein. We speculated that hydrolysis of ATP to ADP and its subsequent release from Akt requires a conformational change in the protein. Indeed, incubation with ADP increased the accessibility of both phosphorylated Thr³⁰⁸ and Ser⁴⁷³ to their respective antibodies, as well as to the PH domain-specific Akt1 antibody (Fig. 5E), suggesting that ADP-bound Akt assumes a different conformation compared to that of the ATP or the ATP-competitive inhibitor-bound state. Using the HTRF sandwich immunoassay described above to analyze the accessibility of phosphorylated Thr³⁰⁸, we found that ADP reduced the time required for the anti-

phosphorylated Thr³⁰⁸ antibody to bind and reach the maximum FRET signal compared to vehicle control (Fig. 5C). The opposite effects of ADP and ATP on the accessibility of the phosphorylated Akt residues, and the high concentration of ADP (in the millimolar range) required for this effect, led us to propose a model in which binding of ATP protects Akt from dephosphorylation until ATP is hydrolyzed into ADP, upon which a conformational change occurs that promotes the dephosphorylation and inactivation of Akt.

Both PP2A and PP1 are inhibited by ADP, ATP, or AMP-PNP in cell-free systems (fig. S2, B to D), consistent with previous reports (28). Therefore, it is difficult to directly assess the effect of these nucleotides on dephosphorylation of Akt. In cells, ATP and ADP are complexed with divalent metal ions that can counteract this inhibitory effect (28). Indeed, 10 mM MgCl₂ reduced ATP-mediated inhibition of PP2A activity as detected with a peptide substrate, especially at ATP concentrations below 1 mM (fig. S2D). When Akt was used as a substrate, however, as low a concentration as 0.2 mM of ATP still inhibited Akt dephosphorylation in the presence of 10 mM MgCl₂, suggesting that this is at least in part due to its stabilization of the active Akt conformation (fig. S2E). We reasoned that triggering of ATP hydrolysis into ADP in the presence of an Akt substrate would counteract this inhibitory effect. Indeed, in the presence of a fusion protein containing a peptide analog of the Akt substrate site in GSK3 (crosstide), the inhibitory effect of ATP was reduced, consistent with hydrolysis of ATP into ADP transiently increasing the exposure of the phosphorylated residues (fig. S2E).

To further address whether ATP hydrolysis into ADP in the presence of an Akt substrate increases the exposure of the phosphorylated residues, we compared the efficiency of anti-phosphorylated Thr³⁰⁸ antibody to pull down Akt in the presence or absence of a GSK3 peptide substrate (GRPRTSFAE) and ATP in a kinase reaction buffer. Indeed, the effect of ATP to block access to phosphorylated Thr³⁰⁸ diminished in the presence of the peptide substrate, resulting in immunoprecipitation of similar amounts of Akt with or without ATP, in contrast to the reduced pull down of phosphorylated Akt with only ATP present (fig. S2F).

ATP-competitive inhibitors selectively target activated Akt

Because the ATP-competitive inhibitors stabilize the phosphorylated active conformation, we expected that ATP-competitive inhibitors would preferentially bind to the activated state of Akt. Comparison of the structure of Akt1 in an inactive state bound to an allosteric inhibitor (9) to the GDC-0068-bound active Akt structure revealed that ATP-competitive inhibitors are sterically hindered from binding to the inactive Akt conformation (Fig. 6, A to D). In the allosterically inhibited inactive Akt1 structure, Tyr²²⁹ is rotated into the ATP binding pocket, blocking binding of nucleotides or ATP-competitive inhibitors. In addition, the inactive, DFG-out loop conformation is incompatible with high-affinity GDC-0068 binding because of steric hindrance from Phe²⁹³. Accordingly, ATP-competitive inhibitors showed ~200-fold higher affinity for the activated Akt compared to inactive Akt in a LanthaScreen displacement binding assay (table S3), which measures the displacement of a tracer molecule bound to Akt by a kinase inhibitor. Furthermore, NIH 3T3 cells expressing an oncogenic mutant Akt1 (Glu¹⁷→Lys; E17K) or a myristoylated Akt1 were more sensitive to growth inhibition by GDC-0068 than those expressing a wild-type Akt1 (Fig. 6E), whereas expression of the E17K mutant decreased cellular sensitivity to the allosteric inhibitor Akti VIII (29) (Fig. 6F). The latter is consistent with previous reports that the allosteric Akt inhibitors are less effective against the E17K mutant of Akt1 (30) or pre-activated Akt (31).

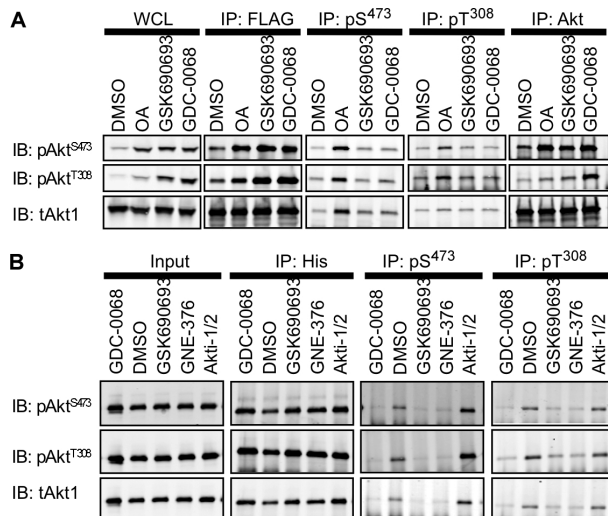


Fig. 4. Akt inhibitors block the accessibility of phosphorylated Thr³⁰⁸ and phosphorylated Ser⁴⁷³ of Akt. (A) ATP-competitive inhibitors block phosphorylated residue accessibility in cell lysates. NIH 3T3 cells expressing FLAG-Akt1 were incubated with DMSO, OA, GSK690693, or GDC-0068. Cells were lysed in nondenaturing lysis buffer and subjected to immunoprecipitation (IP) with monoclonal antibodies to FLAG, phosphorylated Ser⁴⁷³, phosphorylated Thr³⁰⁸, or a monoclonal antibody to Akt. Immune complexes and whole-cell lysates (WCL) were analyzed by IB. **(B)** ATP-competitive inhibitors block phosphorylated residue accessibility in vitro. Purified active His-Akt1 pre-phosphorylated on both Thr³⁰⁸ and Ser⁴⁷³ was incubated with DMSO or the ATP-competitive inhibitors GDC-0068, GSK690693, or GNE-376, or the allosteric inhibitor Akti-1/2, and then subjected to IP with monoclonal antibodies to His, phosphorylated Ser⁴⁷³, or phosphorylated Thr³⁰⁸. Immune complexes or pre-IP inputs were analyzed by IB. Representative data of three experiments are shown. Quantification and statistical analysis are provided in table S4.

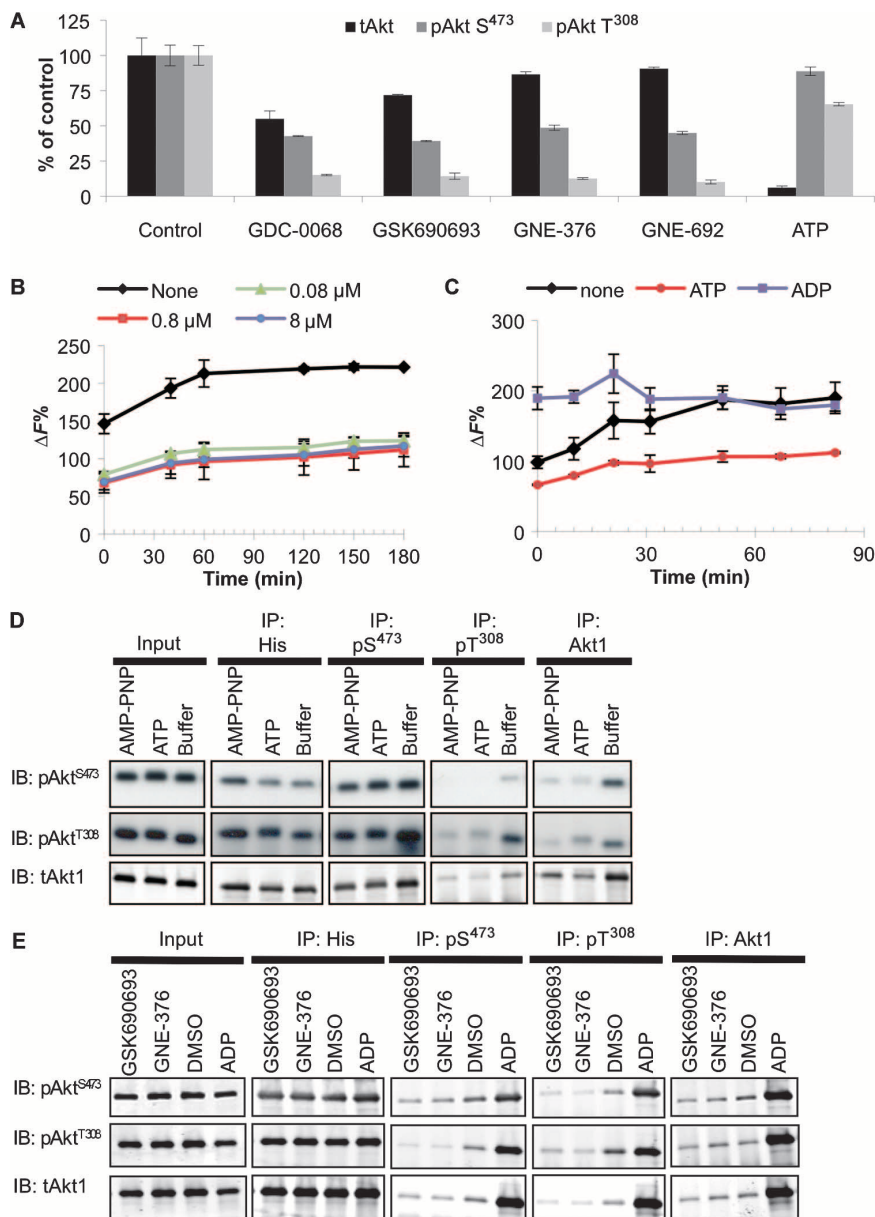


Fig. 5. Effects of Akt inhibitors and nucleotides on the detection of phosphorylated Akt. (A) Purified activated His-Akt1 was incubated with DMSO, the Akt inhibitor compounds, or ATP. Akt phosphorylated at Thr³⁰⁸ or Ser⁴⁷³ and total Akt were then detected with the MSD platform. (B and C) Activated His-Akt1 was incubated with the indicated concentrations of GDC-0068 (B), ATP or ADP (C), and analyzed by the HTRF assay. (D) ATP and AMP-PNP block accessibility to phosphorylated Akt in vitro. Activated His-Akt1 was incubated with buffer or ATP or AMP-PNP and then subjected to IP with monoclonal antibodies to His, phosphorylated Ser⁴⁷³, phosphorylated Thr³⁰⁸, or Akt1. (E) ADP increases accessibility to phosphorylated Akt in vitro. Activated His-Akt1 was incubated with DMSO, Akt inhibitors, or ADP and then subjected to IP with monoclonal antibodies to His, phosphorylated Ser⁴⁷³, phosphorylated Thr³⁰⁸, or Akt1. In (A) to (C), error bars represent the SD of three replicates. For (D) and (E), quantification and statistical analysis are provided in table S4. Representative data from three experiments are shown.

DISCUSSION

Protein kinases constitute an important enzyme family in living systems and also currently are among the most therapeutically targeted proteins involved in human diseases. A large amount of knowledge has been gained on the mechanisms of kinase activation. However, comparatively less attention has been paid to the physiological mechanisms by which kinases are turned off. Protein kinases require ATP hydrolysis for their catalytic activity. Here, we propose a new mechanism in which ATP and ADP serve as “on-off” switches for Akt under physiological conditions (Fig. 7). Specifically, this model suggests that Akt is prone to be inactivated after a single round of catalysis, whereas freshly activated ATP-bound Akt is protected from inactivation until it has catalyzed a kinase reaction. This mechanism enables the cells to tightly control and reset Akt activity, allowing strict temporal coupling of upstream activation and downstream signaling, and suggests that Akt activity would be affected by the nucleotide exchange rate and the ATP/ADP ratio in the cells. ATP-competitive inhibitors disrupt the equilibrium by locking Akt in the phosphorylated but nonfunctional state, whereas allosteric inhibitors lead to the accumulation of dephosphorylated Akt by stabilizing the inactive conformation of Akt and promoting dephosphorylation.

Increased activation of Akt is frequently observed in human cancers by various mechanisms (1), including the E17K activating mutation, which has been observed in a subset of human cancers (30) and identified as the underlying genetic abnormality associated with the Proteus syndrome, a congenital disorder characterized by patchy or segmental overgrowth and hyperplasia of multiple tissues and organs, along with susceptibility to the development of tumors (32). The preferential targeting of activated Akt by selective ATP-competitive inhibitors, such as GDC-0068, can have important therapeutic implications because this activation state-selective form of inhibition could act in concert with the effects of oncogene addiction, the dependency of cancer cells on a particular pathway (the Akt pathway in this case) for survival or proliferation, to increase the therapeutic index by targeting tumor cells with highly activated Akt while sparing normal cells with low Akt activity.

Fig. 6. Differences in the ATP-competitive and allosteric inhibitor binding modes. **(A)** Close-up view of Akt1–GDC-0068 complex structure highlighting residues interacting with the inhibitor. Akt1 residues listed in table S1 are illustrated in yellow sticks and the inhibitor is shown in spheres. **(B)** Superposition of GDC-0068 (yellow spheres) on the inactive Akt1–Akti VIII complex structure [Protein Data Bank (PDB) code 3O96], shown in green, and GDC-0068-interacting Akt1 residues from table S1 illustrated in orange sticks shows that GDC-0068 clashes with several residues in the inactive conformation. **(C)** GDC-0068 (yellow spheres) binds to the DFG-in conformation of Akt1. **(D)** Superposition of GDC-0068 (yellow spheres) on the Akt1–Akti VIII structure (green ribbon and spheres, respectively) reveals that GDC-0068 binding is incompatible with the inactive, DFG-out loop conformation. **(E and F)** CellTiter-Glo dose-response curves of NIH 3T3 cells expressing FLAG-tagged wild-type (WT), myristoylated (Myr), or E17K mutant forms of human Akt1 treated with GDC-0068 (E) or Akti VIII (F). Relative fluorescence units (RLU) are normalized as percentage of DMSO control; error bars represent the SE of quadruplets. Representative data of more than three experiments are shown.

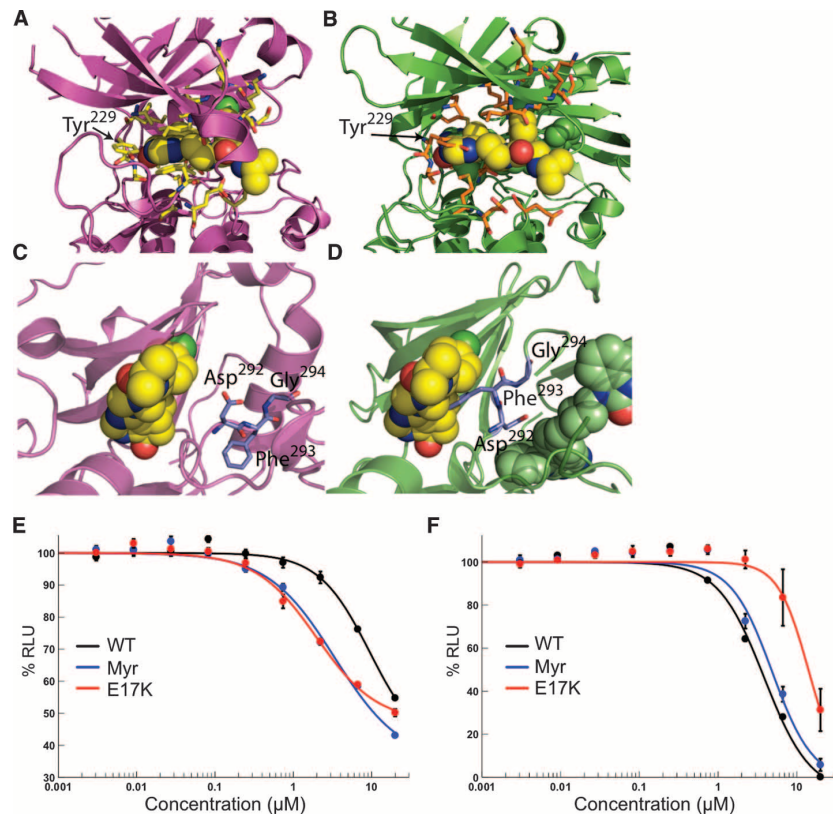
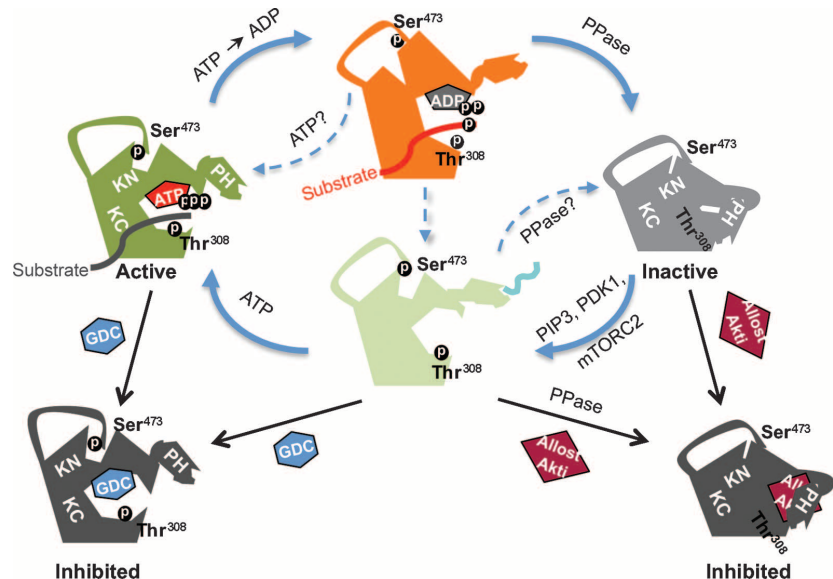


Fig. 7. Schematic model of the Akt on-off cycle. In its quiescent state, Akt is not phosphorylated on either Thr³⁰⁸ or Ser⁴⁷³ and preferentially adopts an inactive “PH-in” conformation, with the PH domain interacting with the inactive kinase domain (light gray). Upon interaction with phospholipids (aqua squiggly line), a conformational shift leads to release of the PH domain from the kinase domain, exposing Thr³⁰⁸ and Ser⁴⁷³ to be phosphorylated by PDK1 and mTORC2, respectively (light green). Phosphorylation of both residues promotes formation of the active site to accommodate ATP (red pentagon). Binding of ATP stabilizes the active conformation, in which the phosphate group of Thr³⁰⁸ forms multiple contacts with basic residues in the active site, making it less accessible to phosphatases. In addition, the hydrophobic motif containing phosphorylated Ser⁴⁷³ engages a binding groove at the back of the N lobe, also reducing its accessibility to phosphatases (darker green). Once ATP is hydrolyzed into ADP (light gray pentagon) with the γ -phosphate transferred to a substrate, another conformational shift occurs (orange), exposing both Thr³⁰⁸ and Ser⁴⁷³ to phosphatases, thus promoting the reset of Akt into the unphosphorylated inactive state, ready for a new round of activation. Akt exists in equilibrium between these states under physiological conditions. Allosteric inhibitors (dark red rhombus) stabilize the inactive conformation and shift the equilibrium toward dephosphorylated state, whereas ATP-competitive inhibitors (blue hexagon) target the active conformation and lock Akt in the phosphorylated but nonfunctional state incapable of ATP binding.



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MATERIALS AND METHODS

Cell culture and reagents

PC3-NCI and MCF7-neo/HER2 {MCF7 human breast cancer cell line [American Type Culture Collection (ATCC)] engineered at Genentech to overexpress HER2} cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). NIH 3T3 cells (ATCC) stably expressing wild-type or mutant FLAG-tagged full-length human Akt1 were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. Cells were maintained at 37°C under 5% CO₂. When indicated, cells were pretreated with 25 µg/ml cycloheximide (Calbiochem) and 30 µM MG-132 for 30 min to block protein synthesis and degradation. Akti VIII was purchased from Calbiochem. GSK690693 and BX-795 were purchased from Symansis. Other compounds were synthesized at Array BioPharma or Genentech.

Plasmids and cell transfection

MyrAkt1 was constructed by fusing an N-terminal myristoylation signal from v-Src (MGSSKSKPK) to human Akt1 to generate Myr-Akt1, which was then cloned into the pTRE2pur vector (Clontech) or pRetro-IRES-GFP vector (Clontech). N-terminally FLAG-tagged Akt1 was constructed in the pRetro-IRES-GFP vector (Clontech). The R273A and S473A mutations in Akt1 were generated in the pTRE2pur vector, and the E17K mutation was cloned into the pRetro-IRES-GFP vector. MEF/3T3 Tet-off cells (Clontech) were transiently transfected with MyrAkt1 (wild-type, R273A, or S473A) in the pTRE2pur vector and serum starved for 5 hours until 30 hours after transfection. Cells were then treated with or without 5 µM GDC-0068 or 0.5 µM okadaic acid for 1 hour, and cell lysates were analyzed by Western blotting. NIH 3T3 cells stably expressing FLAG-tagged Akt1 (wild-type, Myr, or E17K) in the pRetro-IRES-GFP vector were generated by retroviral infection using the Phoenix amphoteric packaging cell line and sorted by flow cytometry based on GFP fluorescence.

Immunoblot analysis and immunoprecipitation

For Western blot analysis, whole-cell lysates or purified recombinant proteins were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose. Antibodies used were anti-Akt1, anti-Akt2, anti-Akt3, anti-total Akt, anti-phosphorylated Akt (Ser⁴⁷³), anti-phosphorylated Akt (Thr³⁰⁸), anti-FoxO1, anti-FoxO3a, anti-phosphorylated FoxO1 (Thr²⁴)/FoxO3a (Thr³²), anti-total PRAS40, anti-total S6, anti-Akt1 that recognizes an epitope within the PH domain, anti-phosphorylated S6 (Ser^{235/236}), anti-phosphorylated MEK1/2 (Ser^{217/221}), anti-total MEK2, anti-phosphorylated GSK3β (Cell Signaling Technology); anti-phosphorylated PRAS40 (Thr²⁴⁶) (Invitrogen); anti-β-actin (Sigma); anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Advanced Immunochemical Inc.); and anti-PP2A C subunit (Millipore). Primary antibodies were detected with IR Dye 800-conjugated (Rockland) and Alexa Fluor 680-conjugated (Molecular Probes) species-selective secondary antibodies. Detection and quantification were performed with an Odyssey infrared scanner (LI-COR) with the manufacturer's software. For immunoprecipitation, cells were lysed in lysis buffer containing 30 mM tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 0.5 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and EDTA-free Protease Inhibitor Cocktail (Roche). Purified recombinant Akt proteins were incubated with the compounds in 25 to 50 mM tris-HCl (pH 7.5), 0.01% Triton X-100, 1 mM dithiothreitol (DTT), 10 mM MgCl₂, 1 mM NaF, 0.5 mM sodium orthovanadate, and EDTA-free Protease Inhibitor Cocktail (Roche). Cell lysates or buffer containing purified recombinant proteins was incubated with Dynabeads Protein G (Invitrogen) according to the manufacturer's protocols. The concentrations

of the Akt inhibitor compounds or nucleotides used to treat cells were also present during immunoprecipitation or wash steps.

PP2A activity and protein dephosphorylation assays

To measure PP2A activity, we used a fluorescence-based ProFluor Ser/Thr PPase Assay kit (Promega) according to the manufacturer's instructions. For the effect of Akt inhibitors on protein dephosphorylation by PP2A, recombinant activated His-Akt1, His-Akt2, His-Akt3 (Invitrogen) or MEK2 (US Biological) in the presence or absence of Akt inhibitors (10 µM unless indicated otherwise) were incubated with PP2A (Millipore) in the phosphatase assay buffer provided by the manufacturer for 30 min at 30°C. Calyculin (2 µM) was used as a positive control as a PP2A inhibitor. To assess the effect of ATP and its hydrolysis on dephosphorylation of Akt by PP2A, we preincubated recombinant activated His-Akt1 with ATP for 10 min at room temperature in 25 mM tris (pH 7.5), 2 mM DTT, 10 mM MgCl₂, and EDTA-free Protease Inhibitor Cocktail Tablets (Roche). A fusion protein containing the GSK3 α/β crossidase (CGPKGPGRGRRRRTSSFAEG) (Cell Signaling Technology) was added and incubated for 5 min at 30°C before incubation with PP2A for another 30 min. Phosphatase reactions were terminated with SDS sample buffer, and phosphorylated Akt and total Akt1 were analyzed by immunoblots.

PDK1 kinase assay

A PDK1 kinase assay kit (Millipore) was used according to the manufacturer's protocol, with inactive Akt1 (Biaffin GmbH & Co KG) as a substrate. One hundred nanograms of recombinant inactive Akt1 was incubated with 5 or 10 ng of PDK1 in the absence or presence of 1 or 10 µM GDC-0068 either added simultaneously or preincubated with Akt for 20 min. Kinase reaction was terminated after 30 min at 30°C with SDS sample buffer, and phosphorylated Akt and total Akt were analyzed by immunoblots.

Akt protein purification and crystallization

Recombinant Akt proteins expressed in insect cells were purified by use of the general protocol described previously (9). Inactive Akt protein was obtained by including PP1 treatment during the purification. Active Akt protein was obtained by incubating Akt with exogenous PDK1 and Mg-ATP during the purification. Akt1 (144–480)/S473D/phosphorylated Thr³⁰⁸ (10 mg/ml) was mixed with 5 mM AMP-PNP, 5 mM MnCl₂, and 5 mM GSK3β peptide and crystallized at 20°C under oil with 20% PEG 4000 (polyethylene glycol, molecular weight 4000), 10% isopropanol, and 0.1 M Hepes (pH 7.5) as the precipitant. The crystal was flash-frozen in the precipitant supplemented with 20% glycerol. The GDC-0068 crystal structure was obtained by soaking an Akt1/AMP-PNP/GSK3β peptide crystal in 0.5 mM GDC-0068, 22.5% PEG 4000, 100 mM Hepes (pH 7.5), 2.5 mM tris(2-carboxyethyl)phosphine (TCEP), 1 mM EDTA, and 0.5 mM GSK3β peptide at 20°C for 23 hours. The crystal was flash-frozen in the soaking solution supplemented with 20% glycerol. Although the GSK3β peptide was present in the soaking solution, the peptide was not visible in the electron density map and appeared to be displaced by the inhibitor.

Data collection and structure refinement

Data were collected at 100 K on a Rigaku FR-E generator and R-Axis IV++ detector (Rigaku America Inc.). Refinement was performed with REFMAC5 (33), and iterative model building was done in Coot (34). In the Ramachandran plot for the Akt1/GDC-0068 structure, 2 of 330 residues were in disallowed regions (0.6%). In Ramachandran plots of both the A and the B chains for the Akt1/AMP-PNP/GSK3β peptide, 3 of 319 residues were in disallowed regions (1%).

Cell proliferation assays

NIH 3T3 cells stably expressing FLAG-tagged wild-type, myristoylated, or E17K mutant forms of human Akt1 were incubated with serial dilutions of GDC-0068 or Akti VIII for 4 days. Cell proliferation and viability was measured with the CellTiter-Glo assay (Promega). Relative fluorescence units (RLUs) are normalized as percentage of DMSO control.

Meso scale assay

For detection of phosphorylation of Akt at Thr³⁰⁸ or Ser⁴⁷³ and of total Akt by the MSD platform, MULTI-ARRAY Phospho-Akt (Thr³⁰⁸) (K111DYD-2), phosphorylated Akt (Ser⁴⁷³) (K151CAD-2), and total Akt (K111CBD-1) assays were used according to the manufacturer's instructions. Recombinant activated His-Akt1 was incubated with DMSO control, 1 μ M of the Akt inhibitor compounds, or 1 mM ATP for 30 min at 30°C. The mixtures were then transferred to the appropriate MSD plates and incubated for 1 hour at room temperature followed by wash and detection antibody incubation steps according to the manufacturer's instructions. Akt inhibitor compounds or ATP was present at the same concentrations during all steps.

HTRF assay

Recombinant activated His-Akt1 was incubated with the indicated concentrations of GDC-0068, ATP, or ADP with or without the GSK3 fusion protein substrate (Cell Signaling Technology) at 30°C for 5 min in a buffer containing 50 mM tris-HCl (pH 7.5), 0.01% Triton X-100, 2 mM DTT, 0.1 mM Na₃VO₄, and 10 mM MgCl₂. Cryptate-labeled anti-phosphorylated Akt (Thr³⁰⁸) and d2-labeled anti-His monoclonal antibodies (Cisbio Bioassays) were added, and HTRF signals were detected with a SpectraMax M5 reader at the indicated time points. Ratios of acceptor emission at 668 nm and donor emission at 620 nm were calculated, and $\Delta F\%$ was calculated by normalizing to the negative control (antibodies only) according to the manufacturer's instructions.

PP1 activity assay

Recombinant PP1A enzyme was incubated for 10 min at 25°C in 100 mM tris (pH 7.5), 200 mM NaCl, 1 mM MnCl₂, 0.2 mM EDTA, 0.5 mM TCEP, 2% DMSO, bovine serum albumin (BSA) (0.1 mg/ml), and 50 mM fluorescein diphosphate substrate. Inhibitors were allowed to incubate with enzyme for 10 min before the initiation of the reaction. In most cases, the inhibitor concentration was kept constant at 10 mM. However, in experiments with nucleotide, concentrations varied from 10 to 0.03 mM. Activity triggered an increase in fluorescence emission at 520 nm.

PP1 protein dephosphorylation assays

Recombinant Akt proteins (1 μ M) were incubated with an equal molar concentration of PP1A enzyme overnight at 4°C in PP1 activity assay buffer. Inhibitor concentrations were fixed at 10 μ M, and nucleotide concentrations were 10 mM. Reactions were subjected to SDS-PAGE and then either stained with Coomassie blue or transferred to nitrocellulose membrane. Membranes were probed with anti-phosphorylated Thr³⁰⁸ (Cell Signaling) and detected by alkaline phosphatase-conjugated secondary antibodies.

PHLPP1 dephosphorylation assay

Recombinant activated His-Akt2 (Invitrogen) was incubated in the presence or absence of 10 μ M GDC-0068 with recombinant full-length PHLPP1 (OriGene Technologies). The dephosphorylation reactions were carried out in a reaction buffer containing 50 mM tris (pH 7.4), 1 mM DTT, and 5 mM MnCl₂ at 30°C for 10 min, as previously described (21).

LanthaScreen displacement binding assay

Akt binding to inhibitors was determined according to the manufacturer's protocol (Invitrogen) with purified N-terminal His6x-tagged recombinant Akt proteins by means of a custom-made tracer dye (Invitrogen) in assay buffer containing 25 mM Hepes (pH 7.5), 10 mM MgCl₂, 0.01% Triton X-100, and 1 mM DTT. The dissociation constants of tracer against individual Akt proteins were determined by displacing the Akt inhibitor VIII (Calbiochem).

Statistical analysis

Paired *t* tests were performed with unnormalized data or the "normalization *i*" method (35).

Note added in proof: While this paper was under review, Chan *et al.* published complementary data showing that occupancy of the ATP site in membrane-bound Akt conferred resistance of Thr³⁰⁸ to dephosphorylation (36).

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/5/223/ra37/DC1

Fig. S1. Structures of Akt inhibitors used in this study.

Fig. S2. The activity of PP2A or PP1 is inhibited by ATP, ADP, and AMP-PNP, but not by Akt inhibitors.

Fig. S3. ATP-competitive inhibitors block dephosphorylation of Akt2 and Akt3.

Fig. S4. ATP-competitive inhibitors block dephosphorylation of Akt by PP1.

Fig. S5. Scenarios to explain the phosphorylation status of Akt in cells treated with upstream kinase inhibitors before or after treatment with the ATP-competitive inhibitors.

Table S1. Data collection and refinement statistics.

Table S2. Akt1 residues within 4 Å of GDC-0068.

Table S3. LanthaScreen displacement binding assay results.

Table S4. Quantification and statistical analysis of Western blotting data.

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