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Article

Identification of a Small-Molecule Inhibitor of Class Ia PI3Ks with Cell-Based Screening

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SUMMARY

The mammalian target of rapamycin (mTOR) signaling network is central to the regulation of cell growth in response to both growth factors and nutrients. We developed a high-throughput, cell-based assay to identify small-molecule modulators of the mTOR signaling network. One such compound, which we name quinostatin, potently inhibits this network by directly targeting the lipid-kinase activity of the catalytic subunits of class Ia PI3Ks. This study illustrates the power of unbiased, phenotypic screening as a means for illuminating cell circuitry, and resulted in the identification of a chemotype for selective inhibition of the class Ia PI3Ks.

INTRODUCTION

The mammalian target of rapamycin (mTOR) signaling network receives inputs from both small molecules and extracellular proteins and plays an important role in regulating cell growth and metabolism in response to environmental cues [1, 2]. Carbon and nitrogen sources, such as glucose and glutamine, are primary stimuli of mTOR, yet the mechanisms by which they stimulate the mTOR pathway are still unknown. The canonical mTOR pathway (TSC/Rheb/mTOR/rictor/raptor) is impinged upon indirectly by mitogen signaling, for example, by mitogen-activated receptor tyrosine kinases (RTKs) such as the insulin and insulin-like growth factor I (IGF-I) receptors. Signals mediated by RTKs regulate mTOR signaling primarily through the PI3K/Akt pathway. RTK signaling may also regulate mTOR, albeit to a lesser extent, through the mitogen-activated protein kinase (MAPK) pathway involving ERK and RSK (also known as p90 S6K) [3–5]. It is believed that signals from these two pathways converge at the protein complex consisting of TSC1 (hamartin) and TSC2 (tuberin). TSC2 is the GTPase-activating protein (GAP) for the Ras-family GTP-binding protein Rheb, which has been reported to bind directly and to activate the rapamycin-sensitive raptor-mTOR complex. mTOR is also found in a rapamycin-insensitive rictor-mTOR complex, and this complex has been reported to be responsible for the phosphorylation of serine473 of Akt [6], the direct upstream regulator of TSC1/2 in the PI3K/Akt pathway. The raptor-mTOR complex regulates cell growth and proliferation via the downstream targets ribosomal S6 kinase 1 (p70 S6K1) and eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1). Cellular energy status is also believed to regulate mTOR signaling through the TSC1/2 complex. However, nutrient-derived signals might be regulating mTOR signaling independent of TSC1/2 [7]. In accord with its critical roles in integrating growth factor, nutrient, and energy signalings, dysregulation of the mTOR pathway is associated with certain cancers, cardiovascular disease, autoimmunity disorders, and metabolic disorders.

The study of the mTOR signaling network has relied extensively on the use of small molecules. Rapamycin enabled the discovery and characterization of mTOR [8, 9], while wortmannin and, more recently, a family of small-molecule inhibitors of lipid kinases have illuminated PI3K/Akt signaling [10]. Small molecules that target downstream elements of TOR signaling, e.g., uretupamine, which targets Ure2p, and that either enhance or suppress the cellular phenotypes of cells treated with rapamycin have shined a bright light on mTOR circuitry [11–13]. Here, we have extended these studies by developing a high-throughput, cell-based assay to screen for additional small-molecule modulators of mTOR signaling. We report the discovery of quinostatin (named after its quinoline core structure and its antiproliferative effect), which was identified from a collection of ~20,000 compounds as a potent modulator of signals that impinge on the S6 protein, whose phosphorylation is under the control of both PI3K/Akt and mTOR signaling. We show that quinostatin prevents the phosphorylation of S6 in cells by inhibiting the lipid-kinase activity of the class Ia phosphatidylinositol 3-kinase (PI3K). PI3K is a signaling protein of considerable interest from a therapeutic perspective; thus, these studies also reveal a completely new, to our knowledge, type of small-molecule structure (chemotype) that can serve as a starting point for therapeutic discovery.

RESULTS

Identification of Quinostatin as an Inhibitor of Cellular S6 Phosphorylation by Cell-Based Screening

mTOR regulates the activity of the ribosomal protein kinase p70 S6K, thereby controlling phosphorylation of

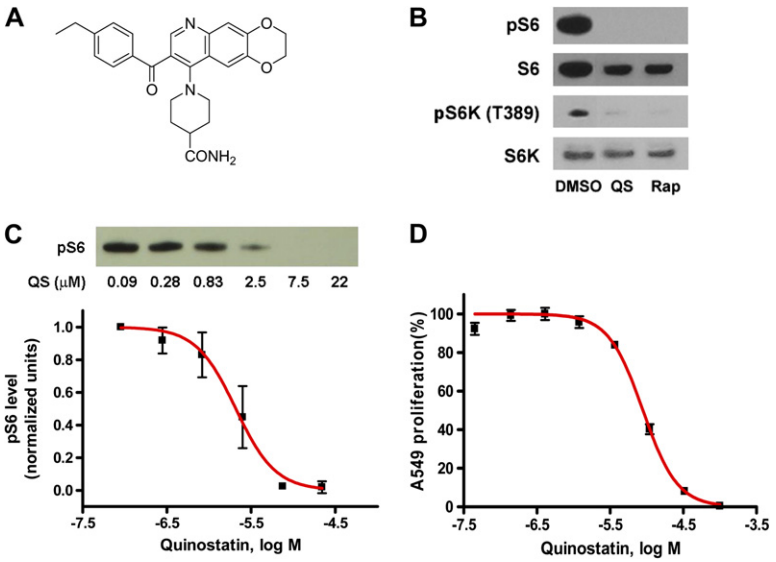

Figure 1. Quinostatin Inhibited S6 Phosphorylation

(A) The structure of quinostatin.

(B) Immunoblotting showed that the phosphorylation of the S6 and p70 S6K proteins in A549 cells was inhibited by quinostatin. The S6 protein level was also reduced. The p70 S6K protein level was not significantly affected. Rapamycin was used as the positive control. QS, quinostatin; Rap, rapamycin.

(C) Immunodetection showed that the phosphorylation of S6 protein in MCF7 cells was dose-dependently inhibited by quinostatin treatment. Data presented are means of three independent experiments with standard errors.

(D) Quinostatin also does-dependently inhibited A549 proliferation as measured by CellTiter-Glo. Data presented are means of three independent experiments with standard errors.

the 40S ribosomal protein S6 [14]. Phosphorylation of S6 at serine235/236 is therefore frequently used as readout for the activity of the mTOR signaling pathway. A high-throughput cytoblot assay [15] was developed to monitor the phosphorylation of the S6 protein by immunodetection. Briefly, A549 lung carcinoma cells in complete growth media were treated with compounds at a final concentration of 10 μg/ml in 384-well assay plates. The cells were allowed to incubate for 5 hr before S6 phosphorylation levels were examined by immunodetection with a phospho-S6 (serine235/236) primary antibody and an HRP-conjugated secondary antibody. Approximately 20,000 compounds were screened for their ability to decrease the phosphorylation level of S6. The positives from this primary screen were validated in a secondary cytoblot assay by using an α-tubulin primary antibody/HRP-conjugated secondary antibody detection system. This assay helped to identify false positives that caused cells to detach from the assay plates. A number of compounds, including quinostatin (Figure 1A), were identified as potent antagonists of S6 phosphorylation. The inhibition of S6 phosphorylation by quinostatin was verified by immunoblotting (Figure 1B). The phosphorylation level of p70 S6K, which directly correlates with its kinase activity, was also reduced. To a lesser extent, the total S6 protein level was also reduced upon quinostatin treatment. This is consistent with the observation that mTOR signaling regulates the translation of a subset of mRNAs that contains a 5′ tract of oligopyrimidine (TOP) [1, 2]. The 5′ TOP mRNAs encode components of the translation apparatus, such as ribosomal proteins and elongation factors. The total p70 S6K level was not significantly affected. The effect of quinostatin proved to be general in all cell lines tested, including A549, MCF7, HeLa, Jurkat, HCT116, and U2OS. An IC_{50} of ~2 μM (MCF7, Figure 1C) to 8 μM (A549) was observed. Consistent with its biochemical effect on S6 phosphorylation, quinostatin also inhibited A549 proliferation in a dose-dependent manner (IC_{50} of ~8 μM, Figure 1D).

Structure-Activity Relationship of Quinostatin

A collection of structural analogs of quinostatin was assembled, and the compounds were tested for their ability to inhibit S6 phosphorylation (Figure 2). (The compounds were either purchased from commercial vendors or were synthesized in house. The protocol for synthesizing the compounds will be published in due course.) The 4′-substituted piperidine appears to be important for quinostatin function: replacing the 4′-substituted piperidine with pyrrolidine, azepane, or unsubstituted piperidine caused a loss of activity, while polar substitutions at the 4′ position of piperidine helped to potentiate the compound. The highest activity was observed when the 4′ position was substituted with an amidocarbonyl group as in quinostatin. It is possible that hydrogen bonds formed between the 4′-amidocarbonyl and amino acids at the binding site of the target protein help to stabilize the small-molecule/protein interaction. The *p*-ethylphenyl substitution of C3-carbonyl proved not to be essential, and it can be replaced by an ethoxy without any detrimental effect on the activity. However, the C3-carbonyl is critical since replacement of the C3-carbonyl with a cyano or converting the carbonyl to an oxime abolished the activity. Interestingly, compound **A**, a known inhibitor of EGF receptor [16], did not inhibit S6 phosphorylation.

Quinostatin Targets the PI3K/Akt Pathway

Since the molecular target of quinostatin was unknown, we set out to characterize the effects of quinostatin on both PI3K/Akt and MAPK signaling, the two major pathways responsible for regulating mTOR signaling in response to growth-factor stimulations.

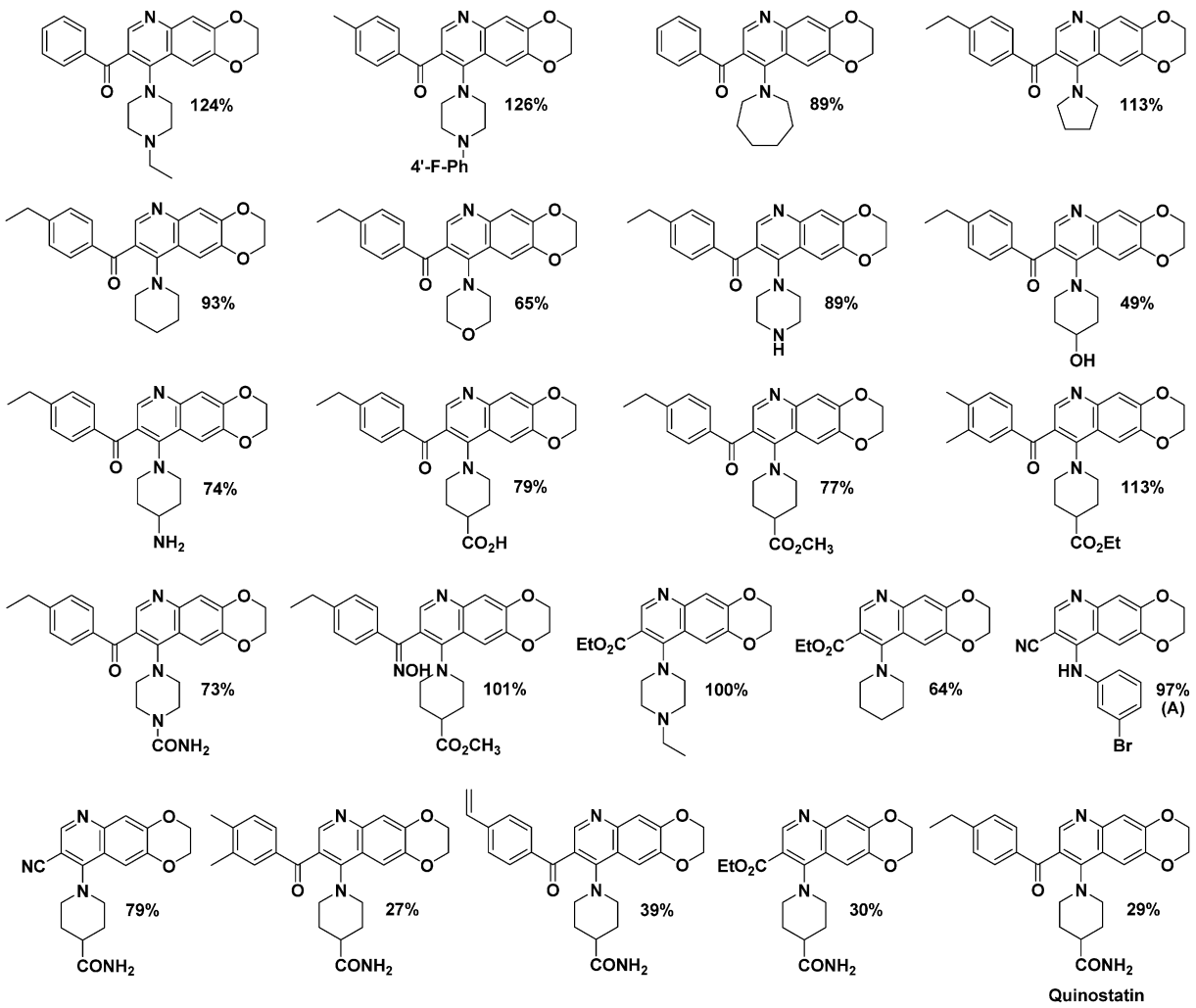

Figure 2. Structure/Activity Relationships of Quinostatin

The results are presented as the S6 phosphorylation level in the presence of the test compound (~20 μM) as a percentage of that in control incubations in A549 pS6-cytoblot assays.

The S6 protein was phosphorylated under complete growth media conditions, reflecting the basal activity of mitogen-activated signaling. This phosphorylation of the S6 protein in A549 cells was inhibited by wortmannin treatment (results not shown). Wortmannin covalently modifies the ATP-binding site of the catalytic subunit of class Ia PI3Ks (p110) and inhibits the lipid-kinase activity of the catalytic subunit [17]. Strong inhibition of S6 phosphorylation by wortmannin suggests that the PI3K/Akt pathway regulates mitogen-mediated mTOR signaling under the complete growth media conditions. Similar inhibition of S6 phosphorylation by wortmannin treatment was also observed when the cells were stimulated with insulin (Figure 3). Quinostatin abolished S6 phosphorylation both in the presence or absence of insulin stimulation. On the other hand, the MEK1/2 inhibitor U0126 [18] has no effect on insulin-stimulated S6 phosphorylation. To investigate whether the MAPK pathway was involved in quinostatin function, we also examined the effect of quinostatin on MAPK signaling. Phorbol 12-myristate 13-acetate (PMA) is known to activate MAPK signaling by stimulating the kinase activity of PKC, which, in turn, activates MAPK signaling through Ras and Raf proteins [3]. Consistent with the parallel roles of the PI3K/Akt and MAPK pathways in regulating mTOR signaling [3–5], wortmannin or U0126 alone only partially inhibited S6 phosphorylation caused by PMA treatment. Simultaneous inhibition of both the PI3K/Akt and MAPK pathways by wortmannin and U0126 was required to abolish PMA-stimulated S6 phosphorylation. A combination of quinostatin and U0126 also strongly inhibited PMA-stimulated S6 phosphorylation. On the other hand, quinostatin alone or in combination with wortmannin only partially inhibited S6 phosphorylation caused by PMA treatment. These results suggest that quinostatin exerts its effect on S6 phosphorylation through the PI3K/Akt pathway.

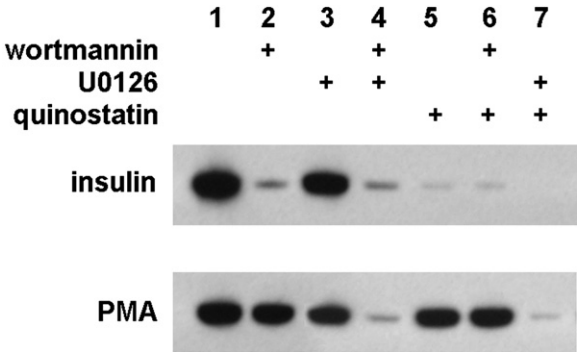

Figure 3. Immunoblot of Phospho-S6 in A549 Cells Stimulated with Insulin or PMA

Wortmannin or quinostatin alone was able to inhibit insulin-stimulated S6 phosphorylation. However, a combination of U0126 and wortmannin or quinostatin was required to inhibit PMA-stimulated S6 phosphorylation.

Class Ia PI3K Is the Molecular Target of Quinostatin

To investigate quinostatin's mechanism of action further, we carried out affinity chromatography purification to identify the protein target of quinostatin. Specifically, an analog of quinostatin was prepared by attaching a polyethylene glycol linker to the C3-carbonyl through an amide bond (Figure 4A, compound **B**). This modified compound was found to be 100-fold less active than quinostatin in the S6 phosphorylation assay (data not shown). Since substitution at the C3-carbonyl was shown to be inconsequential to quinostatin's activity, this dramatic reduction of potency might be attributed to poor cell permeability caused by the polyethylene glycol appendage instead of a disruption of the interactions at the protein binding site. The affinity reagent was prepared by immobilizing the polyethylene glycol-modified molecule onto agarose beads through a second amide bond. In order to isolate the proteins that interact with quinostatin, MCF7 cell lysate was incubated with the affinity reagent. After the resin had been extensively washed, the associated proteins were analyzed by SDS-PAGE. A number of bands were identified by silver staining. Performing the affinity capture in the presence of 100 μM quinostatin to compete with the resin for binding caused a single ~85 kD band to disappear, suggesting that it corresponded to a possible target (Figure 4B). After in-gel trypsin digestion and LC/MS analysis, the band was found to consist of class Ia PI3K regulatory subunits p85α and p85β. This result was independently confirmed by immunoblotting with a p85-specific antibody (Figure 4C). The class Ia PI3K consists of a regulatory subunit, p85, and a catalytic subunit, p110 [19]. The two subunits form a functional heterodimeric complex through noncovalent interactions. Since staining caused by nonspecific binding proteins overlapped the region corresponding to p110, the affinity-purified proteins were probed for p110 by immunobloting with a p110-specific antibody. Indeed, the p110 catalytic subunit was also affinity purified in a manner that was sensitive to the presence of quinostatin (Figure 4C). The interaction between p85/p110 and the affinity resin was sensitive to the presence of LY294002, a noncovalent PI3K inhibitor [20] that targets the ATP-binding site of p110 [21]. In addition, the affinity resin was also able to pull down a recombinant p110 from a solution of the purified protein, and this affinity was sensitive to the presence of quinostatin (Figure 4D). The affinity between quinostatin and the recombinant p85α/p110α complex was quantified by using surface plasma resonance, and a K_D of ~0.6 μM was observed at 5°C. The effect of quinostatin on the lipid-kinase activity of the recombinant p85α/p110α complex was tested by using an in vitro lipid-kinase assay. Quinostatin dose-dependently inhibited the lipid-kinase activity with an IC_{50} of ~15 μM (Figure 4E). Quinostatin also inhibited the lipid-kinase activity of recombinant p110γ, an isoform of p110α, with a slightly lower potency (IC_{50} of ~30 μM).

DISCUSSION

Class Ia PI3Ks exert their physiological functions by catalyzing the phosphorylation of phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2) to generate phosphatidylinositol-3,4,5-triphosphate (PI(3,4,5)P3), a second messenger with several downstream effectors [22]. This lipid-kinase activity is countered by the phosphatase activity of PTEN and SHIP1/2, which dampens the effect of class Ia PI3Ks by hydrolyzing PI(3,4,5)P3 to PI(4,5)P2 and PI(3,4)P2, respectively. PI3K signaling is crucial to many aspects of cell growth and survival. Dysregulation of this signaling pathway by genetic aberrations such as mutation, amplification, and rearrangement is frequently observed in human cancer [22]. Indeed, the loss-of-function mutation of PTEN and the gain-of-function mutation of class Ia PI3Ks are observed in both sporadic tumors and those that arise as a result of predisposition syndromes. Theoretically, such tumors could be treated by inhibition of PI3K signaling. In fact, small molecules that directly or indirectly modulate PI3K signaling have been actively pursued as potential cancer therapies, and some of the inhibitors (including some mTOR inhibitors) are currently in clinical trials [23, 24]. Additional molecular entities that target these pathways could be useful as tools by which to gain a better understanding of the signaling pathways or as therapeutics for treating relevant diseases.

We developed an assay for high-throughput identification of small molecules modulating the posttranslational modification status of the 40S ribosomal protein S6, a downstream effector of mTOR signaling. Compared with in vitro biochemical assays that measure the activity of specific protein targets, this cell-based assay can be used without bias to identify small-molecule regulators affecting proteins in the pathway without prior knowledge of their function.

Using this cell-based assay, quinostatin was identified as a potent inhibitor of S6 phosphorylation. Differential stimulation of the PI3K/Akt or MAPK pathways showed that the former was the major regulator of signaling to S6. Quinostatin phenocopied wortmannin in terms of its effect on S6 phosphorylation. Through structure-activity

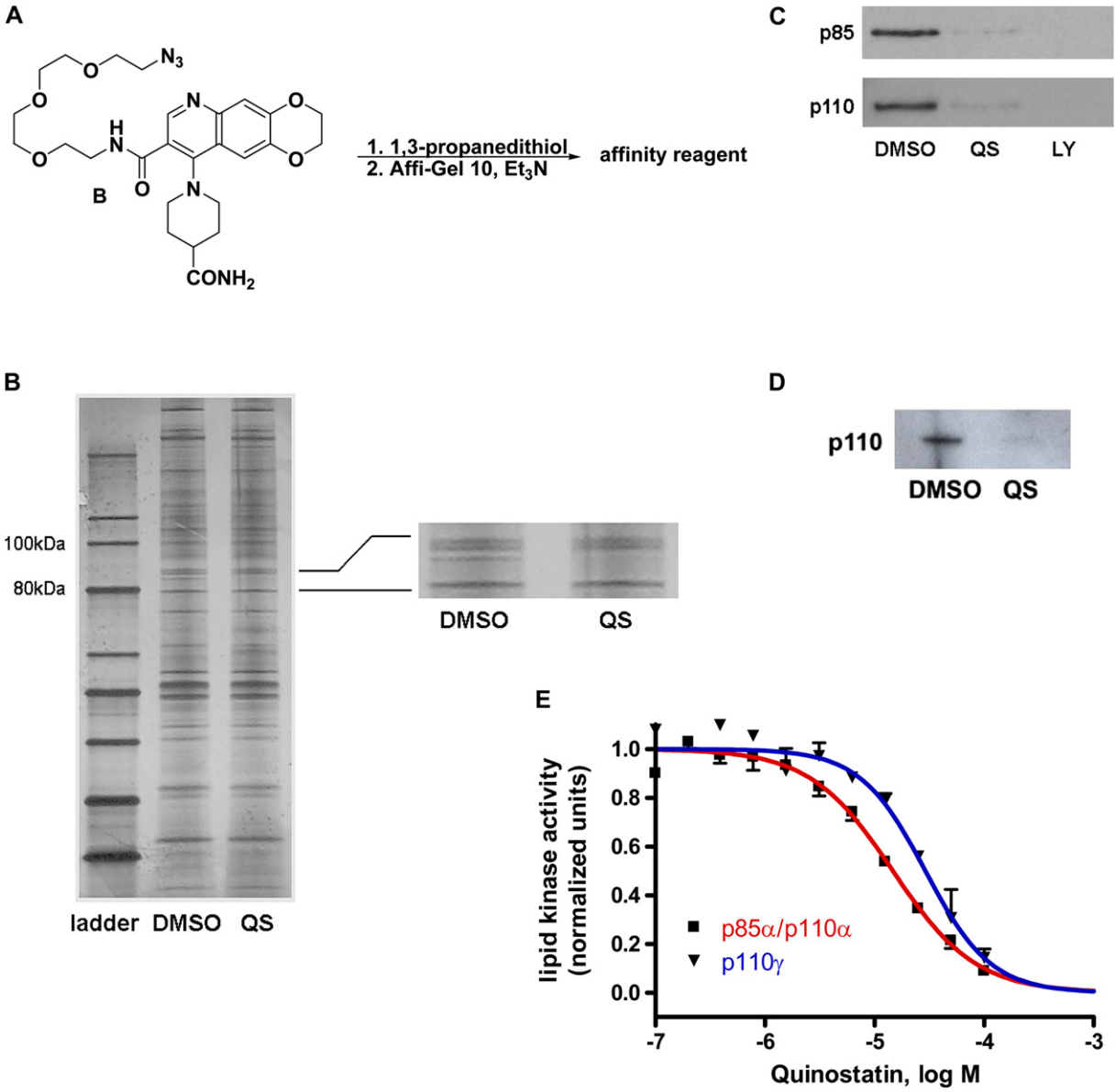

Figure 4. Quinostatin Targets the Catalytic Subunit of PI3K

(A) Preparation of the affinity resin.

(B) Affinity-purified proteins were separated by SDS/PAGE and visualized by silver staining. The staining of an ~85 kD band was sensitive to quinostatin treatment.

(C) Immunoblotting showed that the ~85 kD band was reactive toward p85 antibody. This band disappeared when affinity chromatography was performed in the presence of quinostatin or LY294002. Immunoblotting with p110 antibody showed that p110 was also affinity purified in a manner that was sensitive to quinostatin or LY294002 treatment. QS, quinostatin; LY, LY294002.

(D) Immunoblotting showed that a recombinant p110 was pulled down from a solution of the purified protein by the affinity resin.

(E) In vitro kinase assays showed that quinostatin dose-dependently inhibited the lipid-kinase activities of the recombinant p85α/p110α and p110γ proteins. Data presented are means of three independent experiments with standard errors.

relationship studies, we identified a permissive site for quinostatin immobilization. The corresponding affinity resin was synthesized and used to purify quinostatin-interacting proteins. Both the catalytic and regulatory subunits of the class Ia PI3Ks were affinity purified. The binding between quinostatin and a recombinant class Ia PI3K (p85α/p110α) was independently verified by surface plasma resonance. Quinostatin was shown to inhibit the lipid-kinase activity of the recombinant PI3K. In addition, it also inhibited the lipid-kinase activity of p110γ, an isoform of p110α. Since no regulatory subunit was involved in the latter case, this result suggested that quinostatin targeted the catalytic subunit of the PI3Ks. The sensitivity of the affinity purification of p85/p110 to the presence of

LY294002 is also consistent with the catalytic subunit being the direct target of quinostatin. This targeting was further corroborated by the selective binding of recombinant p110γ with the quinostatin affinity reagent. While the p110 protein might have been purified through its direct interaction with the immobilized quinostatin, the p85 proteins were copurified in the form of functional heterodimers. A thorough profiling would be required to determine the selectivity of quinostatin against other kinases. However, the data we obtained suggest that quinostatin did not, in cells, target some of the protein kinases relevant to mTOR signaling, such as those involved in relaying PMA stimulation through the MAPK signaling pathway. Thus, quinostatin represents a new, to our knowledge, chemotype for the selective inhibition of PI3K. In light of a recent report of unexpected synergistic effects of kinase inhibitors with multiple targets [25], it will be useful to profile quinostatin against the whole kinome in the future.

SIGNIFICANCE

Using the posttranslational modification status of the 40S ribosomal S6 protein as the readout, we developed a cellular assay for unbiased, high-throughput identification of small-molecule modulators of mTOR signaling. Quinostatin was identified as an antagonist of mTOR signaling. Its molecular target was found to be the catalytic subunit of the class Ia PI3Ks. The molecular scaffold represented by quinostatin constitutes a new, to our knowledge, chemotype by which therapeutically relevant kinase inhibitors may be developed.

EXPERIMENTAL PROCEDURES

Materials

All cell culture reagents and the silver staining kit were purchased from GIBCO/Invitrogen. Wortmannin, LY294002, phosphatidylinosotol, and the recombinant p110γ were purchased from Sigma. Quinostatin can be purchased from ChemDiv (catalog number: CDL C768-0445). In-house-synthesized and ChemDiv compound libraries were used in the screening. The recombinant p85α/p110α protein and p85 antibody were purchased from Upstate. Other antibodies were purchased from Cell Signaling Technology. AffiGel-10 was purchased from BioRad. The EDTA-free protease inhibitor cocktail was purchased from Roche. The CellTiter-Glo luminescent cell viability assay kit was purchased from Promega.

Screening of Small-Molecule Libraries

A549 cells were seeded in 384-well assay plates at a density of 4,000 cells/well in 50 μl complete growth media (DMEM plus 10% FBS) and were incubated overnight. Compounds in DMSO (100 nl, 5 mg/ml) were robotically pinned into the media. The cells were cultured for another 5 hr and fixed with 4% formaldehyde in PBS. Immunodetection of S6 phosphorylation, with a phospho-S6 (serine235/236) primary antibody and an HRP-conjugated secondary antibody, was carried out by following a protocol similar to that reported previously [15].

Affinity Chromatography

Affinity chromatography was performed as described by Wan et al. [26] with some modifications. MCF7 cells were lysed with a modified RIPA buffer (50 mM Tris-HCl [pH 7.4], 1% NP40, 250 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4, and supplemented with an EDTA-free protease inhibitor cocktail) at 4°C for 10 min, and the cell lysate was cleared by centrifugation. The cleared lysate (1 ml) was tumbled with the affinity resin (30 μl) at 4°C for 2 hr. The suspension was centrifuged, and the supernatant was discarded. The resin was washed with RIPA buffer (1 ml) four times. After the final wash, the supernatant was removed, and SDS sample buffer (30 μl) was added to the resin. The affinity-purified proteins were heat denatured and separated by SDS-PAGE. The proteins were visualized by silver staining following manufacturer's protocol.

Lipid-Kinase Assay

A reaction mixture containing the recombinant p85α/p110α (or p110γ) protein, quinostatin (in 1 μl DMSO), and phosphatidylinositol (20 μg, suspended by sonification) in the kinase reaction buffer (45 μl, 120 mM HEPES [pH 7.5], 10 mM $MgCl_2$) was treated with a solution of ATP (100 μM, 5 μl) containing γ-^{32}P-ATP (10 μCi) in the same kinase reaction buffer. The reaction mixture was incubated at room temperature for 20 min before the reaction was quenched with 1 N HCl (100 μl). The resulting mixture was extracted with $CHCl_3$:CH_3OH (1:1, 160 μl). The organic phase was separated and allowed to dry. The residue was taken into $CHCl_3$ (20 μl) and spotted onto a TLC plate. The plate was developed in a boric acid developing buffer [27] and then dried. It was exposed to a film, and the image was quantitated.

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