Inhibition of Mammalian Target of Rapamycin Signaling by 2-(Morpholin-1-yl)pyrimido[2,1-α]isoquinolin-4-one*  

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From the ¹Department of Medicine, the ⁶Department of Chemistry and the Institute of Chemical Biology & Drug Discovery, and the ⁵Department of Physiology and Biophysics and the Institute of Molecular Cardiology, Stony Brook University, Stony Brook, New York 11794 and the ¹Department of Veterans Affairs Medical Center, Northport, New York 11768  

Signaling through the mammalian target of rapamycin (mTOR) is hyperactivated in many human tumors, including hamartomas associated with tuberous sclerosis complex (TSC). Several small molecules such as LY294002 inhibit mTOR kinase activity, but they also inhibit phosphatidylinositol 3-kinase (PI3K) at similar concentrations. Compound 401 is a synthetic inhibitor of DNA-dependent protein kinase (DNA-PK) that also targets mTOR but not PI3K in vitro (Griffin, R. J., Fontana, G., Golding, B. T., Guiard, S., Hardcastle, I. R., Leahy, J. J., Martin, N., Richardson, C., Rigoreau, L., Stockley, M., and Smith, G. C. (2005) J. Med. Chem. 48, 569–585). We used 401 to test the cellular effect of mTOR inhibition without the complicating side effects on PI3K. Treatment of cells with 401 blocked the phosphorylation of sites modified by mTOR-Raptor and mTOR-Rictor complexes (ribosomal protein S6 kinase 1 Thr389 and Akt Ser473, respectively). By contrast, there was no direct inhibition of Akt Thr308 phosphorylation, which is dependent on PI3K. Similar effects were also observed in cells that lack DNA-PK. The proliferation of TSC1¹/⁻ fibroblasts was inhibited in the presence of 401, but TSC1¹/⁺ cells were resistant. In contrast to rapamycin, long-term treatment of TSC1¹/⁻ cells with 401 did not up-regulate phospho-Akt Ser473. Because increased Akt activity promotes survival, this may explain why the level of apoptosis was increased in the presence of 401 but not rapamycin. These results suggest that mTOR kinase inhibitors might be more effective than rapamycins in controlling the growth of TSC hamartomas and other tumors that depend on elevated mTOR activity.

The mammalian target of rapamycin (mTOR)² is a protein kinase that occupies a central position in a signaling network that regulates cell proliferation, size and survival. This signaling network integrates growth factor-activated signals with permissive signals in the presence of sufficient amino acids and energy to result in mTOR activation. Many genetic defects found in human cancers lead to hyperactivation of mTOR signaling (1). It is believed that certain tumor cells become dependent on this pathway because it provides a growth or survival advantage. Therefore, drugs that inhibit mTOR have great therapeutic potential for the treatment of cancer. Rapamycin, a macrolide antibiotic produced by the bacterium Streptomyces hygroscopicus, binds to its intracellular receptor FKBP12 to form a complex that inhibits mTOR function. Rapamycin and its analogs are under clinical development as anti-cancer agents, but these drugs have two disadvantages: they block only some of the functions of mTOR, and they can activate the protein kinase Akt that promotes cell survival. By contrast, a small molecule designed to compete with ATP in the catalytic site of mTOR would be expected to inhibit all of the kinase-dependent signaling functions of the enzyme without enhancing the survival pathway.

The mTOR kinase domain is most closely related to the one found in phosphatidylinositol (PI) 3-kinases (PI3Ks) (2). However, unlike PI3Ks, mTOR phosphorylates proteins, not lipids. The unusual mTOR kinase domain defines the PIKK (PI3K-related kinase) family of protein serine/threonine kinases, which includes ATM (ataxia telangiectasia-mutated), ATR (ATM and Rad3-related), and DNA-PK (DNA-dependent protein kinase) (3). Until recently, rapamycin sensitivity was the major criterion used in mammalian systems to identify mTOR-controlled events. However, it is now found that mTOR binds to different regulatory subunits to produce multiprotein complexes with distinct signaling functions and rapamycin sensitivity (4). Complexes containing mTOR and Raptor (mTORC1) phosphorylate ribosomal protein S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and are rapamycin-sensitive (5). Complexes that contain mTOR and Rictor (mTORC2) phosphorylate Akt and are insensitive to the drug (6). Because mTOR has rapamycin-insensitive functions, it is thought that direct inhibitors of mTOR kinase activity will display broader anti-tumor activity than rapamycins.

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3. The abbreviations used are: mTOR, mammalian target of rapamycin; PI, phosphatidylinositol; PI3K, PI 3-kinase; PIKK, PI3K-related kinase; ATM, ataxia telangiectasia-mutated; ATR, ATM and Rad3-related; DNA-PK, DNA-dependent protein kinase; PDGF, platelet-derived growth factor; IC₅₀, concentration that gives 50% inhibition; S6K1, ribosomal protein S6 kinase 1; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; GST, glutathione S-transferase; PDK1, 3-phosphoinositide-dependent protein kinase-1; MEF, mouse embryo fibroblast; TSC, tuberous sclerosis complex; IGF-I, insulin-like growth factor-I; PLD, phospholipase D; PA, phosphatidic acid; AMPK, AMP-activated kinase.
Most kinase inhibitors that have been developed for clinical applications work by competing with ATP in the catalytic site. LY294002 (Fig. 1) is a synthetic morpholino compound that was designed as a PI3K inhibitor (7). Kinetic studies showed that this compound behaves as a competitive inhibitor with ATP (7), and x-ray crystallographic structures have revealed how LY294002 fits into the active site of the p110y isoform of PI3K (8). Not surprisingly, considering the structural similarity between the PI3K and mTOR catalytic domains, the concentration of LY294002 that gives 50% inhibition (IC50) of mTOR (IC50 ~ 5 μM) is almost the same as for PI3K (IC50 ~ 3 μM) (9). This compound also blocks the activity of DNA-PK (10). PI3Ks regulate a wide range of cellular functions including growth, metabolism and motility, and PIKKs regulate processes such as cell cycle progression and genome maintenance. Therefore, use of LY294002 as a non-selective mTOR inhibitor might have undesirable toxic side effects.

Recently, Griffin et al. (10) used LY294002 as a template for the design of DNA-PK inhibitors. One derivative (compound 13 in Ref. 10; compound 401 in Fig. 1) was reported to be a poor inhibitor of PI3K, ATM, and ATR in vitro, but it was active against mTOR. 401 was not subjected to a biological evaluation. In this study, we tested the ability of compound 401 to inhibit mTOR signaling in vivo and evaluated its effect on proliferation and apoptosis in cells with hyperactivated mTOR signaling. Our results support the idea that inhibitors of mTOR kinase activity might be more effective than rapamycins in treating some proliferative disorders.

**EXPERIMENTAL PROCEDURES**

**Materials**—Platelet-derived growth factor (PDGF) A/B, phenylephrine, and insulin-like growth factor 1 (IGF-I) were from Sigma. Rapamycin, AMA37, Akt inhibitor VIII and LY294002 were from Calbiochem. Phosphospecific antibodies were from Cell Signaling Technology (Danvers, MA). Antibodies to Akt1/2, S6K1, and Erk2 were from Santa Cruz Biotechnology (Santa Cruz, CA). Raptor antibody was from Bethyl Laboratories (Montgomery, TX) and AU1 antibody was from Covance (Berkeley, CA). 2-(Morpholin-1-yl)pyrimido[2,1-α]isoquinolin-4-one (compound 401) was synthesized as described earlier (10). Its identity and purity were confirmed by 1H NMR. Akt T308D-myc-His and Akt S473D-myc-His were described earlier (11). Purification of the p110α/p85α complex from insect cells was previously described (11). The cDNA for the human p110β PI3K catalytic subunit was purchased from Invitrogen. It was found to contain a mutation (C at 2772 was changed to A) that rendered the enzyme catalytically inactive. The mutation was repaired by PCR and the coding region was subcloned into pBlueBacHis2B (Invitrogen). A baculovirus was produced using the Bac-N-Blue kit (Invitrogen). A baculovirus expressing the p85α regulatory subunit of PI3K was purchased from Orbigen (San Diego, CA). The p110β/p85α PI3K complex was purified from infected insect cells as described for p110α/p85α (11).

**Cell Culture**—FreeStyle 293-F cells (Invitrogen) were grown in FreeStyle 293 Expression Medium. Rat-1 fibroblasts stably transfected with the human α1,α2-adrenergic receptor (12), COS7 cells and mouse embryo fibroblasts (MEFs) expressing or lacking the tuberous sclerosis complex 1 (TSC1) gene (a gift from Dr. D. Kwiatkowski, Harvard University) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) containing antibiotics and 10% fetal bovine serum. M059J human glioma cells (American Type Culture Collection, Manassas, VA) were kept in complete growth medium (a 1:1 mixture of DMEM and Ham’s F12 medium plus supplements) according to the vendor’s instructions.

**mTOR Assays**—FreeStyle 293-F cells were transfected with cDNA for AU1-mTOR (a gift from Dr. R. T. Abraham, The Burnham Institute, La Jolla, CA) using 293fectin (Invitrogen). Two days later, the cells were lysed and mTOR immunoprecipitates were prepared as described (13) using AU1 antibody. Alternatively, the mTORC1 complex was immunoprecipitated from untransfected cells using Raptor antibody under conditions described earlier (14). Kinase activity in the immunoprecipitates was assayed in the presence of vehicle (dimethyl sulfoxide) or compound as described (13) using bacterially expressed glutathione S-transferase (GST)-4E-BP1 (15) as a substrate. Kinase reactions were stopped by boiling in SDS sample buffer and the samples were subjected to SDS-PAGE. Phosphorylated 4E-BP1 was detected by autoradiography. Radioactivity in the bands was quantified by scintillation counting.

**PI3K Assays**—Vehicle (dimethylformamide) or compound was added to assay tubes and the solvent was evaporated under vacuum. 35 μl of PI3K assay buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, and 0.5 mM EGTA) was added to each tube, and they were placed on a shaker for 10 min at 25 °C to dissolve the compounds. Then 5 μl of enzyme was added, and the mixtures were incubated for 10 min at 25 °C. Kinase assays were started by adding 5 μl of 10 mg/ml L-α-PI (Sigma) sonicated in assay buffer and 5 μl of reaction mix containing 400 μM ATP, 0.25 μl of [γ-32P]ATP (3000 Ci/mmol; PerkinElmer Life Sciences) per assay, and 100 mM MgCl2 in assay buffer. Tubes were heated at 25 °C for 20 min with shaking. The reactions were stopped with 120 μl of CHCl3:CH3OH:HCl (10:20:0.2), and the tubes were put on a shaker for 10 min. After centrifuging for 5 min, 15 μl of the lower phase was spotted onto a silica gel thin layer plate and the reaction product was separated by chromatography for 1.5 h in CHCl3:CH3OH:NH3OH:H2O (86:76:10:14). Radioactive spots containing PI 3-phosphate were visualized by autoradiography, cut out of the plate and quantified by scintillation counting.

For PI3K autophosphorylation, 20 μl of autophosphorylation buffer (20 mM HEPES, pH 7.4, and 100 mM NaCl) was added to tubes containing dried vehicle or compound and they were
placed on a shaker for 10 min at 25 °C. Then 5 μl of p110α/p85α PI3K was added, and the mixtures were incubated for 10 min at 25 °C. Reactions were started by adding 5 μl of autophosphorylation buffer containing 60 μM ATP, 0.25 μl of [γ-32P]ATP per assay, and 60 mM MnCl2. Tubes were heated at 25 °C for 20 min. The reactions were stopped by boiling in SDS sample buffer, and the samples were subjected to SDS-PAGE. Phosphorylated p85α was detected by autoradiography.

Western Blots—After treatments, cells were rinsed on ice with cold phosphate-buffered saline and scraped into lysis buffer containing 50 mM HEPES, pH 7.5, 1% Triton X-100, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and a mixture of protease inhibitors. After centrifugation at 20,000 × g, equal quantities of supernatant protein were subjected to SDS gel electrophoresis and Western blotting. Signals were detected using horseradish peroxidase-linked secondary antibodies (GE Healthcare) and chemiluminescence reagents (PerkinElmer Life Sciences). Protein expression was assessed in the presence of the indicated concentrations (in μM) of compound 401. Autoradiographs are shown. B, PI3K activity of p110α/p85α (circles) or p110β/p85α (squares) was assayed in the presence of increasing concentrations of 401 (closed symbols) or LY294002 (open symbols). Averages from two experiments are shown. C, autophosphorylation of the p85α subunit of p110α/p85α was assessed in the presence of the indicated concentrations (in μM) of 401 or LY294002. An autoradiograph is shown. C, vehicle control; BI, enzyme blank.

FIGURE 2. Effect of compound 401 on mTOR and PI3K activities in vitro. A, mTOR kinase activity in AU1 or Raptor immunoprecipitates was assayed in the presence of the indicated concentrations (in μM) of compound 401. Autoradiographs are shown. B, PI3K activity of p110α/p85α (circles) or p110β/p85α (squares) was assayed in the presence of increasing concentrations of 401 (closed symbols) or LY294002 (open symbols). Averages from two experiments are shown. C, autophosphorylation of the p85α subunit of p110α/p85α was assessed in the presence of the indicated concentrations (in μM) of 401 or LY294002. An autoradiograph is shown. C, vehicle control; BI, enzyme blank.

Table 1

Inhibition of protein kinases by 401

| Protein kinase % Inhibition | Protein kinase % Inhibition |
|-----------------------------|-----------------------------|
| ABL1                        | 5                           |
| AKT1 (PKBα)                 | 5                           |
| BTK                         | 9                           |
| CDK1/cyclin B               | 3                           |
| CHEK1 (CHK1)                | 9                           |
| CSNK1G2 (CK1γ2)             | 2                           |
| CSNK2A1 (CK2α1)             | 3                           |
| Dyrk3                       | 5                           |
| EGFR (Erbb1)                | 3                           |
| EPHA2                       | 13                          |
| Erbb2 (HER2)                | 3                           |
| FgfR1                       | 0                           |
| Flt3                        | 36                          |
| Gsk3B (GSK3β)               | 0                           |
| IGFIR                       | 8                           |
| Insr                        | 3                           |
| Irak4                       | 5                           |
| Jak3                        | 2                           |
| Kdr (Vegfr2)                | 0                           |
| Kit                         | 13                          |

motif. Thr389 in the hydrophobic motif of S6K1 is phosphorylated by mTORC1 (5, 18). Phosphorylation of Thr389 allows 3-phosphoinositide-dependent protein kinase-1 (PKD1) to phosphorylate Thr223 in the T loop to activate the enzyme (19). Similarly, mTORC2 phosphorylates Akt at Ser473 in the hydrophobic motif and facilitates PKD1 phosphorylation.

RESULTS

Effect of 401 on Kinase Activities in Vitro—Several small molecules have been reported to inhibit mTOR kinase activity at low micromolar concentrations or better, but they show unfavorable selectivity between mTOR and PI3K (9, 10, 16, 17). By contrast, compound 401 shows activity against mTOR (IC50 = 5.3 μM) but not p110α/p85α PI3K (IC50 > 100 μM, Ref. 10). We speculated that 401 might be a useful tool to probe mTOR signaling without the complicating side effect of PI3K inhibition.

First, we performed in vitro kinase assays to confirm that 401 selectively inhibits mTOR over PI3K. In agreement with the results of Griffin et al. (10), we found that 401 inhibited immunoprecipitated epitope-tagged mTOR or endogenous mTOR in Raptor immunoprecipitates (Fig. 2A). In both cases, inhibition of 67% or 78% was observed at 5 μM or 10 μM 401, respectively. By contrast, dose response curves showed that the p110α/p85α or p110β/p85α PI3K complexes were poorly inhibited by 401 at these concentrations (Fig. 2B). LY294002 was much more potent than 401 at inhibiting both PI3Ks (Fig. 2B). We also tested if 401 inhibits the protein kinase activity of p110α by examining autophosphorylation of p85α in the PI3K complex. Compound 401 at 25 μM had no effect on this reaction, whereas inhibition was seen with 5 μM LY294002 (Fig. 2C). Together, these results confirm that compound 401 has increased selectivity for mTOR over PI3K.

The sensitivity of 40 different protein kinases to 5 μM 401 was also determined. Most of these enzymes were inhibited less than 20% by 401 at this concentration (Table 1).

Effect of 401 on mTOR Signaling—S6K1 and Akt are two of the best-studied effectors of mTOR signaling. Both kinases possess highly conserved phosphorylation sites in the T loop of the kinase catalytic domain and in a C-terminal hydrophobic
**mTOR Kinase Inhibitor**

|          | Con | 041 | LY294002 |
|----------|-----|-----|-----------|
| P-Thr<sup>389</sup> S6K1 |       |     |           |
| S6K1     |       |     |           |
| P-Ser<sup>473</sup> Akt |       |     |           |
| P-Thr<sup>308</sup> Akt |       |     |           |
| Akt      |       |     |           |
| P-Thr/Tyr Erk |     |     |           |
| Erk2     |       |     |           |
| PDGF     | -   | +   | +         |
| PE       | +   | +   | +         |

**FIGURE 3. Effect of compound 401 on mTOR signaling in Rat-1 cells.** Cells were cultured in serum-free medium for 20 h and then preincubated for 20 min with 0.1% vehicle (dimethyl sulfoxide; Con) or 10 μM rapamycin (R). Cells were then stimulated for (A) 10 min with 50 ng/ml PDGF or (B) 20 min with 10 μM phenylephrine (PE). Cell lysate protein was analyzed on Western blots probed sequentially with antibodies to S6K1 (P-Thr<sup>389</sup> and total), Akt (P-Ser<sup>473</sup>, P-Thr<sup>308</sup>, and total), or Erk (P-Thr<sup>202/204</sup>Tyr<sup>204</sup> and total). The experiments were repeated with similar results.

Of Thr<sup>308</sup> in the T loop (6). Examination of the phosphorylation state of S6K1 Thr<sup>389</sup> and Akt Ser<sup>473</sup> is a convenient way to assess intracellular mTORC1 and mTORC2 activity, respectively. We expected that treatment of cells with an mTOR kinase inhibitor such as 401 would block phosphorylation of both sites, unlike rapamycin, which only affects S6K1.

To test the effect of 401 on growth factor-activated mTOR signaling, serum-starved Rat-1 fibroblasts were pretreated with inhibitors and then stimulated with PDGF. The phosphorylation of S6K1 and Akt was analyzed on Western blots probed with antibodies that recognize S6K1 phospho-Thr<sup>389</sup> or Akt phospho-Ser<sup>473</sup>. As expected for an mTOR kinase inhibitor, 401 decreased the phosphorylation of both sites (Fig. 3A). Inhibition was nearly complete at a concentration of 401 (10 μM) that has little effect on PI3K activity (see Fig. 2B). Consistent with its ability to target mTOR, LY294002 also reduced the phosphorylation of both sites (Fig. 3A). By contrast, rapamycin affected only the mTORC1 site (S6K1 Thr<sup>389</sup>, Fig. 3A). Compound 401 did not inhibit the phosphorylation of Erk induced by PDGF (Fig. 3A), indicating that it does not block all PDGF receptor-induced responses. The blots were reprobed with general antibodies to S6K1, Akt, or Erk2 to show that similar amounts of each protein were present in each lane (Fig. 3A).

These results are consistent with 401 acting as an mTOR kinase inhibitor in vivo.

Treatment of these Rat-1 cells with phenylephrine to stimulate the α<sub>1A</sub> adrenergic receptor also induced S6K1 Thr<sup>389</sup> phosphorylation that was inhibited in the presence of 401 or LY294002 (Fig. 3B). Because phenylephrine activation of S6K1 occurs without an increase in PI3K activity (20), it is likely that the inhibitory effect of 401 is due to inhibition of mTOR and not PI3K.

The PI3K inhibitor LY294002 blocked the phosphorylation of Akt Thr<sup>308</sup> (Fig. 3A), which was expected because PI 3-phosphates promote the colocalization of Akt and PDK1 at the plasma membrane (21). Surprisingly, Akt Thr<sup>308</sup> phosphorylation was also inhibited in the presence of 401 (Fig. 3A). Two possible explanations for this observation are that 401 inhibits PDK1 or that Thr<sup>308</sup> phosphorylation depends on prior phosphorylation of Ser<sup>473</sup>, as has been reported by others (22). To examine these possibilities, COS7 cells were transfected with Akt mutants that contain the acidic amino acid Asp at either Thr<sup>308</sup> or Ser<sup>473</sup> to mimic phosphorylation. Cells were then treated with insulin to induce phosphorylation of the intact site. Phosphorylation of Ser<sup>473</sup> was inhibited in the presence of 401, but phosphorylation of Thr<sup>308</sup> was not affected (Fig. 4). These results indicate that 401 does not inhibit PDK1, and suggest that decreased Akt Thr<sup>308</sup> phosphorylation is an indirect effect due to inhibition of mTOR-catalyzed Ser<sup>473</sup> phosphorylation.

Because 401 is a potent inhibitor of DNA-PK (IC<sub>50</sub> = 0.28 μM, Ref. 10), the possibility exists that its effects on Akt and S6K1 phosphorylation might be due either directly or indirectly to inhibition of DNA-PK. This possibility was tested in M059J glioma cells, which lack DNA-PK (23). Treatment of M059J
cells with 401 decreased the phosphorylation of Akt Ser\(^{473}\) and S6K1 Thr\(^{389}\) at the same doses that were effective in Rat-1 cells (Fig. 5). Thus, the inhibitory effect of 401 on phosphorylation of these mTOR sites is not due to DNA-PK inhibition.

**Effect of 401 on Growth and Survival of TSC\(^{-/-}\) MEFs—**

Tuberous sclerosis complex (TSC) is a disease characterized by the development of benign tumor-like growths called hamartomas in multiple tissues. This disorder is caused by mutations in the tsc1 or tsc2 tumor suppressor genes. The TSC1 and TSC2 proteins form a complex that suppresses mTOR activity. Loss of either protein leads to constitutive elevation of mTOR signaling (reviewed in Ref. 24).

Recent studies indicate that Akt activity in TSC\(^{-/-}\) or TSC2\(^{-/-}\) MEFs is abnormally low as compared with control MEFs due to feedback inhibition from the hyperactivated mTORC1/S6K1 pathway (25, 26). Long term treatment of these cells with rapamycin turns off the negative feedback program and up-regulates Akt, which can provide a survival signal (25).

To determine if rapamycin and 401 have different effects on Akt, TSC1\(^{-/-}\) MEFs were incubated with drugs for increasing times for up to 24 h. Cells cultured in the presence of rapamycin exhibited an increase in Akt Ser\(^{473}\) phosphorylation, whereas Akt phosphorylation remained low in cells cultured with 401 (Fig. 6A). Both drugs completely blocked S6K1 Thr\(^{389}\) phosphorylation (Fig. 6A). Several breast cancer cell lines (BT-474, MCF-7, and ZR-754) that up-regulated phospho-Akt Ser\(^{473}\) after 24 h in the presence of rapamycin also maintained low Akt phosphorylation levels in the presence of 401 (Ref. 27 and data not shown).

Next, we determined if rapamycin and 401 differentially affect the proliferation of TSC1\(^{-/-}\) cells. Rapamycin treatment for 1 day did not cause a significant decrease in cell number as compared with the control cells, but after 2 days a significant decrease (57%) was evident (Fig. 6B). By contrast, proliferation was strongly inhibited in cells exposed to 401 for 1 day (54%) or 2 days (84%) (Fig. 6B). Growth inhibition by 401 was dose-dependent, with a half-maximal effect at roughly 2 \(\mu M\) (Fig. 6C). The growth inhibitory effect of 401 is not due to general toxicity, as TSC1\(^{+/+}\) MEFs cultured for 2 days in the presence of the drug did not show a significant decrease in cell number (Fig. 6B). The effect of 401 on cell growth is also probably not due to DNA-PK inhibition, because TSC1\(^{-/-}\) MEFs cultured for 2 days in the presence of the drug did not show a significant decrease in cell number (Fig. 6B). The effect of 401 on cell growth is also probably not due to DNA-PK inhibition, because TSC1\(^{-/-}\) MEFs were resistant to AMA37 (Fig. 6D), a compound that inhibits DNA-PK (IC\(_{50} = 0.27 \mu M\)) but not mTOR (IC\(_{50} > 100 \mu M\)) (28).

To determine if the 401-induced decrease in cell number in the proliferation experiments above might be due in part to apoptosis, we measured the level of nucleosomes in the cytoplasm of drug-treated TSC1\(^{-/-}\) MEFs. Whereas the nucleosome level was not changed in rapamycin-treated cells, 401 induced a significant increase in apoptosis (Fig. 6E). Treatment of TSC1\(^{-/-}\) MEFs with an Akt1/2 inhibitor (29) also increased apoptosis, suggesting that the cytotoxic effect of 401 might be due in part to suppression of mTORC2/Akt signaling (Fig. 6E). These results suggest that inhibition of mTOR kinase activity by a small molecule inhibitor is more effective than rapamycin at killing TSC1\(^{-/-}\) MEFs that exhibit hyperactivated mTOR signaling.

**DISCUSSION**

Preclinical studies examining the effect of rapamycin or its analogs (CCI-779, RAD001, and AP23573) on tumor cells have supported mTOR as a therapeutic target in cancer (30). These drugs potently inhibit the proliferation of some tumor cell lines grown in culture or in mice as xenografts (31). Rapamycin also has antiangiogenic properties that can have dramatic antineoplastic effects, as demonstrated in an animal model of metastasis (32). Rapamycin and its derivatives are currently in clinical trials for the treatment of cancer (30). Early reports indicate that they exhibit anti-tumor activity in some patients with renal cell carcinoma and breast cancer (33). Rapamycin also caused regression of astrocytomas associated with TSC (34). Although rapamycin-based drugs show promise in treating cancer, the overall response rate in patients is low and it is becoming clear that alternative inhibitors of mTOR would be beneficial. In addition to a possible clinical benefit, use of mTOR kinase inhibitors as an experimental research tool will increase our understanding of mTORC2 signaling and its role in physiological and pathological cellular processes.

Our data indicate that compound 401 exhibits the expected properties of an mTOR kinase inhibitor on signaling in intact cells. Treatment of cells with 401 blocked growth factor-induced phosphorylation of S6K1 Thr\(^{389}\) and Akt Ser\(^{473}\) (Figs. 3 and 4) at the same doses that inhibit mTOR kinase activity in vitro (Fig. 2A). Use of a cell line that does not express DNA-PK...
were made using Tukey’s post hoc tests.

is defined as an increase in apoptosis. By contrast, TSC1

 Activity for survival. One likely mediator of an mTORC2-de-

resistant to both drugs (Fig. 6). One interpretation of these

findings that are due to inhibition of this enzyme (Fig. 5). Our findings that our compound does not affect the PDGF receptor/Raf/MEK or insulin/IGF-I receptor/PI3K/PDK1 pathways. Indeed, in vitro assays showed that 401 does not inhibit PI3K (Fig. 2B), the PDGF, insulin or IGF-I receptors or MEK1 (Table 1). Together, these data are consistent with an inhibitory effect on mTORC1 and mTORC2.

According to the concepts of “oncogene addiction” and “tumor suppressor hypersensitivity”, cancer cell growth and viability become dependent on pathways that are altered by specific activated oncogenes or loss of tumor suppressor genes (35). Rapamycin had a growth-inhibitory effect on TSC1/−/− MEFs without inducing apoptosis, whereas 401 induced a severe suppression of proliferation that is due at least in part to an increase in apoptosis. By contrast, TSC1+/−/− MEFs were resistant to both drugs (Fig. 6). One interpretation of these results is that TSC deficiency renders MEFs dependent on mTORC1 activity for optimal proliferation and on mTORC2 activity for survival. One likely mediator of an mTORC2-de-

ruled out the possibility that the effects of 401 on S6K1 and Akt are due to inhibition of this enzyme (Fig. 5). Our findings that 401 did not block PDGF-induced Erk phosphorylation (Fig. 3A) or insulin-activated Akt Thr308 phosphorylation (Fig. 4) suggest that the compound does not affect the PDGF receptor/Raf/MEK or insulin/IGF-I receptor/PI3K/PDK1 pathways. Indeed, in vitro assays showed that 401 does not inhibit PI3K (Fig. 2B), the PDGF, insulin or IGF-I receptors or MEK1 (Table 1). Together, these data are consistent with an inhibitory effect on mTORC1 and mTORC2.

A number of different signaling pathways regulate mTORC1 activity, and the best characterized positive effector is the PI3K/Akt pathway. Akt controls mTOR through the TSC1 and TSC2 proteins and Rheb, a small GTP-binding protein related to Ras (41). The TSC1/TSC2 heterodimer opposes mTOR signaling by stimulating the GTPase activity of Rheb (42). In the presence of growth factors that activate PI3K/Akt, Akt phosphorylates TSC2 and inhibits the TSC1/2 complex (43). This allows Rheb to maintain an active GTP-bound form that increases mTORC1 signaling to S6K1 and other effectors (44). Growth factor activation of phospholipase D (PLD) is another mitogen-activated pathway that provides a stimulatory signal to mTOR (45). Data from our laboratory suggest that growth factors can use either PI3K/Akt or PLD to activate mTORC1 signaling (46). PLD catalyzes the hydrolysis of phosphatidylcholine to yield phosphatidic acid (PA) and choline. It was reported that PA
binds to the rapamycin-binding domain of mTOR and is required for mTOR activation (45). Finally, inhibition of mTOR in response to low intracellular energy levels is mediated by AMP-activated kinase (AMPK) and its activator, the protein kinase LKB1 (47). Under conditions in which intracellular ATP is depleted and the level of AMP is increased, AMP binding to AMPK induces a conformational change that allows LKB1 to phosphorylate and activate AMPK (48). AMPK then phosphorylates TSC2 on a site that enhances its ability to turn off mTOR signaling (49). The details of how or if these pathways regulate mTORC2 are not yet known.

Alterations in the three pathways discussed above can lead to hyperactivation of mTOR signaling and have been identified in many human proliferative disorders. The optimal approach for treating mTOR-dependent tumors will probably depend to a certain extent on which pathway is activated. Genetic defects in components of the PI3K/Akt pathway are especially common (1, 50). Mutation or loss of expression of PTEN, a phosphatase that dephosphorylates the 3’-OH position of PI 3-phosphates, is frequently seen, particularly in breast and lung tumors (51). Gain-of-function mutations in the PIK3CA gene that encodes p110α are found in breast, colorectal, brain, and other cancers (52–54). A survey of colorectal tumors showed that nearly 40% of them contain alterations in one of eight PI3K pathway genes, including Akt2 and PDK1 (55). Amplification of the Akt2 gene was also detected in 12% of ovarian carcinomas (56). Currently, there is strong interest in developing isoform-selective PI3K inhibitors as anti-cancer agents. Interestingly, the unique ability of one such compound (PI-103) to block the proliferation of malignant glioma in culture and in xenografts was attributed to its ability to target both the p110α isoform of PI3K and mTOR (57). In light of the multiplicity of cellular processes regulated by PI3Ks, side effects caused by PI3K inhibitors might limit their use as therapeutic agents. For example, PI-103 induced insulin resistance in mice (17). Because mTOR inhibitors such as 401 would block Akt activation without affecting PI3K activity, this type of agent might have a more limited side effect profile than PI3K inhibitors.

Dysregulation of PLD has also been detected in human cancer. PLD activity was reported to be significantly increased in 17 out of 19 breast tumors as compared with normal breast tissue (58), and PLD1 protein and mRNA levels were overexpressed in 14 of 17 breast cancer tissues (59). Elevated PLD activity in breast cancer cell lines confers rapamycin resistance (60) that may be due to competition of PA and the drug for the same binding site on mTOR (45). It would be of interest to determine if tumors that exhibit high PLD activity are sensitive to an mTOR kinase inhibitor such as 401.

Finally, inactivating mutations in LKB1 up-regulate mTOR signaling and are associated with Peutz-Jeghers syndrome, which is characterized by benign hamartomatous polyps in the gastrointestinal tract and an increased risk for cancer (47, 61). Akt activity in LKB1-deficient cells is low, and rapamycin rescues them from apoptosis induced by glucose deprivation (49). We predict that the protective effect of rapamycin in these cells is due to Akt activation, and that 401 would potentiate apoptosis in the absence of glucose. Further development and testing of selective and potent mTOR kinase inhibitors will be needed to address these and other questions about mTOR signaling and its role in cancer.

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