LY303511 (2-Piperazinyl-8-phenyl-4H-1-benzopyran-4-one) Acts via Phosphatidylinositol 3-Kinase-Independent Pathways to Inhibit Cell Proliferation via Mammalian Target of Rapamycin (mTOR)- and Non-mTOR-Dependent Mechanisms

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ABSTRACT

Mammalian target of rapamycin (mTOR), a serine/threonine kinase, regulates cell growth and proliferation in part via the activation of p70 S6 kinase (S6K). Rapamycin is an antineoplastic agent that, in complex with FKBP12, is a specific inhibitor of mTOR through interaction with its FKBP12-rapamycin binding domain, thereby causing G1 cycle arrest. However, cancer cells often develop resistance to rapamycin, and alternative inhibitors of mTOR are desired. 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) blocks mTOR kinase activity, but it also inhibits phosphatidylinositol 3-kinase (PI3K), an enzyme that regulates cellular functions other than proliferation. We hypothesized that a close structural analog, 2-piperazinyl-8-phenyl-4H-1-benzopyran-4-one (LY303511) might inhibit mTOR-dependent cell proliferation without unwanted effects on PI3K. In human lung epithelial adenocarcinoma (A549) cells, LY303511, like rapamycin, inhibited mTOR-dependent phosphorylation of S6K, but not PI3K-dependent phosphorylation of Akt. LY303511 blocked proliferation in A549 as well as in primary pulmonary artery smooth muscle cells, without causing apoptosis. In contrast to rapamycin, LY303511 reduced G2/M progression as well as G2/M-specific cyclins in A549 cells. Consistent with an additional mTOR-independent kinase target, LY303511 inhibited casein kinase 2 activity, a known regulator of G1 and G2/M progression. In addition to its antiproliferative effect in vitro, LY303511 inhibited the growth of human prostate adenocarcinoma tumor implants in athymic mice. Given its inhibition of cell proliferation via mTOR-dependent and independent mechanisms, LY303511 has therapeutic potential with antineoplastic actions that are independent of PI3K inhibition.

In response to mitogens, intracellular kinases regulate numerous biological processes, including cell proliferation and apoptosis. Mammalian target of rapamycin (mTOR) and phosphatidylinositol 3-kinase (PI3K) belong to a family of proteins with C-terminal PI3K-like domains and have emerged as key regulatory enzymes that control cell fate (Blume-Jensen and Hunter, 2001). In response to mitogens and nutrients, mTOR kinase activity regulates cell proliferation in part by activating p70 S6 kinase (S6K) and subsequent cell cycle progression through the G1/S checkpoint (Huang et al., 2003). Drugs that target mTOR are effective antineoplastic agents (Bjornsti and Houghton, 2004) and are used to block smooth muscle cell proliferation and restenosis after coronary angioplasty (Marks, 2003).

Rapamycin blocks the activation of S6K by forming a stable complex between FKBP12 and the FKBP12-rapamycin binding domain of mTOR, which is located N-terminal to the kinase domain (Huang et al., 2003). The resulting net dephosphorylation of S6K at T389 correlates with inhibition of S6K activity. mTOR or S6K regulates cyclin D, cyclin E, cyclin A, and p21 and p27 levels and G1 cell cycle progression (Albers et al., 1993; Chou et al., 2003; Nelsen et al., 2003). By effects on the cyclin-dependent kinases (Cdks), mTOR also increases phosphorylation of the retinoblastoma protein (Rb), ABBREVIATIONS: mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; S6K, p70 S6 kinase; Cdk, cyclin-dependent kinase; Rb, retinoblastoma protein; LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; LY303511, 2-piperazinyl-8-phenyl-4H-1-benzopyran-4-one; PASM, pulmonary artery smooth muscle; CK2, casein kinase 2; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; FN-γ, interferon-γ; BrdU, 5-bromo-2-deoxy-uridine; MOPS, 3-(N-morpholino)propanesulfonic acid; ANOVA, analysis of variance; HSD, honestly significant difference; L/I, lipopolysaccharide and interferon-γ; UCN-01, 7-hydroxystaurosporine; RAD001, everolimus; CCI-779, temsirolimus.
which disinhibits the transcription factor E2F, allowing for the transcription of genes involved in DNA synthesis and progression through the cell cycle (Hidayat et al., 2003).

Activation of PI3K by growth factors or mitogens also plays an important role in cell proliferation and apoptosis, in part by activating Akt (Fruman and Cantley, 2002; Chang et al., 2003). Increased PI3K activity leads to the recruitment of Akt to cell membrane signaling domains, its phosphorylation, and its activation. Phosphorylation of Akt at T308 or S473 correlates closely with its kinase activity. Akt can phosphorylate a variety of effectors, including those that promote cell cycle progression, inhibit apoptosis, or activate genes whose products regulate lipid and glucose metabolism, cell proliferation, and survival. Inhibitors of PI3K-like domain-containing proteins, such as LY294002, have been used in vitro and in vivo to block cell proliferation, tumor growth, and inflammation (Hu et al., 2000; Kwak et al., 2003). However, LY294002, which targets multiple isoforms of PI3K, each with diverse regulatory functions (e.g., metabolism and cytokinesis), has not been a useful therapeutic agent (Luo et al., 2003), perhaps because of unacceptable "side effects" resulting from inhibition of numerous critical PI3K-dependent pathways.

X-ray crystallographic analysis demonstrated that the LY294002 morpholino oxygen forms a hydrogen bond with T882, thereby displacing the ATP adenine from the PI3K active site. Likely via a similar mechanism, LY294002 also potently inhibits mTOR activity (Brunn et al., 1996) and its autophosphorylation (Kristof et al., 2001). LY294002 seems to interact with multiple structurally similar kinase catalytic sites, although its effectiveness differs. In contrast to LY294002, rapamycin inhibits mTOR via binding to the FKBP12-rapamycin binding domain in mTOR (Brown et al., 1995). However, rapamycin does not block mTOR autophosphorylation at S2481, and demonstration of a direct effect on mTOR kinase activity in vivo has been elusive (Peterson et al., 2000; Raught et al., 2001).

Structural analogous of LY294002 differ in their pharmacological profile with respect to effects on PI3K (Ding et al., 1995). Published studies use LY303511 as controls for LY294002 in experiments looking at PI3K effects in cultured cells (Ding et al., 1995; Reddy et al., 2000; Altmann et al., 2003). LY303511 differs from LY294002 by an amine substitution for the morpholino oxygen (Fig. 1A), thus reducing its capacity to inhibit PI3K (Vlahos et al., 1994). Here, we show that, like rapamycin, LY303511 inhibits mTOR-dependent activation of the S6K pathway and blocks proliferation of lung epithelial adenocarcinoma (A549) cells and primary human pulmonary artery smooth muscle (PASM) cells in vitro as well as human prostate adenocarcinoma tumors in vivo. Unlike rapamycin, however, LY303511 caused a combined G1 and G2/M arrest, suggesting an additional kinase target. Like LY294002 (Davies et al., 2000), LY303511 inhibited casein kinase 2 (CK2), an enzyme that can influence G1 and G2/M progression. LY303511 is a novel inhibitor of mTOR-dependent and independent pathways that control the cell cycle.

Fig. 1. LY303511 is a structural analog of LY294002 and inhibits mTOR-dependent signaling. A, chemical structures of LY294002 and LY303511. The morpholino oxygen in LY294002 is replaced by an amine in LY303511. A549 cells were incubated without or with 100 nM W/262LY294002, 200 ng/ml rapamycin, or 200 nM wortmannin (B) or 0 to 100 nM LY303511 (C and D) for 1 h before addition of L/I for 30 min and preparation of cell lysates. Levels of phospho-p70 S6 kinase (pS6K), phospho-Akt (pAkt), total p70 S6 kinase (S6K), total Akt (Akt), phospho-mTOR (pmTOR), or total mTOR (mTOR) were analyzed by Western blot. For D, mean integrated band density measurements (pmTOR normalized to total mTOR) s E.M. are represented graphically. Data are means of values from five experiments (s E.M.), s, p < 0.05 by Student's t test.
Materials and Methods

Cell Culture. A549 cells (CCL 185, American Type Culture Collection, Manassas, VA), a human alveolar type II epithelial cell-like lung adenocarcinoma cell line, were grown at 37°C with 5% CO₂ in Ham’s F-12 K medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml penicillin, and 100 μM streptomycin (all from Biosoft, Rockville, MD). Human PASM cells (Cambrex Bio Science Walkersville, Inc., Walkersville, MD) were grown at 37°C with 5% CO₂ in SMG2 medium supplemented with 5% FBS, insulin, fibroblast growth factor, epidermal growth factor, and gentamicin/amphotericin as per the manufacturer’s instructions. Pharmacological Inhibitors and Antibodies. LY303511 and rapamycin (Calbiochem, San Diego, CA) or LY294002 and wortmannin (BIOMOL Research Laboratories, Plymouth Meeting, PA) were dissolved in DMSO. Antibodies against phospho-S6K T389, phospho-Akt S473, phospho-mTOR S2448, phospho-Rb S807/S811, S6K, and Akt were purchased from Cell Signaling Technology Inc. (Beverly, MA). Monoclonal antibodies against mTOR (RAFT1), cyclin A, cyclin B, cyclin D, cyclin E, p27 Kip1, and p21 Cip1 were purchased from BD Biosciences (San Diego, CA).

Measurement of Protein Phosphorylation. A549 cells were incubated in serum-free medium without or with inhibitors for 1 h, as indicated, before incubation for 30 min with a mixture of 100 μg/ml lipopolysaccharide (Sigma, St. Louis, MO) and 100 U/ml IFN-γ (Hoffman-La Roche, Nutley, NJ). PASM cells were incubated in serum-free medium without or with inhibitors for 24 h, as indicated, before incubation for 30 min with 10% FBS. A549 or PASM cells were washed once with ice-cold phosphate-buffered saline and incubated for 15 min on ice in lysis buffer (20 mM Tris, pH 8.0, 1% Nonidet P-40, 1 mM EDTA, 5 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml trypsin soybean inhibitor, 1 mM phenylmethylsulfonyl fluoride, 50 mM sodium fluoride, 100 μM sodium orthovanadate, and 1 mM dithiothreitol). After freezing and thawing, lysates were centrifuged for 30 min at 16,000 g at −80°C. Equal amounts of total protein were separated by SDS-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane, before immunoblotting with primary antibodies as indicated. Membranes were treated with anti-rabbit IgG or anti-mouse IgG antibodies (Promega, Madison, WI) linked to horseradish peroxidase, developed using an enhanced chemiluminescence detection kit (SuperSignal West Pico; Pierce Chemical, Rockford, IL), and exposed to X-ray film, which was scanned using an Epson Expression 636 scanner. Integrated band densities were quantified using Scion Image beta 3b software (Scion Corporation, Frederick, MD) or with imaging software (BD Biosciences). The 488-nm line from an argon laser was used for excitation of the propidium iodide, and the emitted fluorescence was collected using a 585-nm band pass filter (FL2). Listmode data were collected on a linear scale using Cell Quest software. Propidium iodide-stained cells were counted by flow cytometry, and the percentage of cells in G₀, S, or G₂/M phase was determined.

Measurement of CK2 Activity. CK2 activity (counts per minute) was measured using a CK2 activity kit (Upstate Biotechnology, Charlottesville, VA) by incubating 100 ng of recombinant CK2, magnesium/ATP cocktail (10 μM of γ-32P]ATP, 0.675 μM of MgCl₂, and 4.5 nmol of ATP), and 10 μmol of peptide substrate (amino acid sequence RRDDDDSDDDD) in 50 μl of assay buffer (20 mM MOPS, pH 7.2, 5 mM EGTA, 25 mM β-glycerol phosphate, 1 mM sodium orthovanadate, and 1 mM dithiothreitol) without or with the indicated concentrations of LY294002. LY303511, wortmannin, or rapamycin in 1% DMSO for 10 min at 30°C. Assays were stopped with 20 μl of 40% trichloroacetic acid, and samples (25 μl) of supernatant were applied to PS1 phosphocellulose paper squares, which were then washed three times with 0.75% phosphoric acid and once with acetone. 32P in the substrate peptide was quantified using a PerkinElmer Life and Analytical Sciences 100 Tri-Carb liquid scintillation counter (Boston, MA) on phosphocellulose squares in 5 ml of scintillation fluid. Activity is expressed as picomoles of phosphate incorporated per 10 min. CK2 activity measured in the presence of inhibitors was divided by that measured for the DMSO control (100% control).

Evaluation of Tumor Growth in Vivo. Human prostate adenocarcinoma (PC-3) cells (ATCC CRL-1435) were cultured in vitro before harvesting and implantation of 1 × 10⁶ cells in 20% Matrigel/α-phenethrynic NCR nude mouse by subcutaneous injection at the flank. Inoculated mice were subdivided into four groups of 10. Administration of vehicle or LY303511, 10 mg/kg/day, was begun (day 1) when tumors reached ~150 mm³ (n = 35). At each time point. Data are expressed as the average tumor volumes ± S.E.M. for each treatment group and time point. Experimental procedures were in accordance with the institutional animal care and use committee.

Statistical Analysis. Statistical analysis was performed using JMP 5.0.1a (SAS Institute, Cary, NC). Two-way ANOVA with Tukey-Kramer honestly significant difference (HSD) post hoc analysis was performed to detect statistically significant differences in tumor volume related to treatment group or time. Repeated measures ANOVA was performed to confirm the inter- and intragroup interactions. In addition, the rate of tumor growth for each mouse was estimated by linear regression analysis. Linearity was confirmed by evaluating residual plots. Mean growth rates (slopes of the regression lines) for each treatment group was compared by one-way ANOVA and Tukey-Kramer HSD post hoc analysis. For ethical reasons, the protocol required euthanasia of mice with tumor volumes greater than 1000 mm³, and excess variability was observed for all groups after day 21. All data collected after day 21 were censored. Finally, a multiple linear regression model was used to examine the effects of initial tumor volume, time, and treatment group on tumor volume.

Results

LY303511 Inhibits Mitogen-Activated Phosphorylation of S6K and mTOR in A549 Cells. In previous experiments in A549 cells, lipopolysaccharide and interferon-γ (L/I) stimulated mTOR activity, as reflected by its autophosphorylation at S2481 (Kristof et al., 2003); phosphorylation of this site requires autokinase activity, and the S2481 moiety does not seem to be phosphorylated by other kinases in intact cells (Peterson et al., 2000). Moreover, L/I stimulated mTOR-dependent phosphorylation of S6K (Kristof et al., 2003). LY294002 was a potent inhibitor of mTOR autophosphorylation, whereas the PI3K inhibitor wortmannin had
minimal effect. We used L/I-stimulated A549 cells to test the ability of LY303511 to inhibit signaling via mTOR.

LY303511 differs from LY294002 by a substitution that replaces the morpholino oxygen with an amine (Fig. 1A) and has no apparent effect on PI3K activity (Ding et al., 1995). We hypothesized that the structural difference might enable LY303511 to inhibit other kinase sites with different specificity and thus have a different pharmacological profile.

LY303511 as well as wortmannin and the mTOR inhibitor rapamycin blocked L/I-stimulated phosphorylation of S6K at T389 (Fig. 1B). In contrast to wortmannin, which inhibits PI3K, LY303511 and rapamycin increased basal and L/I-stimulated phosphorylation of Akt at S473 (Fig. 1, B and C). The opposing effects of LY303511 on S6K and Akt phosphorylation are consistent with its potential selectivity for mTOR-dependent signaling events and were similar to rapamycin.

In L/I-stimulated cells, 1 to 100 μM LY303511 inhibited autophosphorylation of mTOR in a concentration-dependent manner (Fig. 1D). The inhibitory effect was greater in stimulated than in unstimulated cells. Unlike rapamycin, LY303511 had minimal effects on basal phosphorylation of S6K (Fig. 1B, lane 1 versus 2). Thus, LY303511 inhibited LPS/IFN-γ-stimulated mTOR activity and mTOR-dependent phosphorylation of S6K in PI3K-independent manner, and at concentrations as low as 1 μM.

**LY303511 Inhibits A549 Cell Proliferation.** Since mTOR and S6K are important regulators of cell cycle progression, we next measured the effect of LY303511 on cell proliferation. A549 cells (80,000 cells per well) were grown for 24 h before addition of inhibitors (Fig. 2A). After 24 h, cells incubated with trypan blue were counted using a hemocytometer. Cell viability was >99% under all conditions (data not shown). In cells exposed to vehicle alone, cell number increased by ~50% over 24 h. Rapamycin slightly attenuated cell proliferation, whereas LY303511 had a significant inhibitory effect, almost equal to that of LY294002. Consistent with PI3K-independent effects of LY303511 and LY294002, wortmannin did not significantly reduce A549 cell proliferation. LY303511 did not induce apoptosis or necrosis as determined by flow cytometric analysis of propidium iodide-stained cells (data not shown). In separate experiments, LY303511 significantly inhibited A549 cell proliferation, but it did not cause cytotoxicity by LDH release assay (Supplemental Fig. 1).

The effect of LY303511 on proliferation accompanied a marked inhibition of DNA synthesis over 24 h as assessed by BrdU incorporation (Fig. 2B). Whereas rapamycin had a
weak inhibitory effect on DNA synthesis, administration of LY303511 led to a concentration-dependent reduction (Fig. 2B). There was no additional effect when LY303511 was combined with rapamycin. DMSO alone had no effect on A549 cell proliferation (data not shown). At low concentrations (1–10 μM), LY303511 inhibited phosphorylation of S6K or mTOR but not cell proliferation or cell cycle (Figs. 1 and 2), suggesting an additional mTOR- or G1-independent mechanism for the inhibition of cell proliferation by LY303511.

Effect of LY303511 on Cell Cycle. To determine the mechanism by which LY303511 inhibited cell proliferation, A549 cells were exposed to inhibitors for 24 h before harvesting, staining with propidium iodide, and counting by flow cytometry. Cells were gated by intensity of propidium iodide staining to determine the proportions of cells in G1, S, or G2/M phase of the cell cycle. Consistent with an effect on DNA synthesis, 100 μM LY303511 significantly reduced the fraction of cells in S phase (Fig. 2C). The proportion of cells in G2/M remained unchanged, indicating that cells were arrested in both G1 and G2/M. In contrast, rapamycin increased the G1 population by reducing the proportion of cells in both S and G2/M. The effects of 10 μM LY303511 and rapamycin on the reduction in S phase cells were additive to that of 10 μM LY303511 alone (P = 0.056; Fig. 2C). The greater reduction in S phase cells produced by adding LY303511 to rapamycin occurred without a further decrease in G2/M, indicating that LY303511 blocks the cell cycle by an additional mechanism that differs from that used by rapamycin.

Effect of LY303511 on Cell Cycle Markers. To define better the mechanism by which LY303511 inhibited the cell cycle, we assessed its effect on the levels of cyclins and cell cycle inhibitors. Consistent with a reduction in mTOR kinase activity was the inhibition of phosphorylation of S6K at T389 12 and 24 h after addition of either LY303511 or rapamycin (Fig. 3B). Like rapamycin, LY303511 increased cyclin D levels (Fig. 3B). LY303511 also increased p27 Kip1 (p27) levels, indicating an effect on inhibitors of G1/S transition similar to that of rapamycin (Fig. 3A).

In contrast to rapamycin, however, LY303511 caused a significant increase in p21 Cip1 (p21) (Fig. 3A), which, in
addition to regulating the G1 checkpoint, can inhibit cyclin B-dependent entry into mitosis (Vermeulen et al., 2003). LY303511, but not rapamycin, decreased levels of cyclin A and B, which are also regulators of S and G2/M phase progression (Fig. 3B) (Vermeulen et al., 2003). LY303511 reduced the phosphorylation of Rb, suggesting that the mechanism of cell cycle inhibition was, in part, due to inhibition of E2F-dependent genes. These results support a role for LY303511-sensitive kinase(s) in late S and G2/M progression, in addition to G1/S transition.

**LY303511 Inhibits Serum-Activated Phosphorylation of S6K and Akt in Pulmonary Artery Smooth Muscle Cells.** Because of the demonstrated role of mTOR and PI3K in smooth muscle cell growth and proliferation (Ammit and Panettieri Jr., 2001), and to evaluate the relative effects of LY303511 on cancer and primary cells, we assessed its effect on proliferation, cell cycle, and phosphorylation of S6K or Akt in PASM cells. Like rapamycin, LY303511 decreased basal and FBS-stimulated phosphorylation of S6K in a dose-dependent manner (Fig. 4, A and B). LY303511, unlike its effect in A549 cells, also decreased the phosphorylation of Akt, albeit to a lesser extent than that of S6K (Fig. 4B). Rapamycin, however, increased phosphorylation of Akt, suggesting that, in these cells, apart from inhibiting mTOR-dependent phosphorylation of S6K, LY303511 might inhibit PI3K or another kinase that regulates the phosphorylation of Akt at S473.

**LY303511 Inhibits Cell Proliferation and Cell Cycle Progression in Pulmonary Artery Smooth Muscle Cells.** In PASM cells incubated with serum before addition of inhibitors and BrdU, rapamycin had little effect on proliferation, whereas LY303511 inhibited in concentration-dependent manner (Fig. 4C). The effect of rapamycin on proliferation, without or with LY303511, was enhanced when PASM cells were incubated in serum-free medium for 24 h before addition of FBS (Fig. 4D). The effect of LY303511 on PASM cell proliferation, like that of rapamycin, was enhanced in the absence of serum. The effect on proliferation of 10 μM LY303511 and 200 ng/ml rapamycin were additive to that of 10 μM LY303511 alone (p < 0.05; Fig. 4, C and D). Although

![Fig. 4. LY303511 inhibits serum-stimulated phosphorylation of S6K and Akt as well as proliferation in PASM cells. After incubation in serum-free medium for 24 h, PASM cells were incubated without or with 100 μM LY303511, 200 ng/ml rapamycin, or 200 nM wortmannin (A) or 0 to 100 μM LY303511 (B) for 1 h before the addition of 10% FBS for 30 min. Phospho-p70 S6 kinase T389 (pS6K) or phospho-Akt S473 (pAkt) were detected by Western blot. Data are from one experiment representative of five. PASM cells (4000 per well) were grown for 24 h before incubation for 24 h in medium with (C) or without (D) 10% FBS and then addition of 10% FBS, 10 μM BrdU, and inhibitors as indicated for 24 h. BrdU incorporation was measured by in situ enzyme-linked immunosorbent assay as per the manufacturer’s instructions (BrdU detection kit; Hoffman-La Roche). Data are means of BrdU content relative to DMSO control (% control) ± S.E.M. from three experiments with assays in sextuplet. *, p < 0.05 versus DMSO control or †, p < 0.05 versus 10 μM LY303511, by Student’s t test.
Akt phosphorylation was inhibited by LY303511 (Fig. 4B), wortmannin did not inhibit cell proliferation, indicating that this effect of LY303511 was independent of PI3K.

As expected from previous studies on primary smooth muscle cells, incubation with rapamycin or serum-free medium led to G1 arrest (Fig. 5A). In contrast, and consistent with A549 cells, LY303511 reduced the proportion of cells in S phase by increasing the fraction in G1 and G2/M phase. The effects of rapamycin plus LY303511 on the reduction of S phase cells were additive. The increase in number of smooth muscle cells in G1 in response to LY303511 was smaller than that observed in A549 cells, perhaps reflecting the analysis of cell cycle in cell types with different basal rates of proliferation. As was the case for A549 cells, LY303511 did not significantly affect cell viability by propidium iodide staining of sub-G1 cells. In separate experiments, LY303511 significantly inhibited PASM cell proliferation, but it did not cause cytotoxicity by LDH release assay (Supplemental Fig. 1).

The Effect of LY303511 on CK2. We recently reported microarray gene expression analyses in which A549 cells were incubated without or with LY294002 or wortmannin for 1 h before the addition of L/I for 6 h (Kristof et al., 2003). By cellular feedback mechanisms, the levels of mRNA encoding proteins that are directly inhibited by a pharmacological agent might be increased in the presence of that agent. To identify additional kinase targets for LY294002 that are PI3K-independent, mRNAs that were increased by LY294002, and not by wortmannin, were identified from the data set by filtering and statistical analysis. As expected, the levels of mTOR mRNA were increased in the presence of LY294002 (1.7-fold) and not by wortmannin. The mRNAs of other kinases that were increased by LY294002 included that encoding CK2α (1.8-fold; GenBank accession no. M55268).

In a previous study, LY294002 inhibited CK2 activity in vitro (Davies et al., 2000). Since CK2 can regulate both G1 and G2/M cell cycle transitions (Litchfield, 2003), we tested the effects of LY294002 and LY303511 on CK2 activity.

Incubation of recombinant CK2 with LY303511 or LY294002 led to a concentration-dependent inhibition of CK2 activity (Fig. 5B). The approximate IC50 for 10 μM LY294002 was one-tenth that for LY303511. Neither wortmannin nor rapamycin affected CK2 activity. These data indicate that CK2 might be another kinase target for LY303511 in intact cells.

The Effect of LY303511 on Tumor Growth in Vivo. We next tested the ability of LY303511 to inhibit proliferation in vivo. A mouse xenograft model of human prostate adenocarcinoma (PC-3) tumor growth was chosen because inhibition of PC-3 cell proliferation by rapamycin was demonstrated previously (Grunwald et al., 2002). Intraperitoneal administration of vehicle or LY303511 was begun when tumors reached a volume of ~150 mm3, at which time 35 mice had developed a tumor. After 21 days, >15% of the mice required euthanasia because of excessive tumor growth, and these data were censored due to unreliable estimates of average tumor volume. Since LY303511 suppressed growth in vitro, and it did not induce apoptosis, we chose to assess the effect of duration of treatment on the suppression of tumor growth.

Despite the requirement for micromolar concentrations in vitro, the administration of LY303511, 10 mg/kg/day, was sufficient to inhibit PC-3 tumor growth in vivo (Fig. 6, A and B). The effects of time and duration of treatment were statistically significant by two-way ANOVA (Fig. 6A; p < 0.0001). The same was true for the means of data normalized to initial tumor volume (i.e., percentage of increase in tumor volume; data not shown). Longer duration of therapy (treatment for 10 or 20 days; groups 3 or 4) led to greater suppression of tumor growth. By Tukey-Kramer HSD, the effect of time on tumor volume was statistically significant when comparing each of days 10 to 21 versus day 1 (Fig. 6A; p < 0.05).

In addition, for all time points, the effect of treatment for 10 or 20 days (groups 3 and 4) was significantly different from that due to treatment for 0 or 5 days (groups 1 and 2), but not from each other (p < 0.05). The effect of group 2 was statistically different from those of groups 1, 3, and 4, respectively (p < 0.05). Perhaps due to variability in the data, tumor growth rates over the first 5 days of treatment seemed different among the groups (Fig. 6A); however, these differences were not statistically significant.

Over the 21-day study period, the effect of treatment group...
mTOR and PI3K share a homologous C-terminal domain, the structure of which is common to members of the PI3- and 4-kinase family of proteins (PFAM accession no. PF00454). This family includes other cell cycle regulatory proteins, such as DNA-dependent protein kinase, ataxia telangiectasia mutated protein kinase, and ataxia telangiectasia and Rad3-related protein kinase. Others demonstrated PI3K-independent effects of LY294002 (Kristol et al., 2003) or LY303511 (Choi et al., 2004). Our group showed that LY294002 inhibited mTOR-dependent activation of the proinflammatory transcription factor signal transducer and activator of transcription-1 (Kristol et al., 2003). Here, we further identify the molecular mechanism by which LY303511 inhibits cell proliferation. LY303511, an analog of LY294002 (Fig. 1A), preferentially inhibited mTOR-dependent activation of S6K, mTOR autophosphorylation, and proliferation of A549 cells. Unlike rapamycin, LY303511 inhibited cell proliferation by inducing G2/M arrest in addition to G1 arrest and had a different effect on G1 and G2/M cyclins. The ability of LY303511 and LY294002 to inhibit CK2 in vitro suggests a second mTOR- and PI3K-independent mechanism by which these inhibitors might block cell proliferation. These results establish a novel family of compounds that might be useful for the treatment of neoplastic disorders.

By a variety of mechanisms, some tumors and cell lines are resistant to the antiproliferative effects of rapamycin (Huang et al., 2003). The effects of LY303511 on the S6K and PI3K pathways were similar to that of rapamycin, suggesting that LY303511 targeted the mTOR kinase domain. However, LY303511 was a more efficient inhibitor of cell proliferation than rapamycin. In contrast to rapamycin, LY303511 decreased cyclin A and B levels and induced G2/M arrest. LY303511 also increased the levels of cell cycle inhibitor p21; whereas, consistent with previous studies, rapamycin decreased p21 (Nelsen et al., 2003) (Fig. 3A). These differences between LY303511 and rapamycin support our conclusion that LY303511 inhibited a pathway distinct from mTOR.

The finding that LY303511 and LY294002 block CK2 activity suggests a new alternative target for this class of drugs and is consistent with an mTOR-independent mechanism for the inhibition of cell proliferation and cell cycle regulation. CK2 is a ubiquitous and highly conserved serine/threonine kinase that is required for cell survival (Ahmed et al., 2002). In general, tumor cells exhibit high levels of CK2 activity, and is consistent with an mTOR-independent mechanism for the inhibition of cell proliferation and cell cycle regulation. CK2 is a ubiquitous and highly conserved serine/threonine kinase that is required for cell survival (Ahmed et al., 2002; Litchfield, 2003). Although its molecular mechanisms are poorly understood, CK2 protects cells from apoptosis by directly phosphorylating proteins such as p53, BH3-only proapoptosis protein, β-catenin, or Fas-associated factor (Ahmed et al., 2002; Litchfield, 2003). The inhibition of cell proliferation and cell cycle regulation by CK2 is likely to occur in a variety of tumors and cell lines.

**Discussion**

The mTOR signaling pathway regulates cell cycle checkpoint control and the initiation of translation in response to mitogens. Rapamycin is a relatively specific inhibitor of mTOR, and its antiproliferative effects underlie its immunosuppressive and antineoplastic properties. However, many cancer cells are resistant to rapamycin, and it is desirable to identify other agents that directly target the mTOR kinase domain (Huang et al., 2003). Small molecule inhibitors that act at its C-terminal PI3K-like domain might better inhibit mTOR-dependent signaling when administered alone or in combination with rapamycin.

**Fig. 6.** Effect of LY303511 on tumor growth in vivo. A, 1 × 10⁶ human prostate adenocarcinoma (PC-3) cells (ATCC CRL-1435) in 20% Matrigel were implanted in each of 40 nude mice by subcutaneous injection at the flank. Administration of vehicle (n = 10) or LY303511, 10 mg/kg/day (n = 3 groups of 10) began (day 1) when tumors reached ~150 mm³. Tumor volumes (caliper length × width²/2) were measured for 21 days at the indicated times. Data are expressed as the average tumor volumes ± S.E.M. for each treatment group and time point. *p < 0.0001 for the effects of time and treatment group by two-way ANOVA. +, p < 0.05 versus day 1 by Tukey-Kramer HSD; †, p < 0.05 versus group 1 or §, p < 0.05 versus group 1, 2, or 3, by Tukey-Kramer HSD. B, rate of tumor growth was estimated by linear regression analysis for each mouse, and the means ± S.E.M. are shown for each treatment group. *p < 0.0001 by one-way ANOVA. *p < 0.05 versus group 1 by Tukey-Kramer HSD.

*on the rate of tumor growth was statistically significant by one-way ANOVA (Fig. 6B). By Tukey-Kramer HSD, mean rate of tumor growth was significantly inhibited in mice undergoing treatment with LY303511 for 10 or 20 days compared with untreated mice. Increased tumor growth rates were not observed upon cessation of treatment (Fig. 6A, groups 2 and 3), possibly due to persistent effects of LY303511 or suboptimal sensitivity of the outcome measure. There was no overt toxicity related to the administration of LY303511 (e.g., weight loss and decreased activity) attributed to the daily administration of 10 mg/kg LY303511 for up to 20 days. Via mTOR-dependent and -independent inhibition of G1 and G2/M cell cycle progression, LY303511 inhibits cancer cell proliferation in vitro and in vivo as well as smooth muscle cell proliferation in vitro.
as well as the increase in p21 and p27 levels is consistent with CK2-dependent blockade of the cell cycle. A549 cells were relatively resistant to rapamycin but not to LY303511. In rapamycin-sensitive cells, rapamycin decreased proliferation and cyclin D and E levels via an effect on protein turnover (Nelsen et al., 2003). Rapamycin also inhibited S6K-dependent induction of cyclin E transcription, an event that is required for G1/S progression (Chou et al., 2003). In A549 cells, however, despite the fact that both rapamycin and LY303511 increased p27 levels and blocked phosphorylation of S6K at T389, rapamycin had no effect on cyclin D (Fig. 3, A and B). Consistent with this observation, others have demonstrated an association between high cyclin D levels and resistance to rapamycin (Gera et al., 2004). Moreover, rapamycin did not inhibit phosphorylation of Rb, possibly as a result of its inability to reduce levels of cyclin D (Fig. 3, A and B). It would therefore seem that the relative resistance of A549 cells to the antiproliferative effect of rapamycin occurred via a mechanism downstream of S6K. The additive effect of LY303511 on proliferation and cell cycle indicates that, in addition to inhibiting mTOR phosphorylation of S6K, LY303511 can overcome resistance to rapamycin by inhibiting a pathway other than S6K, such as CK2. In A549 cells, LY303511-induced G1 arrest correlated with increased G1 cyclin inhibitors and reduced Cdk2-dependent phosphorylation of Rb (Fig. 3A; p27 Kip1, p21 Cip1, and pRb). However, levels of cyclin D and E were unexpectedly increased (Fig. 3B). Consistent with this finding, others have observed similar cell type-dependent effects of pharmacological agents that target Cdk activity. For example, the Cdk inhibitor UCN-01 inhibited Cdk2 activity and Rb phosphorylation but increased cyclin D levels in immortalized mammary epithelial cells (Chen et al., 1999). Flavopiridol, a Cdk1/ Cdk2 inhibitor, increased cyclin E levels in squamous cell carcinoma cells (Patel et al., 1998). In A549 cells, although inhibition of Rb phosphorylation at S807/811 likely reflected blockade of Cdk2 activity (Knudsen and Wang, 1996), a simultaneous increase in G/M cells suggests that LY303511, like flavopiridol or UCN-01, inhibits other Cdns directly or indirectly.

LY303511 represents a new class of kinase inhibitor that targets mTOR and CK2 with lesser effects on PI3K activity. The lack of effect on PI3K in A549 cells might explain the ability of LY303511 to inhibit cell proliferation without causing apoptosis. This pharmacological profile might limit the toxicity of LY303511 in vivo and confer a favorable side effect profile.

Like rapamycin, LY303511 inhibits cell proliferation, in part, by increasing the number of cells in G1. Rapamycin is commonly used as an immunesuppressive and is Food and Drug Administration-approved as an antineoplastic agent. Other rapamycin analogs (e.g., CCI-779 and RAD001) are currently being tested in phase 1 or 2 chemotherapy trials (Dancey and Sausville, 2003). In clinical trials, compounds that cause G1 or G0/M arrest, such as flavopiridol, also seem to be effective antineoplastic agents (Swanton, 2004). In addition to promoting cell cycle arrest, these compounds seem to promote apoptosis (Patel et al., 1998). In contrast to LY303511 or LY294002, the benzopyran ring in flavopiridol is predicted to displace the ATP adenine from the cyclin-dependent kinase 2 active site (De et al., 1996).

By acting at the mTOR kinase domain, or by inhibiting additional kinases such as CK2, LY303511 has a pharmacodynamic profile different from either that of rapamycin or LY294002. Given its efficacy in vivo (Fig. 6), LY303511 (or its derivatives) is a potential antineoplastic agent in humans with rapamycin-resistant or -sensitive neoplasms. LY303511 exhibits antiproliferative effects in primary human smooth muscle cells, and future studies will assess its ability to prevent abnormal smooth muscle proliferation in vivo (e.g., postcoronary angioplasty restenosis).

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