Biochemical, Cellular, and In vivo Activity of Novel ATP-Competitive and Selective Inhibitors of the Mammalian Target of Rapamycin


Abstract

The mammalian target of rapamycin (mTOR) is centrally involved in cell growth, metabolism, and angiogenesis. While showing clinical efficacy in a subset of tumors, rapamycin and rapalogs are specific and allosteric inhibitors of mTOR complex 1 (mTORC1), but they do not directly inhibit mTOR complex 2 (mTORC2), an emerging player in cancer. Here, we report chemical structure and biological characterization of three pyrazolopyrimidine ATP-competitive mTOR inhibitors, WAY-600, WYE-687, and WYE-354 (IC50 5–9 nmol/L), with significant selectivity over phosphatidylinositol 3-kinase (PI3K) isoforms (>100-fold). Unlike the rapalogs, these inhibitors acutely blocked substrate phosphorylation by mTORC1 and mTORC2 in vitro and in cells in response to growth factor, amino acids, and hyperactive PI3K/AKT. Unlike the inhibitors of PI3K or dual-pan P13K/mTOR, cellular inhibition of P-S6K1(T389) and P-AKT(S473) by the pyrazolopyrimidines occurred at significantly lower inhibitor concentrations than those of P-AKT(T380) (PI3K-PDK1 readout), showing mTOR selectivity in cellular setting. mTOR kinase inhibitors reduced AKT downstream function and inhibited proliferation of diverse cancer cell lines. These effects correlated with a strong G1 cell cycle arrest in both the rapamycin-sensitive and rapamycin-resistant cells, selective induction of apoptosis, repression of global protein synthesis, and down-regulation of angiogenic factors. When injected into tumor-bearing mice, WYE-354 inhibited mTORC1 and mTORC2 and displayed robust antitumor activity in PTEN-null tumors. Together, our results highlight mechanistic differentiation between rapalogs and mTOR kinase inhibitors in targeting cancer cell growth and survival and provide support for clinical development of mTOR kinase inhibitors as new cancer therapy. [Cancer Res 2009;69(15):6232–40]

Introduction

The historical discovery of the macrolide rapamycin (1) and subsequent biological elucidation of its mammalian target of rapamycin (mTOR) have highlighted a great example of the modern chemical biology that has illuminated human disease and therapy. Today, whereas rapamycin and rapalog therapies are already used clinically for treating allograft rejection, coronary heart disease, and renal cancer, mTOR has attracted even more research for new therapies, particularly for treating major solid tumors. mTOR is the founding member of a family of unconventional high molecular mass serine/threonine protein kinases termed phosphatidylinositol 3-kinase (PI3K)-related kinases (PIKK; ref. 2). The catalytic sites of the PIKK family resemble those of the PI3Ks but differ from those of the broad-spectrum conventional protein kinases. These distinctive structural features, coupled with the essential biological functions and scarcity of PIKKs in the entire human kinome of ~500 kinases, highlight mTOR and the PIKK family as exciting drug targets for the development of potent and selective inhibitor therapy.

mTOR resides in at least two functional multiprotein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), mediating diverse signals from growth factors, nutrients, and energy supply (3–5). A prominent role in promoting cellular translation is well established for mTORC1 through its direct phosphorylation of the ribosomal protein S6 kinase 1 (S6K1) and the eukaryotic translation initiation factor eIF4E-binding protein 1 (4E-BP1). The recently identified mTORC2 phosphorylates the serine/threonine kinase AKT, thereby increasing its activity. mTOR is a critical mediator of the canonical pathways of PI3K/AKT and Ras/extracellular signal-regulated kinase (ERK), the two pathways that are most frequently dysregulated in human malignancy (6–8). Evidence from both epidemiologic and experimental studies indicate that mTOR signaling contributes to both the tumorigenic effects by numerous oncogenes such as PI3K, AKT, epidermal growth factor receptor, HER2/neu, and BCR-Abl as well as the effects due to loss of tumor suppressor genes such as PTEN, tuberous sclerosis complex, von Hippel-Lindau, and neurofibromatosis 1 (6–8). The positive regulation of AKT by mTORC2 implicates mTOR as acting both upstream and downstream of AKT and its diverse and complex roles in cancer cell growth, survival, and resistance to chemotherapy.

The rapamycins (rapamycin and rapalogs) are allosteric inhibitors that, in complex with FKBP12, target the FRB domain adjacent to the catalytic site of mTOR (9, 10). Intriguingly, both in vitro and in vivo studies showed that the FRB in mTORC2 is inaccessible to rapamycins as indicated by the lack of suppression of the mTORC2 substrate AKT (11, 12). Thus, the rapamycins are allosteric inhibitors of mTORC1, leaving the possibility of undiscovered rapamycin-resistant mTORC1 substrates. Targeting mTORC1 by the rapamycins is associated with clinical efficacy in a subset of cancers (13–15). However, it is also increasingly recognized that the rapamycin mechanism of action may not be sufficient for achieving a broad and robust anticancer effect due to failure to inhibit mTORC2 in some tumor types. Furthermore,
rapamycins can induce feedback activation of PI3K and ERK/ 
mitogen-activated protein kinase (MAPK) in certain cells (6, 7, 16). The antiproliferative effects of the rapamycins are generally 
modest and variable in cancer cells, which may reflect their 
inaccessibility to mTORC2 and the complex feedback activation of 
PI3K/AKT and/or ERK/MAPK. To further explore the therapeutic 
potential of targeting mTOR in cancer, a strong interest now exists 
for small-molecule ATP-competitive inhibitors of mTOR kinase as 
anticancer agents. Such inhibitors will target mTORC1 and 
mTORC2 and can minimize the feedback activation of PI3K and/or 
ERK/MAPK. In this regard, dual-pan PI3K/mTOR inhibitors 
haveshownenhancedantiproliferativeeffectsagainstsomecancer 
cells with strong G1 cell cycle arrest (17, 18). Nevertheless, these 
agents do not permit a direct assessment on the antitumor 
activity of representative molecules.

Materials and Methods

Chemicals. WAY-600, WYE-687, and WYE-354 were synthesized by Wyeth Discovery Medicinal Chemistry (19). PI-103 was synthesized by Wyeth Discovery Synthetic Chemistry. Rapamycin and HWT (17-hydroxysteromtamin) were provided by Wyeth Chemical and Pharmaceutical Development. All general chemical reagents used for buffers and assays were obtained from Sigma-Aldrich unless specified.

mTOR enzyme assay and immunocomplex kinase assay of mTOR complexes. mTOR kinase assays via DELFIA (20) and immunocomplex kinase assays were performed (Supplementary Methods).

Assays of other kinases. Enzyme assays of PI3K isoforms used PI3Kα and PI3Kγ (Upstate Biotechnology) and were performed as described (21). Assays of 24 protein kinases were performed (Supplementary Methods).

Cell culture, tumor cell growth assays, and cell cycle analysis. Cell lines of Rat1, HEK293, MDAMB-361, MDAMB-468, MDAMB-231, LNCap, DU145, U87MG, A498, HT116, and HT29 were obtained from the American Type Culture Collection. PCLSMM2 was obtained from Dr. Carolyn Discanfani (Wyeth Discovery Oncology). All cells were cultured using standard cell culture methods. Tumor cell growth assays and cell cycle analysis were performed (Supplementary Methods).

Assays of protein synthesis. For protein synthesis assays, methionine incorporation (22), 7-methyl-GTP pull-down (23), and polysome analysis (22) were performed (Supplementary Methods).

Protein lysates and immunoblotting. For in vitro cell culture treatment with various inhibitors, total cellular lysates were prepared using NuPAGE-LDS sample buffer (Invitrogen). Crude lysates were sonicated and then clarified by centrifugation. Protein concentrations were quantified using the RCDC protein assay (Bio-Rad). Equal amounts of proteins were subjected to immunoblotting analysis using NuPAGE electrophoresis system (Invitrogen). Various antibodies were obtained as follows: raport (Cell Signaling); P-AKT(S473) and P-AKT(T384), AKT, P-S6K(T389), S6K1, P-GSK3a/S9, GSK3, P-ERK(T202/Y204), ERK, elF4E, P-4E-BP1(T70), P-4E-BP1(T37/T46), eIF4B, eIF4F, cyclin D1, P-p38(S15), P-Chk1(S345), P-1KBs(S32), 1KB, P-c-Jun(S63), and P-P38 MAPK (Cell Signaling Technology); mTOR (N-19), poly(ADP-ribose) polymerase (PARP), elF4G, and elF3b (Santa Cruz Biotechnology); P-FKHRL1(T32) and FKHR1L (Upstate Biotechnology); hypoxia-inducible factor-1α (HIF-1α; BD Biosciences); β-actin (Chemicon International); Hif6 clone-1 (Sigma-Aldrich); and rictor (Novus Biologicals).

Vascular endothelial growth factor assay. U87MG cells were grown to confluence and then fed with fresh growth medium with or without inhibitors for 24 h. Culture supernatants were collected and assayed for vascular endothelial growth factor (VEGF) levels by ELISA using the Endogen Human VEGF ELISA kit (Pierce Biotechnology). Corresponding cell viabilities were determined and used in calculation for the normalized VEGF levels.

Inhibition of mTOR in xenograft tumors and efficacy studies. PCLSMM2 tumors (400 mm³) grown s.c. in BALB/c nu/nu female mice were dosed by a single i.p. injection with vehicle or 50 mg/kg WYE-354 formulated in 5% ethanol, 5% polysorbate 80, 5% polyethylene glycol-400. Tumor lysates were prepared and immunoblotted (24). For efficacy experiments, s.c. U87MG tumors were staged (200 mm³) and randomized into treatment groups (n = 10). Mice were dosed i.p. with vehicle or 50 mg/kg WYE-354 on days 0 to 4 once daily or twice daily. Tumor growth was monitored twice weekly and analyzed (24).

Results

Discovery of novel pyrazolopyrimidines as ATP-competitive and selective inhibitors of mTORC1 and mTORC2. High-throughput screening of chemical library against a recombinant mTOR enzyme discovered a small-molecule pyrazolopyrimidine WAY-001 (IC50, 0.22 μmol/L), a lead-like compound that served as the starting point for further optimization. WAY-001 was 6-fold more potent against PI3Ka than against mTOR, and its phenolic group was a potential glucuronidation site. Replacement of the phenol with isosteric groups such as indole or methyl carbamate overcame this potential metabolic liability while retaining the ability of these groups to function as hydrogen bond donors. Chemical synthesis varying the phenol isosteres as well as the piperidine substituents identified potent and selective mTOR kinase inhibitors as exemplified by the structures WAY-600, WYE-687, and WYE-354 (Fig. 1; ref. 19). As depicted in Fig. 1B, although rapamycin and rapalogs in complex with FKBP12 target the FRB domain in mTORC1, these inhibitors target the catalytic sites of both mTORC1 and mTORC2. In the DELFIA measuring His6-S6K1 T389 phosphorylation, WAY-600, WYE-687, and WYE-354 inhibited recombinant mTOR enzyme with IC50 values of 0.009, 0.007, and 0.005 μmol/L, respectively. Significantly, the pyrazolopyrimidines were selective for mTOR over PI3Ka (>100-fold) and PI3Kγ (>500-fold) and were poorly active in a panel of 24 protein kinases (Supplementary Table S1).

In a representative ATP matrix assay with WAY-354, double-reciprocal plots of the initial enzyme rate versus ATP concentration were well fit to competitive inhibition (Fig. 1C). To test directly the inhibition of mTORC2 catalytic activity in vitro, we immunoprecipitated mTORC2 and mTORC1 from HEK293 cells and performed immune-complex kinase assay of the mTORC2-specific substrate His6-ACK or the mTORC1 substrate S6S-S6K. AKT(S473) phosphorylation was dose dependently inhibited by all three inhibitors but not by the rapamycin-FKBP12 complex (Fig. 1D). As expected, both the kinase inhibitors and rapamycin-FKBP12 complex efficiently blocked the mTORC1 substrate S6K(T389) phosphorylation (Fig. 1D). Together, the results in Fig. 1 and Supplementary Table S1 identify WAY-600, WYE-687, and WYE-354 as potent, ATP-competitive, and selective inhibitors of mTOR kinase activity.

Global targeting of mTOR signaling and down-regulation of AKT function in cellular models. We assayed the pyrazolopyrimidines in cells stimulated with insulin-like growth factor-I (IGF-I), amino acids, and in tumor cells expressing hyperactive PI3K/AKT/ 
mTOR. IGF-I–stimulated P-S6K1(T389) and P-AKT(S473) in Rat1 cells were both inhibited by these compounds (Fig. 2A, left). Inhibition of P-AKT(S473) required higher inhibitor concentration.

P. Nowak et al. and A. Zask et al., manuscripts in preparation.
A mol/L) than that of P-S6K1 (T389) (0.3 A mol/L). Notably, P-AKT (T308), an activity marker for PI3K-PDK1, was fully inhibited by the PI3K inhibitor HWT or a dual-pan PI3K/mTOR inhibitor PI-103 but not by the pyrazolopyrimidines at doses up to 3 μmol/L, showing selectivity for targeting mTORC2 over PI3K. In HEK293 cells, amino acid–induced P-S6K1 and P-4EBP1 were blocked by the pyrazolopyrimidines (Fig. 2A, right). mTOR inhibition in cancer cell lines MDA361 (PIK3CA; HER2++) and U87MG (PTEN null) was examined after 6-hour inhibitor exposure. Similar to the action of rapamycin, the pyrazolopyrimidines potently inhibited the mTORC1 substrates P-S6K1 (T389) and P-4EBP1 (T70) (≤0.3 μmol/L) in MDA361 (Fig. 2B) and U87MG (data not shown). Notably, the pyrazolopyrimidines but not rapamycin inhibited P-4EBP1 (T37/46) (Fig. 2B). These differential inhibitory effects on 4EBP1 phosphorylation likely play a role in regulation of protein synthesis (see below). In MDA361, the pyrazolopyrimidines inhibited P-AKT (S473) at low concentrations (≤0.3 μmol/L), whereas they substantially inhibited P-AKT (T308) at considerably higher concentrations (≥10 μmol/L), showing a 10- to 30-fold selectivity for mTORC2 over PI3K (Fig. 2C, left, top). A similar trend for selective inhibition of P-AKT (S473) over P-AKT (T308) was also seen in U87MG (Fig. 2C, left, bottom), whereas HWT readily inhibited both S473 and T308 in these cell lines (Fig. 2C). A dual-pan PI3K/mTOR inhibitor PI-103 displayed a nearly identical dose response in suppression of P-S6K, P-4EBP1, P-AKT (S473), and P-AKT (T308) (Fig. 2C, right). Additionally, we found that a prolonged exposure (20 hours) of U87MG, MDA361, and LNCap cells to 2 μmol/L pyrazolopyrimidines did not significantly diminish P-AKT (T308), whereas P-AKT (S473) remained suppressed (Supplementary Fig. S1A). It was further noted that the AKT from the pyrazolopyrimidine-treated cells exhibited a further increased electrophoresis mobility (downward band shift) when compared with that of HWT-treated cells, implying the possibility of additional phosphorylation site(s) targeted by mTORC2 (Supplementary Fig. S1A).

The pyrazolopyrimidines dose dependently reduced phosphorylation of the AKT substrates P-FKHRL1 (T32) and P-GSK3 (S21/9) in MDA361 (Fig. 2D, top) and U87MG (Fig. 2D, bottom), whereas P-ERK was not inhibited. Targeting of mTORC1 by rapamycin did not lead to suppression of these markers (Fig. 2D). Notably, the pyrazolopyrimidines inhibited P-FKHRL1 and P-GSK3 at higher...
concentrations than those of P-AKT(S473), and the inhibition of P-GSK3 was not complete even at optimal inhibitor concentrations (Fig. 2C and D; data not shown). P-FKHRL1 and P-GSK3 remained significantly suppressed after a prolonged treatment, which correlated with the loss of P-AKT(S473) but not P-AKT(T308) (Supplementary Fig. S1B). In a cell-based assay, several members of the PIKK family can be activated by the cancer drug etoposide (VP-16). VP-16–induced PIKK substrate phosphorylations of P-

p53(S15) and P-Chk1(S345) were not blocked by these inhibitors at 3 μmol/L, indicating that the underlying PIKKs were not targeted (Supplementary Fig. S2A). In separate cell assays, the inhibitors did not block tumor necrosis factor-α (TNF-α)–induced activation of IKKβ (Supplementary Fig. S2B, left) or TNF-α–induced phosphorylations of MAPK cascades (Supplementary Fig. S2B, right). Taken together, these results confirm the pyrazolopyrimidines as selectively inhibiting cellular AKT activity via targeting mTORC2.

Figure 2. Cellular mTORC1 and mTORC2 substrate phosphorylation and AKT functions are suppressed by mTOR kinase inhibitors. A, left, IGF-I–induced mTOR substrate phosphorylation. Serum-starved Rat1 cells were pretreated for 1 h with inhibitors followed by 30-min stimulation with IGF-I. Right, amino acid (AA)–induced mTOR substrate phosphorylation. Amino acid–starved HEK293 cells were pretreated for 1 h with inhibitors followed by 2-h stimulation with amino acid.

B to D, inhibition of mTOR in cancer cells. MDA361 and U87MG cells were treated for 6 h with inhibitors in growth medium. Total lysates of cells in A to D were immunoblotted with antibodies against P-S6K(T389), S6K, P-4EBP1(T70), P-4EBP1(T37/46), 4EBP1, P-AKT(S473), P-AKT(T308), AKT, P-FKHRL1(T32), FKHRL1, P-GSK3(S21/9), GSK3, P-ERK, ERK, and β-actin.
Profound inhibition of cap-dependent and global protein synthesis. As rapamycin is a known inhibitor of cap-dependent translation (25), we compared the pyrazolopyrimidines with rapamycin in three separate translation studies (Fig. 3). The inhibitors were first examined for effect on global protein synthesis in MDA361 cells (Fig. 3A, left). Methionine incorporation in cells treated with a cytotoxic dose of Taxol (92% of control) or rapamycin (93% of control) was not significantly reduced. Interestingly, cells treated with pyrazolopyrimidines all displayed a significant reduction, ranging from 44.7% to 59% of control, albeit less pronounced than the value by cycloheximide (16% of control). Immunoblotting of these cells after 6-hour exposure to 1 or 10 μmol/L of rapamycin indicated no suppression of P-AKT(S473) (Fig. 3A, right). Protein lysates from inhibitor-treated cells were subjected to 7-methyl-GTP pull-down for examination of the cap-dependent initiation eIF4F protein complex (Fig. 3B). The pyrazolopyrimidines induced a substantially higher increase than rapamycin in binding of 4EBP1 to eIF4E and caused a nearly complete loss of eIF4G compared with a moderate loss of eIF4G caused by rapamycin. Both events are well known to disrupt the eIF4F complex, thereby inhibiting cap-dependent translation. Lastly, the inhibitor effects on actively translating ribosomes were examined (Fig. 3C). The P/M ratios (polyribosomes versus the 60/80S monosomes) for DMSO-treated, rapamycin-treated, and WAY-600–treated HEK293 cells were 1.85, 1.65, and 0.93, respectively, indicating a 50% inhibition of polysomes by WAY-600 compared with the 11% suppression by rapamycin. Thus, results in Fig. 3 indicate that inhibition of both mTORC1 and mTORC2 is accompanied by a profound suppression of both the cap-dependent and global translation.

Antiproliferative effects in cancer cells involving G1 cell cycle arrest and selective induction of apoptosis. In representative growth inhibition assays with the rapamycin-sensitive MDA361 and U87MG cells, all three pyrazolopyrimidines elicited a considerably more profound growth suppression than rapamycin (Fig. 4A), which was accompanied by reduced cyclin D1 levels (Fig. 4B). The pyrazolopyrimidines inhibited a panel of diverse cancer cell lines with IC50 values generally in the submicromolar to single-digit micromolar range (Supplementary Table S2). Cell cycle profiles were analyzed in both the rapamycin-sensitive MDA361 and rapamycin-resistant HCT116 cells. After 24-hour treatment, the
pyrazolopyrimidines caused a dose-dependent strong G1 arrest in both cell lines, whereas rapamycin induced a modest G1 arrest in MDA361 and was inactive in HCT116 (Fig. 4C). Rapamycin at higher concentrations (up to 10 μmol/L) did not further increase G1 cells (data not shown). Furthermore, treatment of MDA361 with the pyrazolopyrimidines also led to cell death, which was readily observed at 24 hours and peaked at 48 hours. Cell death was not induced by rapamycin even after a prolonged exposure (Fig. 4D).

Immunoblot analysis showed PARP cleavage in the pyrazolopyrimidine-treated MDA361 cells, indicating activation of apoptotic caspases (Fig. 4E, left). WYE-354 also induced a small but significant amount of PARP cleavage in the PTEN-null LNCap cells but not in HCT116 or HT29 cells (Fig. 4E, right). The results in Fig. 4 indicate that inhibition of mTORC1 and mTORC2 is associated with a profound suppression of proliferation and selective apoptosis in subsets of cancer cells.

**Inhibition of cancer cell angiogenic factors.** Rapalogs can reduce the angiogenic properties of cancer cells by down-regulating the expression and activity of HIF-1α (26–30). The PTEN-negative U87MG glioma cells express elevated levels of VEGF when cultured in normoxic condition in vitro and form highly vascularized tumors in vivo. These cells were therefore examined for effects of mTOR kinase inhibition. After 24-hour treatment, whereas rapamycin potently but partially inhibited the level of secreted VEGF, the pyrazolopyrimidines elicited a similar inhibition at the doses that inhibited mTOR (Fig. 5A). Consistent with the inhibition of VEGF, both rapamycin and the pyrazolopyrimidines caused a similar reduction in the expression of HIF-1α in U87MG, MDA361, and LNCap cells (Fig. 5B). The observation that both the rapamycin and pyrazolopyrimidines induced similar inhibition of VEGF and HIF-1α indicates that these events are largely mediated by mTORC1. Together, the results in Fig. 5 reinforce the role of mTOR in regulation of angiogenic signaling in cancer cells.

**Inhibition of mTOR signaling and antitumor efficacy in vivo.** WYE-354 achieved significant in vivo exposure and was hence selected for in vivo studies. Nude mice bearing the PTEN-null PC3MM2 tumors were administered ip. with vehicle or 50 mg/kg WYE-354. Tumor lysates prepared at 1, 2, 4, and 6 hours after dosing were immunoblotted for levels of P-S6K(T389), P-AKT(S473), and P-AKT(T308) (Fig. 6A). Quantification of the immunoblotting results indicated that WYE-354 completely inhibited P-S6K(T389) for at least 6 hours and substantially inhibited P-AKT(S473) for 6 hours (Fig. 6B). As expected, P-AKT(T308) in the same tumors was variably but not significantly inhibited (Fig. 6B), indicating that
consistent with the in vitro study results, WYE-354 selectively inhibited P-AKT(S473) via targeting mTORC2. The PTEN-null U87MG glioma grows rapidly in nude mice and serves as a robust and convenient efficacy model for evaluating mTOR inhibitors. In a representative study, WYE-354 dosed at 50 mg/kg twice daily and once daily for 5 days achieved dose-dependent suppression of tumor growth with treated over control (T/C) values of 0.14 and 0.62 (P < 0.05), respectively (Fig. 6C). The results in Fig. 6 show that selective targeting of mTOR via ATP-competitive mTOR kinase inhibitor results in a substantial antitumor efficacy.

Discussion

Heightened mTOR activity, as indicated by an elevated phosphorylation of its downstream substrates, is frequently observed in clinical samples of various solid tumors as well as hematopoietic malignancies. Although the rapalogics are efficacious in a subset of clinical tumors, the lack of direct inhibition of mTORC2 and feedback activation of PI3K and/or ERK/MAPK associated with these compounds may limit their anticancer potential (6, 7). Interestingly, prolonged rapamycin treatment can inhibit P-AKT(S473) in some cells (31), and reducing level of phosphatidic acid in renal 786-O cells can sensitize P-AKT(S473) suppression by rapamycin (32). These reports and future studies will help further elucidate the biochemical basis for the differential accessibility of rapamycin to mTOR complexes. Ongoing widespread efforts in targeting the PI3K/AKT/mTOR pathways have led to the discovery of numerous small-molecule inhibitors of PI3K, AKT, and dual-pan PI3K/mTOR (17, 18, 33–35) with several lead candidates in cancer trials (35). To our knowledge, our study is among the first in biological characterization of ATP-competitive and selective inhibitors of mTOR in cancer cells.

Unlike the rapalogics, WAY-600, WYE-687, and WYE-354 acutely blocked mTORC2-dependent phosphorylation of AKT in vitro and in vivo. In various cancer cells, these compounds inhibited the mTORC1 substrate P-S6K(T389) and mTORC2 substrate...
P-AKT(S473) at submicromolar concentrations without significant inhibition of P-AKT(T308). These results, together with the data obtained from kinase panel profiling, show that WAY-600, WYE-687, and WYE-354 represent a new class of selective inhibitors of mTOR kinase. Targeting of P-AKT(S473) by these compounds reduced AKT downstream function, a property particularly relevant for control of tumors with hyperactive PI3K/AKT/mTOR.

Although mTOR is a well-known positive regulator of the cap-dependent mRNA translation (25, 36, 37), we found that rapamycin at the commonly studied drug concentrations did not acutely block global protein synthesis in actively proliferating breast cancer cells or in other cancer cells (data not shown). In sharp contrast, mTOR kinase inhibitors significantly reduced global protein synthesis. More detailed examination of the cap-dependent eIF4F protein complex revealed a much stronger perturbation by the kinase inhibitors than rapamycin, as shown by a drastic increase in the inhibitory binding of 4EBP1 to eIF4E as well as a nearly complete loss of the scaffolding protein eIF4G. The substantial disruption of the eIF4F complex by the kinase inhibitors may in part reflect their ability to efficiently suppress P-4EBP1(T37/46), two regulatory sites that are directly phosphorylated by mTOR but are resistant to inhibition by rapamycin (38). Additional studies indicated that cellular polysomes were also more profoundly inhibited by the kinase inhibitors than rapamycin. Overall, these results reconfirm mTOR as an essential regulator of cap-dependent translation and further reveal potential new role(s) of mTOR in protein synthesis via mTORC2 and/or rapamycin-insensitive function of mTORC1.

Targeting tumor angiogenesis is integral to the successful treatment of solid tumors. Indeed, deregulated overproduction of angiogenic factors is a hallmark of cancer cell signaling that often involves mTOR (3–5). The antitumor efficacy of the rapalogs is in part attributed to their antiangiogenic properties mediated by the inhibition of HIF-1α, a well-known activator of the VEGF gene transcription (26, 28, 29, 39–43). In the highly angiogenic U87MG and numerous prostate and breast cancer cell lines cultured in normoxic condition, both rapamycin and mTOR kinase inhibitors similarly reduced VEGF expression. Consistent with a role of mTOR in growth factor–regulated production of HIF-1α, both rapamycin and the kinase inhibitors profoundly inhibited HIF-1α in majority of cell lines examined. These observations therefore support previous reports and further suggest that the HIF-1α/VEGF-promoting function in normoxic growth culture primarily requires mTORC1 in these cells.

mTOR kinase inhibitors are potent antiproliferative agents. In several cell lines expressing hyperactive PI3K/AKT/mTOR, these compounds achieved submicromolar IC50. These antiproliferative effects correlated with a loss of cyclin D1 and strong G1 cell cycle arrest in both the rapamycin-sensitive and rapamycin-resistant cells. Interestingly, dual-pan PI3K/mTOR inhibitors have been shown to cause strong G1 arrest in PTEN-null glioma cells (17, 18). In our studies, a substantial G1 arrest can be induced in MDA361 and HCT116 at the inhibitor concentrations that do not target P-AKT(T308). It therefore seems likely that suppression of mTORC2 and/or rapamycin-insensitive function of mTORC1 critically interfere with the G1 cell cycle progression. Although the substantial loss of cyclin D1 in the kinase inhibitor–treated cells is consistent with G1 arrest, it is presently unknown whether this is a direct drug effect or a consequence of the reduced growth. Strikingly, the global targeting of mTOR resulted in apoptosis in some cancer cell lines with hyperactive PI3K/AKT/mTOR. These observations highlight the fundamental importance of mTOR in growth and cell cycle progression and reinforce the notion that subsets of PI3K/AKT-deregulated cancers are dependent on mTOR for growth and survival. These mTOR-dependent survival effects are expected to involve the mTORC2-mediated AKT functions that can escape from the rapalogs therapy but are susceptible to mTOR kinase inhibitors. Consistent with the in vitro pharmacology profile, WYE-354 caused a rapid, sustained, and selective inhibition of P-S6k(T389) and P-AKT(S473) in PTEN-null tumors in vivo. WYE-354 displayed a robust antitumor activity against the PTEN-null U87MG glioma. The antitumor efficacy was dose dependent, related to the plasma and tumor exposures of the inhibitor concentration and to the suppression of mTOR kinase activity in tumor tissue. In the regimens and doses studied, WYE-354 was well tolerated with no significant body weight loss or adverse effects.

In summary, the present mTOR kinase inhibitors have provided new tools for elucidating the novel roles of mTOR in tumorigenesis and for exploring selective and global mTOR inhibitors as antitumor agents. Medicinal chemistry efforts are under way to further improve the mTOR inhibition potency, selectivity, and pharmaceutical properties of this inhibitor class and may lead to new anticancer therapy.

While this article was under review, Thoreen and colleagues (44) reported the biochemical and cellular activity of a potent, ATP-competitive, and selective mTOR inhibitor with undisclosed chemical structure. Feldman and colleagues (45) also reported novel pyrazolopyrimidine derivatives as active site inhibitors of mTOR. Although the studies on these chemically distinct compounds with various different systems may not be directly compared, several observations from the current study agree with these two reports (44, 45).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 1/26/09; revised 4/28/09; accepted 5/1/09; published OnlineFirst 7/7/09.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Wyeth HTS group for the mTOR inhibitor HTS and Rob Mahoney, Michael Cinque, Christine Gaydos, Irwin Hollander, and Inder Chaudhary for technical support.

References

30. Land SC, Tee AR. Hypoxia-inducible factor 1α is regulated by the mammalian target of rapamycin (mTOR) via an mTOR signaling motif. J Biol Chem 2007;282:20534–43.
Biochemical, Cellular, and In vivo Activity of Novel ATP-Competitive and Selective Inhibitors of the Mammalian Target of Rapamycin

Ker Yu, Lourdes Toral-Barza, Celine Shi, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-09-0299

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2009/07/06/0008-5472.CAN-09-0299.DC1

Cited articles
This article cites 44 articles, 13 of which you can access for free at:
http://cancerres.aacrjournals.org/content/69/15/6232.full#ref-list-1

Citing articles
This article has been cited by 43 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/69/15/6232.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/69/15/6232.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.