Beyond Rapalog Therapy: Preclinical Pharmacology and Antitumor Activity of WYE-125132, an ATP-Competitive and Specific Inhibitor of mTORC1 and mTORC2

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Abstract

The mammalian target of rapamycin (mTOR) is a major component of the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway that is dysregulated in 50% of all human malignancies. Rapamycin and its analogues (rapalogs) partially inhibit mTOR through allosteric binding to mTOR complex 1 (mTORC1) but not mTOR complex 2 (mTORC2), an emerging player in cancer. Here, we report WYE-125132 (WYE-132), a highly potent, ATP-competitive, and specific mTOR kinase inhibitor (IC50: 0.19 ± 0.07 nmol/L; >5,000-fold selective versus PI3Ks). WYE-132 inhibited mTORC1 and mTORC2 in diverse cancer models in vitro and in vivo. Importantly, consistent with genetic ablation of mTORC2, WYE-132 targeted P-AKT(S473) and AKT function without significantly reducing the steady-state level of the PI3K/PDK1 activity biomarker P-AKT(T308), highlighting a prominent and direct regulation of AKT by mTORC2 in cancer cells. Compared with the rapalog temsirolimus/CCI-779, WYE-132 elicited a substantially stronger inhibition of cancer cell growth and survival, protein synthesis, cell size, bioenergetic metabolism, and adaptation to hypoxia. Oral administration of WYE-132 to tumor-bearing mice showed potent single-agent antitumor activity against MDA361 breast, U87MG glioma, A549 and H1975 lung, as well as A498 and 786-O renal tumors. An optimal dose of WYE-132 achieved a substantial regression of MDA361 and A549 large tumors and caused complete regression of A498 large tumors when coadministered with bevacizumab. Our results further validate mTOR as a critical driver for tumor growth, establish WYE-132 as a potent and profound anticancer agent, and provide a strong rationale for clinical development of specific mTOR kinase inhibitors as new cancer therapy.

Introduction

Mammalian target of rapamycin (mTOR) is an unconventional serine/threonine protein kinase related to the phosphoinositide 3-kinase (PI3K) family (1, 2). As a master regulator of growth, mTOR resides in at least two functional multiprotein complexes, mTORC1 and mTORC2, mediating diverse signals from growth factors, nutrients, and energy supply (3–5). mTORC1 is an essential mediator of PI3K/AKT through its direct phosphorylation of the ribosomal protein S6 kinase 1 (S6K1) and the eukaryotic translation initiation factor eIF4E-binding protein 1 (4EBP1). The more recently identified mTORC2 phosphorylates the survival kinase Akt and regulates the cytoskeleton network (6–8). The positive regulation of Akt by mTORC2 implicates mTOR in acting both upstream and downstream of AKT. The signaling network engaged by the PI3K-AKT-mTOR axis is frequently dysregulated in human malignancy (9–11). Although the mTOR gene locus is not known to be oncogenically mutated or amplified in cancer, the mTOR kinase is a pivotal downstream target of numerous oncogenic proteins, such as PI3K, Akt, epidermal growth factor receptor (EGFR), HER2/neu, and BCR-Abl. mTOR is also hyperactivated in response to the loss of tumor suppressor genes, such as PTEN, tuberous sclerosis complex, von Hippel-Lindau (VHL), and neurofibromatosis 1. A heightened level of mTOR activity, as indicated by an elevated phosphorylation of its downstream substrates P-S6K1, P-S6, and P-AKT, is frequently observed in clinical samples of various solid tumors and hematopoietic malignancies. Compelling preclinical and a limited body of clinical evidence indicate that certain cancers with hyperactive PI3K/AKT/mTOR status are particularly susceptible to mTOR inhibition. Clinically used rapamycins (rapamycin and rapalogs) partially inhibit mTOR through allosteric binding to the FKBP12-rapamycin binding domain adjacent to the catalytic site of mTORC1 (12, 13). These compounds do not directly
target mTORC2 and do not completely block all mTORC1 outputs. Although rapalog therapies have shown clinical efficacy in a subset of cancers (14–16), this mode of drug action does not fully exploit the antitumor potential of mTOR targeting in cancer. In particular, emerging roles of mTORC2 in hyperphosphorylation (activation) of AKT in major solid cancers are vital to tumor maintenance and progression, but they are resistant to the rapamycins. Additionally, mTORC1 can also negatively regulate PI3K or extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK), implicating potential feedback activation of PI3K and/or ERK/MAPK signaling by rapamycins in certain cancer cells (10, 17). Consequently, intense efforts are now under way to develop inhibitors of the PI3K/AKT/mTOR pathways, including ATP-competitive inhibitors of mTOR (18, 19). Several recent reports have described biochemical and cellular properties of ATP-competitive and selective inhibitors of mTOR (20–23). These chemically distinctive compounds all show suppression of mTORC2 substrate P-AKT(S473) and attenuate AKT downstream biomarkers. Although their anticancer activities have not been reported (20–22), these mTOR kinase inhibitors induce a stronger suppression than rapamycin of protein synthesis and cell growth (20, 21, 23).

We recently reported the discovery of pyrazolopyrimidine inhibitors of mTOR (23). Extensive structure-activity relationship studies of this chemical series led to the discovery of WYE-125132 (WYE-132), a highly potent, ATP-competitive, and specific inhibitor of mTOR. We now report in vitro and in vivo anticancer activity of WYE-132 in major solid tumors. The profound anticancer activity of WYE-132 is mediated by specific and global targeting of mTORC1 and mTORC2 without affecting the PI3K/PDK1 activity biomarker P-AKT (T308), further establishing a fundamental role of mTOR in tumor growth. Comparative studies highlight mechanistic differentiation between WYE-132 and rapalog temsirolimus/CCI-779 in targeting cancer cell growth and survival, protein synthesis, bioenergetic metabolism, and hypoxia adaptation, supporting the rationale for clinical use of specific mTOR kinase inhibitors as new cancer therapy.

Materials and Methods

**Inhibitors.** WYE-125132, PI-103, 17-hydroxywortmannin, and temsirolimus/CCI-779 were provided by Wyeth Discovery Synthetic Chemistry. For in vitro studies, inhibitors were dissolved in DMSO as 20 mmol/L stocks and diluted before assays. Bevacizumab was obtained from MedWorld Pharmacy.

**Kinase assays.** mTOR enzyme assays via dissociation-enhanced lanthanide fluorescence immunosay (DELFIA), ATP matrix assays, and mTOR immune-complex kinase assays were performed as described previously (24). Assays of PI3Ks were performed (25). hSMG1 and ATR were assayed using glutathione S-transferase–p53 as a substrate, detected via DELFIA using an anti–P-p53 (S15) antibody. Assays of a panel of 230 protein kinases were performed by SelectScreen profiling (Invitrogen).

**Tumor cell growth inhibition assays, protein lysates, and immunoblotting.** Cell lines of MDA-MB-361, MDA-MB-231, MDA-MB-468, BT549, LNCap, A549, H1975, H157, H460, U87MG, A498, 786-O, HCT116, MG63, Rat1, HEK293, and HeLa were obtained from the American Type Culture Collection. PC3MM2 was described (23). Cell growth assays and IC50 determination were described (23). For immunoblotting, cultured cells were treated as indicated. Total cellular lysates were prepared using NuPAGE lithium dodecyl sulfate sample buffer (Invitrogen) and immunoblotted with various antibodies as described (23).

**Assays of protein synthesis.** To measure global protein synthesis, MDA361 cells were fed with methionine-free RPMI 1640 (Invitrogen) with 10% fetal bovine serum (FBS) and treated with inhibitors for 3 h. Cells were then labeled for 2 h with 1 μCi per well of l-[methyl-3H]methionine (Perkin-Elmer) and assayed (26). For examination of the cap-dependent translation initiation complex, MDA361 cells were treated for 16 h with inhibitors. Methionine incorporation and [3H]methyl-GTP pull-down were performed (26, 27). For polysome analysis, HEK293 cells were treated for 16 h with the indicated inhibitors and analyzed (26).

**Analyses of cell cycle and cell size.** For cell cycle and cell size experiments, various cells were treated for 12, 24, or 48 h with inhibitors. Cell cycle distribution was analyzed in a Becton Dickinson FACScalibur flow cytometer using CellQuest software, and mean FSC-H population was determined as a measure of relative cell size (28). In some experiments, cells were also analyzed on Guava PCA-96 instrument (23).

**Assays of lactate and vascular endothelial growth factor in normoxia and hypoxia.** For normoxic culture measurements, confluent cells were fed with fresh medium with inhibitors for 24 h. For hypoxia response, cells were plated overnight and fed with fresh growth medium with inhibitors immediately before incubating without or with hypoxia (1% O2) for 24 h. Culture supernatants were assayed for vascular endothelial growth factor (VEGF) using the Endogen Human VEGF ELISA kit (Pierce Biotechnology) or lactate using the Lactate Reagent (Trinity Biotech).

**Oxygen consumption.** MDA361 cells were suspended in glucose-free DMEM (Invitrogen) with 10% dialyzed FBS, treated for 1 h with inhibitors, and placed into the oxygen biosensor system (BD Biosciences). Fluorescence was measured using Envision 2100 reader (Perkin-Elmer) at excitation/emission of 485/630 nm every 20 min up to 5 h. The oxygen consumption rates were calculated based on the slope within the linear range, converted into fluorescence arbitrary unit per minute, and normalized with total protein (29).

**In vivo tumor studies.** For mTOR biomarker studies, various tumors (400 mm3) grown s.c. in female nude mice were dosed by a single i.v. or oral injection with vehicle or WYE-132 formulated in 5% ethanol, 2% Tween 80, and 5% polyethylene glycol–400. Tumor lysates were prepared and immunoblotted (30). For efficacy studies, nude mice bearing U87MG, MDA361, H1975, A549, A498, or 786-O tumors were staged and randomized into treatment groups (n = 10). Mice were dosed orally with vehicle or WYE-132 following qd x5 cycle regimen (5 d on, 2 d off) for up to four cycles. Temsirolimus/CCI-779 was formulated as WYE-132 and dosed i.v. once weekly. Bevacizumab was formulated in PBS and dosed i.p. via its clinical...
regimen (200 μg/mouse; once weekly). Tumor growth was monitored and analyzed (30).

Results

WYE-132 is a potent, specific, and ATP-competitive inhibitor of mTORC1 and mTORC2. Our efforts in the mTOR inhibitor lead optimization led to the discovery of WYE-132, a novel pyrazolopyrimidine substituted with a bridged morpholine (Fig. 1A; ref. 31). WYE-132 potently inhibited recombinant mTOR (IC\textsubscript{50}: 0.19 ± 0.07 nmol/L) via an ATP-competitive mechanism (Supplementary Fig. S1). WYE-132 was highly selective over various PI3Ks, the PI3K-related kinases hSMG1 and ATR (>5,000-fold; Fig. 1A), and was largely inactive against a panel of 230 protein kinases (Supplementary Table S1). In immune-complex assays, it inhibited mTORC1-dependent phosphorylation of S6K(T389) and mTORC2-dependent phosphorylation of AKT(S473) (Fig. 1B), whereas CCI-779–FKBP12 complex only inhibited mTORC1 (23). In cellular settings, P-S6K(T389) and P-AKT(S473) in insulin-like growth factor-I (IGF-I)–induced Rat1, the PIK3CA mutant MDA361, or PTEN-null U87MG cells were dose dependently inhibited.
by WYE-132 with ED50 in low nanomolar range (Fig. 1C and D). The steady-state levels of the PI3K/PDK1 activity biomarker P-AKT(T308) were not reduced by WYE-132 in these cells and in others (Fig. 1C and D, Supplementary Fig. S2A), confirming its mTOR selectivity versus PI3K in cells. Notably, in MDA361 cells, WYE-132 transiently and partially reduced P-AKT(T308) at 1 hour but the level of P-AKT(T308) recovered by 3 hours and remained uninhibited (Supplementary Fig. S2B). WYE-132 reduced AKT substrates P-FKHRL1(T32) and P-GSK3(S21/9) but did not alter P-ERK/MAPK (Fig. 1D, Supplementary Fig. S2C). These results establish WYE-132 as a potent and highly selective inhibitor of mTORC1 and mTORC2 in vitro and in cells.

**Inhibition of cancer cell proliferation, cell cycle progression, and induction of apoptosis.** WYE-132 is a potent anti-proliferative agent against a panel of cancer cell lines with IC50 values generally in the nanomolar range (Supplementary Table S2). In the typical 3-day dose-response studies, WYE-132 exhibited a more profound antiproliferative activity than CCI-779 in MDA361 and other cells, as shown by the sharper inhibition at doses up to 10 μmol/L; CCI-779 and rapalogs in general elicit a flat, modest (≤50%) suppression of cell growth (Fig. 2A; Supplementary Fig. S3A). Both WYE-132 and paclitaxel showed, as expected, higher IC50 values after 1- or 2-day treatment than the 3-day treatment (Supplementary Fig. S3B). Fluorescence-activated cell sorting (FACS) analysis of inhibitor-treated (1 μmol/L, 24 hours) MDA468, PC3MM2, U87MG, A549, and HCT116 cells indicated that WYE-132 elicited a more profound increase in G1-phase and a reduction in S-phase cells than CCI-779 (Fig. 2B). Strikingly, WYE-132 but not CCI-779 caused cell death in the PI3K/mTOR- and HER2-hyperactive MDA361 cells, which was readily detectable at 24 hours and became massively at 48 hours (Fig. 2C). The WYE-132–induced cell death was evident at 10 and 30 nmol/L (62% and 13%, respectively) and was dose dependent, reaching 47% at 1 μmol/L and 59% at 3 μmol/L (Fig. 2C, right). Immunoblotting detected apoptotic poly(ADP-ribose) polymerase (PARP) cleavage in MDA361 cells treated with WYE-132 but not CCI-779 (Fig. 2D, top). WYE-132 also induced PARP cleavage in LNCap, H1975, and BT474 cells but not in HCT116 and HT29 cells (Fig. 2D, bottom). Notably, the induction of PARP cleavage in these cells correlated with the loss of P-AKT(S473) but not P-AKT(T308) (Fig. 2D, top; data not shown). We next compared WYE-132 with a dual-pan PI3K/mTOR inhibitor PI-103 (32) in cell cycle profile and caspase activation. Whereas both inhibitors had little effect at 12-hour exposure, treatment of MDA361 for 24 hours indicated similar increases in G1 by both inhibitors but a more profound cell death by PI-103 than WYE-132 (27% versus 12% at 5 μmol/L; Supplementary Fig. S4A). Similarly, PI-103 caused a more rapid and larger increase in caspase-3/caspase-7 activity in MDA361 and LNCap cells (Supplementary Fig. S4B). Collectively, these results indicate that WYE-132 induces apoptosis in subsets of cancer cells with a slower kinetics than that of PI-103.

**Profound inhibition of protein synthesis and cell size.** In MDA361 cells, global translation as measured by methionine incorporation was unaffected by 3 μmol/L CCI-779, whereas WYE-132 at 3.1, and 0.3 μmol/L all showed ~50% inhibition (Fig. 3A). mTOR promotes cap-dependent translation via phosphorylation of 4EBP1, a repressor of the cap-binding factor eIF4E. CCI-779 partially inhibited P-4EBP1 at S65 and 170 but not at the T37/46 sites. In contrast, WYE-132 completely inhibited phosphorylation of all four sites (Fig. 3B, left). "methyl-GTP pull-down analysis of the cap-initiation complex indicated that WYE-132 caused a substantially higher increase than CCI-779 in the eIF4E-bound 4EBP1 and a complete loss of eIF4G (Fig. 3B, right), indicating a total disruption of eIF4F complex. The ratio of polysomes to 60/80S monosomes is directly related to cellular translational activity. In an analysis with HEK293 polysomes, the polysome/monosome ratios for cells treated with DMSO, 0.5 μmol/L CCI-779, and 0.5 μmol/L WYE-132 were 1.62, 1.02, and 0.58, respectively, indicating a larger reduction in polysomes by WYE-132 than CCI-779 (Fig. 3C). Consistent with the more effective suppression of protein synthesis, WYE-132 caused a more profound reduction in cell size than CCI-779 (Fig. 3D, left). The relative cell sizes (mean FSC-H) for CCI-779–treated and WYE-132–treated renal cancer A498 cells were 97% and 83% of control, respectively (Fig. 3D, right). Collectively, these results document a stronger suppression of protein synthesis and cell size by WYE-132 than CCI-779.

**Tumor cell metabolism and hypoxia response modulated by WYE-132.** Aerobic glycolysis is a hallmark of cancer cell metabolism that involves PI3K/AKT/mTOR pathways. Treatment of the highly glycolytic U87MG cells led to a dose-dependent and more pronounced reduction in lactate by WYE-132 (48% of control at 1 μmol/L) than that of CCI-779 (80% of control at 1 μmol/L; Fig. 4A, left). WYE-132 also potently and acutely reduced levels of hypoxia-inducible factor (HIF)-1α, a principal regulator of glycolysis, which was noticeably more complete than that of CCI-779 or 17-hydroxyxwortmannin (Fig. 4A, right). The effect of mTOR inhibition on mitochondrial respiration was examined in MDA361 cells, as they display a robust increase in oxygen consumption on switching to a glucose-free, pyruvate-containing culture medium. Oxygen consumption rate was again more strongly reduced by WYE-132 (35% of control) than CCI-779 (82% of control; Fig. 4B). Hypoxic stress response is important for cancer cell survival and tumor-induced angiogenesis, which requires HIF-1α and HIF-2α. WYE-132 was more effective in blocking CCI-779 in blocking the accumulation of HIF-1α and HIF-2α in low oxygen (Fig. 4C). Accordingly, WYE-132 was more effective in blocking the hypoxia-induced lactate (Fig. 4D, left) and hypoxia-induced VEGF (Fig. 4D, right). Therefore, WYE-132 seems to exert a stronger effect than CCI-779 in blocking cancer cell adaptation to hypoxia.

**Inhibition of mTOR signaling and tumor growth in PI3K/AKT/mTOR-hyperactive glioma and breast tumor models.** A single i.v. administration of 50 mg/kg WYE-132 into tumor-bearing mice led to suppression of P66K(T389) and P-AKT(S473) for at least 8 hours in PC3MM2, MDA361, HCT116, and HT29 tumors, whereas the steady-state level of P-AKT(T308) was not significantly reduced (Supplementary Fig. S5), indicating that the antitumor
efficacy of WYE-132 under such dosing regimens reflects the suppression of mTOR rather than PI3K. Oral administration of WYE-132 caused dose-dependent tumor growth delay in the PI3K/mTOR- and HER2-hyperactive MDA361 tumors with significant antitumor activity at 5 mg/kg (Fig. 5A, left), which correlated with a suppression of P-S6 and P-AKT(S473) but not P-AKT(T308) (Fig. 5A, middle and right). An optimal dose of 50 mg/kg WYE-132 induced a substantial regression of large MDA361 tumors, which was not achieved with a clinical regimen of bevacizumab (Fig. 5B, left). Notably, this dose regimen achieved a more sustained suppression of mTOR activity and induced PARP cleavage in the early hours after dosing (Fig. 5B, right), indicating that apoptotic tumor cell death *in vivo* may contribute to tumor regression. WYE-132 also caused a potent and substantial tumor growth delay in the PTEN-null U87MG glioma (Fig. 5C).

**Figure 2.** Antiproliferative effects of CCI-779 and WYE-132. **A,** MDA361 cells were treated for 3 d with inhibitors. Relative cell viabilities are plotted. **B,** the indicated cells were treated for 24 h with DMSO or 1 μmol/L inhibitors and analyzed by FACS. Percent of G0-G1-phase and S-phase cells is plotted. **C,** MDA361 cells were treated for 24 or 48 h with the indicated CCI-779 or WYE-132 and analyzed by FACS. Percent of cells in each cell cycle stage is plotted. **D,** the indicated cells were treated for 48 h with WYE-132 or CCI-779 and analyzed by immunoblotting.
these results support the idea that mTOR kinase inhibitors may be particularly effective against subsets of tumors bearing an oncogenically activated PI3K/AKT/mTOR signaling cascade.

In vivo antitumor efficacy in lung and renal tumors. In a model for EGFR-dependent non–small cell lung cancer (NSCLC), growth of the orthotopically implanted H1975 tumors was significantly attenuated by 10 mg/kg WYE-132,

Figure 3. CCI-779 and WYE-132 differentially inhibit protein synthesis and cell size. A, MDA361 cells were pretreated for 3 h with DMSO, 10 μg/mL cycloheximide (CHX), CCI-779, or WYE-132 in methionine-free medium and assayed for [3H]methionine incorporation. Mean values of protein synthesis are plotted. B, MDA361 cells were treated for 16 h with inhibitors. Total cell lysate and the γmethyl-GTP pull-down protein complex were analyzed by immunoblotting. C, HEK293 cells were treated for 16 h with DMSO and 0.5 μmol/L of CCI-779 or WYE-132 and analyzed for polysome/monosome (P/M) profile. The absorbance profiles at 294 nm (A294) recorded during gradient fractionation of the samples are shown. The diagram (WYE-132) was stretched proportionally to normalize the vertical scale. The ratio of polysomes to monosomes was calculated. D, A498 cells were treated for 48 h with DMSO or 1 μmol/L inhibitors and analyzed by flow cytometry for relative cell size. Histograms of the forward scatter (FSC-H) for the treated cells and mean FSC-H values are shown.
resulting in improved animal survival (Fig. 6A). WYE-132 also dose dependently inhibited the growth of NSCLC A549 tumors, achieving a complete or nearly complete suppression at 50 and 25 mg/kg and a partial suppression at 10 mg/kg (Fig. 6B, top). An optimal dose of WYE-132 caused a rapid and substantial regression of large A549 tumors, which was not achieved with a clinical regimen of CCI-779 or bevacizumab (Fig. 6B, bottom).

Majority of the renal cell carcinomas (RCC) are characterized by the loss of E3 ubiquitin ligase VHL and stabilization of HIF-1/2 that promotes tumor angiogenesis. CCI-779 and related rapalogs exhibit clinical efficacy in RCC, which is in part mediated by targeting tumor angiogenesis. In vivo growth of the RCC A498 (VHL null) tumors was only partially inhibited by 25 mg/kg CCI-779 (five times the clinical dose) but was completely suppressed by 25 mg/kg WYE-132 (Fig. 6C, top). WYE-132 also completely suppressed the tumor growth of RCC 786-O (VHL null; PTEN null; Fig. 6C, bottom). WYE-132 was tested in combination with bevacizumab in A498 for tumor regression. Interestingly, 50 mg/kg WYE-132 alone elicited a significant tumor regression. Combination of WYE-132 with bevacizumab caused a most dramatic regression so that tumors continue to shrink over the course of therapy (Fig. 6D, top). Representative tumors on day 20 of this study are shown (Fig. 6D, bottom). These results show that WYE-132 is more effective than CCI-779 in the preclinical models of RCC.

Discussion

Although clinical studies with rapalogs have validated mTOR as a cancer target, the effectiveness of these drugs may be limited due to several pharmacologic parameters, including the resistance of mTORC2 signaling functions and the rebound activation of PI3K/AKT induced in certain tumor cells. These considerations led to intensive efforts to develop mTOR-selective kinase inhibitors that induce a deeper and broader suppression of both mTORC1 and mTORC2 activities in cancer cells. In this report, we have provided biochemical, cellular, and in vivo evidence that WYE-132 is a highly potent, ATP-competitive, and selective inhibitor of mTORC1 and mTORC2. mTORC2 phosphorylates AKT S473, and dysregulated
mTORC2 contributes to the malignant phenotype (6, 33). Phosphorylation of S473 and T308 can occur independently (34), and genetic ablation of mTORC2 leads to selective loss of P-AKT S473 without affecting T308 (35, 36). Likewise, WYE-132 selectively targeted the steady-state levels of P-AKT S473 versus T308 in diverse cancer cell lines. This property of WYE-132 reflects a further improvement from its earlier chemical analogues (23) and differs somewhat from other mTOR-selective inhibitors PP242 (21) and Ku-0063794 (22) that substantially reduced P-AKT T308 in the described cell assay systems (21, 22). Although more detailed comparisons of these mTOR-selective inhibitors are clearly needed, the actions of WYE-132 are largely consistent with those predicted for a specific inhibitor of mTORC1 and mTORC2 based on previously published genetic studies (34–36).

WYE-132 potently inhibited proliferation of diverse cancer cell lines with IC_{50} values generally in the low nanomolar range. It was previously suggested that the strong G_{1} cell cycle arrest induced by the dual-pan PI3K/mTOR inhibitor PI-103 is attributed to the dual targeting of mTOR and PI3K (32). In the present study, WYE-132 induced a strong G_{1} arrest in both the CCI-779–sensitive and the CCI-779–resistant cells at the inhibitor concentrations that do not target P-AKT (T308), indicating a critical dependence of mTORC2 and/or rapamycin-resistant function of mTORC1 in G_{1} progression. Furthermore, WYE-132 induced a substantial apoptotic response in breast MDA361, BT474, prostate LNCap, and lung H1975 cancer cells, all of which express hyperactive PI3K/AKT/mTOR and/or oncogenic EGFR/HER2. It is particularly noteworthy that although the WYE-132–induced cell death occurred more slowly than that of PI-103, these cells underwent massive apoptosis in spite of an undiminished phosphorylation of AKT T308, underscoring a specific and unequivocal role of mTOR in survival. These observations not only highlight the critical importance of mTOR in growth and cell cycle progression but also strongly suggest that subsets of PI3K/AKT/mTOR deregulated cancers are critically dependent on mTOR for survival. These mTOR-dependent survival effects likely involve AKT and/or the rapamycin-resistant function of mTORC1.

The substantial reduction in global protein synthesis and cell size distinguishes WYE-132 form CCI-779 and may be relevant for its antitumor activity. Whereas mTOR regulates
Figure 6. WYE-132 antitumor efficacy in lung and renal tumor models. A, mice bearing orthotopic NSCLC H1975 tumors were treated with vehicle or 10 mg/kg WYE-132 (seven cycles). Tumor burden–related animal deaths are plotted. B, top, mice bearing A549 tumors were treated for four cycles with vehicle or WYE-132 as indicated. *, $P < 0.05$, WYE-132 versus vehicle. Bottom, mice bearing large A549 tumors were treated orally with 50 mg/kg WYE-132 and the clinical regimens of CCI-779 (5 mg/kg, i.v.) and bevacizumab (200 μg/mouse). *, $P < 0.05$, WYE-132 versus CCI-779 or bevacizumab. C, mice bearing A498 (top) or 786-O (bottom) tumors were treated with vehicle, 25 mg/kg WYE-132, and 25 mg/kg CCI-779. *, $P < 0.05$, WYE-132 versus CCI-779 or vehicle. D, A498 tumor-bearing mice were treated with vehicle, 50 mg/kg WYE-132 alone, bevacizumab (200 μg/mouse), combination of bevacizumab and WYE-132 (BEV+132), or combination of bevacizumab and 5 mg/kg CCI-779 (BEV+CCI). *, $P < 0.05$, bevacizumab plus WYE-132 versus WYE-132, bevacizumab, or bevacizumab plus 5 mg/kg CCI-779. Tumor growth curves (top) and representative tumors on day 20 (bottom) are shown.
cap-dependent mRNA translation (37–39), rapamycins at the commonly studied drug concentrations do not provoke a substantial reduction in global protein synthesis. WYE-132 and other mTOR-selective inhibitors (20, 21, 23) strongly perturbed the cap-dependent translation initiation eIF4F complex, as shown by a drastic increase in the inhibitory binding of 4EBP1 to the cap-binding factor eIF-4E and a nearly complete loss of the scaffolding protein eIF4G. These effects reflect in part their ability to efficiently suppress the rapamycin-resistant P-4EBP1(T37/46) (40). The stronger suppression of initiation is also reflected by a profound loss of polysomal fractions. Additionally, reduction in global protein synthesis may also involve an attenuated translation elongation, as we observed a dramatically increased phosphorylation (inactivation) of the elongation factor eEF2 T56 in the WYE-132–treated MDA361 cells as compared with a moderate increase in CCI-779–treated cells (data not shown). Further studies are needed to elucidate additional novel function(s) of mTOR in protein synthesis that might be resistant to rapamycin.

Hyperactive PI3K/AKT/mTOR is strongly implicated in cancer cell metabolism, favoring bioenergetic pathways that control cell growth and proliferation (41, 42). Compared with CCI-779, WYE-132 more profoundly inhibited both aerobic glycolysis and mitochondrial respiration in various cancer cell lines, establishing WYE-132 as a stronger inhibitor than CCI-779 in attenuating bioenergetic metabolism. HIF-1α is a master regulator of glucose metabolism and tumor angiogenesis in response to growth factors and hypoxia (41–43). WYE-132 and CCI-779 similarly blocked growth factor–dependent HIF-1α in glioma cells and in other cell lines (data not shown). These two inhibitors also similarly reduced VEGF levels in the highly angiogenic glioma and prostate cells (data not shown). These results are in line with earlier reports on antiangiogenic properties of rapalogs in cancer treatment (44, 45) and further suggest that the growth factor–mediated HIF-1α/VEGF primarily requires mTORC1 in these cells. Importantly, WYE-132 but not CCI-779 strongly inhibited hypoxia-induced accumulation of HIF-1α and HIF-2α. Accordingly, WYE-132 more profoundly inhibited hypoxia-induced production of lactate and VEGF. It therefore seems that global targeting of mTOR leads to a stronger effect than the mTORC1–selective CCI-779 in blocking cancer cell adaptation to hypoxia, a key feature of the tumor microenvironment in vivo.

WYE-132 showed single-agent antitumor activity in multiple tumor models when administered at dosing regimens that do not target the PI3K/PDK1 activity biomarker P-AKT(T308). These observations not only show the high specificity of WYE-132 in vivo but also highlight the critical importance of mTOR as a driver of tumor growth in vivo. WYE-132 was particularly effective against subsets of tumors harboring PTEN deficiency (U87MG and 786-O), mutational activation of PI3K p110α PIK3CA (MDA361), oncogenic EGFR (H1975), or HER2/neu (MDA361), as well as LKB1/STK11 deficiency with mutant K-RAS (A549). In these studies, WYE-132 potently inhibited tumor growth, and an optimal dose of WYE-132 achieved substantial regression of large tumors, an outcome that was not observed with a clinical regimen of CCI-779 or the anti-VEGF therapy bevacizumab. Notably, an optimal dose of WYE-132 induced a rapid PARP cleavage in large-size MDA361 tumors in vivo, implicating apoptotic death in tumor regression. RCC tumors are often characterized by the loss of E3 ubiquitin ligase VHL and stabilization of HIF-1/2 (46). Rapalogs show clinical efficacy in RCC, achieving stable disease and improvement in progression-free survival (47, 48). The rapalog efficacy in RCC is in part mediated by targeting mTOR-dependent HIF-1/2 expression (49, 50). Importantly, we found that in vivo growth of the VHL-null RCC A498 tumors was more profoundly inhibited by WYE-132 than CCI-779 and that WYE-132 in combination with bevacizumab induced a nearly complete regression of very large tumors. These results implicate a critical and novel role(s) of mTOR in RCC tumor growth.

In conclusion, specific and global targeting of mTOR in cancer cells by WYE-132 has further validated essential roles of mTOR in tumor growth, survival, protein synthesis, and tumor metabolism. WYE-132 inhibits growth of numerous human tumor models of brain, breast, lung, and renal cancer. The preclinical pharmacology and efficacy of WYE-132 support its use as an anticancer agent and provide a strong rationale for clinical development of ATP-competitive and specific mTOR kinase inhibitors as new anticancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Michael Cinque, Vad Buklan, Stanley Jones, Irwin Hollander, William Hu, and Inder Chaudhary for performing various studies.

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Received 7/1/09; revised 10/9/09; accepted 11/2/09; published OnlineFirst 1/12/10.

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Beyond Rapalog Therapy: Preclinical Pharmacology and Antitumor Activity of WYE-125132, an ATP-Competitive and Specific Inhibitor of mTORC1 and mTORC2

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Cancer Res 2010;70:621-631. Published OnlineFirst January 12, 2010.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-09-2340

Supplementary Material
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