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REVIEW

mTOR and cancer therapy

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Proteins regulating the mammalian target of rapamycin (mTOR), as well as some of the targets of the mTOR kinase, are overexpressed or mutated in cancer. Rapamycin, the naturally occurring inhibitor of mTOR, along with a number of recently developed rapamycin analogs (rapalogs) consisting of synthetically derived compounds containing minor chemical modifications to the parent structure, inhibit the growth of cell lines derived from multiple tumor types *in vitro*, and tumor models *in vivo*. Results from clinical trials indicate that the rapalogs may be useful for the treatment of subsets of certain types of cancer. The sporadic responses from the initial clinical trials, based on the hypothesis of general translation inhibition of cancer cells are now beginning to be understood owing to a more complete understanding of the dynamics of mTOR regulation and the function of mTOR in the tumor microenvironment. This review will summarize the preclinical and clinical data and recent discoveries of the function of mTOR in cancer and growth regulation.
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mTOR activation and cancer

The signaling pathways that activate mammalian target of rapamycin (mTOR) are altered in many human cancers. The amplification of the genomic region containing PIK3CA, the gene coding for the p110 alpha subunit of phosphatidylinositol 3′-kinase (PI3K), has been identified in 40% of the cases of ovarian cancer (Shayesteh *et al*., 1999). Activating mutations may occur in as many as 35% of the cases of breast cancer and is associated with a poor prognosis (Li *et al*., 2006). Modifications of PIK3CA have also been identified in colon, brain and lung cancers (Samuels *et al*., 2004).

The dual function phosphatase that negatively regulates PI3K, phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is mutated, silenced or deleted in a number of tumor types including glioblastoma, hepatocellular carcinoma, lung carcinoma, melanoma, endometrial carcinomas and prostate cancer (Li *et al*., 1997; Risinger *et al*., 1997; Steck *et al*., 1997). These changes result in constitutive activation of AKT and consequently mTOR signaling.

Inactivating mutations in the serine/threonine kinase 11 (STK11/LKB1) gene are associated with the Peutz-Jeghers cancer prone syndrome (Giardiello *et al*., 1987). The LKB1 kinase activates the adenosine monophosphate (AMP)-dependent kinase (AMPK) which in response to low energy levels inhibits the function of mTOR via tuberous sclerosis complex protein 2 (TSC2) (Shaw *et al*., 2004). The observed mutations in Peutz-Jeghers result in a failure to inhibit mTOR under low energy conditions (Shaw *et al*., 2004).

Mutations in the TSC protein, TSC2, which inhibits mTOR function under hypoxic conditions as well as conditions of energy deprivation, leads to tuberous sclerosis syndrome (Inoki *et al*., 2003; Liu *et al*., 2006). This syndrome is associated with well-vascularized hamartomas (benign lesions) as well as an increased risk of renal cell carcinoma (RCC) (Yeung *et al*., 1994; Kwiatkowski, 2003).

Cancer related changes in mTOR kinase substrates and their associated proteins are also reported. For instance, S6K1 is overexpressed or constitutively active in several tumor cell lines, and in the early stages of transformation in ovarian surface epithelium with BRCA1 mutations (Wong *et al*., 2001). S6K1 is also amplified in some breast carcinomas (Couch *et al*., 1999). Generally, for tumors that have an amplification of S6K1, there is a corresponding increase in the level of S6K1 protein (Couch *et al*., 1999).

The gene coding for eukaryotic initiation factor 4E (eIF4E) that is positively regulated by mTOR, is altered in a number of tumors. Progressive amplification of the eIF4E gene is associated with late-stage head and neck carcinoma, ductal cell breast carcinoma and thyroid carcinoma (Sorrells *et al*., 1999; Haydon *et al*., 2000; Wang *et al*., 2001). Levels of eIF4E are elevated in some colon carcinomas in comparison to normal colon cells (Rosenwald *et al*., 1999; Berkel *et al*., 2001). The levels of eIF4E are also increased in some bladder and breast cancers that have a poor outcome (Crew *et al*., 2000; Li *et al*., 2002). In these cancers, a corresponding increase in vascular endothelial growth factor (VEGF) was also observed (Scott *et al*., 1998; Crew *et al*., 2000).

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In a lymphomagenesis mouse model, when Myc is overexpressed in lympohoid tissue eIF4E cooperates with c-Myc in the genesis of B-cell lymphoma, accelerating the formation of tumors (Wendel *et al*., 2004). When the Myc mice were crossed with p53± mice the incidence of lymphoma was increased owing to the loss of the remaining allele, unless eIF4E was overexpressed. If eIF4E is overexpressed in the p53± mice, the mice still developed lymphomas but the wild-type p53 allele is maintained (Wendel *et al*., 2004). So a moderate increase in the level of eIF4E appears to abrogate the requirement for suppressing p53-mediated apoptosis in this model system. Overexpression of eIF4E also results in increased frequency of late-onset cancers (Ruggero *et al*., 2004). These data point to the possibility that at least under some conditions eIF4E may act as an oncogene.

In certain types of cancer the relationship between the levels of the mTOR substrate 4EBP1, and the protein whose function it inhibits, eIF4E, might be a predictor of metastasis. In colon carcinoma both eIF4E and 4EBP1 are frequently overexpressed, but the 4EBP1 levels are the most elevated in patients that have little or no metastatic disease (Martin *et al*., 2000).

Rapamycin analogs

Treatment with rapamycin or its analogs (rapalogs) inhibits proliferation in a large number of cell lines and in some instances leads to apoptosis. Cell lines effected by rapamycin treatment have been derived from a number of tumor types including rhabdomyosarcoma, neuroblastoma, glioblastoma, small-cell lung carcinoma, osteosarcoma, pancreatic carcinoma, RCC, Ewing sarcoma, prostate cancer and breast cancer (Bjornsti and Houghton, 2004).

The rapalogs are the only specific small molecule inhibitors of mTOR yet reported**.** Rapamycin forms a ternary complex with FK506-binding protein (FKBP)12 and mTOR, resulting in potent inhibition of mTOR signaling. Structural studies indicate that there are relatively few contacts between the two proteins (Choi *et al*., 1996). FKBP12 binding to rapamycin may induce a favorable conformation for the association of rapamycin with mTOR. All of the rapalogs contain relatively minor modifications to the structure of rapamycin with the primary advantage being increased solubility and stability.

To date all of the compounds that have progressed to clinical trials involve substitution at the C40 hydroxyl of rapamycin. The majority of these compounds substitute esters or ethers. AP23573 substitutes a phosphonate for the C40 hydroxyl. This compound is not a prodrug but it inhibits mTOR activity and binds FKBP12 at concentrations similar to that of rapamycin, in contrast to CCI-779 which is a prodrug that is metabolized to rapamcyin in the body.

Current rapalogs in clinical trials include rapamycin/sirolimus (Wyeth), CCI-779/temsirolimus (Wyeth), RAD-001/everolimus (Novartis) and AP23573 (Ariad).

Cellular responses to rapalogs

The principal effect of rapamycin in most normal cells and many tumor cell lines is growth retardation, but in some tumor cell lines as well as certain primary cells treatment with rapamycin leads to apoptosis. Among the primary cells that undergo apoptosis are certain populations of dendritic cells, and renal tubular cells (Lieberthal *et al*., 2001; Woltman *et al*., 2003). In some instances some of mechanisms responsible for this apoptotic effect have been characterized. It appears that mTOR-regulated components of translation and their targets such as MYC, as well as the prosurvival properties of AKT, which is also a positive regulator of mTOR are involved in this process.

MYC has been proposed to have both antiapoptotic and proapoptotic functions (Secombe *et al*., 2004). The translation of MYC is regulated in part by m^7GTP cap-dependent mechanisms and is therefore dependent on eIF4E and sensitive to inhibitors of mTOR. Recent data indicate that MYC translation is also controlled by the activity of an internal ribosome entry site (IRES) present within the promoter region. IRES-dependent translation of MYC has been linked to the proapoptotic effects of MYC expression, and may be inhibited by AKT activity (Stoneley *et al*., 2000; Shi *et al*., 2005a).

AKT is known to phosphorylate the proapoptotic protein BAD as well as GSK-3 and the FOXO family of transcription factors (Cross *et al*., 1995; Datta *et al*., 1997; Brunet *et al*., 1999). Phosphorylation of BAD by AKT results in its sequestration by 14-3-3 thereby blocking its apoptotic function (Zha *et al*., 1996). As AKT is an activating component of mTOR, it contributes to downstream events such as the m^7GTP cap-dependent regulation of MYC. So mutations or amplifications that activate AKT in addition to suppressing the proapoptotic substrates that are directly phosphorylated by AKT also (via activation of mTOR) effect MYC-dependent regulation of apoptosis. Support for this concept is provided by the eμ-Myc mouse model described earlier in which it was shown that inhibition of mTOR reverses the chemo resistance in lymphomas overexpressing AKT (Wendel *et al*., 2004). The overexpression of eIF4E mimicked the chemo resistance observed when AKT was overexpressed. Rapamycin could reverse the chemo resistance in cells overexpressing AKT but not the cells overexpressing eIF4E. Indicating at least in this setting, the observed chemoresistance was dependent on mTOR-dependent prosurvival signals.

Rapamycin sensitizes multiple myeloma cell lines to dexamethasone treatment, dramatically increasing the degree of apoptosis both *in vitro* and *in vivo* (Yan *et al*., 2006). The sensitivity of cells was independent of activation of the stress pathways, bad phosphorylation, PTEN, or p53 status. Overexpression of a constitutively active mutant of 4EBP1 that cannot be phosphorylated by mTOR resulted in increased sensitivity to dexamethasone similar to the results observed in cells treated with rapamycin, implying that inhibition of translation mediates this effect (Yan *et al*., 2006).

The relationship between mTOR signaling, p53, and apoptosis has also been examined. Under serum free conditions rapamycin treatment causes apoptosis in tumor cell lines with mutated p53. Overexpression of wild-type p53 or $p21^{Cip1}$ protects these cells from rapamycin-dependent apoptosis (Huang *et al*., 2001). The apoptotic effect is a result of the activation of the stress response pathway in cells treated with rapamycin, and is also dependent on the expression of 4EBP1 and concomitant inhibition of eIF4E (Huang *et al*., 2003). So the activation of cap-dependent translation is involved in both p53- dependent and independent mechanisms that inhibit apoptosis and promote growth and survival of transformed cells.

Murine embryo fibroblasts from $PTEN^{+/-}$ mice have increased levels of phosphorylated 4EBP1 and active S6K, which is consistent with increased mTOR signaling (Podsypanina *et al*., 2001). Some $PTEN^{-/-}$ tumor cell lines are extremely sensitive to apoptosis, and it has been proposed that this effect is from a dependence on the increased PI3K activity and subsequent mTOR activity in $PTEN^{-/-}$ cells during tumor development (Neshat *et al*., 2001). Similarly, multiple myeloma cells and glioblastoma cell lines lacking PTEN appear more sensitive to rapalogs, although there are exceptions. The dependence of transformed cells on pathway mutations that provide a growth advantage as part of the transformation process has been termed 'oncogene addiction' (Weinstein, 2002).

mTOR and cell proliferation

In its activated state, mTOR, as part of mTORC1 complex, phosphorylates 4EBP1, allowing for efficient m^7GTP cap-dependent translation. Inhibiting mTOR results in a block of phosphorylation of 4EBP1 resulting in sequestration of eIF4E and a failure to form the m^7GTP cap-dependent preinitiation complex, greatly reducing translation of m^7GTP cap containing transcripts. Many of these m^7GTP cap-containing transcripts encode proteins required for cell cycle progression, including ornithine decarboxylase and cyclin D (Shantz and Pegg, 1994; Rosenwald, 1996). Several lines of evidence indicate that inhibiting this mechanism of translational control contributes to the cytostatic effects resulting from mTOR inhibition. Tumor cell lines that overexpress eIF4E are able to partially overcome the effects of the G1 delay induced by rapamycin (Dilling *et al*., 2002). In cell lines selected for resistance to rapamycin, 4EBP1 levels are reduced thereby creating a stoichometric deficit with eIF4E and relieving inhibition of eIF4E. Cells that have reverted back to rapamycin sensitivity have levels of 4EBP1 similar to those observed in wild-type cells (Dilling *et al*., 2002).

The inhibition of mTOR leads to increased levels of the cyclin-dependent kinase 2 (CDK2) inhibitor, $p27^{kip1}$, an inhibitor of G1-S cell cycle progression (Nourse *et al*., 1994; Law *et al*., 2002). Induction of $p27^{kip1}$ appears to be a significant contributor to the block in cell proliferation observed *in vivo* and in some cell lines treated with rapalogs (Barata *et al*., 2001; Law *et al*., 2002). If a constitutively active form of 4EBP1 is expressed in MCF7 breast cancer cells the level of $p27^{kip1}$ increases and proliferation is inhibited indicating that control of $p27^{kip1}$ is regulated in part by cap-dependent translation (Bader *et al*., 2003).

Bone marrow malignancies display an angiogenic phenotype with increased numbers of bone marrow endothelial cells (Lyden *et al*., 2001). Stimulation of bone marrow endothelial cells with growth factors or association of these cells with leukemia cells activates mTOR, resulting in phosphorylation of the mTOR substrates 4E-BP1 and S6K1. Rapamycin, or CCI-779 significantly inhibits growth factor and leukemia-dependent proliferation of bone marrow endothelium by inducing G0/G1 cell cycle arrest. This effect is correlated with a downregulation of cyclin D1 and upregulation of the CDK inhibitors $p27^{kip1}$ and $p21^{cip1}$ (Costa *et al*., 2006).

The extent of the G1 arrest resulting from rapamycin treatment varies between cell lines. This is likely to be a function of both cell type and the genetic modifications present in the cell line. However, the above results indicate that certain tumor types as well as certain types of primary cells that support tumorogenesis may be inhibited by treatment with rapalogs (Figure 1).

Cancer stem cells and mTOR

The idea that tumors arise from a rare population of self-renewing cancer stem cells capable of generating a hierarchy of more differentiated cells with limited potential to replicate was first demonstrated in leukemia (Lapidot *et al*., 1994). This population is highly enriched when sorted using markers that identify hematopoietic stem cells ($Flk2^-$ $Sca\text{-}1^+$ Lin^- $c\text{-}Kit^+$ $CD48^-$). When these cells were isolated from human patients and then introduced into severe-combined immunodeficient (SCID) mice it was found that the leukemia with its various hierarchy of differentiated cells was reproduced. If leukemic cells from patients outside of this 'stem cell' population were introduced into mice it rarely led to leukemia.

More recently a leukemia mouse model was developed where PTEN was conditionally deleted from hematopoietic cells. If the PTEN tumor suppressor is inactivated in hematopoietic cells in adult mice, these mice immediately developed myeloproliferative disease and go on to develop leukemia in 4–6 weeks (Yilmaz *et al*., 2006). Immediately after the conditional deletion of PTEN, there was a dramatic but transient proliferation of hematopoietic stem cells. When these cells were transplanted into irradiated mice, they were unable to stably reconstitute the hematopoietic program indicating that the PTEN deleted stem cell population is limited in its ability to self-renew. However, if the transfer of the stem cell marker-positive cells was delayed until the

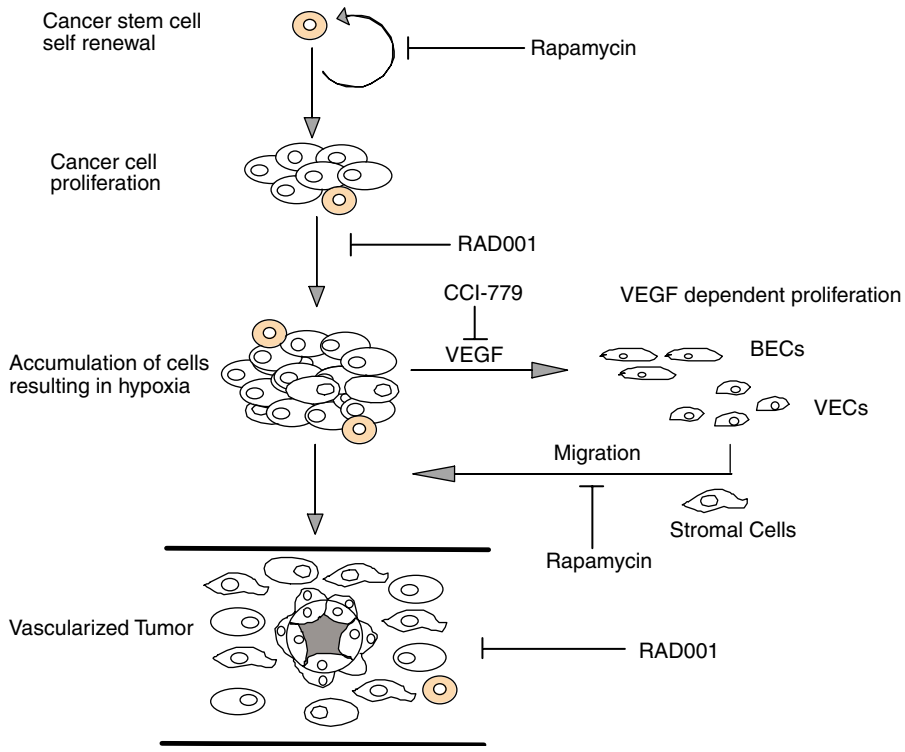

Figure 1 Potential mechanisms of action of rapalogs in cancer. The above diagram illustrates the proposed mechanisms of action of rapalogs from recent publications using *in vivo* and *in vitro* models for specific subsets of cancer (see text for details). Blocked lines indicate inhibition and arrows indicate activation. The abbreviations used are vascular endothelial growth factor (VEGF), Bone marrow endothelial cells (BECs) and vascular endothelial cells (VECs).

donor mice had developed leukemia, the SCID-recipient mice also develop leukemia. Introduction of the non-stem leukemic cells resulted in a much lower rate of leukemic development in the recipient mice. As the deletion of PTEN effectively mimics the overexpression of PI3K, a number of possible PI3K-dependent functions could be responsible for this effect. Thus, the contribution of mTOR to the genesis of these effects might be anticipated to be limited. Surprisingly, the inhibition of mTOR by rapamycin was sufficient to dramatically reduce the effects on stem cell proliferation, and leukemia development resulting from the PTEN deletion. Both the myeloproliferative phenotype as well as the development of leukemias was blocked in mice treated with rapamycin. The transient proliferation of hematopoietic stem cells upon PTEN deletion was also abrogated. Perhaps most significant was that hematopoietic stem cells transplanted into irradiated mice treated with rapamycin were able to reconstitute the hematopoietic population in the donor animals indicating that there was a selective inhibition of the cancer stem cells and not a general inhibition of all stem cells. These results imply that it may possible to selectively target certain populations of cancer stem cells that have alterations in receptor kinase signaling using mTOR inhibitors, even though they have many of the same properties of the stem cells they are derived from or resemble (Figure 1).

mTOR, autophagy and cancer

Autophagy is clearly implicated in the genesis of cancer. Both tumor derived cells and transformed cells exhibit lower levels of protein turnover than their normal cell counterparts. Although a reduction in autophagy appears to be common in tumor cells, some level of autophagy may be required for the development of cancer. Evidence for this is provided in breast cancer in which one copy of the essential autophagy gene BECN1, which codes for the protein BECLIN 1, is frequently deleted, while the remaining copy of BECN1 is wild-type, indicating that BECLIN 1 may be acting as a haploinsufficent oncogene (Liang *et al*., 1999). Further confirmation that BECLIN 1 acts as a haploinsufficent oncogene is provided by a mouse model where deletion of one allele of BECN1 resulted in an increased incidence of lung cancer, hepatocellular carcinoma and lymphoma (Yue *et al*., 2003). Interestingly, this is a similar spectrum of tumors as shown to arise in transgenic mice overexpressing eIF4E (Ruggero *et al*., 2004). In mammals BECLIN 1 was originally identified as a protein interacting with the antiapoptotic protein Bcl-2 (Liang *et al*., 1998). Recent experiments indicate that Bcl-2 is a potential-negative regulator of BECLIN-1, implying a relationship between autophagy and apoptosis (Pattingre *et al*., 2005).

The role of mTOR in autophagy is conserved from yeast to mammals where it acts to regulate the induction of the autophagic process (Noda and Ohsumi, 1998; Levine and Klionsky, 2004). If mTOR is inactive autophagy proceeds, and conversely, when mTOR is activated the autophagic process is inhibited. In mammals this process may be mediated in part through mTOR-dependent phosphorylation of eukaryotic translation elongation factor 2 kinase (eEF-2K). The chain promoting activity of activity of eEF-2 is inhibited when eEF-2 is phosphoryated by eEF-2K. Phosphorylation of eEF-2K by mTOR-dependent pathways inhibits eEF-2K acitivity. In glioblastoma cell lines the knockdown of eEF-2K by RNA interference (RNAi) inhibits autophagy whereas the overexpression of eEF-2K increases autophagy (Wu *et al*., 2006). Hence the inhibition of mTOR results in the activation of eEF-2K, the inhibition of eEF-2 and induction of autophagy. Perhaps one reason that activating mutations of mTOR have not been observed in tumors is that this results in a reduced level of autophagy that may be required to promote survival of tumor cells in later stages of tumor development were energy and nutrients may be limited. However, in breast cancer uncoupling oxygen-responsive signaling pathways from mTOR function may abrogate control of protein synthesis mediated by this pathway (Connolly *et al*., 2006).

Treatment with many of the chemotherapy agents currently in use, or with radiation therapy results in the formation autophagosomes in tumor cells (Bursch *et al*., 1996; Paglin *et al*., 2001; Kanzawa *et al*., 2004). What has not yet been resolved is whether this increase in autophagasomes is a survival mechanism to sequester damaged organelles, or part of an autophagic precursor to apoptotic, or non-apoptotic cell death. For instance, treatment of the MCF-7 breast cancer cell line with tamoxifen increases the levels of autophagy and cell death (Bursch *et al*., 1996). Both cell death and autophagy can be inhibited by the addition of the class-III-PI3K inhibitor 3-methyladenine that functions by blocking preautophagosome formation (Bursch *et al*., 1996). The implication of this data is that a block in the formation of autophagic bodies is sufficient to inhibit the action of the antiestrogen. When MCF-7 cells are modified to overexpress AKT they become more resistant to tamoxifen, but sensitivity to tamoxifen can restored if the cells are treated with rapamycin (deGraffenried *et al*., 2004). Gamma-irradiation of MCF 7 breast cancer cells promotes the formation of autophagosomes and cell death (Paglin *et al*., 2001). Irradiation of these cells inhibits phosphorylation of 4EBP1 and p70S6K1 (Paglin *et al*., 2005). Rapamycin treatment of these tumor cells also promoted autophagy, and combining rapamycin with gamma irradiation resulted in an increase in the amount of cell death.

The constitutive expression of AKT1 in the luminal epithelial cells of the mouse ventral prostate rapidly results in the development of a highly penetrant prostatic intraepithelial neoplasia (PIN) (Majumder *et al*., 2004). This AKT1 induced PIN phenotype is completely dependent on mTOR. When the mice are treated with the mTOR inhibitor RAD001, there is a rapid loss of intraluminal epithelial cells, marked apoptosis, and a reversal of the PIN phenotype within 14 days. Although hypoxia inducible factor 1 alpha (HIF1 alpha) appears to be both the principal mediator of the transcriptional response to elevated AKT1 activity leading to PIN, and the target of RAD001-dependent inhibition of tumor development; the apoptotic component, and the growth inhibitory component may be controlled by a different mechanisms. Overexpression of BCL2 in these cells blocked the apoptotic effects of RAD001, but did not overcome the inhibition of proliferation. This raises the question as to whether inhibition of BECLIN 1 by BCL2 and the consequent inhibition of autophagy might contribute to the block in RAD001 apoptosis. This would imply that the induction of autophagy resulting from mTOR inhibition would be secondary to the inhibition resulting from downregulation of BECLIN 1. Of course, an alternative explanation would be that overexpression of BCL2 counteracts the activation of any proapoptotic proteins that might interact with BCL2 thereby promoting survival.

The above results imply that the use of rapamycin to increase autophagy might be useful in facilitating the effectiveness of other chemotherapeutic agents, but a great deal more work needs to be done to verify this effect *in vivo*.

Hypoxia the tumor microenvironment and mTOR

If cells in culture are exposed to hypoxic conditions, mTOR activity is rapidly inhibited, as measured by both autophosphorylation of mTOR, and phosphorylation of the mTOR substrates S6K1 and 4EBP1 (Arsham *et al*., 2003). Hypoxia inhibits eIF2 and eEF2, as well as the mTOR targets 4EBP1, p70S6K and rpS6 (Liu *et al*., 2006). Initial reports indicated that inhibition of mTOR under hypoxic conditions was the result of the HIF1 alpha-dependent transcript, REDD1, which functions in a TSC2-dependent manner (Brugarolas *et al*., 2004). More recent reports indicate that there is also a HIF-1 alpha/REDD1-independent mechanism that involves activation of AMPK. The activation of AMPK results in the phosphorylation of TSC2 and inhibition of the mTOR activator, RHEB (Liu *et al*., 2006). The function of AMPK within the cell is to sense low energy levels as defined by the amount of cellular AMP. The data indicating that hypoxia is acting in these cells through AMPK links the mechanism by which cellular energy levels and hypoxia regulate mTOR. However, recent data suggest that breast cancer cells acquire resistance to hypoxia by uncoupling oxygen-responsive signaling pathways (e.g. TSC2) from mTOR function, thus abrogating control of protein synthesis mediated by 4E-BP1 and eEF2 (Connolly *et al*., 2006).

The relative contribution of the HIF1alpha/REDD1/TSC2 and AMPK/TSC2 to the inhibition of mTOR may depend upon the cellular context. In cancers such

as RCC as many as 50% of the tumors have inactivating mutations in von Hippel-Lindau tumor suppressor (VHL) that result in a reduction or elemination of its ability to degrade HIF1 alpha (Kim and Kaelin, 2004) under normal oxygen levels. In isogenic pairs of RCC cell lines, a reduction in the level of VHL by RNAi resulted in an increase in the amount HIF1 alpha protein levels and increased rates of cell proliferation when compared with a scrambled small interfering RNA control (Thomas *et al*., 2006). The levels of VEGF, a HIF1 alpha-regulated gene were increased not only under hypoxic conditions but also under normoxic conditions. The treatment of cell lines expressing reduced levels of VHL with CCI-779 resulted in 70% reduction in growth. This growth inhibitory effect was not observed in the cells in which VHL proteins were not downregulated. In subcutaneous xenografts in SCID mice, cell lines with a knockdown of VHL conferred a growth advantage and increased sensitivity to mTOR inhibition, even though there was efficient biochemical blockade of mTOR in both the expressing and VHL knockdown tumors. VEGF levels were increased in VHL knockdown cells, and reduced by CCI-779 treatment *in vitro* and *in vivo*. This effect correlated with inhibition of HIF1 alpha expression, and the expression of HIF1 alpha mutants defective in VHL binding which suppressed the tumor inhibitory effects of CCI-779.

The extent of mTOR inhibition under hypoxic conditions is not as great as is observed when the cells expressing low levels of VHL are treated with CCI-779 (Treins *et al*., 2002). This incomplete inhibition of mTOR under hypoxia has been proposed as an explanation as to why the HIF1 alpha is still activated under hypoxic conditions. The 5′TOP-containing sequence present in the HIF1 alpha transcript contributes to inhibition of HIF1 alpha expression by rapamycin. Eliminating the 5′TOP sequence in the HIF1 alpha sequence rescues the cells from the growth inhibitory effects of CCI-779.

In contrast to the above reports, it was found that murine Lewis Lung carcinoma cells are protected from massive cell death at high densities under hypoxic conditions by the addition of rapamycin. These rapamycin-treated cells had much higher levels of adenosine triphosphate (ATP) and moderately higher levels of glucose under hypoxic conditions than the untreated cells (Hamanaka *et al*., 2005). Autophagy might also be a possible contributor to this survival, but regardless of the mechanism this result indicates that there may be tissue- or species-specific differences in the response to rapalog treatment under conditions of cellular stress.

One of the questions raised from the above data is whether there are classes of cells *in vivo* whose growth is absolutely dependent on mTOR-mediated regulation of HIF-1 alpha and whether these cells are cancer cells, or stromal cells supporting tumor growth. There is now accumulating evidence that the tumor-inhibitory actions of rapamycins *in vivo* may be in part due to antiangiogenic activity (Guba *et al*., 2002).

Thus, inhibition of mTOR can have direct effects on tumor cells manifest by slowing proliferation, increasing apoptosis or inhibiting tumor-derived vascular endothelial growth factor (VEGF), or indirect effects on tumor cells by directly targeting the proliferation and survival of vascular and smooth muscle stromal cells.

mTOR inhibitors: results from clinical trials

A number of clinical trials with the rapalogs have now been completed. The phase I trial evaluating the safety of CCI-779 was examined for both daily and weekly intravenous (i.v.) treatment in patients with a number of different types of tumors (Dancey, 2002). The results indicated that with daily i.v. treatment there are significant grade 3 toxicities, including hypocalcaemia, vomiting, thrombocytopenia and increase in the level of hepatic transaminases. One patient had an objective response (non-small-cell carcinoma), whereas a number of patients had minor responses or stable disease (cervical carcinoma, uterine carcinoma, RCC and soft tissue sarcoma). On a weekly treatment schedule patients experienced no grade 3 toxicities regardless of dosage. Three patients had a partial tumor regression (renal cell, neuroendecrine and breast carcinomas).

Based on the phase I results a number of phase II trials were initiated to study the effects treating advanced stage refractory RCC, refractory mantle cell lymphoma and refractory metastatic breast cancer with CCI-779 (Atkins *et al*., 2004; Chan *et al*., 2005; Witzig *et al*., 2005).

The completed trials for RCC yielded an objective response rate of 5–7%, a minor response rate of 26–29%, and stable disease in approximately 40% of the patients (Atkins *et al*., 2004). Treatment is associated with a increase in survival time of approximately 4 months.

The phase II trial for mantle cell lymphoma consisted of weekly treatment with 250 mg (fixed dose) of CCI-779 i.v., resulted in an overall response rate of 38%, (3% complete response, and 35% partial response) with a median time to progression of 6.9 months in responders versus 6.5 months for all patients treated (Witzig *et al*., 2005).

For the phase II trial in pretreated patients with advanced breast cancer dosages of 75 mg or 250 mg of CCI-779 were given i.v. weekly. The overall response rate of 9.2%, (all partial responses) was similar for both dosage levels but toxicity was decreased in the patients treated with the 75 mg dosage (Chan *et al*., 2005). CCI-779 appears to have significant activity in endometrial cancer, irrespective of PTEN status, yielding objective responses in 25% of patients, and causing disease stabilization in over half of the patients on study.

Results from the initial dose finding and safety trials for RAD001 indicated that the drug is generally well tolerated with effects on tumors similar to that observed for CCI-779.

These initial results prompted the development of a number of additional phase II trials for various types of cancer for both RAD001 and CCI-779. These include trials for breast cancer, prostate cancer, pancreatic cancer, malignant gliomas, leukemia, lymphoma, multiple myeloma, melanoma and RCC (summarized in Table 1).

As rapamycin cannot be given parenterally there are fewer trials examining its efficacy, However, there is an ongoing phase II trial examining the effect of rapamycin treatment on refractory RCC as well as a phase I trial establishing safety in treatment of pediatric patients with refractory acute leukemia or lymphoma.

For AP23573 there is currently a phase I trial to determine the maximum tolerated dose (MTD) by oral administration in advanced cancers, and phase II evaluation against sarcomas. There are also trials to determine the maximum oral tolerated dose in combination with doxorubicin in the treatment of sarcomas, as well as a trial to determince the MTD dose in patients with multiple myelomas, and a phase II trial of patients with recurrent endometrial cancer. Phase II results suggest significant activity against various subtypes of sarcoma.

Phase III trials are in progress to test the efficacy of CCI-779 either alone or in combination with interferon-α as a first-line treatment of RCC, and the use of the oral form of CCI-779 in combination with the aromatase inhibitor letrozole for the treatment of locally advanced or metastatic breast cancer.

Targeting mTOR or the mTOR pathway?

Many of so called 'molecularly targeted' therapies in preclinical and clinical development that target signaling pathways may also inhibit mTOR activity. Thus, is there potential benefit in inhibiting multiple sites within the mTOR pathway over that of inhibiting only mTOR *per se*? The answer is likely to depend on the properties of the individual tumor.

Pathway strategies include the small molecule receptor inhibitors such as the IGF1R inhibitor NVAEW541 as well as neutralizing monoclonal antibodies against receptors such as those directed against the IGF-1 receptor. The logic of this approach is that tumor cells that overexpress these receptors have become dependent on their activation for survival, the so called 'oncogene addiction'. Unfortunately, targeted therapy data indicates that this does not necessarily hold true for all cancers, or possibly the correct target has not been identified. However, this strategy may still be successful for some receptors and subsets of cancers.

Other strategies target a specific cellular function common to many receptors. For instance the small molecule inhibitors of AKT not only inhibit the direct prosurvival functions of AKT that are mediated through a number of receptors, they also potently inhibit the activation of mTOR, at least in the context of mTORC1 (Thimmaiah *et al*., 2005). Preclinical data indicates that this strategy may prove effective; especially in cells were PTEN is deleted and AKT is constitutively activated.

Inhibitors for proteins with broader functions in receptor activation, such as PI3K, have also been developed. But as the cellular function becomes more universal, the risk of toxicity increases, and this toxicity combined with solubility issues have inhibited the development of this class of compounds.

So is there any advantage to inhibiting mTOR directly, given that mTOR inhibition in most instances

Table 1 Summary of clinical trials using rapamycin or its analogs

Condition	*Rapalogs*	*Monotherapy*	*Additional concurrent therapies*
Endometrial cancer	CCI-779	Yes	
	RAD001	Yes	
	AP23573	Yes	
Sarcoma	AP23573	No	Doxorubicin
Recurrent or refractory B-cell lymphoma, non-Hodgkin's lymphoma, CML, CLL, ALL and AML	CCI-779	Yes	Imatinib mesylate aracytin
	RAD001	Yes	
	Rapamycin	Yes	
Hormonally treated prostate cancer patients	CCI-779	No	Surgery
Mantle cell lymphoma	CCI-779	Yes	Rituximab
	RAD001	Yes	
Refractory or advanced non-small cell lung cancer	CCI-779	Yes	
	Rapamycin	No	Gefitinib
Relapsed or refractory multiple myeloma	CCI-779	Yes	
Metastatic or unresectable low grade neuroendocrine carcinoma	RAD001	No	Octreotide depot
Advanced clear-cell renal carcinoma	RAD001	No	Bevacizumab
RCC	CCI-779	Yes	Interferon-α
Metastatic and refractory breast cancer	RAD001	Yes	Docetaxel Trastuzumab Erlotinib Letrozole
	CCI-779	Yes	
Pediatric patients with recurrent or refractory tumors	CCI-779	Yes	
	RAD001	Yes	
Glioblastoma multiforme	Rapamycin	Yes	

Abbreviations: ALL, acute lymphocytic leukaemia; AML, acute myeloid leukaemia; CLL, chronic lymphocytic leukaemia; CML, chronic myeloid leukaemia; RCC, renal cell carcinoma.

is cytostatic rather than cytotoxic? As described above there are *in vitro* and *in vivo* models where mTOR inhibitors act through both cytostatic and cytotoxic mechanisms. Examples include models for some types of leukemia, prostate neoplasms and RCCs. Although this represents a subset of these cancers in each case, this therapy may prove effective in these cases if the properties of the sensitive tumors can be fully characterized.

Recent clinical results indicate that in tumor tissue from patients receiving RAD001 there is hyperphosphorylation of Akt (O'Reilly *et al*., 2006). This appears to be a consequence of increased stability of IRS-1, and upregulation of IGF-1 signaling. Specific inhibition of IGF-I receptor in conjunction with RAD001 led to increased apoptosis, particularly in PTEN-deficient cells. Also given the high level of specificity of the rapalogs and their cytostatic properties, they may prove effective in combination with other therapeutic drugs.

Combination of mTOR inhibitors with agents used in first line therapy

A number of approaches with combination therapy targeting different signaling pathways are possible. For proliferation-dependent or S-phase-specific cytotoxic agents, the anticipated result would be antagonism if rapalogs arrest cells in G1 phase. However, treatment with rapamycin followed by the timed addition of drugs targeting S phase such as irinotecan may have additive effect in tumors, and recent data suggest that mTOR may play a role in determining cellular responses to DNA damage, at least in yeast. For tumors where treatment with mTOR inhibitors may cause a general slowing of growth, concomitant therapy with compounds such as interferon-α or other compounds inducing general apoptosis may prove more appropriate. This combination is currently a component of the current phase III trials to determine efficacy of CCI-779 alone or combined with interferon-α (standard of care) in the treatment of RCC.

In multiple myeloma cell lines and cells from patients, treatment with the combination of CC-5013 (lenalidomide), a thalidomide derivative, and rapamycin resulted in a synergistic apoptotic effect (Raje *et al*., 2004). This combination of drugs was also able to overcome growth advantages conferred by the addition of growth factors, growth on stromal cells, or drug resistance. Although, CCI-5013 and rapamycin appear to have somewhat similar physiological effects (immune modulator, and antiangiogenic), the mechanism of action of these drugs appears to be through different pathways, perhaps accounting for their synergistic effects (Raje *et al*., 2004).

For chronic myeloid leukemia patients that have developed resistance to imatinib, in the vast majority of cases the resistance is the result of a point mutation in BCR-ABL (Gorre *et al*., 2001). Activation of the tyrosine kinase fusion product BCR-ABL is absolutely required for the development of this type of leukemia, and molecularly defines this disease. As the activity of BCR-ABL is dependent on up regulation of PI3K activity and its downstream effectors, mTOR inhibitors might be predicted have a significant effect in either stabilizing or regressing leukemias that have acquired BCR-ABL mutations. Recent preclinical data has added support to this hypothesis (Mohi *et al*., 2004).

Another possible approach to targeting the same pathway would be to target both proteins simultaneously in first line treatment to reduce the likelihood of developing resistant tumor cells. In the case of imatinib this would be accomplished by slowing proliferation of the initial tumor cell population by cotreatment with rapamycin.

When targeting multiple proteins in the same pathway to bypass drug resistance, it is important to determine if the second drug eliminates the effects of the mutation creating the resistance. A good example of the importance of this effect is illustrated by preclinical studies using the epidermal growth factor receptor inhibitor gefitinib, and rapamycin in RCC cell lines where it was discovered that these two agents acted synergistically to inhibit cell growth only in cell lines that contained the wild-type E3 ubiquitin ligase complex protein, VHL (Gemmill *et al*., 2005). However, caution should be used, as inhibiting two major signaling pathways (Erbb1/mTOR) would be anticipated to have effects in normal tissues. Indeed the combination of RAD001 and erlotinib, another Erbb1 inhibitor, causes considerable toxicity manifest by severe mucositis.

Inhibition of the PI3K/Akt signaling pathway sensitizes tumor vasculature to radiation (Edwards *et al*., 2002). Recently it has been determined that mTOR inhibitors also sensitizes tumor vasculature to ionizing radiation (Shinohara *et al*., 2005) (Figure 1). Indicating that for some types of cancer combining mTOR inhibitors with radiation may have some efficacy as a radiosensitizing agent.

Non-rapalog inhibitors of mTOR

Currently, no specific inhibitors of mTOR that are not based on rapamycin have been described. The most common target for small molecule inhibitors of kinases is the ATP binding pocket. The catalytic domain (CD) of mTOR contains autophosphorylation sites as well as the ATP binding site. Small molecules that target the ATP binding site of mTOR would almost certainly inhibit the function of mTOR in the context of both TORC1 and TORC2.

In yeast, preventing the formation of both TORC1 and TORC2 is lethal, and in mice deletion of mTOR results in an embryonic lethal phenotype (Hentges *et al*., 2001). Conditional knockouts of mTOR have not yet been described so it is not clear what the effect of inhibiting mTOR would be in adult mice or in selective tissues. Despite the recent report about the ability of rapamcyin to inhibit the mTORC2 complex as well as the mTORC1 complex, it is not clear that this is a universal effect in all cells (Sarbassov dos *et al*., 2006).

A specific inhibitor targeting the ATP binding domain of mTOR would provide a great deal of information about the contribution of mTOR containing complexes other than mTORC1 to tumorigenesis as well as cellular and tissue functions of these complexes, regardless of its effectiveness as a therapeutic agent.

Another potential approach to disrupt mTOR signaling would be to block the formation of active mTORC by creating small molecules that interfere with the association of mTOR complex proteins such as mLST8 or RAPTOR. In theory, by targeting protein partners that are unique to either mTORC1, or mTORC2, it would be possible to generate selective TOR complex inhibitors. Small molecule inhibitors might also be developed that block the interaction of the mTOR kinase with substrates containing the TOR signaling motif.

Employing RNAi to downregulate components of the mTOR pathway that are overexpressed or amplified may also be possible. Because of delivery considerations, however, this therapy would currently be restricted to certain types of cancer.

The determination of the efficacy of inhibiting mTOR in the treatment of various types of cancer is still being evaluated, and there are many possibilities that have yet to be explored in identifying areas where rapamycin might prove to be an effective treatment for cancer.

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