Review
New targets for therapy in breast cancer
Mammalian target of rapamycin (mTOR) antagonists
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Abstract

Mammalian target of rapamycin (mTOR) is a serine-threonine kinase member of the cellular phosphatidylinositol 3-kinase (PI3K) pathway, which is involved in multiple biologic functions such as transcriptional and translational control. mTOR is a downstream mediator in the PI3K/Akt signaling pathway and plays a critical role in cell survival. In breast cancer this pathway can be activated by membrane receptors, including the HER (or ErbB) family of growth factor receptors, the insulin-like growth factor receptor, and the estrogen receptor. There is evidence suggesting that Akt promotes breast cancer cell survival and resistance to chemotherapy, trastuzumab, and tamoxifen. Rapamycin is a specific mTOR antagonist that targets this pathway and blocks the downstream signaling elements, resulting in cell cycle arrest in the G_1 phase. Targeting the Akt/PI3K pathway with mTOR antagonists may increase the therapeutic efficacy of breast cancer therapy.

Keywords CCI-779, epidermal growth factor receptor, mammalian target of rapamycin, phosphatidylinositol 3-kinase pathway, *PTEN*

Introduction

Mammalian target of rapamycin (mTOR) is a serine-threonine kinase member of the cellular phosphatidyl-inositol 3-kinase (PI3K) pathway, which is involved in multiple functions such as transcriptional and translational control. Activation of mTOR as a consequence of nutrients and growth factors results in the phosphorylation and activation of the 40S ribosomal protein S6 kinase (p70[S6K]) and the eukaryotic initiation factor 4E-binding protein-1 (4EBP1; Fig. 1). These proteins play a key role in ribosomal biogenesis and cap-dependent translation, which result in increased translation of mRNAs that are important to the control and progression of the cell cycle. mTOR is a downstream mediator in the PI3K/Akt signaling pathway and plays a critical role in cell survival.

It has been shown that Akt regulates mTOR through the tuberous sclerosis (TSC) complex [1]. Under non-stimulated conditions, the TSC complex acts as a negative

regulator of mTOR. Phosphorylation of TSC2 (tuberin) by Akt inactivates the complex, releasing its inhibitory effects on mTOR and resulting in mTOR activation. In addition, TSC regulation of mTOR is mediated by the small G protein Rheb. When in its GTP state, Rheb is a potent activator of mTOR. Phosphorylated TSC shifts Rheb to the inactive GDP state [2].

In breast cancer the PI3K/Akt pathway can be activated by membrane receptors, including the HER (or ErbB) family of growth factor receptors, the insulin-like growth factor (IGF) receptor, and the estrogen receptor (ER) [3]. Stimulation of the PI3K/Akt pathway can also occur through oncogenic Ras. There is evidence suggesting that Akt promotes breast cancer cell survival and resistance to chemotherapy, trastuzumab, and tamoxifen [4–7]. This suggests that targeting the Akt/PI3K pathway with mTOR antagonists may increase the therapeutic efficacy of breast cancer therapy. Rapamycin and rapamycin analogs

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ER = estrogen receptor; IGF = insulin-like growth factor; mTOR = mammalian target of rapamycin; PI3K = phosphatidylinositol 3-kinase; TSC = tuberous sclerosis.

Figure 1
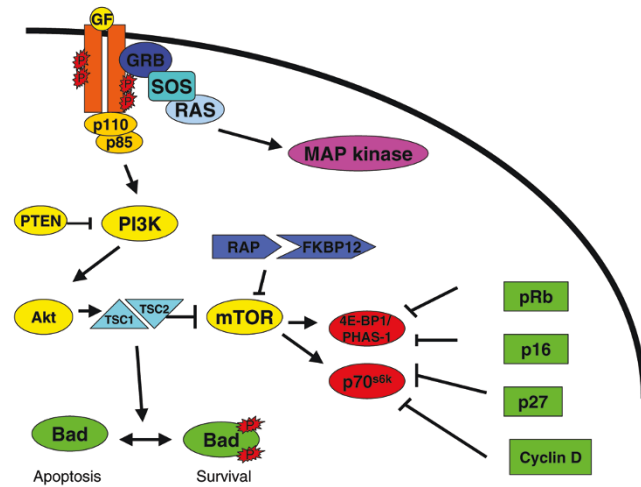
Rapamycin-sensitive signal transduction pathways. Both rapamycin and rapamycin analogs bind to the immunophilin FK506 binding protein-12 (FKBP-12). The rapamycin-FKBP12 complex binds to mammalian target of rapamycin (mTOR), inhibiting its kinase activity, which in turn inhibits the phosphorylation and activation of the downstream translational regulators 4EBP1/PHAS-1 and p70^{S6K}. These downstream effects decrease the translational processing of mRNA for specific proteins that are essential for G_1 to S phase transition. 4E-BP1, 4E binding protein-1; GF, growth factor; GPB, growth factor receptor bound; MAP, mitogen activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PHAS, phosphorylated heat and acid stable protein; pRb, retinoblastoma protein; PTEN, phosphatase and tensin homologue deleted from chromosome 10; RAP, rapamycin; SOS, son-of-sevenless; TSC, tuberous sclerosis complex.

(CCI-779, RAD001, AP23573) are specific mTOR antagonists that are used to target this pathway and block the downstream signaling elements and result in cell cycle arrest in the G_1 phase. These agents have exhibited impressive growth inhibitory effects against a broad range of human cancers, including breast cancer, in preclinical and early clinical evaluations [8,9].

Rapamycin is a macrolytic lactone produced by *Streptomyces hygroscopicus*, which has immuno-suppressive, antimicrobial, and antitumor properties. Rapamycin binds intracellularly to FK506 binding protein-12 (tacrolimus-binding protein) and targets a principal protein kinase that was named mTOR. Other names include FKBP-rapamycin associated protein (FRAP), rapamycin FKBP12 target (RAFT1), and rapamycin target (RAPT1). Inhibition of the phosphorylation of mTOR by rapamycin specifically blocks the activation of the 40S ribosomal protein S6 kinase and 4E-binding protein-1, and directly reduces the translation of mRNAs that encode essential components of the protein synthesis machinery, including growth factors, oncoproteins, and cell cycle regulators. Rapamycin treatment also results in prevention

of cyclin-dependent kinase activation, inhibition of phosphorylation of the retinoblastoma protein, and acceleration of the turnover of cyclin D_1 mRNA and protein, leading to a deficiency of active cyclin-dependent kinase 4/cyclin D1 complexes. The combination of these events likely contributes to the prominent inhibitory effects of rapamycin at the G_1/S boundary of the cell cycle, induction of apoptosis, and inhibition of angiogenesis in several preclinical cancer models [10].

Rapamycin was found to be a potent fungicide, particularly against *Candida albicans* and other filamentous fungi. Later, another related derivative was identified and found to be a potent immunosuppressant (tacrolimus). In bone marrow transplant research, further evaluation of the immunosuppressive qualities of rapamycin revealed successful activity in reversing acute allograft rejection and enhancing long-term donor-specific allograft tolerance. Because this drug is a potent immuno-suppressant with negligible toxicity, it has regulatory approval for use in the prevention of allograft rejection after organ transplantation.

Rapamycin was found to have antiproliferative actions in a diverse range of experimental tumors, including lymphoma, small cell lung cancer, and rhabdomyosarcoma [11–13]. Antitumor actions of rapamycin have been principally attributed to its ability to modulate the synthesis of critical proteins that are required for ribosome biosynthesis, protein translation, and G_1 to S cell cycle phase progression. Rapamycin's poor aqueous solubility and chemical stability limited its development as an anticancer agent, and consequently rapamycin analogs with more favorable pharmaceutical characteristics were developed, including CCI-779, RAD 001, and AP23573.

Although not all of the relevant elements of rapamycin-sensitive signal transduction pathways have been elucidated, PI3K/Akt appears to be the key modulating element in the upstream pathway by which interactions between growth factors and growth factor receptors affect the phosphorylation state of mTOR. The enzyme PI3K, which plays a central role in cellular proliferation, motility, neovascularization, viability and senescence, is upregulated in many types of malignant cells. The best characterized effector of PI3K is the serine/threonine kinase Akt. Both PI3K and Akt are proto-oncogenes, because they have been demonstrated to possess cell-transforming properties. PI3K has other downstream effectors, but the Akt pathway is of particular interest because of its role in inhibiting apoptosis and promoting cell proliferation.

With regard to breast cancer pathogenesis, elements of the PI3K/Akt pathway have been demonstrated to be activated by the ErbB family, IGF receptor, and oncogenic Ras [14–17]. Over-expression of IGF-I receptor and IGF-I

commonly occurs in breast cancers, and circulating IGF-I has been related to the risk for developing breast cancer [18–21]. Furthermore, elements of the IGF signaling pathway are found to be highly expressed in primary breast tumors, and overexpression of IGF pathway elements has been associated with poor prognosis [22]. Upon ligand binding, IGF-I receptor activates insulin receptor substrates that activate elements that are involved in signaling through the PI3K/Akt pathway. Breast tumors can possess genetic alterations in the PI3K/Akt pathway and often exhibit high levels of constitutive Akt activity. Recently, a high frequency of mutations of the *PIK3CA* gene was reported in 32% of colon cancers and almost 10% of breast cancers [23]. This may be a useful tool to identify which patients may respond to rapamycin-directed therapy.

ER activation can directly drive the PI3K/Akt pathway [24]. Cross-talk between ErB1 and ER has been shown to activate the PI3K/Akt pathway, which has been associated with estrogen-independent transcriptional activity and anti-estrogen resistance [2–5]. In support of this hypothesis, breast cancer cell lines with constitutively active Akt can proliferate in the absence of exogenous estrogen and are resistant to the growth inhibitory and proapoptotic effects of tamoxifen *in vitro* and *in vivo* [25]. The role played by the PI3K/Akt pathway in the development of estrogen resistance is also supported by studies of IGF. For example, IGF-I has been demonstrated to downregulate the progesterone receptor via a transcriptional mechanism that involves the PI3K/Akt pathway and is independent of the ER. These data may provide an explanation for why progesterone receptor negative tumors are particularly insensitive to estrogen deprivation [26].

Additional evidence points to a rationale for targeting the PI3K/Akt pathway in the treatment of breast cancer. Over-expression of D-type cyclin has been reported in approximately 50% of invasive breast cancers and was found to be associated with tumor progression [27,28].

Upstream regulators of the PI3K/Akt pathway include the tumor suppressor gene *PTEN* (phosphatase and tensin homolog deleted from chromosome 10). *PTEN* inhibits the activity of PI3K. Thus, loss of *PTEN* suppressor gene function has been associated with Akt activation. A familial syndrome characterized by germline mutations in *PTEN* appear to be responsible for Cowden's syndrome, which predisposes to the development of several types of malignant neoplasms, including breast cancer. Another familial syndrome is Bannayan–Zonana, which has similar features. It is important to note that fewer than 5% of breast tumors harbor a *PTEN* mutation. However, hemizygous deletions of the *PTEN* locus and subsequent lack of PTEN protein occurs in about 30–40% of patients with sporadic breast cancer. This heterozygosity of the *PTEN*

locus is associated with an increased activation of Akt [29–33]. The hyperactivation of PI3K/Akt signaling elements in *PTEN*-deficient malignancies suggests that these cancers are dependent on this pathway for growth and maintenance. Furthermore, experiments conducted in *PTEN* knockout mice indicate that *PTEN*-deficient cancers are extraordinarily sensitive to the growth inhibitory effects of rapamycin and rapamycin analogs [34]. Interestingly, treatment of doxorubicin-resistant and *PTEN*-defective prostate cancer cells with CCI-779 has been shown to reverse doxorubicin resistance [35]. Transfection of those prostate cancer cells with functional *PTEN* produced a similar modulatory effect, suggesting that doxorubicin resistance in this model is mediated through the downstream activation of mTOR.

In breast cancer cells, PI3K/Akt and mTOR pathways appear to be critical for the proliferative responses mediated by epidermal growth factor receptor, the IGF receptor and the ER. These findings suggest that it may be rational to pursue the use of pharmacologic inhibitors of mTOR in treating patients with breast cancers, particularly because these malignancies have evidence of hyperactive PI3K/Akt signaling elements and/or aberrations in tumor suppressor proteins such as PTEN.

Clinical studies with rapamycin/rapamycin analogs

The safety and efficacy of rapamycin in the prevention of organ rejection have been demonstrated in two randomized, double-blind, multicenter, controlled trials involving over 1000 adult patients according to the investigator brochure for CCI-779 formulation. Typical dosing was 2 mg or 5 mg administered daily. In these and most other trials, rapamycin has been administered with cyclosporine and corticosteroids, and limited pharmacokinetic PK data are available alone in this setting. Major side effects noted in these studies included thrombocytopenia, hypercholesterolemia, hypertriglyceridemia, and diarrhea. Renal function was not impaired.

The water-soluble rapamycin ester CCI-779 was selected for clinical development through collaborative efforts between Wyeth-Ayerst Laboratories and the National Cancer Institute. In the US National Cancer Institute's 60 tumor type-specific cell line screening panel, CCI-779 and rapamycin had nearly identical growth inhibitory profiles and 50% inhibitory concentration (IC_{50}) values that were frequently in the nanomolar range according to the investigator brochure for CCI-779 formulation. Cell lines derived from breast, prostate, and small cell lung cancer were among the most sensitive to CCI-779. In further laboratory-based studies by Wyeth, breast cancer cell lines (BT-474, SK BR-3, and MCF-7) exhibited extraordinary sensitivity to CCI-779, with IC_{50} values ranging from 0.0006 to 0.001 μmol/l [34]. Most breast cancer cell lines

highly sensitive to CCl-779 were found to be estrogen dependent, over-expressed Erb-2, and/or had *PTEN* deletions [21]. The resistant breast cancer cell lines lacked these features. Furthermore, sensitive breast cancer cell lines generally had higher levels of activated Akt, perhaps leading to downstream activation of mTOR and subsequent sensitivity to mTOR inhibitors. Laboratory data have also supported the synergistic growth inhibitory effects of MCF-7 breast cancer cell lines and xenografts with combinations of CCl-779 and ER antagonists. Recently, both control MCF-7 and MCF-7/Aro (aromatase expressing) cells were found to be sensitive to treatment with the rapamycin analog RAD001 *in vitro*. RAD001 was found to almost completely inhibit estradiol induced proliferation in both control MCF-7 cells as well as estradiol and androstenedione induced proliferation in MCF-7/Aro cells, suggesting that mTOR signaling is required for estrogen proliferative response in MCF-7 cells. Further-more, combination therapy with letrozole and RAD001 resulted in increased inhibition of cell proliferation as well as a synergistic effect, even at suboptimal levels of RAD001. No evidence of antagonism was observed [36].

Human pharmacokinetics

The pharmacokinetic properties of rapamycin have been studied in healthy individuals, pediatric dialysis patients, hepatically impaired adult patients, and adult renal transplant patients. Oral doses of both liquid and pill forms of rapamycin are rapidly but variably absorbed. Mean time to peak concentrations range from 1 hour in healthy individuals to 2 hours in renal transplant patients. Half-life is upwards of 2.5 days. Metabolism is by the intestinal and hepatic CYP3A4 enzyme family, and 91% of the elimination of the drug is via the gastrointestinal tract. The area under the curve correlates well with peak and trough concentrations.

Patients who ingested the drug after a high fat breakfast did have a delayed time to maximum concentration (Cmax), and it is recommended that rapamycin be consistently taken with or without food. In a phase I pharmacokinetic study conducted in renal transplant patients, doses ranging from 0.5 to 6.5 mg/m^2 were administered every 12 hours. Phase III studies to date have had concomitant use of cyclosporine or steroid, or both. At a dose of 2 mg/day the rapamycin trough concentration was 8.58 ± 4 ng/ml and at 5 mg/day the trough was 17.3 ± 7.4 ng/ml. Rapamycin concentrations in stable renal transplant patients are dose proportional between 3 and 12 mg/m^2. Also, in this population a loading dose of three times the maintenance dose provided a near steady state concentration within 1 day in most patients. Stable renal transplant recipients have received single doses of up to 21 mg/m^2. No toxicity has been observed in any of several single dosing studies with rapamycin doses ranging from 3 to 21 mg/m^2.

Development of mammalian target of rapamycin inhibitors for cancer treatment

Based on preclinical studies suggesting that inhibition of mTOR has antiproliferative effects in a variety of cancer models, clinical studies were initiated with two rapamycin analogs, namely RAD001 and CCl-779. Preliminary results from these studies indicate that these drugs are well tolerated and have promising anticancer activity [37]. The principal toxicities of CCl-779 – the agent for which more clinical data are available – include thrombo-cytopenia, hyperlipidemia, skin toxicity, and elevation in liver function tests. These side effects were in general mild to moderate in intensity and not necessarily associated with administered dose of the drug. In addition, antitumor activity was demonstrated in a variety of tumor types, including breast cancer and renal cancer. A randomized phase II study of multiple dose levels of CCl-779 in patients with advanced refractory renal cell carcinoma has shown antitumor activity and encouraging survival as well as drug tolerability [38].

Additionally, a multicenter European phase II study was conducted in 109 patients with advanced breast cancer [39]. Patients who had progressed on taxanes and anthracyclins were allowed to receive a weekly intravenous dose of CCl-779 at either 75 mg or 225 mg. Clinical benefit was observed in 37% of patients, including 10 partial responses and 26 patients with stable disease for longer than 8 weeks. Activity was seen at both dose levels, with a low toxicity profile. It is interesting to note that activity was even seen in patients with liver metastases at both dose levels but no response was seen in any of the 33 HER-2 negative patients. Further studies were initiated because of these promising results. Because of the association of hormone resistance and activation of the PI3K and mTOR pathways, development of clinical trials combining mTOR inhibitors and hormonal agents are logical. A randomized phase III, placebo-controlled, double-blind study of oral CCl-779 administered in combination with letrozole versus letrozole alone in patients with estrogen-dependent breast cancer is ongoing. Combinations of mTOR inhibitors and cytotoxic agents are also expected.

Conclusion

The PI3K/Akt signaling pathway regulates many normal cellular processes including cellular proliferation, survival, growth and motility, all of which are critical processes for tumorigenesis. It is clear that alteration of this pathway occurs in many cancerous states, and perhaps targeted manipulation to optimize control of this pathway will mitigate its contribution to oncogenic activity. In several cancers, mTOR inhibitors have been shown to be promising agents in reducing tumor growth in vitro and in vivo, including renal cancer and breast cancer. Hopefully, with better understanding of these pathways and improved

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'profiling' of individual patients tumors, future clinical trials and treatment options will identify and target those patients who will benefit from these directed therapies.

Competing interests

HC declares that she has no competing interests. MH has received reimbursements, fees, and funding from Genentec, Astra-Zeneca, OSI pharmaceuticals, Weth, and Brystol Myers Squibb.

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