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“Overcoming breast cancer drug resistance with mTOR inhibitors”. Could it be a myth or a real possibility in the short term future?

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Abstract (250 words):

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Abstract

The mTOR pathway is pivotal not only in tumorigenesis but also in chemotherapy and hormonal drug sensitivity. It is clear that improvements in using new targeted therapies are required to improve breast cancer (BC) outcome. Nevertheless, to achieve this, new molecular biomarkers are required to define the potential sensitivity or resistance of cancer cells. By targeting the mTOR pathway, several critical central transduction pathways that sustain breast cancer are abrogated (HER-2/Neu and the estrogen receptor pathway). Thus the compounds that inhibit mTOR have a double mechanism of toxicity on BC cells when used in combination with a currently used drug: 1) overcoming primary drug resistance and 2) restoring sensitivity when resistance arises after long-term exposure. This review covers the utility of inhibitors of the mTOR pathway in BC and emphasizes the new paradigm of close symbiosis between oncology and molecular biology to better profiling and treating BC with a targeted approach. In particular, we focused on the new drug RAD001 (Everolimus) due to the great results from preclinical and clinical trials make it the most hopeful compound among mTOR inhibitors for the treatment of BC.

Introduction

The central component of mTOR pathway, the serine/threonine kinase mTOR (*Mammalian Target of Rapamycin*), is ubiquitously expressed and highly conserved in eukaryotic cells and functions as the cell gatekeeper. Due to its strategic position, mTOR is a key regulator of multiple cell stimuli, integrating growth factor and cytokine signals with nutrient and energy status. Furthermore, signals emanating from mTOR regulate cell growth, proliferation and survival. Rapamycin (Sirolimus) is the archetypical mTOR-inhibitor: it specifically complexes with FKBP12 (FK506-binding protein 12) which interacts with and inhibits the mTOR protein. The anti-tumour activity of rapamycin has been principally attributed to its ability to modulate the synthesis of critical proteins that are required for ribosome biosynthesis, protein translation, and G1 to S cell cycle phase progression.

Presently there are at least three rapamycin-derived mTOR inhibitors that have been characterized in preclinical models and are in clinical development as anti-cancer agents. These compounds are CCI-779 (Temsirolimus), AP23573 (Deforolimus or Ridaforolimus) and RAD001 (Everolimus). They all have comparable activity against mTOR in biochemical and biological assays but they differ mainly in their bioavailability and formulation. The effect of these drugs on proliferation is cytostatic and not cytotoxic, as they inhibit cell proliferation by causing G1 phase arrest. These agents are thought to have an acceptable safety profile, as generally treatment-related toxicities are mild to moderate in severity and reversible with interruption of therapy or symptom treatment. Everolimus in particular was developed in an attempt to improve the pharmacokinetic characteristics of Sirolimus, wishing to increase its oral bioavailability [18]. Temsirolimus was approved in 2007 by the FDA and EMEA for the treatment of renal cell carcinoma (RCC), while deforolimus is mainly studied for the treatment of sarcoma.

The mTOR signalling pathway, central regulator of cell growth and metabolism

The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase and it is placed downstream of the phosphatidylinositol-3-kinase (PI3K)/protein-kinase B (Akt) pathway. The mTOR pathway is mainly involved in the regulation of cell growth and proliferation by controlling these processes at the translational level. In fact, it has two main downstream messengers involved in controlling translation: the ribosomal p70 S6 kinase (S6K1) and the eukaryotic translation initiation factor 4E-binding protein (4E-BP1). The mTOR-driven phosphorylation of S6K1 causes the subsequent phosphorylation of the 40S ribosomal protein S6, enhancing translation of mRNAs that carries a 5'-terminal oligopyrimidine tract (TOP) such as elongation factor-1 α and ribosomal proteins. On the other hand, the activation of 4E-BP1 results in its dissociation from the cap-binding protein eIF4E, which is thought to be the rare-limiting component for cap-dependent translation. The increased bioavailability of eIF4E levels thus leads to more efficient cap-dependent protein translation initiation, increasing the synthesis of proteins from

that mRNAs with long and highly structured 5'untranslated regions (UTRs), such as cyclin D1 and *c-myc*. Although mTOR is placed directly downstream of PI3K/Akt in a linear pathway, Akt may also regulate mTOR in a more complex fashion, because the tuberous sclerosis complex (TSC), that is formed by the proteins Tsc1 and Tsc2 and that inhibits mTOR, is also target for Akt. In addition to its effect on protein translation mediated by S6K1 and 4E-BP1, mTOR activation leads to the phosphorylation of several downstream effectors and transcription factors, which in turn influence cell proliferation, survival and angiogenesis (Figure 1).

Involvement of the mTOR pathway in drug resistance

The PI3K/Akt signalling pathway, that is dysregulated in a large number of human cancers, up-regulates the downstream mTOR pathway, resulting in enhanced mRNA translation and increased cellular proliferation, as it was previously described. Genetic alterations in this pathway often occur in breast tumours. In fact, a high frequency of mutations of the *PI3KCA* gene was reported in breast cancers (18-26%) as for the p-AKT (42%). PTEN mutations are instead rare in sporadic cases of breast cancers, but this is a site of frequent allelic imbalance and the related protein is absent or decreased in a significant number of breast cancers (15%), while germ-line mutations in PTEN are responsible for Cowden's Syndrome, which predisposes to breast cancer.

It is recognised that cancer cells develop resistance to a variety of standard hormone-therapy and chemotherapeutic regimens through the activation of this same pathway. The current hypothesis, supported by pre-clinical and early clinical studies, is that the addition of an mTOR inhibitor to a conventional therapeutic regimen can enhance the efficacy of established chemotherapeutic and anti-hormonal agents and in some instances can even restore the sensitivity of previously resistant tumour cells, thus improving disease treatment and overall survival. In the case of breast cancer, this is true for tamoxifen, letrozole and trastuzumab (Table 1), but further studies are being performed to establish the efficacy of these combination regimens and also to define new drug associations.

Target pathways for mTOR inhibitors

Current evidences indicate that mTOR inhibitors show efficacy in a subgroup of tumours, suggesting that molecular biomarkers predictive of drug sensitivity or resistance will be critical to enable the development of targeted combination regimens. Currently the only known requirements for the appropriate selection of breast cancer patients who can successfully be treated with an mTOR inhibitor are the overexpression of HER-2 and/or the oestrogen receptor positivity. These should be supplemented by direct measures of mTOR activation to appropriately stratify patients for mTOR inhibitors, since mTOR is responsible for the sensitivity (S) or resistance (R) to both conventional drugs (see Table 1) and mTOR-inhibitors. This can be further expanded using the hypothetical patterns of interactions between a generic chemotherapeutic drug or anti-hormonal agent and an mTOR inhibitor, to design treatment strategies to develop target therapies with mTOR inhibitors. Such postulates, together with breast tumour profiling and mTOR pathway genotypic and/or phenotypic features, will help predict the likelihood of tumor response.

Mammalian TOR and the HER-2/neu pathway

HER-2/neu (ErbB2) is a member of the EGFR (Epidermal Growth Factor Receptor) family and it is amplified in about 15% of breast cancer. Overall survival and time to progression are shorter in patients whose tumours over-express HER-2. Several studies confirmed *in vitro* the causal relationship between mTOR activation and HER-2 over-expression, indicating an underlying role of Akt/mTOR pathway activation in ErbB2-mediated breast cancer progression. Indeed, loss of PTEN and the subsequent Akt/mTOR hyperactivation have been reported to mediate resistance to trastuzumab. Thus rapamycin inhibits multiple stages of tumour progression, including established lung metastases. Since in one study it was used a mouse model that develops spontaneous mammary gland tumours, due to aberrant up-regulation of a non-mutated c-Neu/ErbB2 oncogene comparable to that found in human tumours, it was suggested that inhibition of the mTOR pathway might be an effective treatment for early-stage and late-stage HER-2 positive breast cancer. It may

be also possible that mTOR dependency occurs early in the c-Neu/ErbB2-induced tumourigenic process, suggesting that treatment of hyperplastic regions with rapamycin could prevent or delay primary tumour formation and may be even used as a pharmacopreventive agent. Although trastuzumab has been shown to be a successful example of a rationally designed therapeutic antibody, only 30% of patients with HER-2 over-expressing breast cancer respond to trastuzumab as a single agent, while the majority of patients initially responding develop resistance to this drug within a year. Based on data from pre-clinical studies on cell cultures and xenograft models, which demonstrated that RAD001 enhances the efficacy of trastuzumab [19], it was hypothesized that RAD001 could reverse trastuzumab resistance in patients with HER2-overexpressing breast cancer. Surprisingly, early results from two phase I trials in which RAD001 was combined with trastuzumab (Herceptin®) and chemotherapy agents (paclitaxel or vinorelbine), suggest that the addition of RAD001 overcomes resistance to trastuzumab in patients with HER-2 over-expressing metastatic breast cancer with prior resistance to the above mentioned drug. This combination regiment appeared to be highly efficacious, achieving complete responses in a few patients and partial responses or stable disease in a majority of patients. These preliminary results are very important, also given that the women were heavily pre-treated, had documented resistance to trastuzumab and most were even taxane-resistant.

Interaction of growth factors with their receptors activates not only the mTOR pathway, but also multiple downstream kinase pathways that are not less crucial for cell proliferation and survival. Among them, the most important is maybe represented by the MAP kinase cascade, which exerts downstream effects on Elk-1, a transcription factor related to cell proliferation. For this reason, by simultaneously inhibiting multiple pathways or blocking signal transduction at a nodal point where multiple pathways converge, might be needed to provide an effective strategy for treatment of hormone-dependent breast cancer. They should be tested new compounds capable of inhibiting several pathways, as for example the Farnesylthiosalicylic acid (FTS), or strategies that combines different agents to get the same result [28].

We only have till now information from *in vitro* models, so the next step is to study this phenomenon *in vivo*.

Mammalian TOR and the efficacy of anti-hormonal agents

The estrogen receptor (ER) is expressed in about 60% of breast cancers and is an important predictive and prognostic marker in human breast cancer. However up to 50% of patients with ER positive BC who relapses with distant metastasis not respond to endocrine therapy (*de novo* resistance) and the remainder will eventually relapse despite an initial response (acquired resistance). Efforts to understand the various biologic mechanisms responsible for the development of endocrine resistance are aimed at the development of new strategies to improve anti-ER therapy [1]. Evidence is emerging to suggest both genomic and non-genomic mechanisms for cross-talk in endocrine resistance despite the present of tamoxifen or aromatase inhibitor: an increase of growth factor expression occur during prolonged endocrine therapy in ER positive breast cancer treatment leading to endocrine resistance. Yue *at al* demonstrated that different mechanisms are involved when breast cancer cells adapt themselves to escape from the manipulations blocking the estrogen receptor signalling [27]. This signalling includes EGFR/HER2, Ras/MAPK, ERK1/2 pathway and IGF/PI3K/Akt pathway [2]. In experimental models of LTED, interrupting signalling pathway with drug targeting EGFR, or HER2, or MAPK or mTOR signal restored endocrine responsiveness providing a strong rationale for the clinical use of their combination with hormone therapy in order to prevent or overcome resistance [21]. Using FTS as a tool, we found that the mTOR pathway is a predominant pathway in mediation of proliferation of Long Term Eestrogen Deprivation (LTED) cells. The specific mTOR inhibitor, rapamycin, effectively inhibited growth of LTED cells. Over the last 5 years many studies have established that combining mTOR inhibitors such as CCI-779 or RAD001 with an anti-hormonal agent is synergistic in breast cancer cell *in vitro* models. For example, both control MCF-7 and MCF-7/Aro (aromatase expressing) cells were found to be sensitive to RAD001 treatment *in vitro*. Indeed RAD001 almost completely inhibited estradiol

induced proliferation in both control MCF-7 cells as well as estradiol and androstenedione induced proliferation in MCF-7/Aro cells. The results showed that a combination treatment with letrozole and RAD001 results in increased inhibition of MCF-7/Aro cell proliferation in an additive manner, even at suboptimal levels of RAD001 [17]. Furthermore, the effect of the combination of letrozole with RAD001 in two models of breast carcinoma (MCF-7 and T47D cells) demonstrated that estrogen-induced proliferation is largely dependent on mTOR signaling and that RAD001 in combination with letrozole has more profound effects on aromatase-mediated estrogen-induced proliferation than either agent alone [5]. Similar findings on growth inhibition have been reported for MCF-7 cells treated with RAD001 and tamoxifen [25]. Recent clinical findings from a phase II study conducted in 270 postmenopausal women with ER positive breast cancer in the neoadjuvant setting, showed that RAD001 caused tumour shrinkage when given in combination with letrozole. The combination of letrozole and RAD001 for 4 months before surgery resulted in significantly greater tumour shrinkage, as assessed by ultrasound, and a greater reduction in cell proliferation, as measured by changes in Ki67 after 15 days therapy. Even the overall clinical response rate was significantly greater than letrozole alone treatment in this subset of patients [3].

Can mTOR inhibitors really prevent endocrine resistance?

De novo and acquired resistance to endocrine therapy is a major clinical problem in the treatment of breast cancer. Several studies have shown that the up-regulation of the PI3K/Akt pathway in breast cancers from endocrine-treated patients is responsible for an aggressive clinical phenotype and a short relapse-free and overall survival outcome, by mediating in resistance to anti-estrogen therapeutics. Since the PI3K/Akt is frequently deregulated in breast cancer with up-regulation of this pathway associated with increased sensitivity to mTOR inhibition, this patient population is especially suitable for the administration of mTOR inhibitor rapamycin-like drugs. Both mTOR inhibitors and PI3K inhibitors that are still in the early stages of development are being testing in breast cancer in combination with endocrine therapies. Pre-clinical and clinical studies

have demonstrated a clear association between PI3Kinase downstream Akt signalling and tamoxifen resistance. Thus, a constitutively active Akt in MCF-7 cells renders these normally hormone-dependent breast cancer cells resistant to the inhibitory effect of tamoxifen. This also applies to other hormone inhibitors, including Letrozole® or Fulvestrant®. However, RAD001 was able to restore endocrine therapy sensitivity to refractory breast cancer cells with high Akt activity and also re-sensitize breast cancer cells not responsive to Letrozole as single agents. Interestingly, these results were independent of estrogen receptor α (ER α) activity, indicating that Akt-induced resistance is mediated, at least in part, by ER α -independent mechanisms. These studies demonstrated that the combination of mTOR inhibition and endocrine agents have excellent anticancer activity in pre-clinical models of Akt-induced resistance and provide a rational basis for the following development of clinical trials with mTOR inhibitors in breast cancer [12].

The mechanisms by which endocrine resistance occurs remain unclear, but several studies have demonstrated that estrogen-independent growth properties are mediated at least in part through the PI3K/Akt/mTOR pathway and that hyperactivation of this pathway can protect breast cancer cells from tamoxifen-induced apoptosis, suggesting a significant role in hormone-refractory breast cancer. A recent study in research of mechanism involved into endocrine resistance showed that S6K1, a downstream target of mTOR, is responsible for phosphorylating the Ser¹⁶⁷ of ER α , regulating its transcriptional activity. By the activation of ER α , S6K1 controls the proliferation of ER α positive breast cancer cells [26]. Considering the over-expression of S6K1 is related to an higher sensitivity to rapamycin of breast cancer cells, this finding is important as the detection of S6K1 expression in breast cancer could be a representative way to select whose patients need to be treated with the association of endocrine therapy with an mTOR inhibitor. Nevertheless, there are no clinical studies that demonstrate whether mTOR inhibitors are able to overcome endocrine resistance. However, we have tried to address this question in a phase II clinical trial of 113 patients treated with letrozole, administered alone or with cyclophosphamide. We demonstrated that nuclear ER α phosphorylated on serine 118 (pER α) is a strong marker of endocrine treatment response. This

is an improving of the currently used IHC assessment for the ER expression, as it predict response to hormone therapy in only 36% to 55% of patients, with a small benefit with the progesterone receptor [11] marker evaluation. The molecules p44/42 MAPK and HIF-1, instead, was shown to be significantly associated with endocrine resistance [15].

We observed that the PI3K/Akt/mTOR pathway is significantly down-regulated following estrogen deprivation. Nevertheless, although the observed changes in p-mTOR levels were not significantly associated with tumour shrinkage nor with reduction in proliferation activity, it was tightly associated with a longer disease free survival [14].

It still remains to unfold if the use of mTOR inhibitors in combination with anti-hormonal agents can delay, prevent or overcome the development of endocrine resistance in breast tumors.

Enhancing the anti-tumour activity of mTOR inhibitors

Since AMP-Activated Protein Kinase (AMPK) activity is regulated by the cellular energy level through the balance in ATP:AMP ratio, activated AMPK on its turn can phosphorylate TSC2 at multiple serine and threonine sites, leading to the inactivation of mTOR (Figure 1). Thus, the inhibition of translation through both AMPK activation and mTOR inhibition represents an alternative mechanism to further reduce cancer cell growth. Thus the anti-tumour activity of rapamycin and its derivatives such as metformin, most commonly used for the treatment of type II diabetes, is being explored. Although the molecular mechanism of metformin have been only studied in tissues such as liver, muscle and fat in relation to glucose homeostasis and insulin action, there are occasional reports of anti-neoplastic activity of metformin in various cancer experimental models. A recent work examined the previously uncharacterized direct action of metformin on cancer cells and its potential indirect action due to the overall reduction of insulin levels. Metformin was shown to act as an inhibitor of proliferation *in vitro* in MCF-7 breast cancer cells and in other kinds of epithelial cells. Intracellular control systems, physiologically activated by nutrient deprivation, were induced after treatment with metformin. They were down-regulated processes that

consume energy, such as protein translation and cell division, and they were up-regulated those that generate energy. The growth inhibition was associated with decreased mammalian target of rapamycin and S6 kinase activation and a general decrease in mRNA translation, suggesting potential roles for activators of AMP kinase such as metformin in cancer prevention and treatment [29]. A more recent study on MCF-7 cells shows that metformin inhibits the initiation step in translation via mTOR and that metformin requires two major tumour suppressors, LKB1 and TSC2, to mediate its effects on translation. The results demonstrate that metformin-mediated AMPK activation leads to the inhibition of mTOR and to a reduction in translation initiation, thus providing a possible mechanism of action for metformin-driven inhibition of cancer cell growth. Furthermore, translation in MDA-MB-231 cells, which lack the LKB1 kinase (the kinase responsible for phosphorylating AMPK), and in tuberous sclerosis complex 2 null (TSC2^{-/-}) mouse embryonic fibroblasts, was unaffected by metformin. This indicates that both LKB1 and TSC2 are involved in metformin mechanism of action, in accordance with classical experimental observation on rodents in which severe dietary restriction protects from a variety of carcinogenic influences (22). There is also evidence that this protection is mediated at least in part by the suppressive effect on the circulating level of Insulin-like Growth Factor 1 (IGF-1), which play a role in cancer risk [16]. At the cellular level, physiologic or pharmacological activation of AMP kinase would further attenuate signalling in networks downstream of insulin and IGF-1 receptors, particularly at the level of mTOR. Further studies will be needed to determine the relative importance of direct (AMP kinase pathway activation) and indirect (reduction of insulin levels) mechanisms by which metformin acts as an antiproliferative agent for epithelial cells *in vivo*.

It is now starting a pilot study *in vitro* and *in vivo* which combines rapamycin with metformin, to enhance mTOR inhibition in cancer cells. Furthermore, this study will try to understand if this combination can help overcoming resistance to HER-2 target therapies (trastuzumab and lapatinib) in HER-2 positive breast cancer, given than the PI3K/Akt/mTOR pathway is involved.

Resistance to mTOR Inhibitors

New evidences regarding compensatory pathways involved into mTOR inhibitors resistance are emerging. mTOR inhibition blocks the negative feed-back on IGF-1R signalling interfering on Akt/PI3K signalling. The result is an increase in Akt phosphorylation, protein kinase activity and downstream signaling, which could potentially counteract the inhibition of mTOR. Either *in vivo* or *in vitro* data showed that treatment with mTOR inhibitors increase significantly the p-Akt expression in tumor biopsy or in breast cancer cell lines [22]. However, their understanding is therefore critical to optimize targeted therapies. Based on preclinical models, dual inhibition of both IGF1R – with either monoclonal antibodies or tyrosine kinase inhibitors – and mTOR results in a superior anti-proliferative effect over each single strategy [9], and this combination is now under evaluation in phase I/II trials in patients with breast cancer.

Conclusion

The described findings have brought to light the great potential of the mTOR-inhibitor class of compounds. mTOR inhibitors are ahead in their development providing evidence of anticancer activity in multiple tumor types; rapamycin (sirolimus) derivatives such as temsirolimus, everolimus and deferolimus are potent inhibitor of mTOR without showed the same pharmacokinetic problems of rapamycin. Phase I safety analysis showed that the mTOR inhibitors are generally well tolerated. Class-specific adverse events are consistently observed with each of the three agents including stomatitis, skin rash, erythema, hyperglycaemia and hyperlipidemia. Temsirolimus and everolimus are currently selected for clinical trials. In the case of everolimus, on breast cancer, the Phase I trial identified the dose of 10 mg/day as optimal for further clinical development [3, 11]. In the phase II, double-blind, randomized study of everolimus in combination with letrozole versus placebo and letrozole in the neoadjuvant setting [13], the combination arm proved to be superior over letrozole and placebo with a higher significant response rate (68% vs. 59%) [13]. Based on encouraging preliminary results where everolimus was associated to trastuzumab showing an anticancer activity,

an ongoing phase III trial is evaluating the addition of everolimus to HER2 targeted-therapy and paclitaxel in order to overcome/delay trastuzumab resistance in first-line therapy of HER positive breast cancer. Regarding the other mTOR inhibitor, the initial phase II study of letrozole alone or in combination with temsirolimus in metastatic breast cancer patients, after adjustment of treatment schedule, showed benefit from the combination in terms of median progression-free survival (13.2 months vs. 11.6 months); the consequent phase III trial was interrupted due to the lack of efficacy for the combination.

However, it will be necessary not only to further confirm the efficacy of mTOR-inhibitor in reverting trastuzumab resistance and to verify its interactions with the oestrogen receptor pathway, but also to test if mTOR-inhibitor in combination with other drugs, as for example lapatinib (that is a small molecule inhibitor of ErbB1 and ErbB2 tyrosine kinases), will drive the same overcoming effect. Many other questions needs to be solved, as for example the followings: which are the best conditions or the minimal conditions for the treatment of a breast cancer patient with an mTOR inhibitor? Is it possible to predict an on-coming resistance or we can only waiting for it before an mTOR-inhibitor administration? And more again, is metformin really able to amplify the efficacy of mTOR inhibitors? Are there other undiscovered drug associations with mTOR inhibitor? Future experiments based on pre-clinical studies in animal models and/or clinical trials in breast cancer patients would try to define the parameters for the selection of patients. The neoadjuvant model or the basal stratification based on mTOR activity of breast tumours would probably address the clinician in a better selection of whose patients will have benefits from the use of mTOR inhibitor.

New diagnostic markers need to be validated and introduced in the clinical practice, to stratify and carefully select patients on the basis of mTOR activity in tumour tissues, to take advantage the more as possible of this powerful class of drugs. To conclude, the optimization of mTOR inhibitors' drug administration is opening the way for a new era of bio-oncology.

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Table 1. Molecular events in the mTOR pathway involved in breast cancer drug resistance.

Molecular events	Resistance to	References
Activation of PI3K and AKT	Trastuzumab	[14]
Activation of PI3K and AKT	Endocrine therapy	[15]
Constitutive AKT activation	Tamoxifen	[16], [17], [18]
EGFR, AKT1 and ERK1 activation	Cisplatin	[19]
Raf activation	Doxorubicin and Paclitaxel	[20]
ERK activation	Tamoxifen	[21], [22]
JNK activation	Tamoxifen	[21]

Table 2. Tumour Sensibility (S) or Resistance (R) to a generic conventional drug and to an mTOR inhibitor and expected efficacy of a combined therapy. An additive effect is the sum of the individual effects while a synergistic effect is greater than the sum of the single parts.

Conventional Drug	mTOR inhibitor	Efficacy of the combined therapy
S	S	<ul style="list-style-type: none"> Additive effect Synergistic effect
S	R	Not effective, the single conventional drug is recommended
R	S	<ul style="list-style-type: none"> Effective if the mTOR-inhibitor induce the resistance overcoming Not effective (neither the mTOR-inhibitor can not be used, due to its poor effectiveness alone)
R	R	Not effective, none of the two drugs is recommended

Figure 1 mTOR pathway and influence on cell proliferation, survival and angiogenesis.

Figure 1
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mTOR Pathway

