Chronic Inhibition of Mammalian Target of Rapamycin Signaling Downregulates Insulin Receptor Substrates 1 and 2 and AKT Activation: A Crossroad between Cancer and Diabetes?

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Overactivation of the mammalian target of rapamycin (mTOR) branch downstream of the phosphatidylinositol 3-kinase–AKT pathway critically modulates insulin and growth factor signaling by insulin receptor substrates (IRS). On the basis of in vitro studies, the mTOR inhibitor rapamycin has been reported to lead to enhanced activation of AKT by relieving this feedback inhibition on IRS function. In view of the critical role of AKT in insulin signaling and tumorigenesis, the in vivo expression and activation of this kinase and of IRS-1 and IRS-2 were explored in PBMC of 30 patients who were treated long term with rapamycin. A marked decrease of basal and insulin-stimulated AKT phosphorylation, which correlated with the increase of patients’ insulin resistance, and a significant increase of IRS total protein expression, together with a lower (IRS-2) or absent (IRS-1) increase of insulin-induced tyrosine phosphorylation, were found. Therefore, contrary to the expectations, long-term exposure to rapamycin caused the impairment of IRS signaling and AKT activation, and this would help to explain the antiproliferative effect and the possible deterioration of glucose metabolism that are observed in rapamycin-treated patients. These findings may form a novel basis for improved understanding of the role of mTOR inhibition in human diseases, such as diabetes and cancer.


The mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine kinase that integrates signals from nutrients (amino acids and energy) and growth factors to regulate protein translation through downstream effectors such as ribosomal S6 kinase (S6K) and the translation factor inhibitor 4EBP1 (1). This pathway is critical for proper cell growth, cell cycle progression, and regulation of organ size (1).

Most growth factors and cytokines activate mTOR and its downstream targets primarily through the phosphatidylinositol 3-kinase (PI3-K)-AKT pathway (1). The identification of the components of the tuberous sclerosis protein complex, a heterodimer of the proteins hamartin and tuberin, has established the link between upstream growth factor–signaling cascades and mTOR, providing a mechanism for the crosstalk that occurs between these pathways (1,2). The tuberin–hamartin complex potently inhibits mTOR-dependent signaling; AKT-directed phosphorylation of tuberin relieves its inhibition of mTOR via activation of the small G protein Rheb (3).

Building on studies that have examined the forward mode of mTOR signaling that is activated by the growth factors–PI3-K–AKT pathway, recent studies have provided an insight into the particular role of growth factor signaling via mTOR and S6K in the reverse regulation of PI3-K activation and on the principal role of insulin receptor substrate (IRS) proteins as recipients of negative regulation from kinases such as S6K (4). Therefore, physiologic conditions that lead to the activation of mTOR cascade, such as excess nutrient supply or hyperinsulinemia, promote serine/threonine phosphorylation of IRS-1 and IRS-2 that inhibits their function, promotes their degradation, and, possibly, inhibits their transcription (1–5). In this way, the hyperactivation of mTOR/S6K cascade would exert a significant negative effect on the activity of downstream components of the insulin/PI3-K pathway, such as AKT, and in this way would contribute to or exacerbate the insulin resistance that causes metabolic disease and type 2 diabetes.

Several in vitro studies have shown that inhibitors of mTOR pathway, such as rapamycin, relieve the repression of IRS-1–dependent PI3-K/AKT signaling in states of increased activity of mTOR/S6K pathway and lead to enhanced basal and stimulated AKT serine 473 phosphorylation (2,5–8), although one study reported that chronic repression of mTOR signaling by rapamycin in HEK 293 cells caused a significant reduction in AKT phosphorylation and catalytic activity (9). Paradoxically, these findings might represent a caveat for future use of mTOR.
inhibitors, because activation of AKT could provide cancer cells with an increased survival signal that may be clinically undesirable (7,10,11). It is interesting that recent work indicates that tumors that are formed in mouse models of tuberous sclerosis protein complex may be relatively nonaggressive because activation of raptor–mTOR and S6K1 represses the PI3-K/AKT pathway (12,13).

Recent research, however, has furnished new insights into the molecular mechanisms that regulate mTOR and their role in growth and disease. Sorbassov et al. (14) showed that mTOR kinase and its associated protein, rictor, are necessary for serine 473 phosphorylation of AKT and that a reduction in rictor or mTOR expression inhibited AKT. Also, mTOR inhibition by rapamycin was reported to impede insulin-induced phosphorylation of IRS-1 on tyrosine residues, resulting in decreased insulin sensitivity, in this way transforming human adipocytes into a diabetes-like phenotype (15).

Preliminary evidence arising from clinical studies supports the use of rapamycin and its analogues in human cancers with constitutively activated PI3-K/AKT pathway (16–19) but, conversely, seems to disclose an increased association with hyperglycemia (16,19–22). Accordingly, we recently reported that rapamycin inhibits the progression of Kaposi’s sarcoma (23), a human malignancy in which AKT activation has been suggested to exert a central role (24). We also found that chronic mTOR inhibition caused a significant increase of peripheral insulin resistance, along with an impairment of pancreatic β cell response to glucose load, in a cohort of renal transplant recipients who discontinued the calcineurin inhibitor cyclosporin A (CsA) and were converted to rapamycin (25).

Because AKT is a central player in the signal transduction pathways that are activated in response to growth factors or insulin and the alteration of its activity may induce neoplasia and transformation in some tissues or lead to aberrant glucose metabolism and, eventually, diabetes, we designed this study to evaluate the effect of long-term treatment with rapamycin on the in vivo expression and activation of this protein kinase in circulating mononuclear cells (PBMC) of kidney transplanted patients. Then, we extended this study to explore the effect of chronic mTOR inhibition on IRS-1 and IRS-2 activation.

Materials and Methods

Patients

We enrolled 30 CsA-treated renal transplant recipients (16 men/14 women; age 44.2 ± 12.2 yr) who received the histologic diagnosis of chronic allograft nephropathy 37.5 ± 28.0 mo (range 11 to 119) after transplantation and were asked to be converted to rapamycin (trough 8 to 12 ng/ml), without any further modification of the remaining immunosuppressive therapy (low-dose steroids: prednisone 2.5 to 5 mg/d and mycophenolate mofetil 1 to 2 g/d) (25). All patients were asked to give their written informed consent to participate to the study, according to the Guidelines of the Local Ethical Committee.

All patients had anthropometric and laboratory parameters checked at baseline and at the end of the 6-mo follow-up. Rapamycin exposure was evaluated by predose (trough) monitoring. Blood concentration of rapamycin was determined using an HPLC assay with ultraviolet detection, as described previously (26).

In vivo activation of IRS–AKT–mTOR pathway was attained by intravenous administration of 0.1 IU/kg regular insulin. Blood samples (25 ml) were drawn immediately before and 15 min after insulin injection and processed as detailed next.

Western Blotting

Human PBMC were isolated from freshly drawn blood by Ficoll-Hypaque (Amersham Pharmacia, Little Chalfont, UK) gradient centrifugation, in the presence of 1% (vol/vol) protease inhibitor cocktail and 1% (vol/vol) phosphatase inhibitor cocktail. Cells were lysed and subjected to blotting as described (27), with minor modifications. Briefly, aliquots that contained 50 μg of proteins from each lysate were subjected to SDS/PAGE on a 10% gel under reducing conditions and then electrotransferred onto a polyvinylidene difluoride membrane (Immun-Blot PVDF Membrane, 0.2 μm; BioRad, Milan, Italy) using a semidry Transblot apparatus (BioRad). After overnight blocking, the filter was probed with either polyclonal rabbit anti–phospho-AKT (pAKT) antibody targeted to serine 473 (Saint Cruz Biotechnologies, Santa Cruz, CA) or mouse monoclonal anti–phospho-P70 S6K antibody, raised against a peptide that contained the phosphorylated serine 411 (Santa Cruz Biotechnologies), at 1:200 dilution in TBS at 24°C for 2 h. The membranes then were washed in TBS and incubated for 1 h with horseradish peroxidase–conjugated goat anti-rabbit or goat anti-mouse secondary antibody (BioRad) at 1:1500 dilution in TBS. The same membranes then were stripped and immunoblotted with anti-human AKT or anti-human P70 S6K mAb (Santa Cruz Biotechnologies).

The ECL enhanced chemiluminescence system (Amersham Biosciences, Little Chalfont, UK) was used for detection according to the manufacturer’s instructions. The intensity of signals that were detected by ECL was quantitated by densitometric analysis, and results were expressed in arbitrary units (AU).

Cell Immunofluorescence and Confocal Laser Scanning Microscopy

The low abundance of IRS docking proteins in human PBMC precluded the use of Western blotting for the analysis of IRS expression and activation in a clinical study. We therefore chose to perform a double-fluorescence immunolabeling (27) to quantify the total expression of IRS-1 and IRS-2 proteins and the levels of their activated (tyrosine phosphorylated) forms. In preliminary experiments, we used PBMC from buffycoat byproducts of whole blood that was donated by healthy volunteers to compare the quantification of basal and insulin-stimulated IRS tyrosine phosphorylation by immunoblotting with the quantification of signals that were detected by immunocytochemistry. Cells were incubated with 10 nM human insulin for 15 min. Afterward, the cells were extracted and subjected to immunoprecipitation with anti–IRS-1 or IRS-2 antibodies, followed by immunoblotting with anti-phosphotyrosine antibody (28), or underwent immunocytochemical analysis, as described next. By both methods, we found a marked intersubject variation in the total expression and in the extent of basal and insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-2 proteins, which emphasizes the need to use each patient as his or her own control in the analysis. Importantly, signal quantification by immunocytochemistry was in accordance with the results of Western blot analysis. Finally, immunocytochemistry showed an intrasubject variability (three healthy subjects) over a 2-wk period of only 12%.

For immunocytochemical analysis, PBMC (2 × 10^5/slide) were seeded on a cover glass, fixed with 2% paraformaldehyde, and incubated first with 0.2% Triton X-100 in PBS then with a blocking solution of 3% BSA in PBS. Subsequently, the cells were incubated with two primary antibodies: A polyclonal anti–IRS-1 or anti–IRS-2 antibody (1: 500 dilution in 3% BSA/PBS; Upstate Biotechnology, Dundee, UK)
and a monoclonal anti–phospho-tyrosine antibody (1:500 dilution; Upstate Biotechnology) for 2 h at 37°C in a humidified container. After washing, the cells were incubated in a mixture of two secondary antibodies: Alexa Fluor 488 goat anti-mouse IgG-FITC conjugate (1:300 dilution; Molecular Probes, Eugene, OR) and Alexa Fluor 543 goat anti-rabbit IgG-TRITC conjugate (1:600 dilution; Molecular Probes). The slides then were mounted in Gel/Mount (Biomeda, Foster City, CA) and sealed. Finally, PBMC were analyzed by confocal laser scanning microscopy using the Leica TCS SP2 (Leica, Wetzlar, Germany) (27).

Statistical Analyses

Differences between quantitative variables were tested by the Mann-Whitney U test or Wilcoxon signed rank test, as appropriate. The relationship between nonparametric variables was tested by Spearman rank correlation. \( P < 0.05 \) was considered statistically significant. The Statview software package (5.0 version; SAS Inc., Cary, NC) was used for all analyses.

Results

Patients

Throughout the follow-up, care was taken to avoid any major modification of pharmacologic therapy (25). None of the patients studied had an acute rejection episode or clinically relevant infections; neither did they show significant modifications of body mass index (start 25.7 ± 4.7 kg/m²; end 25.4 ± 3.8 kg/m²) and renal function, measured by serum creatinine (SCr; start 1.52 ± 0.47 mg/dl; end 1.60 ± 0.71 mg/dl) and by creatinine clearance (start 63.9 ± 20.8 ml/min; end 59.4 ± 19.52 ml/min), throughout the follow-up period. Specifically, 56.66% of patients had a decrease of SCr values (-0.31 ± 0.21 mg/dl), 11 (36.66%) showed a worsening of graft function (sCr: 0.39 ± 0.22 mg/dl), and two (6.66%) remained stable during the follow-up period.

Effect of Chronic Exposure to Rapamycin on AKT Activation

Six-month therapy with rapamycin was associated with a decrease in the mean levels of both basal and insulin-stimulated AKT phosphorylation (Figure 1, A and B), without any modification of total AKT content. In view of the large intersubject variation in the expression and phosphorylation of the target kinase, we calculated the percentage change from baseline of pAKT, basal and stimulated, in each patient to ensure a more accurate assessment of drug-induced changes. This approach seems to have reinforced the evidence of the depression of AKT phosphorylation after chronic mTOR inhibition by rapamycin (Figure 1C).

The decrease of insulin-stimulated activation of AKT, calculated as percentage change from pretreatment values in each patient, turned out to correlate with the deterioration of peripheral insulin sensitivity, as measured by the \( \Delta \)-change in the kinetic decline of glucose after insulin infusion (25) (Figure 2, top). The \( \Delta \)-change of insulin-stimulated serine 473 phosphorylation of AKT inversely correlated with rapamycin trough levels: The higher the blood concentration of rapamycin, the lower the insulin-stimulated increase of pAKT after rapamycin therapy (Figure 2, bottom). Then, we wondered whether the modifications of kidney graft function over time would affect AKT phosphorylation, but we did not find any significant relationship between the depressed activation of the target kinase and the \( \Delta \)-change of either SCr or creatinine clearance.

Strong evidence suggests that intracellular lipid accumulation can adversely affect IRS-1–PI3-K–AKT signaling pathway, thereby decreasing insulin sensitivity (29). The search for a relationship between the \( \Delta \) change of serum triglyceride levels and the decrease of AKT phosphorylation revealed a trend toward higher triglyceride levels in patients with lower levels of AKT phosphorylation, which was not statistically significant.

Figure 1. Pharmacologic inhibition of mammalian target of rapamycin (mTOR) inhibits AKT activation. (A) Representative immunoblots using antibody that recognizes only the phosphorylated serine 473 form of AKT (pAKT) and antibody that recognizes total (both nonphosphorylated and phosphorylated) AKT in PBMC of renal transplant recipients, under basal and insulin-stimulated conditions. (B) Quantification of pAKT as a ratio of phosphorylated to total AKT, before and after 6 mo of treatment with rapamycin. Results are the mean (SEM) of the absolute values measured in each patient. (C) Delta (\( \Delta \)) change of basal and insulin-stimulated AKT phosphorylation after long-term exposure to rapamycin. Data represent the mean (±SEM) of the percentage change from baseline measured in each patient. *\( P < 0.0001 \).
however ($P = 0.27$). This may reflect simply the lack of a strict correlation between serum lipid levels and intracellular triglyceride levels (30).

Finally, to rule out the possibility that the modification of AKT activation could be accounted for, at least in part, by the withdrawal of CsA, we performed a set of in vitro experiments in which PBMC from control healthy subjects were cultured in complete medium in the presence or in the absence of 200 ng/ml CsA. Then the cells were challenged with 10 nM regular insulin for 15 min, lysed, and subjected to blotting. Neither short-term (2 h) nor long-term (48 h) exposure to the calcineurin inhibitor seemed to influence the basal and insulin-stimulated serine 473 phosphorylation of AKT, as already demonstrated in vitro and in vivo (31,32).

Impact of Chronic Rapamycin Therapy on P70 S6K Phosphorylation

The P70 S6K represents one of the crucial targets of mTOR in the response to nutrients and in the control of cell growth, and, conversely, its inhibition is a measure of the pharmacologic effect of rapamycin. Therefore, to explore further whether circulating mononuclear cells would reflect the biologic effect of the mTOR inhibitor in vivo, we measured the activation of P70 S6K in PBMC that were isolated from patients before and after 6 mo of therapy with rapamycin. Chronic treatment with the mTOR inhibitor caused a $32.4 \pm 5.3 \%$ decrease of basal P70 S6K phosphorylation (from $3498.3 \pm 397.1$ to $2187.5 \pm 294.7$ AU; $P < 0.0001$) and a $62.3 \pm 3.3 \%$ decrease of insulin-stimulated P70 S6K activation (from $7191.6 \pm 788.1$ to $2667.9 \pm 352.5$ AU; $P < 0.0001$).

Modulation of IRS-1 and IRS-2 Expression and Tyrosine Phosphorylation

First, we found a significant increase of the total protein expression of both the docking proteins after 6 mo of exposure to rapamycin (Figure 3). This seemed to confirm the ability of mTOR inhibition to prevent the proteasomal degradation of IRS proteins and, possibly, the inhibition of their transcription (1–5).

Then we evaluated IRS-1 and IRS-2 tyrosine phosphorylation. Chronic mTOR inhibition was associated with a significant increase in the basal phosphorylation of both of the docking proteins (Figures 4 and 5) and with a complete lack of insulin-stimulated increase of IRS-1 tyrosine phosphorylation (Figure 4). Intravenous insulin injection increased IRS-2 phosphorylation 2.1-fold at baseline and 1.5-fold 6 mo after the conversion to rapamycin; in other words, mTOR inhibition was associated with a significant depression of the incremental insulin activation of IRS-2 (Figure 5).

Last, to ascertain whether the effects of rapamycin on IRS-1/2–AKT–mTOR–P70 S6K pathway described herein were reversible, PBMC from three rapamycin-treated patients were rested overnight (18 h), then rechallenged in vitro with 10 nM insulin and finally processed to measure AKT, P70 S6K, and IRS-1 and IRS-2 expression and activation. In each case, the inhibition of target proteins was reversed to pretreatment levels by overnight incubation of the cells.

Discussion

This is the first demonstration that chronic exposure to the mTOR inhibitor rapamycin inhibits basal and insulin-stimulated activation of AKT in a cohort of renal transplant recipients. The data presented herein suggest that rapamycin leads also to a dysregulation of tyrosine phosphorylation (i.e., activation) of IRS-1 and IRS-2 proteins.

Previous in vitro studies demonstrated that the mTOR signaling pathway is crucial in promoting specific serine/threonine phosphorylation of IRS-1 and IRS-2 that inhibits their function and promotes their degradation (1–5). Conversely, rapamycin is expected to relieve IRS from negative regulation, thereby restoring the activation of the PI3-K–AKT pathway. However, serine/threonine phosphorylation long has been
suggested to have both a positive and a negative regulatory role on tyrosine phosphorylation of IRS-1 and IRS-2 by insulin and IGF-1 receptors (33). Recently, Danielsson et al. (15) demonstrated that insulin-stimulated phosphorylation of serine 307 is required for efficient insulin signaling in human adipocytes and is inhibited by rapamycin, which reduced insulin sensitivity and mimicked type 2 diabetes. The ability of rapamycin to inhibit serine 307 phosphorylation obviously points to the mTOR pathway in relaying the phosphorylation. A potential candidate for serine 307 phosphorylation of IRS-1 is P70 S6K (2). Then, serine 307 is located in a consensus sequence for phosphorylation by AKT (34): the depressed activity of this kinase, as reported here, therefore might impinge on IRS-1 serine 307 phosphorylation.

Our findings showed that chronic treatment with rapamycin resulted, as expected, in the increase of IRS-1 and IRS-2 total protein expression (Figure 3). However, mTOR inhibition markedly hampered the incremental increase of IRS-1 and, less, IRS-2 tyrosine phosphorylation after insulin stimulation, along with a significant increase in the basal phosphorylation of both of the docking proteins (Figures 4 and 5). A closely similar pattern has been reported to occur naturally in patients with insulin-resistant type 2 diabetes (35) as well as in glucose-intolerant first-degree relatives of patients with type 2 diabetes (36).
The elevated basal phosphorylation/activity of IRS proteins likely stems from the removal of mTOR-S6K negative feedback regulation. The incremental defect in insulin-stimulated tyrosine phosphorylation of IRS proteins may be accounted for by the elevated basal phosphorylation of the docking proteins, which possibly masks or inhibits any further increase in signal transduction in response to insulin (36). Then it may result from the inhibition of serine 307 phosphorylation, which dampens the steady-state level of IRS-1 tyrosine phosphorylation (15). In conclusion, although a rapamycin-sensitive pathway is involved in the inhibitory mechanisms impinging on the insulin–IRS signaling pathway in response to prolonged insulin exposure and excess nutrient supply, chronic mTOR inhibition would fail to improve but rather deteriorates IRS-1 and IRS-2 function.

The main objective of this study, however, was to ascertain the effect of chronic exposure to rapamycin on AKT activation, in view of its crucial role in the regulation of cell growth and survival, as well as in the cell response to nutrients and growth factors. We found that chronic mTOR inhibition was associated with a marked downregulation of basal and insulin-induced AKT phosphorylation. AKT is responsible primarily for many of the metabolic actions of insulin (37). Not surprising, therefore, the depression of AKT activation significantly correlated with the increase of insulin resistance in renal transplant recipients (Figure 2).

The inhibition of AKT activation may represent the logical result of the disruption of IRS protein function. Moreover, the rictor–mTOR complex was identified recently as a hydrophobic motif kinase for AKT phosphorylation on serine 473, mTOR being the phosphorylating kinase (15). Although rapamycin does not bind to a preformed rictor–mTOR complex (38), during long-term treatment, the drug eventually should sequester many of the newly synthesized mTOR molecules within cells, thereby inhibiting rictor–mTOR activity and AKT phosphorylation (14). In this way, the prolonged presence of rapamycin may inhibit the kinase, and this, in turn, may impede serine 307 phosphorylation of IRS-1 (15). Figure 6 summarizes the role of mTOR protein complexes in insulin signaling and the interference of rapamycin both upstream and downstream of AKT.

Although in vitro experiments showed the lack of any effect of CsA on AKT phosphorylation in human PBMC, our study does not allow us to rule out the possibility that the downregulation of basal and insulin-induced AKT phosphorylation observed in vivo may be accounted for, at least in part, by the withdrawal of CsA.

The model of the S6K1-deficient mouse has shown that lowering S6K1 levels potentiates insulin-induced AKT phosphorylation (39). However, mice that are deficient in S6K1, unlike rapamycin-treated subjects, are expected to exhibit a fully active rictor–mTOR complex and thus a preserved activation of AKT. In addition, at variance with patients studied here, S6K1-deficient mice are markedly hypoinsulinemic (40), and this might protect IRS proteins from uncontrolled basal tyrosine phosphorylation. Taken together, these data seem to suggest that mTOR inhibition may affect cell responsiveness differently from specific S6K inhibition.

In healthy individuals, β cell mass adapts to an increased metabolic load caused by excess nutrient supply and insulin resistance, via the AKT–mTOR–P70 S6K signal transduction pathway, downstream of IRS-2/P13-K activation (40–42). We previously reported that 6 mo of treatment with rapamycin led to a defect in the compensatory β cell response to increased insulin resistance (25). On the basis of the findings presented here, it might be inferred that pharmacologic manipulations that impair IRS-2 function, as well as AKT and S6K activation, could compromise the ability of β cells to compensate for an increased metabolic demand.

AKT and likely also IRS-1 and IRS-2 have been reported to be overexpressed or activated in a variety of human and experi-
mental malignancies (43–45). Consequently, the expected ability of rapamycin to relieve the feedback inhibition on the IRS branch of the growth factor signaling cascade and downstream AKT activation could provide cancer cells with an increased survival signal that may be clinically undesirable (7,10,11). The data presented herein would hamper the above concerns, demonstrating that chronic rapamycin treatment in humans is associated with impaired activation of both IRS adaptor proteins and AKT, and this may represent a novel mechanism in the anticancer effects of the mTOR inhibitor.

This study allows us to explain better and reconcile our previous observations in the clinical setting, namely the regression of Kaposi’s sarcoma lesions and the marked increase of peripheral insulin resistance, together with the impairment of pancreatic β cell response to glucose load, in patients who are treated long term with rapamycin (23,25). However, we are aware that a potential limitation of this study arises from the cellular model that was used to detect the effects of mTOR inhibition on intracellular signaling, i.e., circulating mononuclear cells. Evidence exists that PBMC may represent a valuable cellular model for the investigation of insulin signaling in humans and for the study of postreceptor signaling defects in insulin-resistant states (46,47). Moreover, in cancer patients, the degree of S6K inhibition by the rapamycin-derivative CCI-779 was reported to be identical in PBMC and simultaneously collected tumor tissue, suggesting that the PBMC are an adequate surrogate tissue for studying the activity of mTOR inhibitors in vivo (48). Regardless, it will be important to establish whether the mechanisms described herein are operating in target tissues of insulin, such as muscle, liver, brain, and in islet β cells.

Conclusion

Here we present evidence that chronic inhibition of the mTOR/S6K pathway by rapamycin is associated with an impaired activation of IRS-1, IRS-2, and AKT in PBMC of renal transplant recipients who are challenged in vivo with insulin. This, on the one hand, may favor systemic insulin resistance and deteriorate glucose metabolism, whereas, on the other hand, may contribute to the antiproliferative effects of rapamycin (Figure 6).
References

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