Rapamycin-mediated FOXO1 Inactivation Reduces the Anticancer Efficacy of Rapamycin

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Abstract. Background: Mammalian target of rapamycin (mTOR) inhibitors such as rapamycin have shown modest effects in cancer therapy due in part to the removal of a negative feedback loop leading to the activation of the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) signaling pathway. In this report, we have investigated the role of FOXO1, a downstream substrate of the PI3K/Akt pathway in the anticancer efficacy of rapamycin. Materials and Methods: Colon cancer cells were treated with rapamycin and FOXO1 phosphorylation was determined by Western blot. Colon cancer cells transfected with a constitutively active mutant of FOXO1 or a control plasmid were treated with rapamycin and the antiproliferative efficacy of rapamycin was monitored. Results: Rapamycin induced the phosphorylation of FOXO1 as well as its translocation from the nucleus to the cytoplasm, leading to FOXO1 inactivation. The expression of an active mutant of FOXO1 in colon cancer cells potentiated the antiproliferative efficacy of rapamycin in vitro and its antitumor efficacy in vivo. Conclusion: Taken together these results show that rapamycin-induced FOXO1 inactivation reduces the antitumor efficacy of rapamycin.

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that regulates cell growth and proliferation by forming two distinct complexes, mTORC1 and mTORC2. mTORC1 is activated by mitogens or nutrient availability and phosphorylates two downstream substrates S6K1 and 4E-BP1 which results in mRNA translation necessary for cell cycle progression. mTORC2 senses growth factors and is responsible for the phosphorylation and full activation of Akt (1, 2). Multiple studies have clinically investigated the potential use of mTOR inhibitors for the treatment of cancer. Overall in cancer patients, the use of rapamycin and its derivatives which specifically block mTORC1 has been less successful than expected (3). One reason might be that in several cell types, the inhibition of mTORC1 by rapamycin activates the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) signaling pathway through the removal of a negative feedback loop and the up-regulation of receptor tyrosine kinases or associated proteins such as platelet-derived growth factor receptor (PDGFR) or insulin receptor substrate 1 (IRS-1) (4, 5). Consistent with this, several reports have shown that targeting PI3K or IRS-1 in combination with rapamycin potentiates the antiproliferative properties of rapamycin (6-9).

The forkhead box class O (FOXO) subclass of forkhead transcription factors plays critical roles in different cellular processes, including cell cycle progression, cell survival, cell differentiation, protection from stress and cell metabolism (10). FOXO members belong to a family of evolutionary highly conserved transcription factors, classified based on their conserved DNA-binding domain, the presence of two large loops and a consensus DNA-binding sequence (11). Different signaling pathways regulate the function and activation of FOXO factors. Most importantly, FOXO proteins are negatively regulated by Akt-mediated phosphorylation which inhibits their ability to induce the transcription of target genes by causing the sequestration of FOXO factors in the cytoplasm (12). Therefore, the activity of FOXO factors are mainly regulated via changes in subcellular localization. Several observations showing that FOXO proteins are inhibitors of cell proliferation and survival suggest that FOXO factors inhibit tumor growth (13). Indeed, while the overexpression of FOXO1 and FOXO3a blocks tumor progression, the inhibition of FOXO1 and FOXO3a induces breast and prostate cancer growth (14, 15). In addition, HER2-induced tumorigenesis in nude mice is prevented by the overexpression of an active mutant of FOXO4 (16).

Since FOXO1 is a direct substrate of Akt and since mTOR inhibition by rapamycin induces the activation of the PI3K/Akt signaling pathway, we evaluated the role of FOXO1 in the antitumor properties of rapamycin.
Materials and Methods

Cell lines, antibodies and reagents. The human colon cancer cell lines HT29 and LS174T were obtained from the American Type Culture Collection (ATCC, Molsheim, France) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum. Antibodies directed against phospho-Akt (S473), Akt, phospho-FOXO1 (Thr24), FOXO1, phospho-S6 ribosomal protein (Ser235/236) and S6 ribosomal protein were from Cell Signalling Technology (Danvers, MA, USA). Rapamycin was from Calbiochem (La Jolla, CA, USA) for in vitro experiments and from LC laboratories (Woburn, MA, USA) for in vivo experiments. The dominant negative mutant Akt (plasmid 9031), pcDNA3 (plasmid 10792) and the constitutive active FOXO1 (Flag-pcDNA3-FOXO1-A3, plasmid 13508) were obtained from Addgene (Cambridge, MA, USA) and are described elsewhere (17, 18).

Cell transfection. For transient transfections, HT29 and LS174T cells were transfected with pcDNA3 or a dominant negative mutant of Akt, using the Effectene transfection reagent (Qiagen, Basel, Switzerland) and following the manufacturer’s instructions. For stable transfections, HT29 cells were transfected with pcDNA3 or a flag tagged constitutively active mutant of FOXO1 (FOXO1-Flag-pcDNA3-A3) using the Effectene transfection reagent. Stable transfectants were selected for resistance to G418 (500 μg/ml; PAA laboratories, Pasching, Austria) for 2 weeks and further expanded in the presence of G418 (100 μg/ml).

Cell cycle analysis. HT29 cells were stably transfected with pcDNA3 or a constitutively active mutant of FOXO1 and treated or not with rapamycin (10 ng/ml) for 48 hours. Cells were subsequently collected by trypsin digestion, washed in phosphate-buffered saline (PBS) and incubated for 24 hours in 70% ethanol. HT29 cells were resuspended in PBS containing 20 μg/ml propidium iodide and 200 μg/ml RNase and incubated for 45 minutes at 37°C. DNA content of the cells was analysed with a FACScan II and Cell QUEST software (Beckton Dickinson, Mountain View, CA, USA).

Subcellular fractionation. Cytoplasmic and nuclear fractions of HT29 cells were obtained by using the Qproteome Cell Compartment Kit (Qiagen) and following the manufacturer’s instructions.

Cell proliferation studies. HT29 cells stably transfected with pcDNA3 or a constitutively active mutant of FOXO1 were plated in six-well culture plates at a density of 200,000 cells/well and cultured in DMEM supplemented with 10% fetal calf serum. Twelve hours later, cells were treated with rapamycin (10 ng/ml) or vehicle (dimethylsulfoxide) and incubated for 3, 5 or 7 days. Cells were subsequently collected and trypsin-blue negative cells were counted by light microscopy using a Neubauer hemocytometer.

Western blot analysis. HT29 and LS174T cells were treated with rapamycin (10 ng/ml) or dimethylsulfoxide as a control for 1, 24 or 72 hours. Alternatively, HT29 and LS174T cells were also transfected with a dominant negative mutant of Akt or pcDNA3 as a control. Forty-eight hours post transfection cells were exposed to rapamycin or dimethylsulfoxide as a control for an additional 24 hours. Cells were subsequently harvested in RIPA lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1% SDS, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptine, 1 mM sodium orthovanadate. Protein concentrations were measured using BCA Assay (Pierce, Rockford, IL, USA). Equal amounts of protein (20 μg) were separated on 4-12% polyacrylamide gel and subsequently transferred to a polyvinylidene difluoride membrane (Millipore, Zug, Switzerland). Membranes were blocked with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) and immunoblotted with primary antibodies followed by infrared secondary antibodies. Bands from immunoreactive proteins were visualised by an Odyssey infrared imaging system.

Tumor xenografts. Animal experiments were in accordance with the Swiss Federal Animal Regulations and approved by the local veterinary office. Female nude mice aged 8 weeks were purchased from Charles River (Charles River Laboratories, St. Germain sur l’Arbresle, France). HT29 cells or HT29 cells stably transfected with pcDNA3 or an active mutant of FOXO1 at 2×10^6 were injected subcutaneously (s.c.) into the flank of nude mice. Once the tumour xenografts reached 100 mm³, mice were randomized into different groups (n=5/group) and treated with vehicle (ethanol) or rapamycin (1.5 mg/kg/d) for 20 days. All mice were sacrificed after 20 days of treatment. Tumor volumes were measured using callipers every day and calculated with the formula =π(a×b²/2) where a is the short axis and b the long axis of the tumor.

Statistical analysis. Data were analyzed by Student’s t-test. Values of p<0.05 were considered statistically significant.

Results

Rapamycin inactivates FOXO1 in colon cancer cell lines. To test whether the inhibition of mTORC1 by rapamycin induces the phosphorylation of FOXO1 in colon cancer cells, HT29 and LS174T cells were exposed to rapamycin and FOXO1 phosphorylation was evaluated by Western blot. In addition, we also analysed S6 ribosomal protein phosphorylation, which is downstream of mTORC1, as an indicator of rapamycin-induced mTORC1 inhibition. We observed that rapamycin increased the phosphorylation of FOXO1 (Figure 1a). This effect was already apparent after one hour and persisted after 72 hours of treatment. In addition, rapamycin also induced the phosphorylation of Akt, the upstream effector of FOXO1. S6 ribosomal protein phosphorylation was totally blocked by rapamycin (Figure 1a).

To demonstrate that rapamycin-induced FOXO1 phosphorylation was mediated by Akt, we transfected HT29 and LS174T cells with a plasmid encoding a dominant negative mutant of Akt (18). We observed that rapamycin-mediated FOXO1 phosphorylation was inhibited by the expression of the dominant negative mutant of Akt in HT29 and LS174T cells (Figure 1b).

Finally, to document that rapamycin-induced FOXO1 phosphorylation resulted in FOXO1 translocation from the nucleus to the cytoplasm, we analysed FOXO1 subcellular localization in HT29 cells following rapamycin treatment. Western blot analysis of subcellular fractions revealed that FOXO1 was present in the cytoplasm and the nucleus. However, upon rapamycin treatment, FOXO1 was mainly...
present in the cytoplasm (Figure 1c). Taken together, these results show that rapamycin induces the phosphorylation of FOXO1 and its translocation from the nucleus to the cytoplasm in colon cancer cells.

The antiproliferative effect of rapamycin is potentiated by the overexpression of a dominant active mutant of FOXO1. We next hypothesized that rapamycin-induced FOXO1 phosphorylation and inactivation may be responsible for reducing the antiproliferative efficacy of rapamycin. To investigate this, we stably transfected HT29 cells with an empty vector (pcDNA3) or a flag tagged active mutant of FOXO1 (FOXO1-A3) (18). The constitutive active mutant protein of FOXO1 was mostly located in the nucleus as observed by Western blot analysis using an anti-flag antibody (Figure 2a). In addition, we also found that treatment of transfected HT29 cells with rapamycin did not induce the translocation of FOXO1-A3 from the nucleus to the cytoplasm, thus showing that FOXO1-A3 was resistant to rapamycin treatment (Figure 2a).

We next evaluated the effect of FOXO1-A3 on the antiproliferative efficacy of rapamycin. We found that expression of FOXO1-A3 in transfected HT29 cells reduced cell proliferation as compared with pcDNA3 transfected cells (Figure 2b). In addition, we also found the growth inhibitory effects of rapamycin were significantly greater on cells transfected with FOXO1-A3 than on cells transfected with pcDNA3 (p=0.0045). Consistent with these findings, cell cycle analysis showed that rapamycin treatment of HT29 cells expressing FOXO1-A3 induced a G1 arrest as shown by a marked increase of the G1 fraction (Figure 2c). Taken together, our results show that the active mutant of FOXO1 potentiates the antiproliferative effect of rapamycin.

The expression of an active mutant of FOXO1 in HT29 tumor xenografts potentiates the anticancer efficacy of rapamycin. We next evaluated the role of FOXO1 on the antitumor efficacy of rapamycin in vivo. We first analyzed whether rapamycin increased FOXO1 phosphorylation in a tumor xenograft model. To test this, HT29 cells were injected subcutaneously into immunodeficient mice. We observed that rapamycin treatment significantly reduced the tumor volume compared with vehicle treatment (Figure 3a). We further found by Western blot analysis of the tumor lysates that rapamycin increased FOXO1 phosphorylation (Figure 3b). Consistent with this finding, we also observed that rapamycin increased the phosphorylation of Akt. Finally as a control for rapamycin-mediated inhibition of mTORC1, S6 phosphorylation was totally blocked by rapamycin.

We next analyzed whether the expression of the constitutive active mutant of FOXO1 in HT29 xenografts would enhance the anticancer efficacy of rapamycin. To test this, HT29 cells that were stably transfected with FOXO1-A3 (HT29/FOXO1-A3) or with pcDNA3 (HT29/pcDNA3) were injected subcutaneously into nude mice. As shown in Figure 4, HT29/FOXO1-A3 tumor xenografts grew slower than HT29/pcDNA3 tumors. In addition, during the 20-day period of treatment, the tumor sizes of rapamycin-treated HT29/FOXO1-A3 xenografts were significantly smaller in
comparison with rapamycin-treated HT29/pDNA3 tumors. Taken together, these results show that the expression of a constitutively active mutant of FOXO1 in HT29 xenografts enhances the anticancer efficacy of rapamycin.

Discussion

In our study, we found that rapamycin induced the phosphorylation of FOXO1 in an Akt-dependent manner in colon cancer cells. FOXO1 phosphorylation resulted in its inactivation as observed by its translocation from the nucleus to the cytoplasm. In addition, we also found that the overexpression of a constitutive active mutant of FOXO1 potentiated the antiproliferative effects of rapamycin in vitro and its anticancer efficacy in vivo.

mTORC1 signaling inhibition has been proposed as a promising therapeutic strategy for cancer therapy. However, despite strong experimental evidence, its benefit in clinical trials has been less successful than expected (1, 3). This is partly explained by the observation that the inhibition of mTORC1 results in the activation of the PI3K/Akt signaling pathway that sustains proliferation and survival and thus counteracts the anticancer efficacy of rapamycin. Therefore, the use of rapamycin as single agent in cancer therapy might have limited efficacy, and combination therapy is required to improve the anticancer efficacy of rapamycin. Consistent with this idea, it was demonstrated that combining erlotinib, an epidermal growth factor receptor (EGFR) inhibitor, with rapamycin had a synergistic effect on cell proliferation inhibition and on tumor xenograft growth inhibition (6). Erlotinib is able to block rapamycin-induced Akt phosphorylation and activation. Similarly, inhibitors of the insulin-like growth factor 1 receptor (IGF-1R) sensitize cancer cells to the antiproliferative effects of rapamycin by in part blocking rapamycin-mediated Akt activation (9). Therefore, combining inhibitors of the upstream components of the PI3K/Akt signaling pathway appears to be a successful strategy to improve the anticancer efficacy of rapamycin. Our work

Figure 2. The expression of a constitutively active mutant of FOXO1 in HT29 cells potentiates the antiproliferative effect of rapamycin. a: HT29 cells were stably transfected with a constitutive active mutant of FOXO1 (FOXO1-A3) or an empty vector (pDNA3). Cytoplasmic and nuclear protein fractions of the stable transfectants were isolated and analysed by Western blot for FOXO1-A3 using an anti-flag antibody. GAPDH and Histone H3 were used as loading controls for the cytosolic and nuclear fractions respectively. b: HT29 cells stably transfected with FOXO1-A3 or pDNA3 were plated in six-well culture plates and treated with rapamycin (10 ng/ml) or vehicle for the indicated time. Cells were subsequently collected and trypan blue-negative cells were counted by light microscopy using a Neubauer hemocytometer. Results are illustrated as the mean cell count ± S.D. of three independent experiments. P-values were calculated by using a Student's t-test. c: HT29 cells stably transfected with pDNA3 or FOXO1-A3 were treated or not with rapamycin (Rapa, 10 ng/ml) for 48 hours and processed for cell cycle analysis.
further identifies FOXO1, a downstream effector of the PI3K/Akt pathway as a putative therapeutical target that potentiates the anticancer efficacy of rapamycin. Indeed, we found that rapamycin induced FOXO1 phosphorylation and FOXO1 translocation from the nucleus to the cytoplasm. In addition, we also found that rapamycin-mediated inactivation of FOXO1 reduced the antiproliferative effect of rapamycin. This is based on the observation that a constitutive active mutant of FOXO1 increased the antiproliferative effect of rapamycin both in vitro and in vivo.

To evaluate the mechanisms by which the active mutant of FOXO1 potentiates the antiproliferative effects of rapamycin, we performed cell cycle analysis. While treatment of HT29 cells with rapamycin or expression of the active mutant of FOXO1 had no significant effects on cell cycle distribution, rapamycin treatment of HT29 cells expressing the active mutant of FOXO1 resulted in a G1 arrest. Consistent with our findings, it was observed that targeting the upstream components of the PI3K/Akt signaling pathway potentiated the antiproliferative effects of rapamycin by acting on the G1/S transition. Treatment of breast cancer cell with the combination of IGF-1R and mTOR inhibitors showed additive effects on G1 arrest compared to cells treated with either agent alone (9). FOXO family members are well known to regulate the cell cycle at the G1/S boundary. They promote cell cycle arrest by repressing the expression of cell cycle activators, including...
cyclin D1 and cyclin D2. In addition, they can also up-regulate cell cycle inhibitors such as p21 and p27 (18). In our work, we did not find any significant changes in the expression of these proteins (data not shown). Therefore, the molecular mechanisms by which the active mutant of FOXO1 in combination with rapamycin induces a G1 arrest in HT29 cells is still under investigation.

Our work further provides a rationale for the use of mTOR inhibitors in combination with therapies that restore the activity of FOXO1. Several molecules and strategies aiming to restore activity of FOXO proteins are currently being developed (13). These include the modulation of the subcellular translocation of FOXO factors whereby the nuclear export of FOXO factors is prevented by blocking proteins responsible for nuclear export. Among these proteins, the human nuclear export protein, chromosome region maintenance 1 (CRM1), has been shown to play an important role for the translocation and inactivation of FOXO factors from the nucleus to the cytoplasm (20). CRM1 inhibitors such as leptomycin B have marked antiproliferative effects on various cancer cell lines in vitro, however, clinical development was discontinued due to high toxicity. Recently, new inhibitors of CRM1 have been developed and present much less toxicity than leptomycin B (21). Future studies will determine their effects in cancer therapy either as single agent or in combination with rapamycin.

In conclusion, these results show that rapamycin-induced FOXO1 phosphorylation and inactivation reduces the anticancer efficacy of rapamycin.

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References


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