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IRS-1: Auditing the effectiveness of mTOR inhibitors

Rapamycin analogs that inhibit mTOR signaling have antitumor activity against certain lymphomas, but treatment of solid tumors has been less encouraging despite inhibition of mTOR function. Two recent papers give insight into the potential use of mTOR inhibitors. O'Reilly et al. provide evidence that poor tumor response to rapamycins is the result of relieving mTOR-mediated feedback inhibition of insulin receptor substrate 1, and activation of Akt-mediated survival. In the second paper, Kaper et al. address the impact of pathway activation on hypoxia-mediated downregulation of mTOR signaling, raising the possibility that rapalogs could selectively inhibit hypoxic cells.

Signaling pathways upstream of mTOR are dysregulated in multiple human cancers, which might make them susceptible to mTOR inhibition (so-called "oncogene addiction"). For example, recent data from genomic sequencing of tumor samples identified mutations in *PIK3CA*, the gene encoding the p110α subunit of phosphatidylinositol 3′ kinase (PI3K) in approximately 30% of colon and breast cancers. Although not all of the mutations have been shown to activate PI3K, there is supporting evidence that this might be the case, since the *PIK3CA* and *PTEN* mutations within the analyzed samples were mutually exclusive. This is consistent with the idea that both events lead to an increase in the second messenger 3′ phosphorylated inositol membrane lipids required to activate the kinase signaling cascade. *PTEN* deletions, or inactivating mutations, are frequently present in carcinomas of the breast and prostate and also gliomas.

The insulin-like growth factors (IGF-I/II) are also implicated in numerous cancers. The IGF/insulin pathway is unique in that, upon ligand binding and receptor autophosphorylation, insulin receptor substrate 1 (IRS-1) associates with these receptors. Tyrosine phosphorylation of IRS-1 in turn leads to the binding and activation of PI3K. The phosphorylation of inositol membrane lipids at the 3′ position by PI3K is a critical step in the IGF-IR signaling pathway. A number of kinases have been identified that associate with these 3′ phosphorylated membrane lipids and subsequently participate in the kinase signaling cascade. Akt, one of the kinases in this activation cascade, has a distinct function in promoting cell survival by phosphorylating and blocking the proapoptotic activity of proteins such as BAD, FoxO transcription factors, and GSK-3α/β (reviewed in Plas and Thompson, 2005) (Figure 1). Akt also positively controls cell proliferation via phosphorylation and inactivation of TSC2, thereby blocking the inhibitory effects of the tuberous sclerosis complex (TSC) on the rapamycin-sensitive mTOR complex (mTORC1) (Inoki et al., 2002; Potter et al., 2002; Dan et al., 2002). The TSC complex, along with Rheb which is considered an activator of mTOR, facilitates mTORC1 sensing of the cellular environment. The activity of mTORC1 is negatively regulated by amino acid deprivation, elevated AMP, or low O_2 (Bjornsti and Houghton, 2004). Activated mTORC1 (comprised of mTOR, Raptor, and GβL/mLST8) phosphorylates 4EBP1 and S6K1. Phosphorylation of 4EBP1 results in its release from the translation initiation factor eIF4E, and the assembly of the preinitiation translation complex eIF4G (Pause et al., 1994). Activation of mTORC1 also leads to phosphorylation of S6K1, which is required for its full activation by PDK1, and assembly of the preinitiation complex required for efficient translation of RNAs with secondary 5′ structures (Holz et al., 2005). Importantly, activation of S6K1 represses upstream signaling through phosphorylation of IRS-1 and its subsequent proteasome-mediated degradation (Haruta et al., 2000).

Rationale for combining inhibitors in the mTOR pathway

Rapamycin, a macrocylic antibiotic in complex with a 12 kDa immunophillin (FKBP12) potently inhibits mTORC1 signaling, resulting in cytostatic or cytotoxic effects on cancer cells. Consistent with the "oncogene addiction" hypothesis, several studies have indicated that cells lacking PTEN function, and hence constitutive Akt activation, are hypersensitive to rapamycin (reviewed in Hay, 2005). However, there are clear exceptions, and reexpression of PTEN does not necessarily induce resistance to rapamycin. Other factors clearly modulate sensitivity to rapamycins. IGF-I and, less potently, insulin are unique among growth factors tested in their ability to overcome the inhibitory effects of rapamycin on proliferation and apoptosis (Hosoi et al., 1999). However, for most cancer cells under normal growth conditions with exogenous growth factors, the effect of rapamycin and its analogs is largely cytostatic, leading to decreased transit through G1 phase and

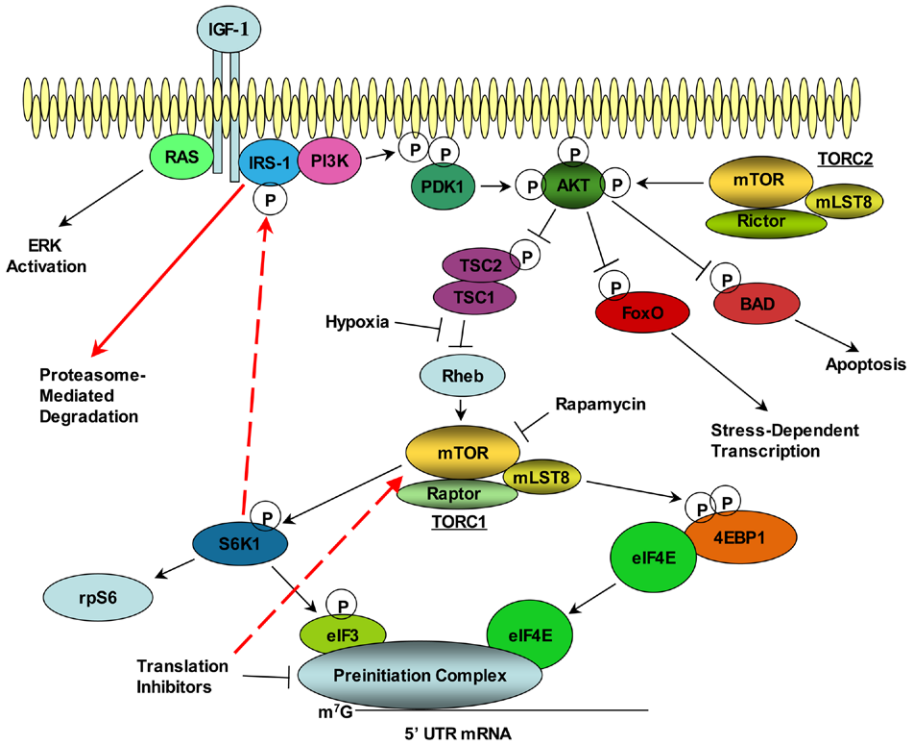

Figure 1. Self-regulation of the IGF-I signaling pathway

Ligand binding induces IGF-IR phosphorylation and association of IRS-1, which in turn is phosphorylated on multiple sites, resulting in the recruitment of the p110 catalytic subunit of PI3K. Akt phosphorylates and inhibits the function of TSC2, thus relieving the negative constraints on Rheb, leading to mTOR activation. Phosphorylation of S6K1 by mTOR leads to phosphorylation of IRS-1 and its degradation via the proteasome (broken red arrow). A second feedback loop that senses inhibition of translation activates mTOR, leading to phosphorylation of ribosomal protein S6 (broken red arrow).

accumulation of cells in G1. The anecdotal finding that inhibition of mTOR increased phosphorylation and activation of Akt in many cell lines thus raised the possibility that this feedback loop in which S6K1 phosphorylated and downregulated IRS-1 may serve to protect cells from rapamycin-induced inhibition of proliferation and potentially apoptosis (Shi et al., 2005). The study from Rosen's laboratory elegantly demonstrates this. Using cancer cells either wild-type or mutant for *PTEN*, it was shown that rapamycin induced rapid and sustained increases in p-Akt (Ser473) and its downstream substrates (FoxO transcription factors and GSK3α/β). Importantly, similar increases in p-Akt in cancer biopsies from patients treated for 4 weeks with the rapalog RAD001 were also found. Increased p-Akt was correlated with increased levels of IRS-1 but not IRS-2. To test the idea that increased p-Akt was a consequence of decreased feedback on the IGF-I signaling pathway, O'Reilly et al. (2006) used a small molecule inhibitor of IGF-IR, NVP-AE541, and an antibody specifically directed against the IGF-IR. Pretreatment with either receptor inhibitor abrogated rapamycin-induced p-Akt induction and substrate phosphorylation. Combination of rapamycin with NVP-AE541 had additive effects on proliferation irrespective of *PTEN* status and increased apoptosis in a *PTEN* mutant cancer cell line. Thus, these results indicate a clear strategy for combining an mTOR inhibitor with an IGF-IR inhibitor or possibly an inhibitor of PI3K, although this latter strategy may be associated with unacceptable hyperglycemia.

The report by Kaper et al. (2006) also addresses activation upstream of mTOR and cellular responses to hypoxia. Inhibition of mTOR by hypoxia (usually 1% O_2) requires the TSC complex and the hypoxia-inducible gene *REDD1*/*RTP801*, the product of which may act upstream of the TSC (Brugarolas et al., 2004). Using U251 cells engineered to inducibly express PTEN, wild-type MEFs, or *TSC2*$^{-/-}$ MEFs, it was shown that activation of the pathway upstream prolonged mTORC1 signaling under hypoxia. Consistent with activation of the mTORC1 pathway, protein synthesis was increased under normoxic conditions, and *PTEN* null or *TSC2* null cells maintained protein synthesis for longer periods under hypoxia. Mutations activating mTOR signaling, or overexpression of mTOR itself, did not maintain DNA synthesis under hypoxia but did result in enhanced transcription and translation of hypoxia-inducible genes. Interestingly, while *TSC* null cells were somewhat resistant to hypoxia-induced apoptosis, rapamycin did not reverse this effect, implicating a TSC-dependent, mTOR-independent survival pathway. Notably, the effect of hypoxia in suppressing downstream phosphorylation of mTOR substrates (4E-BP1 and S6K1) was less pronounced than that of rapamycin treatment, suggesting that repression of mTOR activity by lowered O_2 is not as efficient as repression by rapamycin. However, this neglects an observation made over 30 years ago that inhibition of translation itself leads to enhanced phosphorylation of S6. For example, translation inhibitors such as anisomycin or cycloheximide induce activation of mTOR and rapamycin-sensitive phosphorylation of its substrates (see Brown and Schreiber, 1996). Considering that nutritional deprivation (e.g., amino acid restriction) and hypoxia both reduce mTOR signaling, is it likely that mTOR really functions in hypoxic areas of tumors? To address this issue, xenograft tumors were developed from wild-type or *TSC*$^{-/-}$ MEFs. *TSC*$^{-/-}$ tumors established more rapidly than wild-type, suggesting that enhanced mTOR signaling and translation and expression of hypoxia-induced genes is advantageous only in early establishment of tumors. Importantly, using a pentafluorinated derivative of etanidazole that forms adducts with cellular macromolecules under hypoxic conditions, and immunostaining for phospho-4E-BP1, it was shown that hyperphosphorylated 4E-BP1-positive cells were observed occasionally in hypoxic regions of tumors. Whether this feedback to activate mTOR increases cell survival in hypoxia has not been elucidated. However, it is clear that autoregulatory loops exist to regulate input proximal to mTOR (i.e., feedback inhibition of IRS-1 through S6K1) and distal to mTOR via sensors of translation (Khaleghpour et al., 1999).

Conclusion

The O'Reilly paper demonstrates that effective strategies to target cancer cells require a greater understanding of the effects of disrupting the homeostatic mechanisms of growth control if these pathways are going to be successfully tar-

geted in the treatment of cancer. The work by Kaper et al. focuses our attention on activation of the PI3K pathway upstream of mTOR and the potential role of mTOR inhibitors in selective inhibition of hypoxic cells. A common theme that emerges from these and other recent reports is that signaling pathways are neither linear nor unidirectional, being regulated by compensatory feedback mechanisms to allow cells to survive. This is nicely demonstrated when distinct steps in the PI3K pathway are inhibited.

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