Cellular Assays for Interrogating the PI3K/AKT/mTOR Pathway

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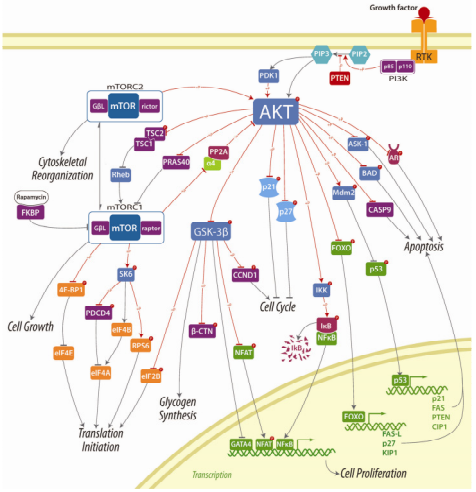
Abstract

Dysregulation of the PI3K/AKT pathway leads to unchecked cellular growth and proliferation. Due to the complexity of this signaling cascade, especially as applied to the regulation of the mammalian target of rapamycin (mTOR), a variety of techniques will be critical for the identification and characterization of small-molecule mediators of this pathway. Since cellular intricacies are often lost when using purified components, we have developed a set of HTS-compatible, fluorescence-based tools to measure target-specific post-translational modification (LanthaScreen™ GFP cellular assays), as well as a pathway-specific readout (CellSensor® cell lines) of the PI3K/AKT pathway in a cellular environment. In combination with traditional biochemical assays, these technologies provide the necessary tools to help evaluate compound effects on PI3K/AKT/mTOR signaling.

Figure 1 – The PI3K/AKT/mTOR pathway



The PI3K/AKT/mTOR pathway is involved in the regulation of metabolism, cell growth and survival, cell-cycle progression, and transcription and translation. AKT resides downstream of phosphoinositide 3-kinase (PI3K) signaling, which is activated upon binding of ligands (such as insulin or other growth factors) to receptor tyrosine kinases (RTKs) on the cell surface. Activated AKT phosphorylates a range of substrates, including PRAS40, BAD, and FOXO3. The kinase mTOR assembles into two distinct complexes inside the cell (mTORC1 and mTORC2), and has been placed on both sides of the AKT signaling hub. mTORC1 (the rapamycin-sensitive complex with raptor) resides downstream of AKT, and mTORC2 (the rapamycin-insensitive complex with rictor) is able to fully activate AKT by direct phosphorylation at Ser473.

Figure 2 – LanthaScreen™ GFP cellular assay technology

To measure target-specific modification in an endogenous signaling pathway, the kinase substrate is expressed as a GFP fusion (FRET acceptor) and an antibody labeled with terbium (FRET donor) is used for detection. Thus, the phosphorylation state of the target can be assessed with a single antibody, which simplifies the assay and improves performance relative to a two-antibody "sandwich" approach.

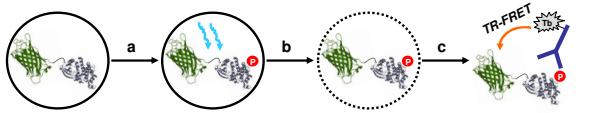
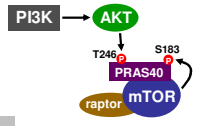
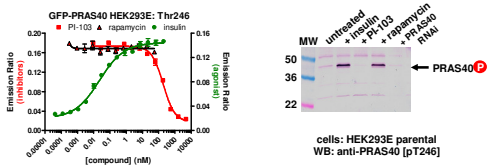


Figure 3 – Assessing compound action on the PI3K/AKT/mTOR pathway

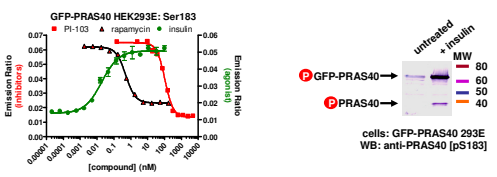
GFP-fusions of key pathway markers were generated as to dissect the complexity of the pathway. The HEK293E cell background is extremely sensitive to insulin signaling, and each of these targets are phosphorylated in a dose-dependent manner upon treatment with insulin or IGF-1. Several known small-molecule inhibitors were profiled using these LanthaScreen™ GFP cellular assays. The dual PI3K and mTOR inhibitor PI-103 shows the expected potency in each assay readout; however, rapamycin only inhibits the phosphorylation of S183 on PRAS40 and S457 on PDCD4 in the mTORC1-dependent assays. Each of these observations has been validated using traditional Western blotting techniques.

PRAS40 [T246]: AKT readout

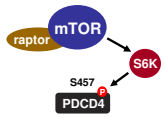


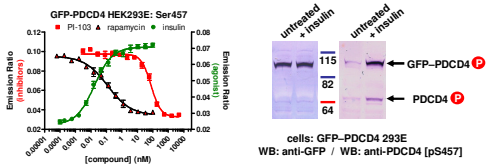


PRAS40 [S183]: mTORC1 readout

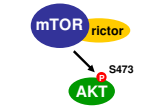


PDCD4 [S457]: mTORC1 readout





AKT [S473]: mTORC2 readout



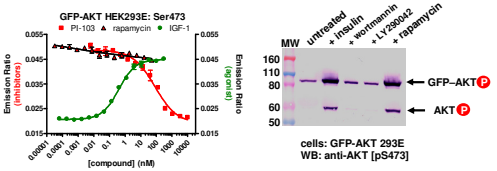
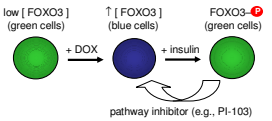
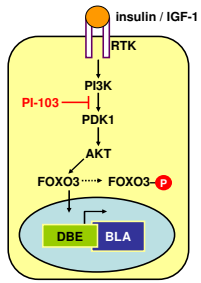


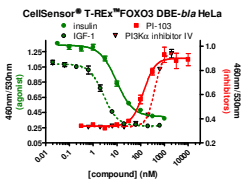
Figure 4 – CellSensor® reporter gene readout for PI3K/AKT pathway analysis



CellSensor® T-REx™ FOXO3 DBE-*bla* HeLa

The CellSensor® T-REx™ FOXO3 DBE-*bla* HeLa cell line has a FOXO3-response element driving beta-lactamase (BLA) expression (DBE-*bla*) along with tetracycline repressor and tetracycline-inducible FOXO3 constructs. Addition of doxycycline (a tetracycline analog) upregulates FOXO3-driven BLA expression. Activation of the endogenous PI3K/AKT signaling cascade with insulin (or IGF-1) leads to phosphorylation / inactivation of FOXO3 and concomitant suppression of BLA. Interruption of the pathway with inhibitors (e.g., PI-103) or Stealth™ RNAi (i.e., against AKT1) restores FOXO3 transcriptional activity and thus BLA expression (green to blue cells).





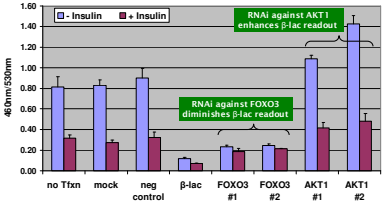


Figure 5 – Comparison of data between assay formats

ligand	LanthaScreen™ GFP Cellular Assays PRAS40 [T246]	PRAS40 [S183]	PDCD4 [S457]	AKT [S473]	CellSensor® FOXO3
PI-103	147	142	103	141	149
rapamycin	>1000	1.5	0.5	>1000	>5000
wortmannin	47	34	34	53	39 (†)
LY294002	2520	2170	2850	3670	1430 (†)
LY303511 (control)	>50000	>50000	>50000	>50000	>50000
PI3Kα inhibitor IV	549	421	445	546	432
PIK-75	443	185	69	132	>10000
TGX 221	>10000	>10000	>10000	>10000	>10000
PI3Kγ inhibitor II	>10000	>10000	>10000	>10000	>10000
IGF-1R inhibitor II	8653	11096	14940	26430	>50000
AKT inhibitor IV	1180	825	740	5360	>10000
Triciribine	90	367	248	126	225
AKTi-1/2	2502	645	703	222	112 (†)
insulin (pM)	3	19	35	270	5000
IGF-1 (pM)	1	n/a	14	44	1600

IC_{50} & EC_{50} values in nM unless indicated; (†) indicates partial antagonist.

Conclusions

- LanthaScreen™ GFP cellular assays (all developed in the same cell background) provide a high-throughput alternative for phospho-protein analysis of specific components within the PI3K/AKT/mOTR pathway.
- CellSensor® cell lines utilize GeneBLAzer® beta-lactamase reporter technology to provide a reliable, rapid, and sensitive method of analyzing the response of signal transduction pathways upon exposure to agonists and antagonists.
- These complementary HTS technologies have been optimized for numerous experimental parameters, and have been validated with a common set of known pathway inhibitors – each displaying the expected potency.