APPLICATION NOTE

Streamlining translational control pathway analysis: Leveraging no-wash HTRF assays for drug discovery.

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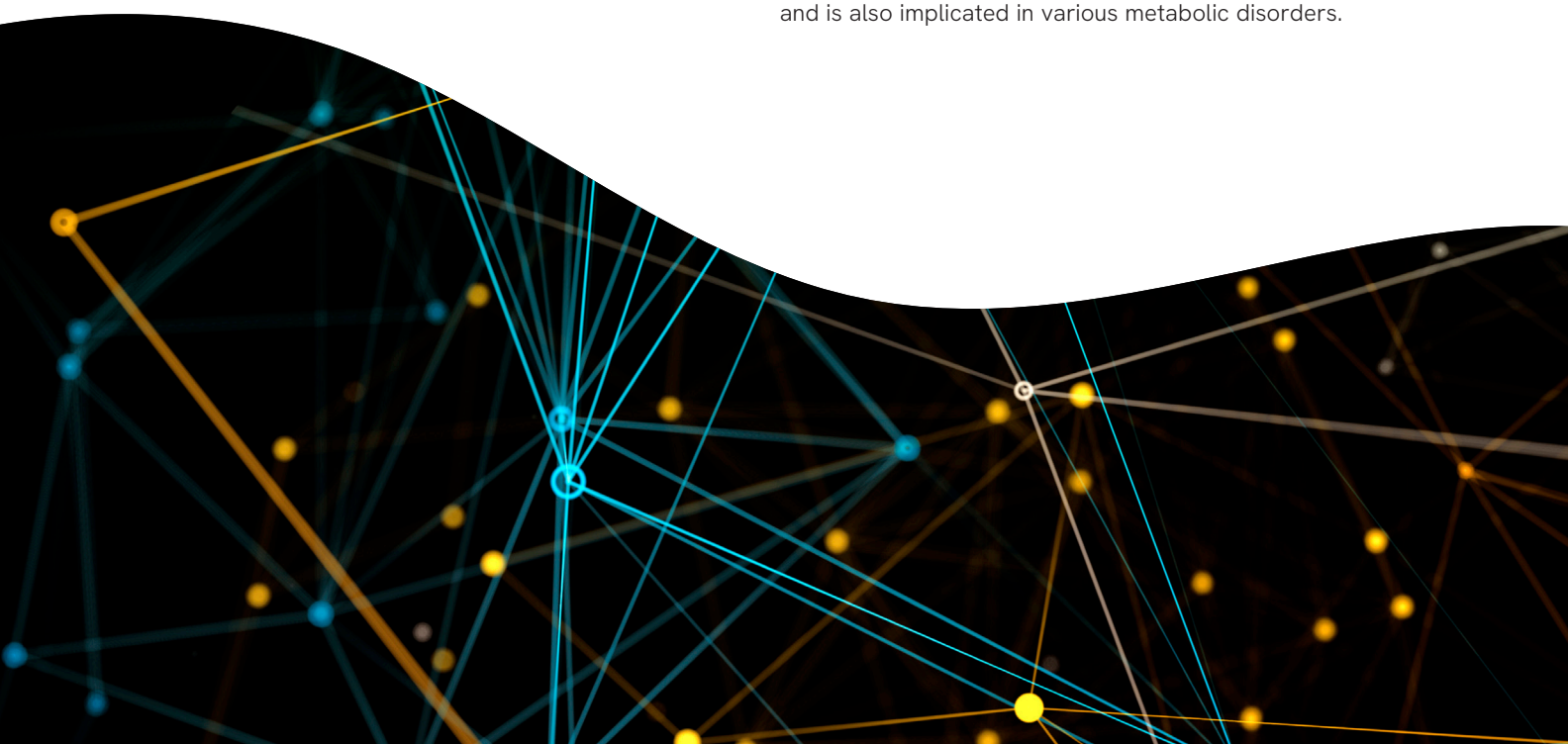
Abstract

In today's challenging environment of budget cuts and the pressing need to reduce attrition, achieving higher throughput while maintaining reproducibility and accuracy is essential. Revvity's HTRF™ cell signaling assay solutions are designed to meet these demands, providing reliable, sensitive, and rapid phospho-protein assays that simplify pathway dissection for drug discovery and research.

This application note highlights the use of HTRF cell-based assays to dissect the PI3K/AKT/mTor translational control pathway across different vertical signaling levels. By measuring the inhibitory effects of various compounds, the assays enable precise classification, and the creation of a detailed pathway map based on their phospho-signatures. The flexibility and robustness of Revvity's HTRF technology make it an ideal choice for researchers focused on cell signaling and drug discovery, offering a streamlined approach to pathway analysis.

Introduction

Phosphatidylinositol 3-kinases (PI3Ks) play a critical role in regulating cellular signal transduction pathways that control key processes such as cell growth, proliferation, survival, apoptosis, and adhesion. Activation of the PI3K/AKT/mTor pathway, a central signaling network, directly influences protein translation. Dysregulation of this pathway is frequently observed in cancer and is also implicated in various metabolic disorders.



To study the pathway's phospho-signature, several well-characterized inhibitors were selected. LY294002 and Wortmannin target and inhibit PI3K activity upstream, while Rapamycin and PP242 block mTor, a key mid-pathway effector. Additionally, PF4708671 and CGP57380 inhibit downstream targets, including ribosomal protein S6 and the translation initiator EIF4E, further dissecting the pathway's regulatory mechanisms.

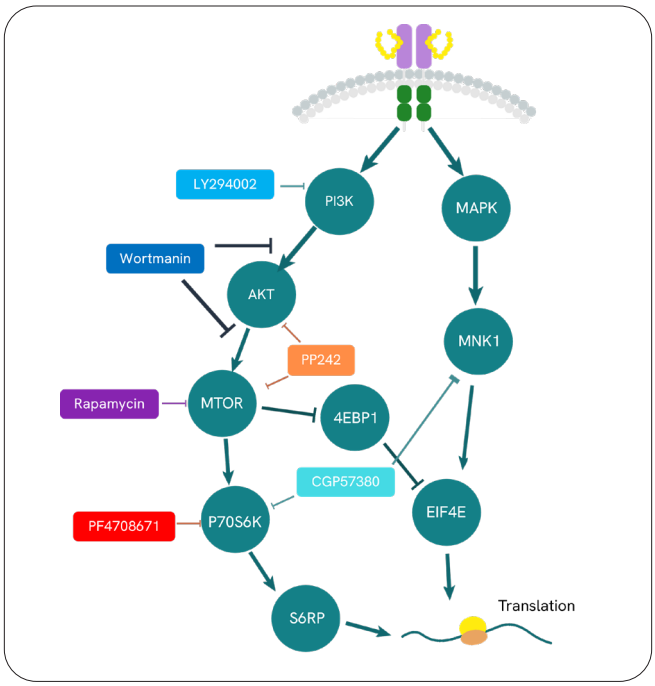


Figure 1: Illustration of the PI3K/AKT/ mTor pathway including reference inhibitors.

Results

Targeting the upstream pathway: LY294002 and wortmannin signatures

LY294002 and Wortmannin are widely used in pharmacological studies to investigate the physiological role of PI3-Kinase (Ref. 11). These compounds represent a class of inhibitors targeting upstream PI3-kinase. Following cell lysis and lysate transfer, six downstream phosphorylated protein markers were analyzed at increasing concentrations of these inhibitors. As shown on Figures 2 and 3, both LY294002 and Wortmannin significantly reduced the phosphorylation of AKT at residues S473, while total AKT expression remained unchanged. Phosphorylation inhibition reached up to 80%, leading to a marked reduction in phosphorylation of downstream targets, including mTor, P70S6K, and S6RP, with inhibition levels ranging from 80% to 95%.

The inhibitors were found to be less potent in reducing the phosphorylation of 4EBP1 and EIF4E. These results are consistent with the literature, which identifies 4EBP1 as a substrate of mTor, while EIF4E is more closely associated with the MAPK-MNK1 pathway (Ref. 6, 7).

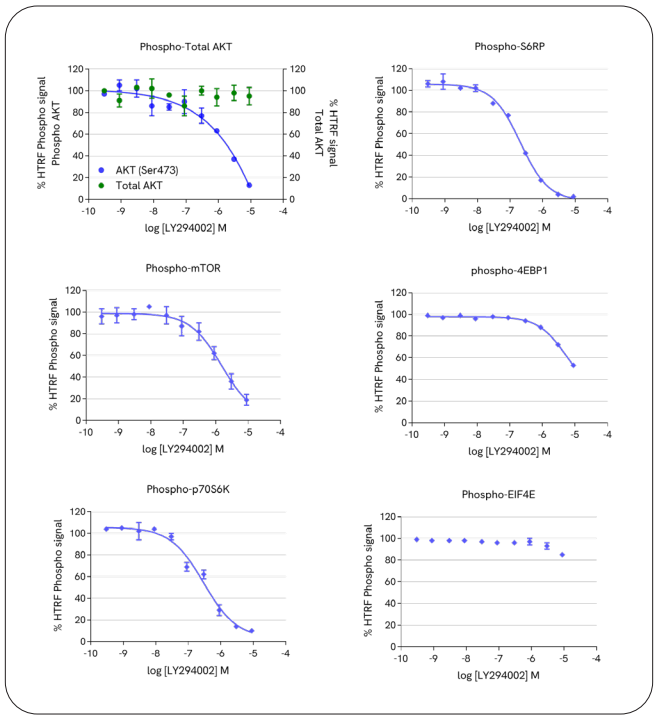


Figure 2: Dose-response analysis of LY294002 on the AKT/mTOR signaling pathway and its downstream effectors.

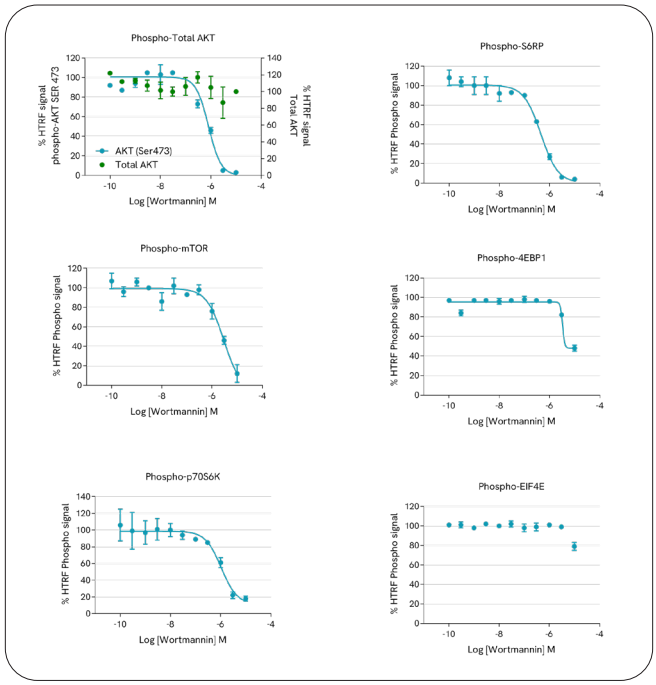


Figure 3: Dose-response analysis of Wortmannin on the AKT/mTOR signaling pathway and its downstream effectors.

Targeting the Mid-Level Pathway: Rapamycin and PP242 Signatures

Rapamycin, an allosteric inhibitor of mTor, prevents its interaction with FKBP-12, thereby specifically inhibiting the activation of the mTor complex 1 (mTorC1). In contrast, PP242, a catalytic inhibitor that competes with ATP at the mTor active site, antagonizes both the mTorC1 and mTorC2 complexes (Ref. 1). As expected, Rapamycin (figure 4) and PP242 (figure 5) strongly inhibited the phosphorylation of mTor, P70S6K, and S6RP, exhibiting similar potencies in the nanomolar range. Despite its inhibitory effect on mTor, Rapamycin did not exhibit any significant inhibition of 4EBP1 phosphorylation or EIF4E phosphorylation.

In contrast to Rapamycin, the active-site inhibitor PP242 effectively inhibits 4EBP1 phosphorylation and demonstrates nearly 40% inhibition of EIF4E phosphorylation (Ref. 3, 4, 5, 10). Since EIF4E phosphorylation is primarily driven by Mnk1 activity via the MAPK pathway, this finding suggests a crosstalk between the PI3K/AKT/mTor and MAPK/ERK/MNK1 pathways.

The most notable difference in the mechanisms of action between Rapamycin and PP242 was observed in AKT phosphorylation levels.

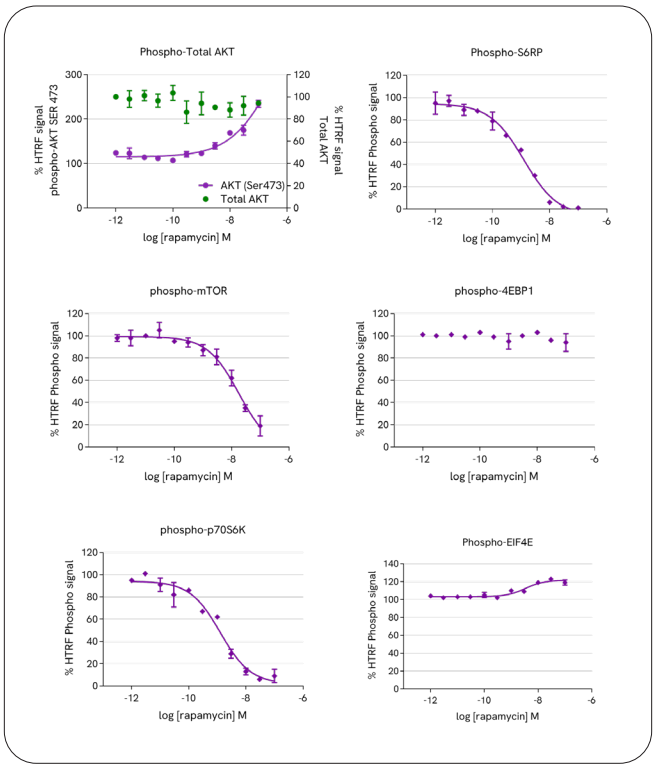


Figure 4: Dose-response analysis of Rapamycin on the AKT/mTOR signaling pathway and its downstream effectors.

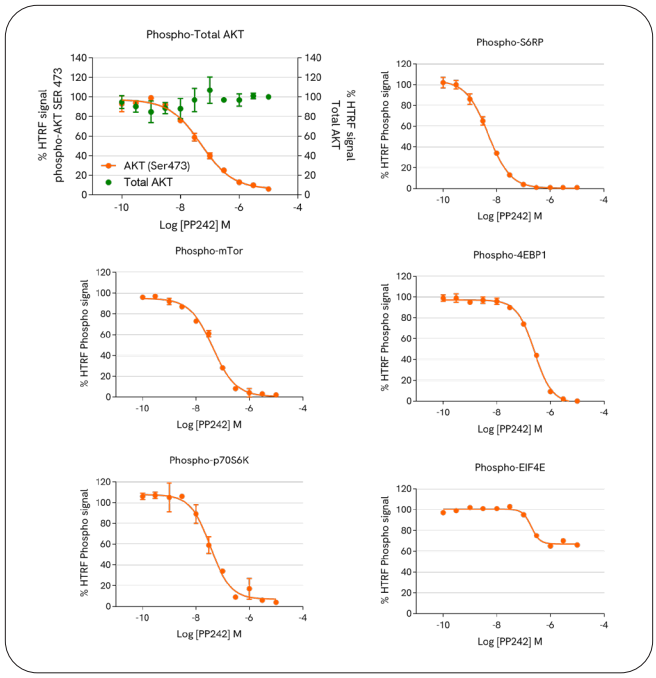


Figure 5: Dose-response analysis of PP242 on the AKT/mTOR signaling pathway and its downstream effectors.

Targeting the basal pathway: PF4708671 and CGP57380 Signatures

PF4708671 has been previously described as an inhibitor of P70S6K activity (Ref. 5). As shown figure 6, our data confirm that PF4708671 effectively inhibits the phosphorylation of both P70S6K and the S6 ribosomal protein. Interestingly, PF4708671 also increases the phosphorylation level of P70S6 Kinase itself. This observation is consistent with reports in the literature (Ref. 5), where it has been shown that although P70S6K phosphorylation levels increase, as detected by Western Blot, its activity is fully suppressed by the compound.

No significant effect of PF4708671 was observed on the upstream mTor kinase, as previously reported (Ref. 5). CGP57380 has been shown to target Mnk1, thereby inhibiting EIF4E phosphorylation as well as P70S6K (Ref. 8, 9). Our data confirm the inhibitory activity of CGP57380 on Mnk1, evidenced by a substantial reduction in EIF4E phosphorylation, along with significant inhibition of P70S6K activity, resulting in decreased phosphorylation of the S6 ribosomal protein (figure 7).

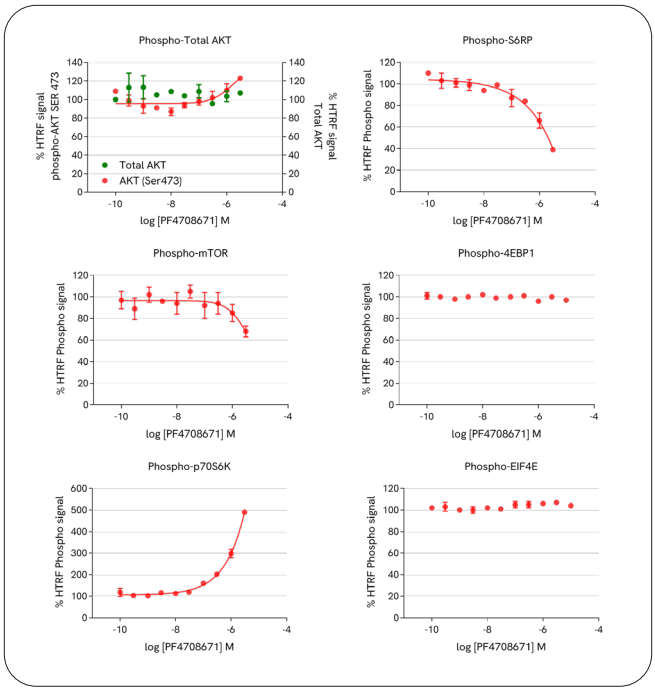


Figure 6: Dose-response analysis of PF4708671 on the AKT/mTOR signaling pathway and its downstream effectors.

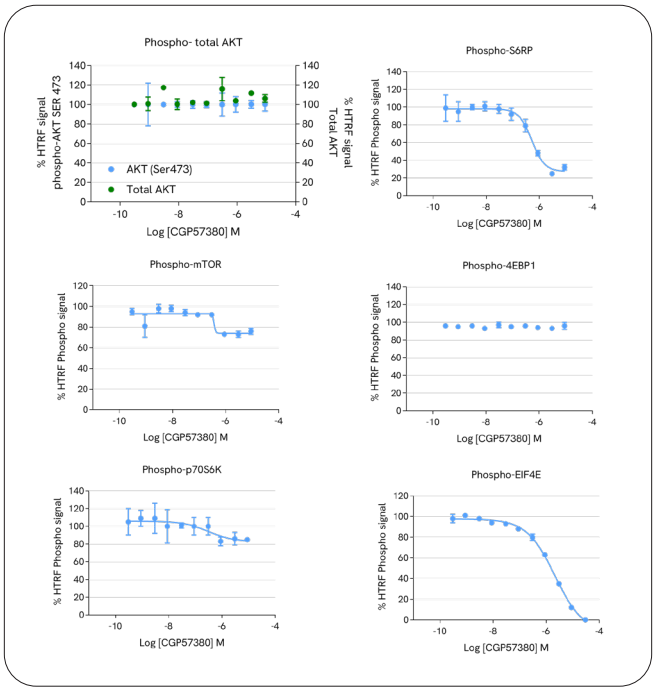


Figure 7: Dose-response analysis of CGP57380 on the AKT/mTOR signaling pathway and its downstream effectors.

Building a signature table

Compounds can be effectively classified based on their inhibition profiles using an IC50 Heat Map. As shown in the table below, LY294002, Wortmannin, and PP242 display identical inhibition patterns, while Rapamycin and PF4708671 exhibit similar behaviors, alongside the Mnk1 inhibitor CGP57380. The differences in IC50 values (nM) between these compounds allow for clear potency discrimination, helping to refine compound classification and optimize pathway targeting.

Inhibitor	LY294002	Wortmannin	PP242	Rapamycin	PF-4708671	CGP57380
Kinase target / Assay	PI3K		PI3K +mTOR	mTOR	p70S6K	Mnk1
AKT Total						
p-AKT Ser473	2,500	500	50	100	>10,000	
p-AKT Thr308	3,000	500	19	100	>10,000	
p-mTor Ser2448	2,000	2,000	40	18	4,000	
p-P70S6K Thr389	330	1,000	15	1	2,300	
p-S6RP Ser235/236	250	500	5	1	3,000	500
p-4EBP1 Thr37/46	~15,000	~10,000	250			
p-EIF4E Ser209			190			2,000
Reference IC50 values (nM)*	500 - 6,000	2 - 400	8-200	0.1-10	200	2,000

partial inhibition | activation
full inhibition | no effect

Conclusion

Smart tools that facilitate pathway profiling and target deconvolution are essential for success in both phenotypic and target-based drug discovery approaches. This study demonstrates how easily the complex PI3K/AKT/mTor translational control pathway can be analyzed using HTRF cellular phospho-assays, breaking it down into individual, measurable steps. The results closely align with the known pharmacology of the reference inhibitors tested. This approach can be applied to a wide range of other pathways and cell types, leveraging Revvity's extensive portfolio of cellular HTRF phospho-assays and custom assay development services.

Experimental procedure

HEK293 cells were plated (60,000 / well) into 96-well plates in 50 µL of cell culture medium and incubated at 37°C under 5% CO2. Following an overnight incubation, cells were treated with 50 µL of increasing concentrations of each compound previously mentioned, ensuring that the final DMSO concentration per well did not exceed 1%. After a 3-hour incubation at 37°C, the cell culture medium was harvested, and 60 µL of complete lysis buffer was added to each well for 30 minutes. Subsequently, 8 or 16 µL of the lysates were transferred to 384 small-volume plates, with an additional fill-in step to 16 µL as needed. Finally, 4 µL of HTRF reagents were added to each well. The phosphorylation levels of AKT, as well as total protein levels, were monitored. The phosphorylation status of mTor, P70S6K, and S6 ribosomal protein, along with 4EBP1 and EIF4E, were also analyzed.

Revvity's HTRF phospho or total protein detection kits:

HTRF AKT phospho-S473 detection kit #64AKSPEG,
HTRF AKT total detection kit #64NKTPEG,
HTRF 4EBP1 phospho-T37/46 detection kit #64EBP1PEG,
HTRF EIF4E phospho-S209 detection kit #64EF4PEG,
HTRF mTor phospho-S2448 detection kit #64TORPEG,
HTRF P70S6K phospho-T389 detection kit # 64S6KPEG,
HTRF S6RP phospho-S235/236 detection kit # 64RP6PEG.

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