APPLICATION NOTE

HTRF Technology

This application note demonstrates that HTRF® assays and CyBio liquid handling form a perfect match offering a straightforward and flexible automated assay platform for drug discovery and cell signaling research.

Simplified Pathway Dissection With HTRF® Phospho-Assays And CyBi®-FeliX Liquid Handling

Abstract

Higher throughput paired with reproducible and accurate results have become a "must" in these tough times of budget cuts and the ever-greater need to fight attrition. To meet this challenge, HTRF® cell signaling assay solutions and our partner CyBio instruments from Analytik Jena have combined their expertise to develop a flexible system to run reliable, sensitive and rapid HTRF phospho-protein assays on the CyBi®- FeliX liquid handling system for simplified pathway dissection.

Here, the dissection of the PI3K/AKT/mTor translational control pathway is shown on different vertical signaling levels. Inhibitory effects of several compounds are measured, enabling their precise classification by elaborating a pathway map of their phospho-signatures. This application note demonstrates that HTRF assays and CyBio liquid handling form a perfect match offering a straightforward and flexible automated assay platform for drug discovery and cell signaling research.

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Material and Method

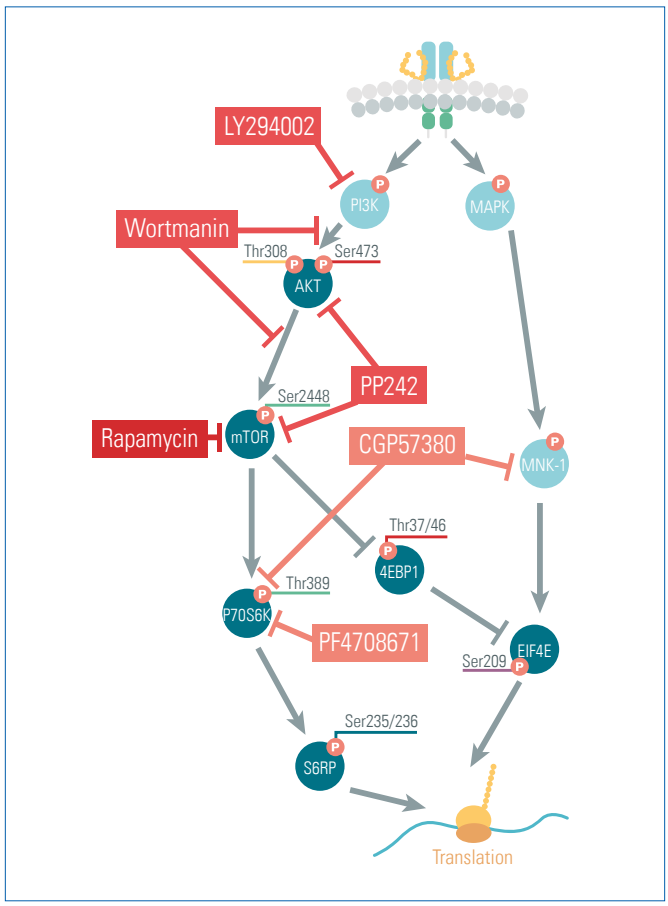

Figure 1: Illustration of the PI3K/AKT/ mTor pathway including reference inhibitors and HTRF phospho-assays used in this application

Biological Background. The phosphatidylinositol 3-kinases (PI3Ks) regulate cellular signal transduction pathways involved in cell growth, proliferation, survival, apoptosis, and adhesion. Leading to the activation of the PI3K/AKT/mTor pathway, the overall network directly participates in the control of protein translation. Dysregulations of these pathways are common in oncogenesis, and are also altered in other metabolic disorders. Several known inhibitors were selected as references to analyse their corresponding phospho-profile in the pathway. LY294002 and Wortmanin inhibit the upstream PI3kinase, while Rapamycin and PP242 inhibit the middle effector mTor. PF4708671 and CGP57380 block the activation of downstream targets such as the ribosomal protein S6 and the initiator of translation EIF4E.

Experimental Procedure. The concept of this approach is illustrated in Fig.2. 60 000 HEK293 cells were dispensed into 96 well plates in 50 µL of cell culture medium, then placed at 37 °C under 5% CO2. After an overnight incubation, cells were treated with 50 µL of increasing concentrations of each previously cited compound, with a final DMSO concentration per well not exceeding 1%. After 3h incubation at 37 °C, cell culture medium was harvested and 60 µL of complete lysis buffer was added to each well for 30 minutes. Then 8 or 16 µL of lysates were transferred to the 384 small volume plates, with an extra fill-in step up to 16 µL when needed. Finally 4 µL of HTRF reagents were dispensed. For each compound, the phosphorylation level of AKT on Ser473 and Thr 308, and the total protein level, were monitored. The phosphorylation status of mTor, P70S6K and S6-ribosomal protein were also analyzed as well as 4EBP1 and EIF4E.

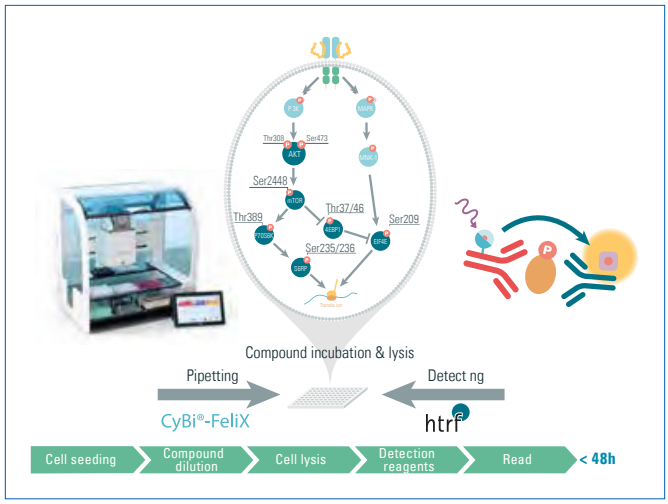

Figure 2: Concept for simplified pathway dissection by combining HTRF cellular phospho-assays and the Cybi-FeliX

HTRF Assays: All phospho-assays used are supplied by PerkinElmer: AKT phospho-S473 kit #64AKSPEG, AKT phospho-T308 kit #64AKTPEG, AKT total kit #64NKTPEG, 4EBP1 phosho-T37/46 kit #64EBP1PEG, EIF4E phospho-S209 kit #64EF4PEG, mTor phospho-S2448 kit #64TORPEG, P70S6K phospho-T389 kit # 64S6KPEG, S6RP phospho-S235/236 kit # 64RP6PEG. For assay principle and detailed protocol please refer to *www.cisbio.com*

CyBi FeliX Pipettor: All HTRF- cellular phospho-assay specific liquid handling tasks were performed under full automation with the CyBi-FeliX Head R 96/60 µL (Fig.3). These include serial dilutions with an 8-channel adapter, 96-channel compound dilution, 96-channel buffer and cell lysate transfers in 96- and 384-well plates, as well as transfers of different kinase reagents into the specified rows of a 384-well assay plate with a 12-channel adapter. Except for the cell seeding step, all further steps were accomplished by a specifically optimized CyBi- FeliX setting dedicated to the HTRF phospho-assay protocol. The pipetting methods were set up in CyBio Composer and can be conveniently controlled via an application specific user interface. For more information please refer to *www.cybio-ag.com*

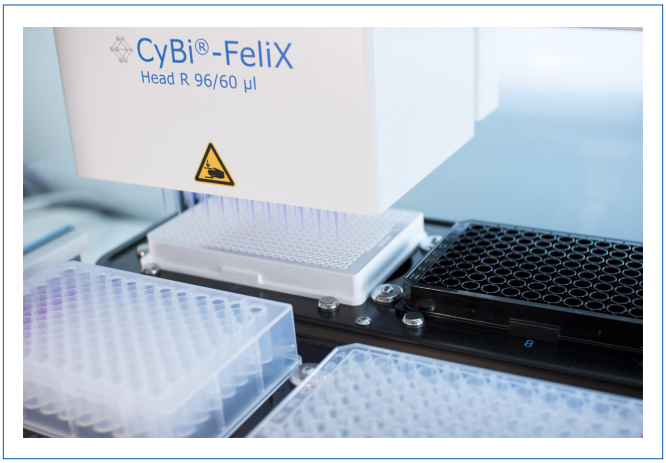

Figure 3: CyBi-FeliX Head R 96/60 µL

Results

Targeting the Upper Pathway Level: Signatures of LY294002 and Wortmanin

LY294002 and Wortmanin have been extensively used in pharmacological studies to elucidate the physiological function of PI3-Kinase (Ref.11) as a representative class of inhibitors, targeting the upstream PI3-kinase. After cell lysis and transfer of the lysates, 8 downstream phospho- and total protein readouts of increasing compound concentrations were analysed. LY294002 and Wortmanin strongly inhibit the phosphorylation of AKT on both residues S473 and T308, whereas the expression level of AKT remains stable as reflected by the HTRF total AKT signal (Fig.4-a). Inhibition of AKT phosphorylation was achieved by up to 80% and consequently the phosphorylation of its downstream targets, mTor (Fig.4-b), P70S6K (Fig.4-c) and S6RP (Fig.4-d), were also strongly reduced, with from 80 up to 95% of inhibition.

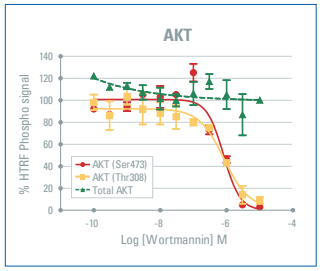

Figure 4-a: Wortmanin effect on AKT

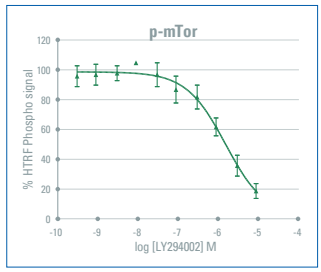

Figure 4-b: LY294002 effect on phospho-mTOR

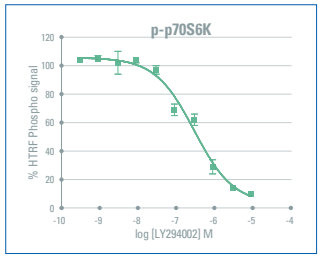

Figure 4-c: LY294002 effect on phospho-P70S6K

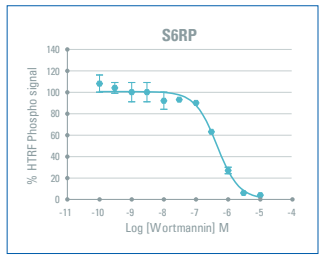

Figure 4-d: Wortmanin effect on phospho-S6RP

The inhibitors were found less potent when measuring the effect on 4EBP1 (Fig.4-e) and EIF4E (Fig.4-f) phosphorylation (Ref.6, 7).

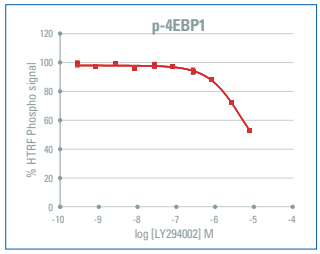

Figure 4-e: LY294002 effect on phospho-4EBP1

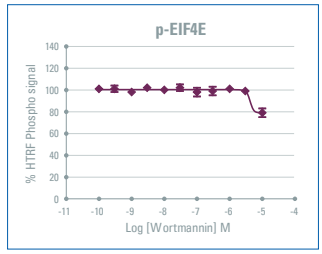

Figure 4-f: Wortmanin effect on phospho-EIF4E

These results obtained here are in agreement with the literature stating 4EBP1 as one of the mTor substrates, whereas EIF4E is rather associated with the MAPK-MNK1 pathway.

Targeting the Medium Pathway Level: Signatures of Rapamycin and PP242

Rapamycin, an allosteric inhibitor of mTor, prevents its interaction with FKBP-12 and thus specifically inhibits the activation of the mTor complex 1 (mTorC1). PP242 which is an active-site inhibitor competing with ATP binding in the ATP pocket of the mTor catalytic site antagonizes the mTorC1 and mTorC2 complexes (Ref.1). As expected, Rapamycin and PP242 strongly inhibit the phosphorylation of mTor (Fig.5-a), P70S6K (Fig.5-b) and S6RP (Fig.5-c) with similar potencies in the nM range.

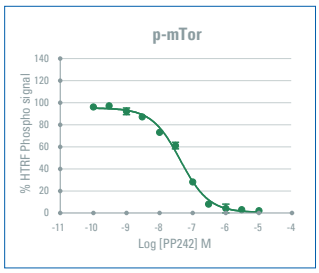

Figure 5-a: PP242 effect on phospho-mTor

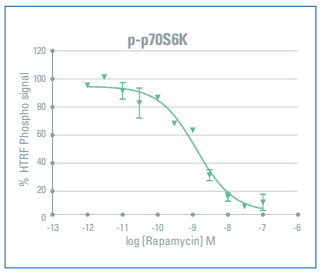

Figure 5-b: Rapamycin effect on phospho-P70S6K

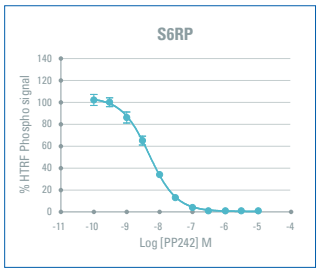

Figure 5-c: PP242 effect on phospho-mTor

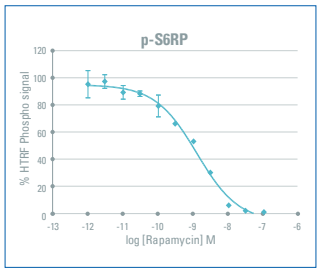

Figure 5-c: Rapamycin effect on phospho-mTorP

Despite its inhibitory activity on mTor, Rapamycin did not reveal any inhibitory behaviour either on 4EBP1 (Fig.5-d) phosphorylation, or on EIF4E (Fig.5-e).

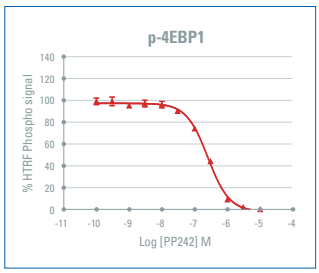

Figure 5-d: PP242 effect on phospho-4EBP1

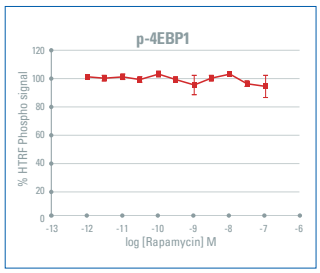

Figure 5-d: Rapamycin effect on phospho-4EBP1

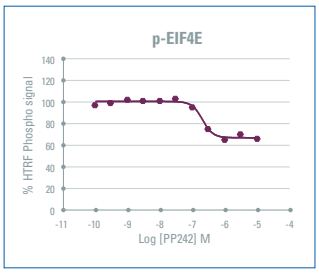

Figure 5-d: PP242 effect on phospho-EIF4E

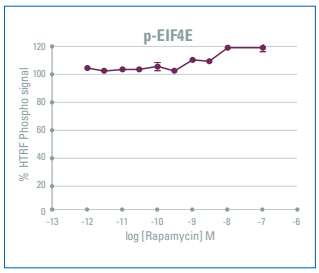

Figure 5-d: Rapamycin effect on phospho-EIF4E

Unlike Rapamycin, the active site inhibitor PP242 efficiently inhibits 4EBP1 phosphorylation and showed almost 40% inhibition of EIF4E phosphorylation (Ref.3, 4, 5, 10). Since EIF4E phosphorylation seems mainly due to Mnk1 activity driven by the MAPK pathway, this result suggests a crosstalk between the pathways , i.e. the PI3K/AKT/mTor and MAPK/ERK/MNK1. The most significant difference in the mechanism of action between Rapamycin and PP242 was revealed on the AKT (Fig.5-f) phosphorylation level.

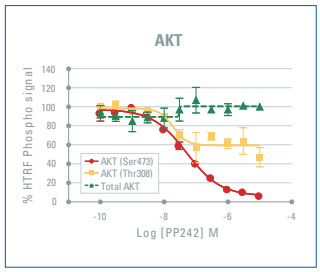

Figure 5-f: PP42 effect on AKT

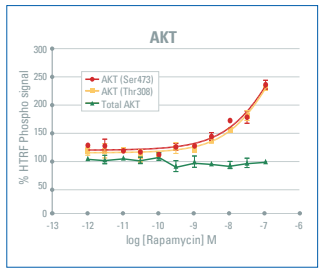

Figure 5-f: Rapamycin effect on AKT

Rapamycin strongly increases AKT phosphorylation at Ser473, while PP242 completely abrogates AKT phosphorylation at this site. This result is consistent with the notion that mTorC2 is the major kinase phosphorylating AKT at Ser473. Such an increase of AKT phosphorylation at Ser473 by rapalogs is well known in various cell types, and has been assigned to the existence of negative feedback loops in the mTorC1/S6K axis, that attenuate cell signaling. Suppression of these negative feedback loops by mTo inhibitors would lead to over-activation of upstream pathways, such as AKT or ERK, and thus could counterbalance the anti-proliferative effects of mTor inhibitors. (Ref.3, 4)

Targeting the Ground Pathway Level: Signatures Of PF4708671 and CGP57380

PF4708671 has previously been described as inhibiting the activity of P70S6K (Ref.5). The data shown here confirm the inhibition effect of PF4708671 on the phosphorylation of P70S6K and S6 ribosomal protein (Fig.6-a). Concomitantly the phosphorylation of P70S6 Kinase (Fig.6-b) itself is increased by PF4708671. This effect is confirmed by the literature (Ref.5), where it has been shown that despite an increase of the P70S6K phosphorylation level, as monitored by Western Blot, its activity was abrogated by the compound.

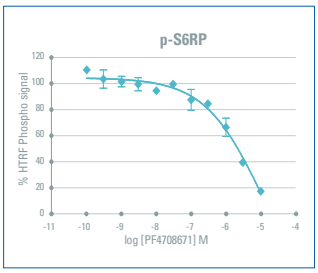

Figure 6-a: PF4708671 effect on phospho-S6RP

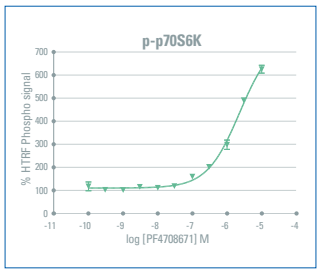

Figure 6-b: PF4708671 effect on phospho-P70S6K

No significant effect of PF480671 was observed on the upstream mTor kinase (Fig.6-c), as also previously reported (Ref.5). CGP57380 was shown to target Mnk1 and thus inhibits EIF4E (Fig.6-d) phosphorylation, but also P70S6K (Ref.8, 9).Our data support both the inhibitory activity of CGP57380 on Mnk1 by a strong diminution of EIF4E phosphorylation, and a significant inhibition of P70S6K activity leading to a reduction of the S6 ribosomal protein phosphorylation.

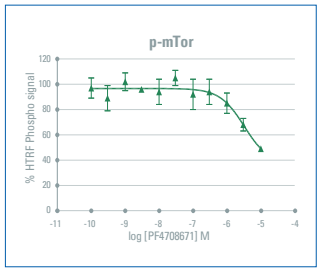

Figure 6-c: PF480671 effect on phospho-mTor

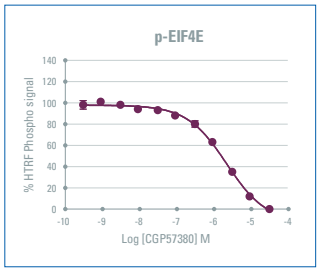

Figure 6-d: CGP57380 effect on phospho-EIF4E

Building a Signature's Table

Compounds can easily be classified in an IC50 Heat Map of signatures with respect to their inhibition profiles.
As indicated in the table below, LY294002, Wortmannin and PP242 lead to an identical inhibition pattern, whereas Rapamycin and PF4708671 showed close behaviors, as well as the Mnk1 inhibitor, CGP57380. Differences of IC50 values between compounds enable potency discrimination.

Inhibitor	LY 294002	Wort mannin	PP242	Rapa mycin	PF-47 08671	CGP 57380
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 the key to success in phenotypic and target-based approaches in drug discovery. This study shows how easy it is to analyze the complex PI3K/AKT/mTor translational control pathway dissected into individual measurable steps with HTRF cellular phospho-assays implemented on the compact and flexible CyBi FeliX pipettor. The results obtained correlate well with the known pharmacology of the reference inhibitors tested. The approach can be extended to many other pathways and other cell types with the broad assay portfolio of cellular HTRF phospho-assays and our custom assay development services.

References

1. Don B *et al.* Rapamycin passes the torch: a new generation of mTOR inhibitors. Nature Reviews Drug Discovery, 2011, 10, 868-80
2. Sun *et al.* Activation of Akt and eIF4E survival pathways by Rapamycin-mediated mammalian Target of Rapamycin Inhibition. Cancer Res, 2005, 65 (16), 7052-8
3. Soares *et al.* Different patterns of AKT and ERK feedback activation in response to rapamycin, active-site mTOR Inhibitors and Metformin in Pancreatic Cancer Cells. PLOS one 2013
4. O'Reilly *et al.* mTOR Inhibition Induces Upstream Receptor Tyrosine Kinase Signaling and Activates Akt.- Cancer Res, 2006, 66(3):1500-8.
5. Pearce *et al.* Characterization of PF-4708671, a novel and highly specific inhibitor of p70 robosomal S6 Kinase. Biochem J, 2010,431(2):245-55
6. J. Werzowa *et al.* Suppression of mTOR complex 2-dependent AKT phosphorylation in melanoma cells by combined treatment with rapamycin and LY294002. British Journal of Dermatology, 2009, 160(5), 955-64
7. Trivigno *et al.* Regulation of protein translation initiation in response to ionizing radiation. Radiation Oncology, 2013,8-35
8. Altman *et al.* Negative Regulatory Effects of Mnk Kinases in the Generation of Chemotherapy-Induced Antileukemic Responses. Mol Pharmacol, 2010, 78(4),778-84.
9. Zhang *et al.* Inhibition of polysome assembly enhances Imatinib Activity against Chronic Myelogenous Leukemia and Overcomes Imatinib Resistance. Mol Cell Biol, 2008,28(20), 6496-509.
10. Feldman *et al.* Active-Site Inhibitors of mTOR Target Rapamycin-Resistant Outputs of mTORC1 and mTORC2. PLOS one 2009
11. Workman *et al.* Drugging the PI3 Kinome: From Chemical Tools to Drugs in the Clinic Review, Canc Research, 2010,70(6),2146-57

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