APPLICATION NOTE

Enhance PROTAC drug discovery with a comprehensive no-wash technological platform: a BTK case study.

Authors

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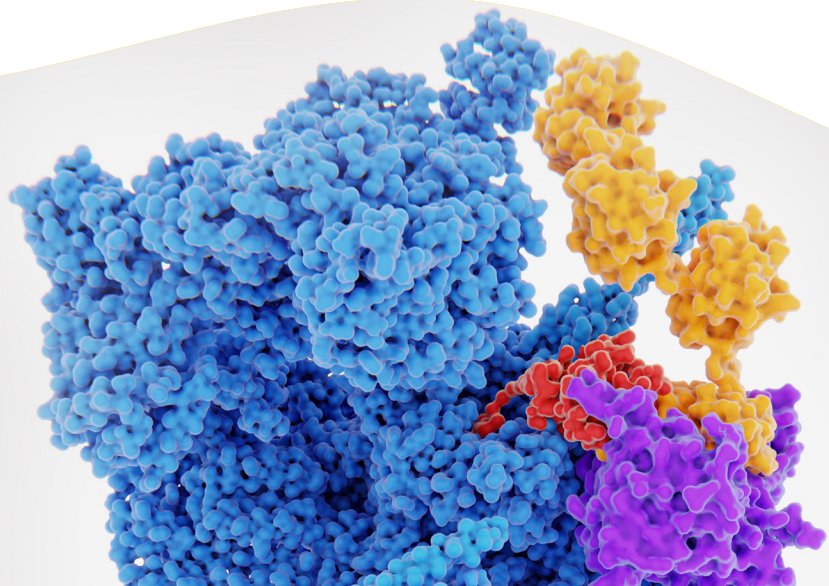
Introduction

Pioneered in the early 2000s by Craig Crews and Ray Deshaies, targeted protein degradation has emerged as a new approach to selectively decrease protein expression levels. Today the pharmaceutical industry is massively investing its drug discovery efforts in this research area referred to as **PROteolysis TArgeting Chimeras** (PROTACs). Unlike conventional small molecules that inhibit protein function, such as kinase inhibitors, PROTACs harness the cell's Ubiquitin Proteasome System (UPS) to destroy undesired proteins. Besides improving potency and efficacy, PROTAC strategy is expected to unlock parts of the proteome that have traditionally been considered "undruggable" (1).

PROTACs are heterobifunctional molecules comprising one ligand referred to as the Warhead that selectively binds to the targeted protein, and a second ligand that binds to an E3 ubiquitin ligase (E3), plus a linker that connect the two ligands. PROTAC acts as a bridge that brings the target of interest into close proximity with an E3 ubiquitin ligase, thereby promoting target ubiquitination and its subsequent proteasomal destruction. Finally, PROTAC molecules can be recycled back and destroy other newly synthesized proteins. This confers PROTACs unique mechanism of action a fundamentally different class of weapon, with novel properties not offered by other drug modalities (2).

This application note showcases how AlphaLISA™ and HTRF™ based assays represent straightforward and reliable methods to identify and study PROTAC compounds.



Here, the use of AlphaLISA and HTRF biochemical and cell- based assays have been exemplified on Bruton Tyrosine Kinase (BTK) as the targeted protein of interest and the MT-802 as the PROTAC compound. As depicted on the scheme, the formation of the binary (steps ②) and ternary (step ③) complexes, as well as cooperativity effects (step ③) were first investigated by biochemical approaches, then cell- based assays were implemented to assess the permeability (step ①) and the induction of BTK proteasomal degradation (step ⑤) of the MT-802 compound.

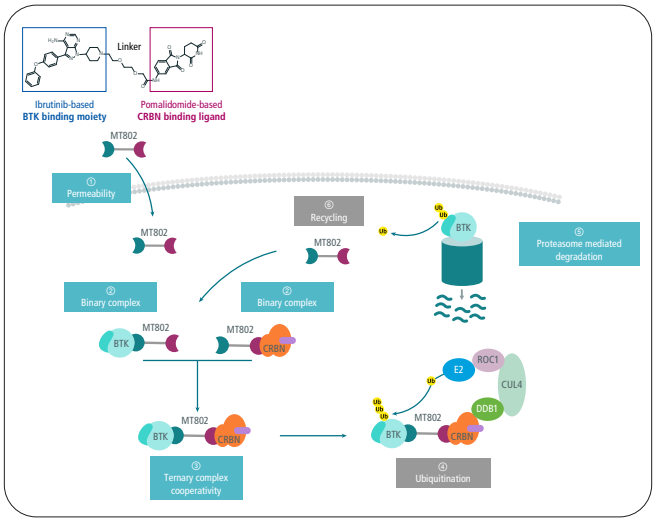


Figure 1: Mechanism of action of PROTAC MT-802.

In vitro investigations with no-wash HTRF and AlphaLISA biochemical assays

The PROTAC MT-802 compound is composed of the well-established BTK inhibitor Ibrutinib as the warhead, and the Pomalidomide Cereblon Ligand (3). First, the binding of MT-802 to BTK on one hand and to Cereblon on the other hand was monitored through biochemical approach. Then, the formation of the BTK:MT-802: Cereblon ternary complex was studied. Finally, the cooperativity effect exerted by BTK on the Cereblon: MT-802 binary complex was assessed.

In vitro experiments were carried out using recombinant tagged BTK and Cereblon proteins, as well as fluorescent probes. The principle and protocol of each assay is represented in the corresponding section below.

Assessment of the binary complexes' formation

BTK: MT-802 complex

Principle and experimental conditions

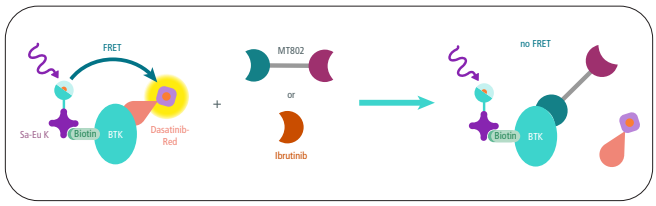


Figure 2: Principle of HTRF kinase binding assay.

A competitive experiment was performed using 5 µL of Biotinylated BTK protein (5 nM), 5 µL of a streptavidin Europium Cryptate (0.5 nM, FRET donor) and 5 µL Dasatinib-Red ligand (1.5 nM, FRET acceptor), plus 5 µL of increasing concentrations of either MT-802 or Ibrutinib, as well as two irrelevant compounds Pomalidomide and dBRD9, which were included as negative controls. All reagents were dispensed in ProxiPlate 384-Plus and incubated 1 h at RT, prior to HTRF signal detection.

In the absence of competitors, a FRET signal occurs between the streptavidin-K and the Dasatinib-Red, while in the presence of competitor, the FRET signal decreases.

Results

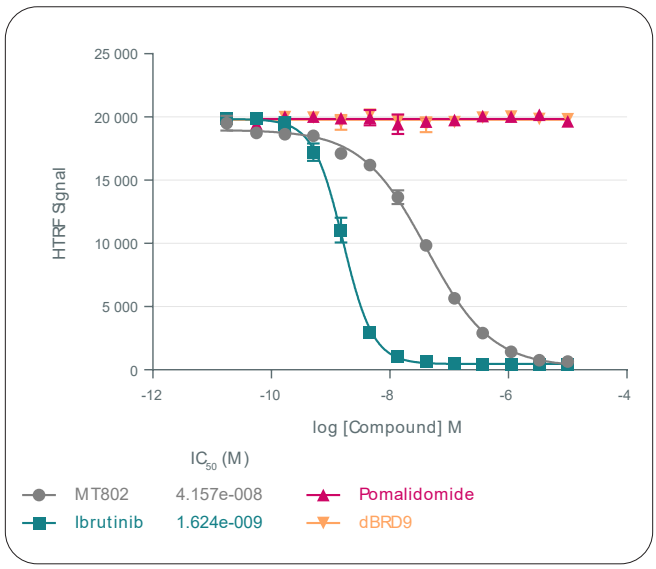


Figure 3: Dose-response inhibition using HTRF Kinase Binding assay. Unlike irrelevant compounds (Pomalidomide and dBrd9) Ibrutinib (blue curve) and MT-802 (Grey curve) efficiently compete with binding of Dasatinib-Red to BTK.

Unlike the two irrelevant compounds Pomalidomide and dBRD9, this result demonstrates that Ibrutinib and MT-802 compete efficiently with the binding of Dasatinib-Red to BTK. Ibrutinib displays a higher affinity for BTK than MT-802, which may result from a sterical hindrance related to MT-802 chemical structure.

Cereblon MT-802 complex

Principle and experimental conditions of HTRF cereblon binding kit

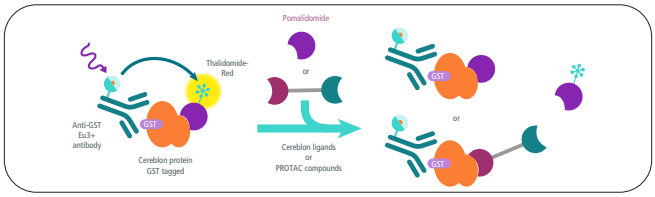


Figure 4: Principle of HTRF cereblon binding kit.

A competitive experiment was performed according to the protocol described (Revvity 64BDCRBNPEG/H). Increasing concentrations of either MT-802 or Pomalidomide, as well as an irrelevant compound VH032 as a negative control. All reagents were dispensed in ProxiPlate 384 and incubated 3 h at RT, prior to HTRF signal detection.

In the absence of competitors, a FRET signal occurs between the anti-GST-K and the Thalidomide-Red, while in the presence of competitor, the FRET signal decreases.

Principle and experimental conditions of AlphaLISA cereblon binding kit

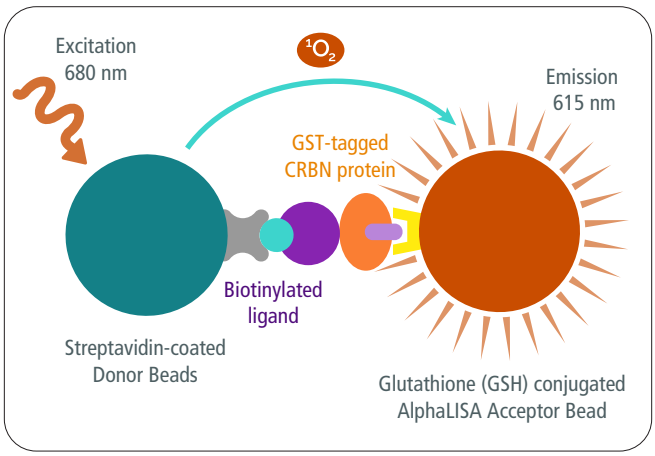


Figure 5: Principle of AlphaLISA cereblon binding kit.

A competitive experiment was performed according to the protocol described (Revvity #AL3147C/F) in the presence of increasing concentrations of MT-802 or Pomalidomide.

All reagents were dispensed in AlphaPlate-384 and incubated 2 h at 23°C in the dark, prior to Alpha signal detection.

In the absence of competitors, Alpha signal occurs between the Streptavidin donor beads and Glutathione AlphaLISA acceptor beads, while in the presence of competitor, the signal decreases.

Results

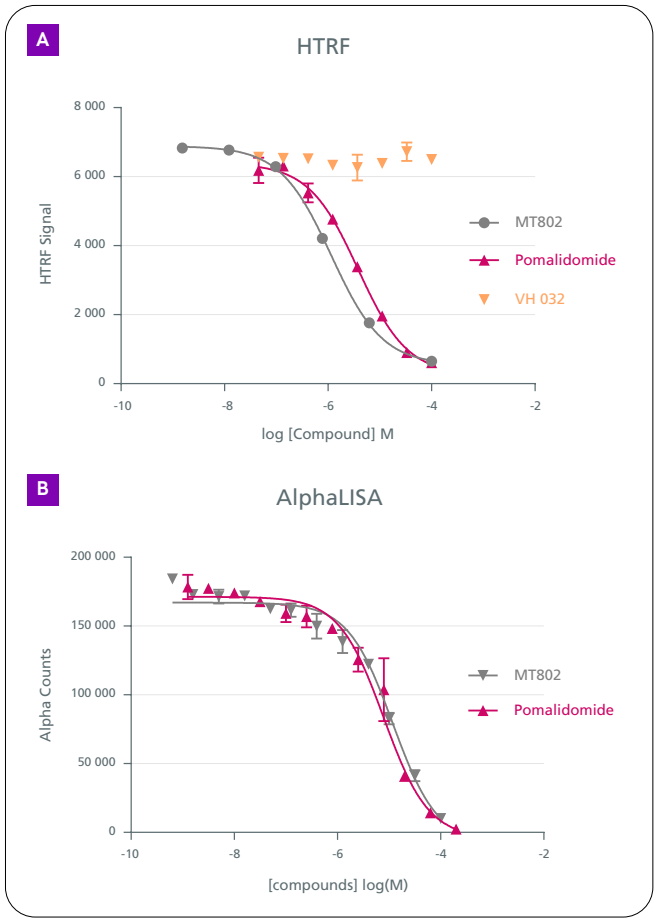


Figure 6: Dose-response inhibition using HTRF and AlphaLISA cereblon binding kit. Unlike the irrelevant compound, VH032, Pomalidomide (Pink curve) and MT-802 (Grey curve) efficiently compete with binding of Thalidomide-Red ligand to Cereblon.

Table 1. AlphaLISA and HTRF IC50 Pomalidomide and MT-802 binding.

IC50	HTRF	AlphaLISA
Pomalidomide	3.8µM	7.5µM
MT802	1.15µM	11µM

Both HTRF and AlphaLISA Cereblon assays nicely evidence Pomalidomide and MT-802 binding to Cereblon with IC50 being in comparable range, as reported in Table 1.

Assessment of the ternary complexes' formation

BTK: MT-802: cereblon complex

Principle and experimental conditions

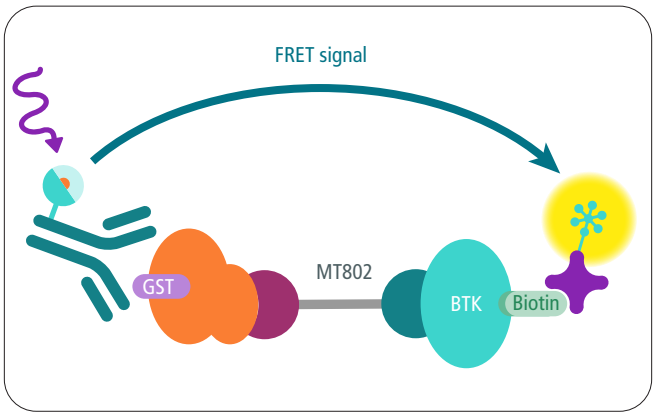


Figure 7: Principle of ternary complex formation assessed by HTRF.

The experiment was carried out using 5µL of GST tagged Cereblon protein (10nM), 5µL of Biotinylated BTK protein (4nM) and 5µL of increasing concentrations of compounds, followed by 5µL of a mix anti GST-Eu cryptate (0.5nM) plus Streptavidin-XL665 (4nM). All reagents were dispensed in ProxiPlate 384-Plus (6008280) and incubated ON at RT, prior to HTRF signal detection.

Results

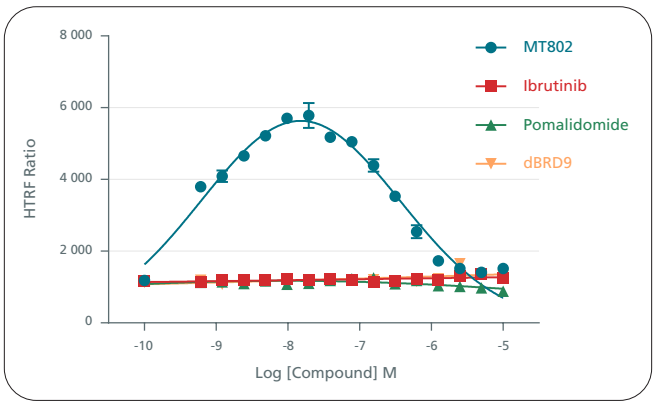


Figure 8: Compound induced ternary complex formation using HTRF. MT-802 compound (blue curve) efficiently promotes the formation of ternary complex only.

Increasing concentrations of MT-802 result in an expected bell-shape curve where a hook effect is obtained at higher concentrations. Thus, this result demonstrates the ability of MT-802 to induce the formation of the ternary complex selectively, bridging the targeted BTK protein and the E3 ligase Cereblon.

Assessment of cooperativity

Principle and experimental conditions

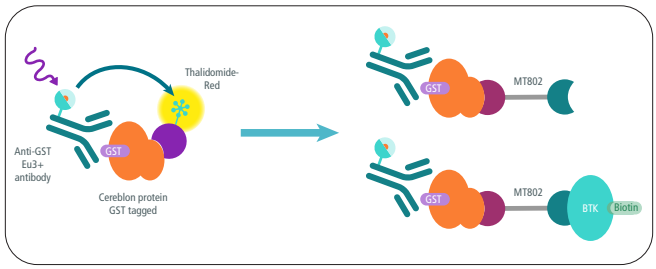


Figure 9: Principle of cooperativity assessed by HTRF.

This experiment was carried out using the protocol described (Revvity 64BDCRBNPEG/H) to prepare the GST-CRBN protein. HTRF Thalidomide-Red ligand (Revvity 64BDCRBNRED) and mAb anti-GST Eu cryptate Kinase Binding (Revvity 62KBGSTKAF) working solutions were prepared by diluting 50-fold the stock solutions in PROTAC binding buffer 1 (Revvity 64BDE31RDF). A range of concentrations of MT802 (from 10 µM to 0.6 nM) were prepared in Diluent 9 (Revvity 62DL9DDC) and increasing concentrations of biotin-BTK protein (100 nM, 1 µM, 2 µM) were prepared in PROTAC binding buffer. 5 µL of buffer or MT802, 5 µL of buffer or biotin-BTK protein, 5 µL of GST-CRBN protein and 5 µL of a mix of Thalidomide-Red plus anti GST-Eu cryptate were dispensed in ProxiPlate 384-Plus and incubated for 3 h at RT, prior to HTRF signal detection.

Results

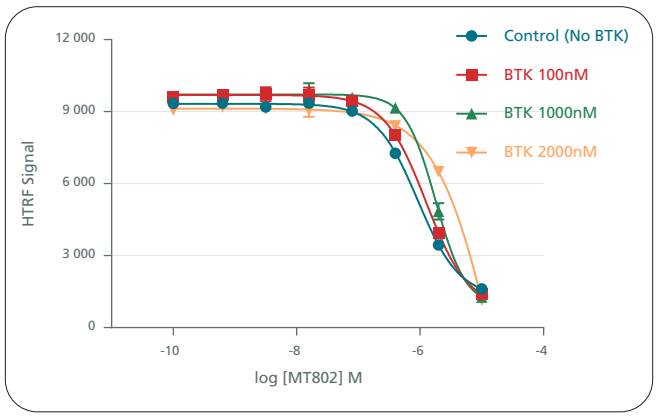


Figure 10: Cooperativity effect of BTK on MT-802 PROTAC compound binding. Increasing concentrations of BTK induce a right shifted IC50 of MT-802.

Cheng & prusoff equation

$$K_i = \frac{IC_{50}}{\left(1 + \left(\frac{\text{Thalidomide - Red}}{K_d}\right)\right)}$$

$$\alpha = \frac{\text{Ki binary complex}}{\text{Ki ternary complex}}$$

Table 2. IC50, Ki MT802 and αfactor under 100 nM, 1 µM and 2 µM of BTK.

	Control (BTK 0 nM)	BTK 100 nM	BTK 1 µM	BTK 2 µM
IC50	919 nM	1241 nM	1778 nM	19030 nM
Ki MT802	352 nM	447 nM	662 nM	7702 nM
α Factor	1	0.74	0.52	0.05

Increasing concentrations of BTK did not change the Kd of Thalidomide-Red on GST-CRBN (data not shown) but reduced the binding affinity of MT-802 to Cereblon as indicated by the calculated Ki established from the Cheng and Prusoff equation. Consequently, the cooperativity factor (α factor) ranges from 0.74 to 0.05 depending on the concentrations of BTK protein. This result demonstrates the negative cooperativity effect of BTK on the MT-802-Cereblon interaction, as reported by Zorba *et al* (3).

In cellulo investigations with no-wash HTRF and AlphaLISA *SureFire Ultra* cell-based assays

HTRF and AlphaLISA Biochemical assays demonstrated the efficacy of MT-802 PROTAC compound to 1) bind to BTK, 2) bind to Cereblon, 3) induce the ternary complex formation. Moreover, cooperativity experiment showed that the α factor presence of BTK reduces the α factor affinity of MT-802 to Cereblon.

Further investigations were performed to address MT-802 efficacy to induce BTK degradation in a cellular environment. To this end, experiments were carried out using Ramos cell line expressing endogenous BTK and Cereblon proteins. The expression level of BTK was determined either with HTRF total BTK assay or AlphaLISA *SureFire Ultra* total BTK assay.

Assessment of MT-802 induced BTK degradation in cells

Principle and experimental conditions

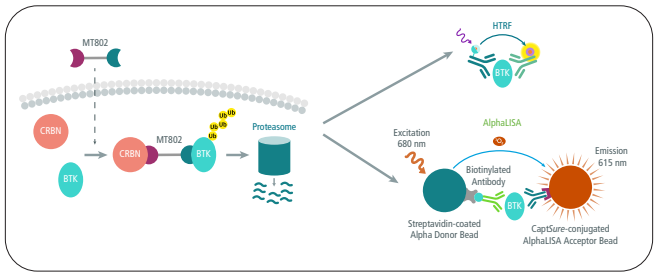


Figure 11: Principle of HTRF and AlphaLISA Total BTK cell-based assays.

HTRF total BTK assay

20 µL of Ramos cells (100,000 cells/well) were seeded in 96 well half area microplate and incubated for 1 h at 37°C - 5 % CO_2. Then cells were treated or not with 5 µL epoxomicin (1 µM - 1h), then 5 µL of MT-802, Ibrutinib or Pomalidomide (0.01 µM - 0.1 µM -1 µM) for 4 h or 24h. Finally, cells were lysed with 10 µL of lysis buffer #4 + blocking reagent 4X for 30 min at RT and 16 µL of lysates were transferred in ProxiPlate-384 Plus white microplates prior to the addition of 4 µL HTRF detection reagents. HTRF signal was recorded after ON incubation at RT.

AlphaLISA *SureFire Ultra* total BTK assay

40 µL of Ramos cells (100,000 cells/well) were seeded in 96 well microplate and incubated for 1h at 37°C - 5 % CO_2. Then cells were treated or not with 20 µL epoxomicin (1 µM - 1 h), then 20 µL of MT-802, Ibrutinib or Pomalidomide (0.01 µM - 0.1 µM -1 µM) for 4 h or 24 h. Finally, cells were lysed with 20 µL Alpha *SureFire Ultra* Lysis Buffer 5X for 10 min at RT and 10 µL of lysates were transferred in AlphaPlate-384 light gray microplates prior to the addition of 5 µL AlphaLISA Acceptor beads (1 h at RT) then 5µL AlphaLISA donor beads. Alpha signal was recorded after ON incubation at RT.

Results

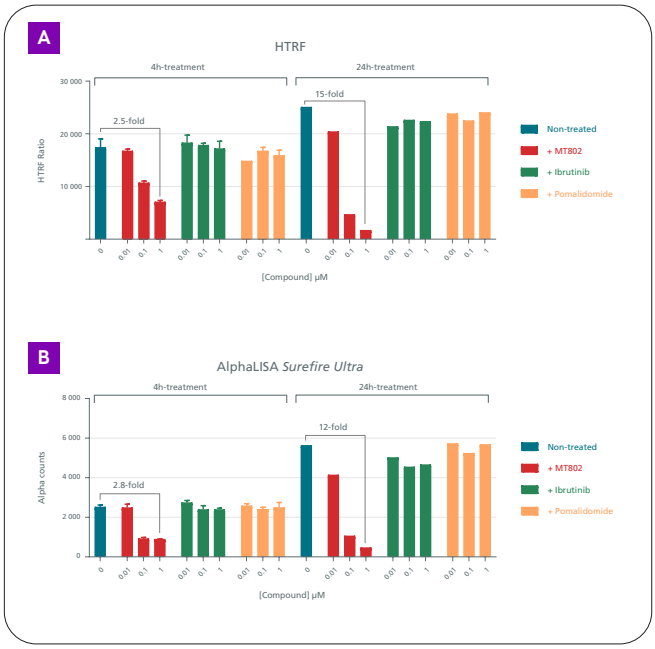


Figure 12: BTK expression level monitored with HTRF and AlphaLISA *SureFire Ultra* kits. Cellular lysates from Ramos cells were treated with different concentrations of MT-802, Ibrutinib or Pomalidomide for 4 or 24 h than BTK were quantified with the indicated assay.

As evidenced on the 2 graphs, MT-802 treatment results in both HTRF and Alpha signal decrease which is more pronounced at 1 µM and 24 h incubation. As expected, no HTRF nor Alpha signal modulation is observed with the two individual moieties, Ibrutinib and Pomalidomide.

This result indicates that MT-802 induced BTK down regulation can be reliably monitored with both technologies (4).

Mechanism of action of MT-802 induced BTK degradation

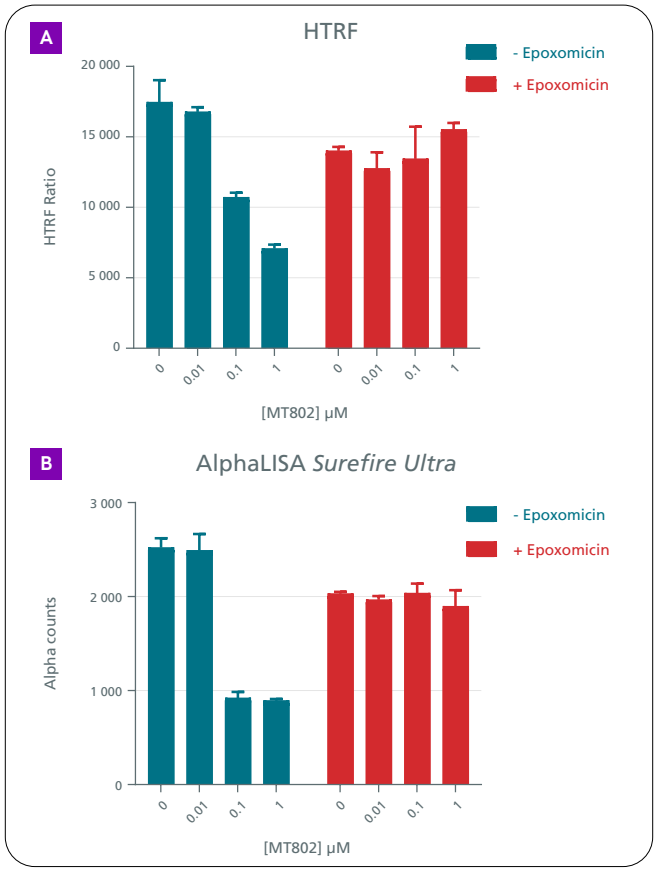


Figure 13: Effect of Epoxomycin mediated proteasome inhibition on BTK modulation upon PROTAC MT-802 treatment. BTK expression level was monitored with HTRF and AlphaLISA *Surefire Ultra* kits. Cellular lysates from Ramos cells were treated with different concentrations of MT-802, in the absence or presence of epoxomycin for 4 h, then BTK were quantified with the indicated assay.

Conclusion

In this application note, consistent results are provided with both HTRF and AlphaLISA technologies in biochemical and cell-based context. Indeed MT-802 PROTAC in binary and ternary complex with BTK and Cereblon can be easily monitored and pharmacological parameters defined. Additionally, cooperativity mechanisms can be studied straightforwardly, and alpha factor accurately determined. HTRF and AlphaLISA *SureFire Ultra* total kits enable the level of endogenous protein expressed in cells to be monitored, can interrogate PROTAC compound' permeability and unravel PROTAC mechanism of action in a very simple way.

Altogether combined HTRF and AlphaLISA technologies offer a versatile and straightforward platform ideally suited for PROTAC drug discovery.

References

1. Kathleen M. Sakamoto, Kyung B. Kim, Akiko Kumagai, Frank Mercurio, Craig M. Crews, and Raymond J. Deshaies PNAS, 98 (15) 8554-8559 (2001)
2. Hijacking protein degradation. Nat Chem Biol 16, 1151 (2020).
3. Adelajda Zorba, Chuong Nguyen, Yingrong Xu, Jeremy Starr, ***et al.*** PNAS, 115(31) (2018)
4. Alexandru D Buhimschi, Haley A Armstrong, Momar Toure, Saul Jaime-Figueroa, ***et al.*** Biochemistry, 57(26) (2018)

Materials and Reagents

	Reagent	Supplier	Part number
Recombinant proteins	GST-tagged BTK	Carna Biosciences	08-180
	Biotinylated BTK	Carna Biosciences	08-480-23N
Microplates	ProxiPlate-384 Plus, White 384-shallow well Microplate	Revvity	6008280/9
	AlphaPlate-384, Shallow well light gray	Revvity	6008350/9
Buffer	HTRF PROTAC Binding Buffer 1	Revvity	64BDE31RDF
	Diluent 9 (5X)	Revvity	62DL9DDC
HTRF detection reagents	Dasatinib-Red	Revvity	62KB02REDC
	Thalidomide-Red	Revvity	64BDCRBNRED
	MAb Anti GST-Eu cryptate Kinase Binding	Revvity	62KBGSTKAF
	Streptavidin-Eu cryptate Kinase Binding	Revvity	62KBSAKAF
	MAb Anti GST-Eu Cryptate (PPI)	Revvity	61GSTKLA
	Streptavidin-XL665 (PPI)	Revvity	610SAXLF
Alpha Beads	AlphaLISA Glutathione Acceptor beads	Revvity	#AL109C/M/R
	Alpha Streptavidin Donor beads	Revvity	#6760002/2S/2B
HTRF kits	HTRF Cereblon binding kit	Revvity	64BDCRBNPEG/H
	HTRF Total BTK kit	Revvity	63ADK064PEG/H
AlphaLISA kits	AlphaLISA Cereblon binding kit	Revvity	AL3147C/F
	AlphaLISA™ *SureFire® Ultra™* total BTK kit	Revvity	ALSU-TBTK-A500
Compounds	MT-802	Revvity	Inhouse synthesis
	Ibrutinib	Selleckchem	S2680
	Pomalidomide	Tocris	6302
	dBRD9	Tocris	6606
	VH 032	Tocris	6462
	Epoxomicin	Sigma	E3652
Cell lines	Ramos (human B-lymphoma cell line)	ATCC	CRL-1596

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