APPLICATION NOTE

Biochemical and cell-based no-wash platform to uncover the full potential of PROTAC®

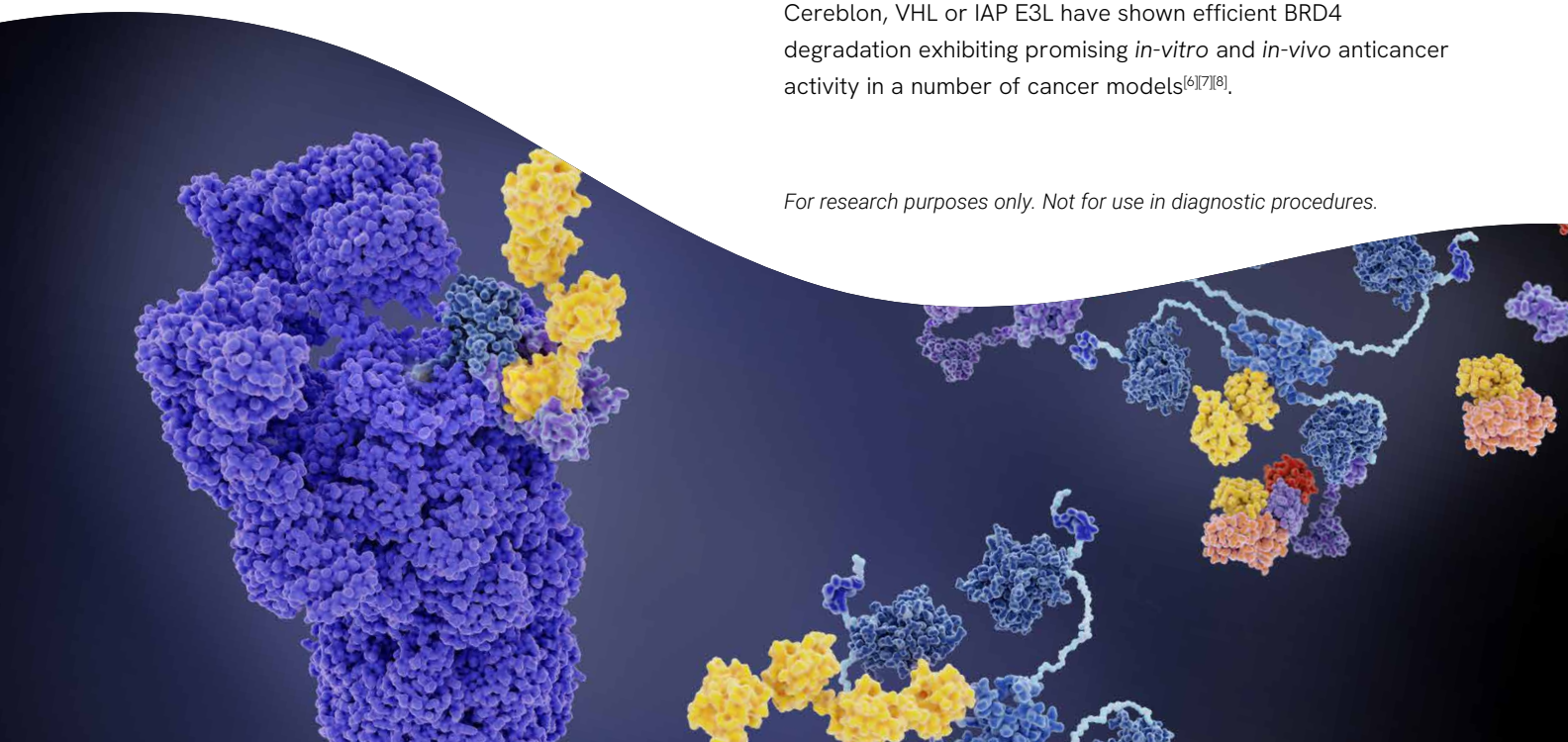
This application note demonstrates through BRD4 case study, PROTAC®s in depth characterization. Potency, selectivity, profiling, mechanism of action and related potential side effects can be deciphered thanks to the combination of Revvity no-wash assay solutions.

Introduction

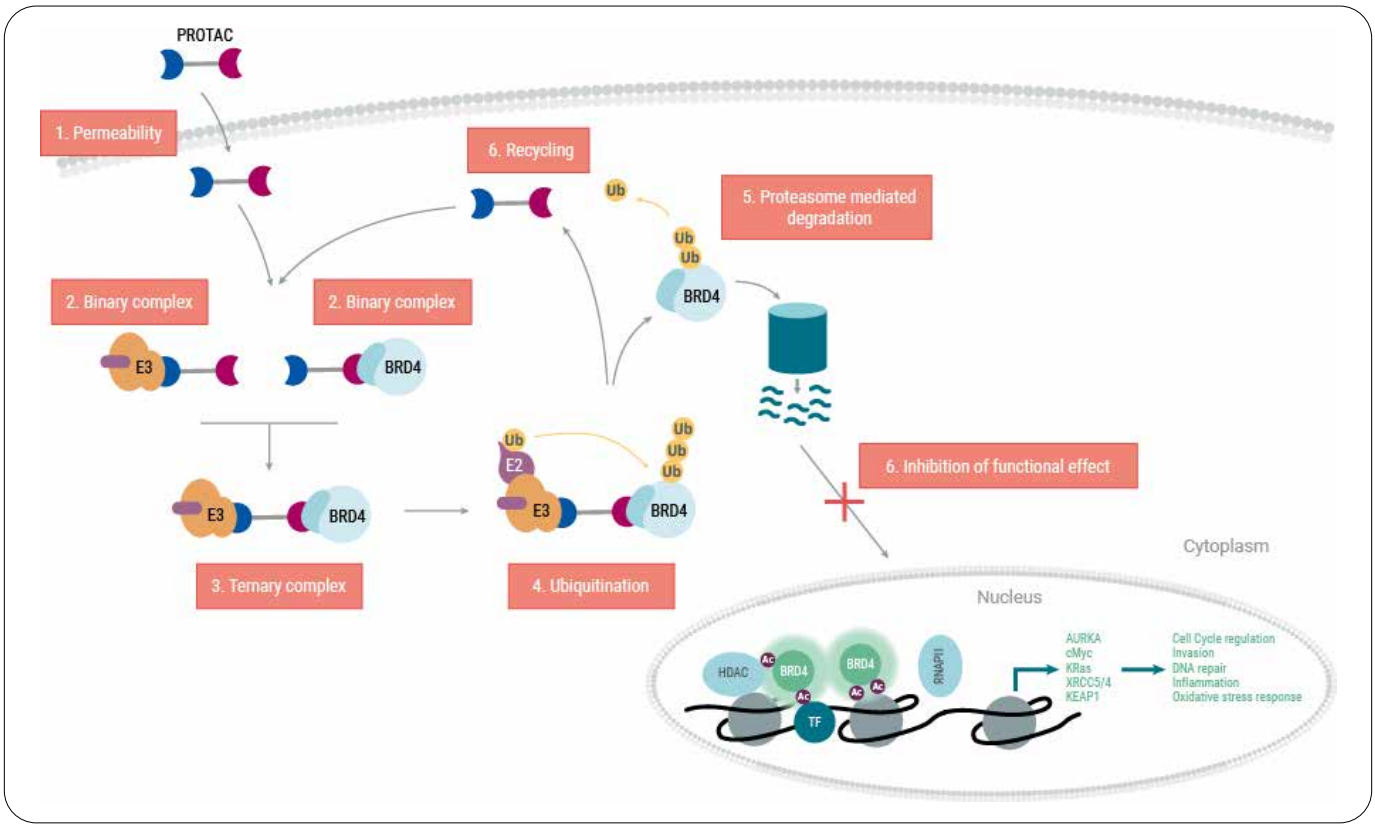
Protein degradation (TPD) has changed the landscape of drug development with the discovery of PROteolysis TArgeting Chimeras (PROTAC®s) as new therapeutic modality twenty years ago[1][2]. It has evolved into a powerful strategy, offering a promising therapeutic approach in many types of diseases, especially in cancers. PROTAC®s, also known as bivalent chemical protein degraders, hijack the ubiquitin proteasome system (UPS) offering a new chemical biology space to control protein function. PROTAC® strategy employs bifunctional molecules with dual functionalities, one is a warhead which binds to a protein of interest (POI), the other one recruits an E3 ligase (E3L), that draws in the POI for ubiquitination and degradation by the proteasome[3][4] (Scheme 1).

Among PROTAC® targeted proteins, Bromodomain-containing protein 4 (BRD4) represents one of most extensively studied protein. BRD4 belongs to the BET family and recruits transcriptional regulatory complexes to acetylated chromatin thereby controlling specific networks of genes involved in cellular proliferation and cell cycle progression including c-Myc proto-oncogene. Alterations in regulation of activities from BRD4, have been associated with number of human diseases such as cancer. Thus, inducing BRD4 degradation constitutes a promising therapeutic strategy offering clear advantage over strategies focused on disrupting the protein–protein interaction with histones by inhibitors, as described by Qin *et al*. and Wang *et al*.[5][6]. In this context, various PROTAC®s recruiting Cereblon, VHL or IAP E3L have shown efficient BRD4 degradation exhibiting promising *in-vitro* and *in-vivo* anticancer activity in a number of cancer models[6][7][8].

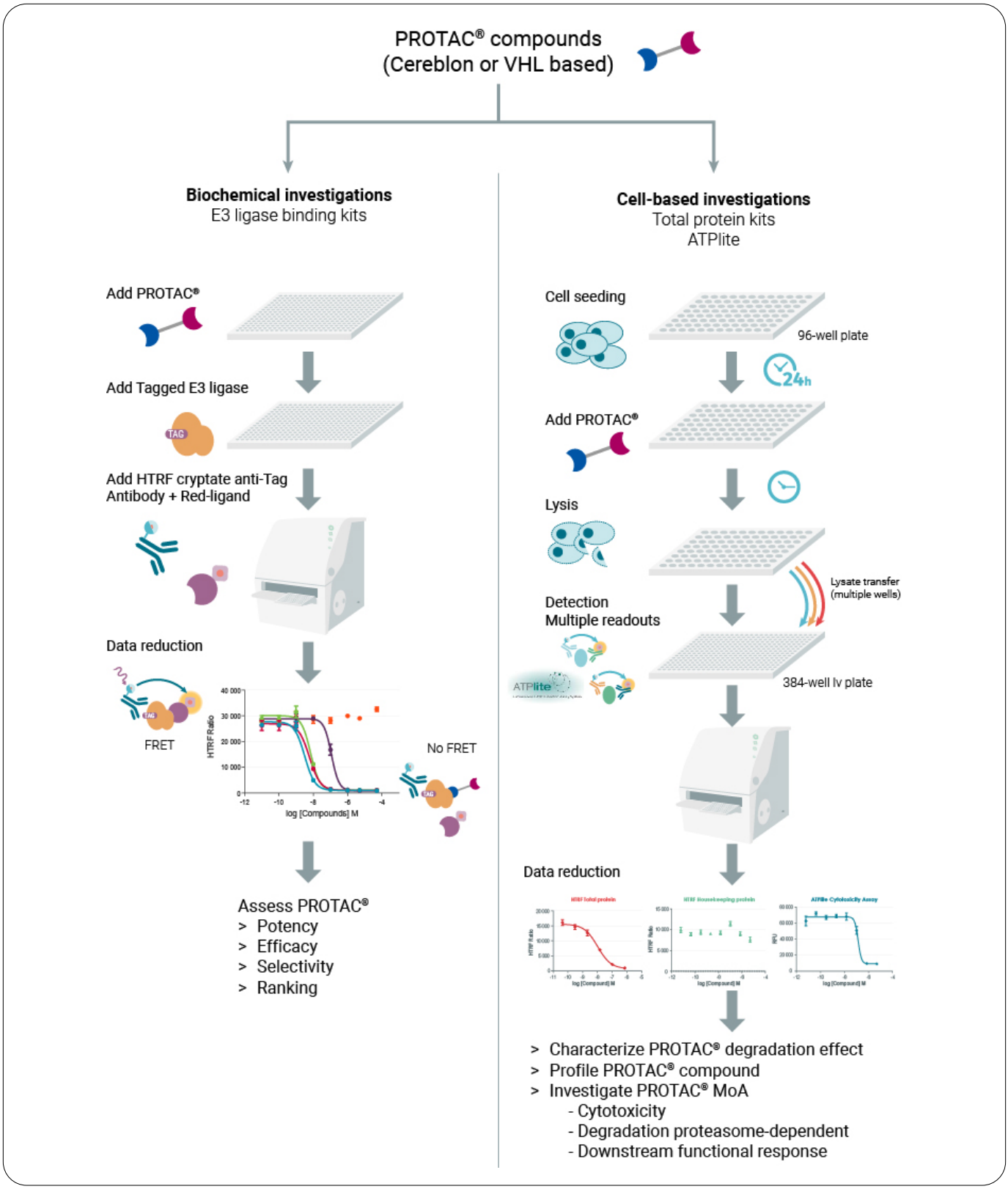
For research purposes only. Not for use in diagnostic procedures.



This Application note describes a method for in-depth characterization of PROTAC®s targeting BRD4 using Revvity biochemical and cell-based HTRF assays. This straightforward technological platform, depicted on Scheme 2, facilitates a comprehensive assessment of key PROTAC®s features such as binding affinity to E3L, POI degradation efficiency, profiling, and their mechanism of actions.



Scheme 1: Schematic representation of PROTAC®s induced BRD4 degradation through the Ubiquitin-Proteasome System (UPS).



Scheme 2: Comprehensive study of PROTAC®s with Revvity' No-wash solutions. **Biochemical solutions:** HTRF E3 ligase competitive binding kits uses an E3 ligase Red Ligand, a tagged recombinant human E3 ligase, and an anti-Tag donor antibody. PROTAC® and E3 ligase binders compete with the Red Ligand and thereby prevent FRET from occurring. HTRF VHL and Cereblon binding assays were carried out according to their respective protocol described in the package insert. **Cell-based solutions:** All HTRF cellular total protein assays are designed to quantify the expression level of a protein in a cellular lysate. These sandwich immunoassays use one antibody coupled to a FRET donor, and a second one coupled to a FRET acceptor. Both antibodies are highly specific and recognize distinct epitope on the protein. In the presence of the protein in a cell extract, the addition of these 2 fluorescent antibodies brings the donor into close proximity with the acceptor. The intensity of FRET signal intensity is directly proportional to the concentration of the protein present in the sample. Cell viability experiments were assessed with ATPlite kit, according to the 1 step-protocol described in the manual.

Table 1: List of PROTAC®s characterized in this study.

PROTAC®s	POI Ligand « Warhead »	E3L Ligand « Anchor »	E3 Ligase	Described Selectivity	Reported Potency (DC50, nM)
SIM1 (Trivalent PROTAC®)	JQ1 derivative	VH032	VHL	BET family	1-3
dBET6	JQ1 derivative	Thalidomide	CRBN	BET family	14-50
MZ-1	JQ1 derivative	VH032	VHL	BET family	5-40
ARV-771	JQ1 derivative	VHL-1	VHL	BET family	8-34
dBET1	JQ1 derivative	Thalidomide	CRBN	BET family	100-800
MZP-54	I-BET726	VH032	VHL	BRD3 & BRD4 (Lower selectivity for BRD2)	50-300
ZXH-3-26	JQ1 derivative	Pomalidomide	CRBN	BRD4	5
VZ185	BI7273	VH-101	VHL	BRD7 and BRD9	4-40
dBRD9	BI7273	Pomalidomide	CRBN	BRD9	10-50
ARV-471 (ERα PROTAC® in clinic)	Lasofoxifene (ERα ligand)	Lenalidomide	CRBN	Irrevelant for BRD	-

Determination of PROTAC®s binding affinity to E3 ligases Cereblon or VHL with HTRF biochemical binding kits

Cereblon, VHL, MDM2 and IAPs are the main E3 ligases for which PROTAC® molecules have been developed. The design of such bi-functional degraders comes with many challenges. Indeed, conjugating two well-established small molecules plus a linker result in a new modality with still unpredictable physicochemical properties, and the nature of each component e.g. E3 ligand, Linker and warhead, influences PROTAC® associated properties and bioactivity[4][9][10][11].

Binding evaluation during PROTAC® design strategy allow proof of concept studies to validate whether the compound is amenable to TPD approach[4][11]. To investigate E3L: PROTAC® binary complex formation, biochemical HTRF VHL or Cereblon binding assays are designed to determine PROTAC® binding affinities.

Reference compounds (cf. Table 1) dose-response curves were performed and IC50 values as well as Ki were determined as reported in Table 2 (Ki calculation is detailed in Appendix 1).

PROTAC®s recruiting VHL such as VZ185 or MZ1 display

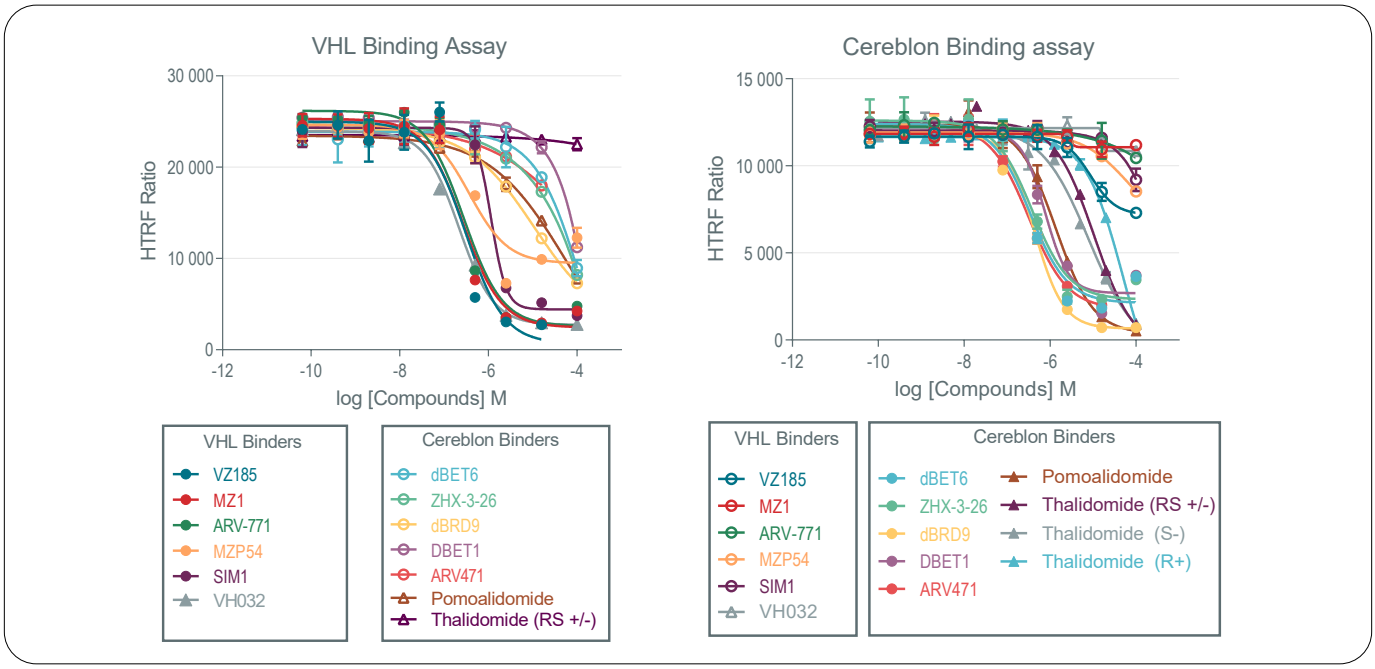


Figure 1: Dose-response inhibition curves using HTRF VHL and Cereblon binding kits.

Table 2: Experimental IC50 and Ki of PROTAC®s determined from the dose-response inhibition curves.

			VHL Binding			Cereblon (CRBN) Binding		
BRD PROTAC®s or E3L ligand	E3 Ligase	E3L Ligand	IC50 (nM)	Ki (nM)	Ranking	IC50 (nM)	Ki (nM)	Ranking
VH032 ligand	VHL	-	218	127	-	No Binding		
VZ185	VHL	VH-101	306	178	1	> 100 µM		
MZ-1	VHL	VH032	267	158	1	No Binding		
ARV-771	VHL	VHL-1	280	163	1	No Binding		
MZP-54	VHL	VH032	376	219	2	No Binding		
SIM 1	VHL	VH032	1150	671	3	No Binding		
Pomalidomide ligand	CRBN	-	No Binding			1375	537	-
Thalidomide (RS +/-) ligand	CRBN	-	No Binding			10660	4115	-
Thalidomide (S-) ligand	CRBN	-	Not determined			7841	3062	-
Thalidomide (R+) ligand	CRBN	-	Not determined			51250	20019	-
dBET6	CRBN	Thalidomide	> 100 µM			345	135	1
ZXH-3-26	CRBN	Pomalidomide	> 100 µM			379	148	1
dBRD9	CRBN	Pomalidomide	> 10 µM			430	168	1
dBET1	CRBN	Thalidomide	> 100 µM			762	297	2
ARV-471 (Erα PROTAC®)	CRBN	Lenalidomide	> 100 µM			315	123	-

binding affinities in the 100-200 nM range. Our results indicate than PROTAC® SIM1 potency is in the µM range, likely due to steric hindrance resulting from its trivalent structure. Cereblon-based PROTAC®s such as dBET6 or ZHX-3-26 display binding affinities comprised between 150 and 300 nM. Moreover, binding affinities of three different Thalidomide sources: Racemic, S-enantiomer associated with teratogenic effects, and R-enantiomers associated with sedative effects were determined[12]. Our results show that the affinity of S-enantiomer is 10 times higher than the R-enantiomer as already described by Mori *et al.*[13].

All together our results demonstrate that HTRF E3 ligase binding kits provide relevant, accurate and reliable results, highly corroborated by the literature. However, PROTAC® potencies may differ whether the PROTAC® has been assessed in a biochemical or biophysical assay or in cell-based assay. Indeed, Imaide *et al.*[14] reported enhanced BRD4 degradation induced by SIM1 in a cellular context, and Winter *et al.*[15] showed that dBET6 is more potent than dBET1 to promote BRD4 degradation in a cellular environment although these two PROTAC® are chemically related (same anchor and warhead with a different linker) and close potency when determined in biochemical context.

Thus, besides the pharmacological characterization of PROTAC®s using biochemical or biophysical techniques, their ability to induce the degradation of the POI in a cellular context must be investigated prior to preclinical and clinical studies. The optimal design of a PROTAC® molecule depends on key parameters such as i) its efficacy to induce the ternary complex formation, ii) its efficacy, potency and kinetic to promote the ubiquitination and the subsequent degradation of the POI as well as iii) its likelihood to mediate cytotoxic or side effects[16]. Versatile and rapid homogeneous total cell-based assays represent a straightforward approach to monitor PROTAC® mediated degradation of a protein of interest and to guiding the design of chemical degrader.

In depth characterization PROTAC®s targeting BRD4: potency, selectivity, cytotoxicity, kinetics, UPS mediated degradation and downstream signaling effects

HTRF Total BRD4 kit: technological prerequisites

Since the Human BRD4 protein shares 60% sequence identity with the Human BRD2 and BRD3 proteins, gene silencing experiments were performed to demonstrate the selective detection of HTRF BRD4 kit over the two other BRD proteins.

In addition, the expression level of BRD4 was assessed in various cell lines to identify a relevant cellular model for the study. These experiments demonstrated the selective measurement of BRD4 in cell lysates, and prompted us to use HeLa cells (Appendix, Figures 9, 10, 11 & 12).

Finally, BRD4 detection with HTRF kit was validated by treating HeLa cells with increasing concentrations of dBET6 and its two chemical entities, namely JQ1 and Thalidomide. As shown in Figure 2 and as expected, JQ1 or Thalidomide compounds did not modulate the HTRF signal whereas the FRET signal is decreased after dBET6 treatment. Moreover, when HeLa cells were incubated with dBET6 in the presence of Epoxomicin, a Ubiquitin Proteasome System inhibitor, the HTRF BRD4 signal remains stable compared to the control condition without Epoxomicin (Figure 7). This result unambiguously demonstrates that the HTRF signal decrease is related to BRD4 degradation process in PROTAC® treated conditions and proves the BRD4 detection consistency with HTRF total BRD4 kit.

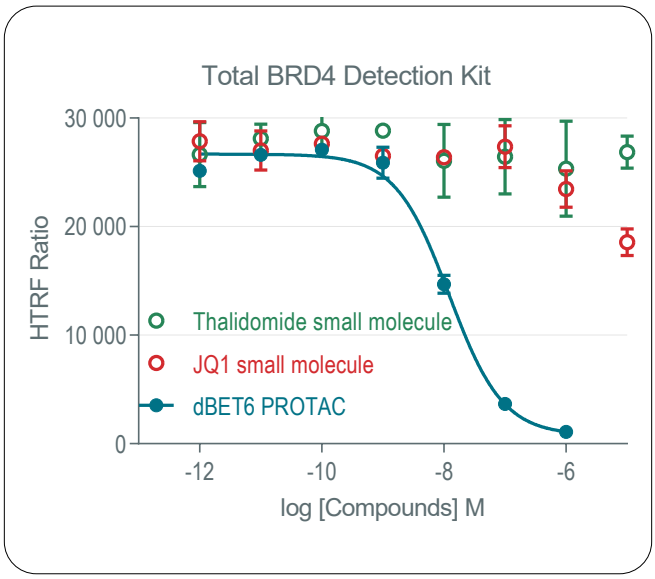


Figure 2: Validation of BRD4 downregulation detected with HTRF total BRD4 kit. HeLa cells were treated with increasing concentrations of dBET6, as well as its two chemical entities: JQ1 and thalidomide derivatives.

Pharmacological study of PROTAC®s induced BRD4 degradation with HTRF Total BRD4 Kit

A panel of PROTAC®s characterized above were assessed for their ability to induce BRD4 degradation. To this end, HeLa cells were treated with increasing concentrations of PROTAC®s for 24h, then BRD4 protein amounts were detected with HTRF Total BRD4 kit. For normalization purposes, the total protein level was assessed with HTRF Housekeeping GAPDH kit.

As shown in Figure 3A, the expression level of BRD4 is significantly decreased in a dose-dependent manner upon HeLa cells treatment with PROTAC®s targeting BET protein family, while no effect of BRD4 level was observed in the presence of non-specific PROTAC®s such as VZ185 and dBRD9 or irrelevant PROTAC® ARV-471. As indicated in the Table 3, experimental DC50 are in perfect agreement with the literature. As such, SIM1 DC50 established with HTRF total BRD4 is 1.8 nM comparable to the study from Imaide *et al.*[14]. Similarly, our results indicate that dBET6 is 10 times more potent than dBET1 in a cellular context as shown by Winter *et al.*[15]. In addition to BRD4 expression level, the amount of the GAPDH protein was quantified (Figure 3B). While GAPDH level remains stable upon certain PROTAC®s treatment (i.e ZXH-3-26, MZP-54), a significant GAPDH decrease was observed with other PROTAC®s such as SIM1 or ARV-771 indicating potential cytotoxic effects.

Finally, HTRF results were further validated by Western-blot in a side-by-side experiment. HeLa cells were treated with increasing concentrations of ZXH-3-26. After lysis step, same quantity of total proteins from the same lysate (10 µg) were analyzed with HTRF or Western-Blot. Figure 4 demonstrates correlated ZXH-3-26 DC50 defined at 24 nM and 96 nM with HTRF and western blot respectively.

Table 3: PROTAC®s DC50 and ranking determined with HTRF total BRD4 kit.

PROTAC®s	Reported affinities DC50 (nM)	HTRF Total BRD4	
		DC50 (nM)	Ranking
SIM 1 (Trivalent PROTAC®)	1-3	1,8	1
ZXH-3-26	5	24	2
ARV-771	8-34	33	2
MZ-1	5-40	37	2
dBET6	15-50	38	2
MZP-54	50-300	63	3
dBET1	100-800	381	4
VZ185 (Irrelevant, BRD family IV PROTAC®)	No specific BET family	No effect	-
dBRD9 (Irrelevant, BRD family IV PROTAC®)	No specific BET family	No effect	-
ARV-471 (Irrelevant, ERα PROTAC®)	Irrelevant for BRD	No effect	-

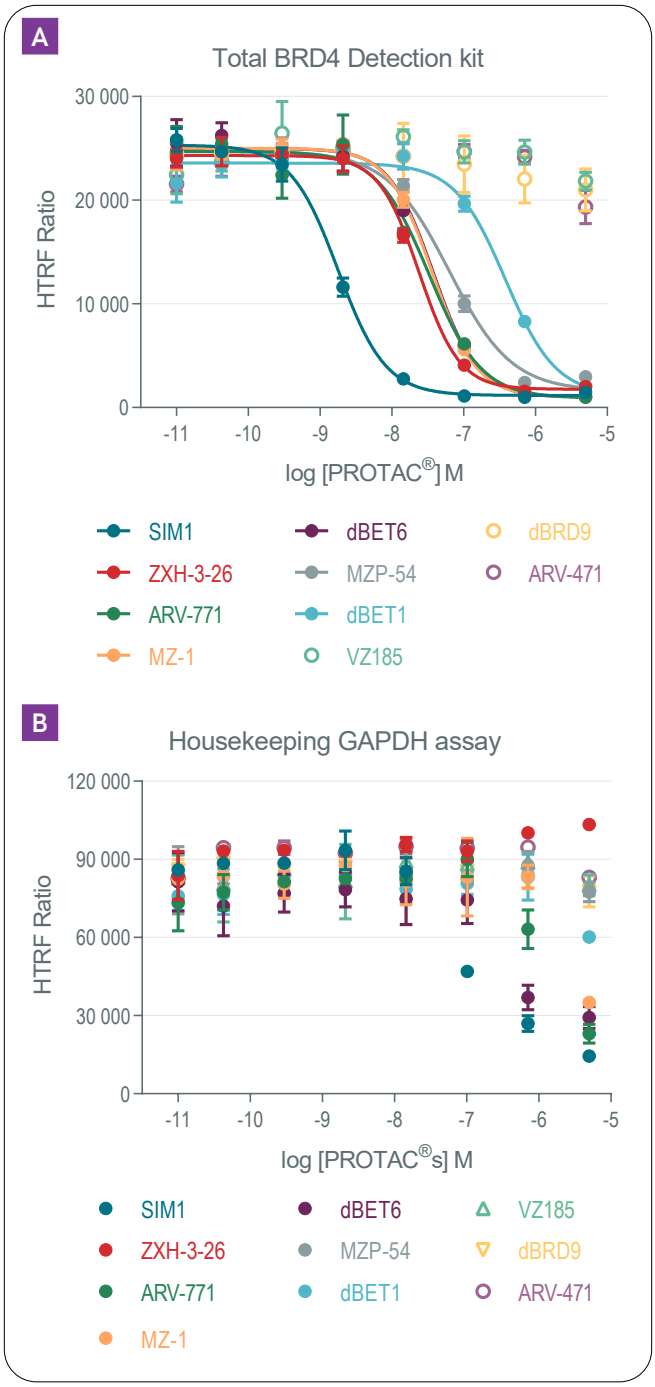


Figure 3: PROTAC® induced BRD4 downregulation monitored with HTRF. HeLa cells (50 kcells/well) were treated overnight with increasing concentrations of compounds. (A) BRD4 protein level assessed with HTRF Total BRD4 kit (A) and GAPDH protein level assessed with HTRF GAPDH housekeeping kit (B).

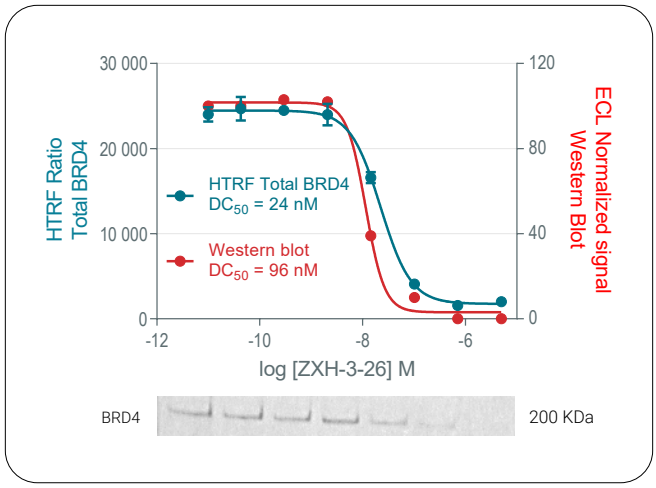


Figure 4: Correlation between Total HTRF BRD4 kit and Western blot. HeLa cells were treated with increasing concentrations of ZXH-3-26 and lysed after overnight treatment. BRD4 detection level in cell lysates was measured with HTRF total BRD4 kit or western blot method.

Profiling of PROTAC®s targeting bromodomain proteins

Clinical development of BRD4-PROTAC®s has stagnated largely due to the safety risks caused by their poor degradation selectivity, leading to the design of selective PROTAC®s in drug discovery[17]. ZXH-3-26 was the first highly selective BRD4 PROTAC® over BRD2 and BRD3 reported by Nowak *et al.*[18].

In this context, PROTAC®s were profiled over different BRD proteins: BET family BRD2/BRD3/BRD4 and Family IV BRD7/BRD9.

As described previously, gene silencing experiments were performed and demonstrated the selective detection of each HTRF BRD kit over the other BRD proteins. Moreover, comfortable quantification of BRD2, 3 and 4 as well as BRD7 and BRD9 was validated in HeLa cells. (Appendix, Figures 9 & 12).

As shown in Figure 5, our results point out four different PROTAC®s behaviors resulting from their expected selectivity and DC50 toward the 5 different BRD proteins tested (Table 4):

i) Pan-BET Family PROTAC®s: SIM1, dBET6, ARV-771, MZ-1, dBET1

ii) Selective BRD3 and BRD4: MZ-54

iii) Selective BRD4: ZXH-3-26

iv) Unspecific BRD4, targeting distinct family IV bromodomain proteins: VZ185 and dBRD9

To gain further insights into BRD4 PROTAC®s mechanism of actions, dBET6 (pan-BET family) and ZXH-3-26 (BRD4 selective) were selected for further investigations.

Figure 5: PROTAC®s profiling and selectivity assessment on BRD2, BRD3, BRD4, BRD7 and BRD9 with HTRF Total kits. HeLa cells were treated with increasing concentrations of PROTAC®s for overnight. BRD expression level (in blue) as well as GAPDH (in red) were quantified with HTRF kits.

Table 4: PROTAC®s DC50 and profiles determined with HTRF total BET Family: BRD2, BRD3, BRD4 and Family IV: BRD7 and BRD9 kits.

PROTAC®s	Warheads	E3L Ligand	E3 Ligase	Reported Selectivity	Reported DC50 (nM)	Experimental Potencies DC50 (nM) Determined with HTRF Kits				
						BET Family			Family IV Bromodomains	
						BRD2	BRD3	BRD4	BRD7	BRD9
SIM1 (Trivalent PROTAC®)	JQ1 deriv	VH032	VHL	BET Family	1-3	0.7	1	2	No effect	No effect
dBET6	JQ1 deriv.	Thalidomide	CRBN	BET Family	14-50	89	13	38	No effect	No effect
ARV-771	JQ1deriv.	VHL-1	VHL	BET Family	8-34	89	9	33	No effect	No effect
MZ-1	JQ1deriv.	VH032	VHL	BET Family	5-40	81	37	37	No effect	No effect
dBET1	JQ1deriv.	Thalidomide	CRBN	BET Family	100-800	> 1000	135	380	No effect	No effect
MZP-54	I-BET726	VH032	CRBN	BRD3 and BRD4 (>> BRD2)	50-300	> 500	37	95	> 10 000	No effect
ZXH-3-26	JQ1deriv.	Pomalidomide	CRBN	BRD4	5	No effect	No effect	24	No effect	No effect
VZ185	BI7273	VH-101	VHL	BRD7and BRD9	4-40	No effect	No effect	No effect	114	51
dBRD9	BI7273	pomalidomide	CRBN	BRD9	10-50	No effect	No effect	No effect	No effect	27

Understanding PROTAC®s' mechanism of action

Cytotoxicity associated with PROTAC® induced BRD4 degradation

Despite being a promising therapeutic approach, PROTAC®s development still face challenges such as adverse effects or cytotoxicity[6]. As such, pan BET inhibitors have been shown to induce toxic effect in pre-clinical as well as in clinical studies. Therefore, selective degradation of BRD4 was investigated in order to limit toxicity[18]. Thus, potential cytotoxicity induced by dBET6 and ZXH-3-26 was assessed using ATPlite kit along with BRD4 degradation efficacy after 4 hours and overnight incubation time.

By combining BRD4 degradation with ATPlite, differences in dBET6 and ZXH-3-26 mechanism of action can be observed as represented in Figure 6. Indeed, dBET6 induces BRD4 degradation from 4h incubation, while it exhibits cytotoxic effect after overnight. Note that dBET6 cytotoxic effect was previously observed by Qin *et al.*[5]. Interestingly ZXH-3-26 promotes BRD4 degradation without significant impact on cell viability, even after overnight treatment. These results suggest BRD4-independent controls of cell viability, and one can hypothesize on existing compensatory mechanisms exerted by other BET proteins such as BRD2 and BRD3 in the absence of BRD4. These data nicely demonstrate that HTRF Total BRD4 in combination with ATPlite kit provide additional and valuable information on PROTAC®s characterization and their mechanism of action.

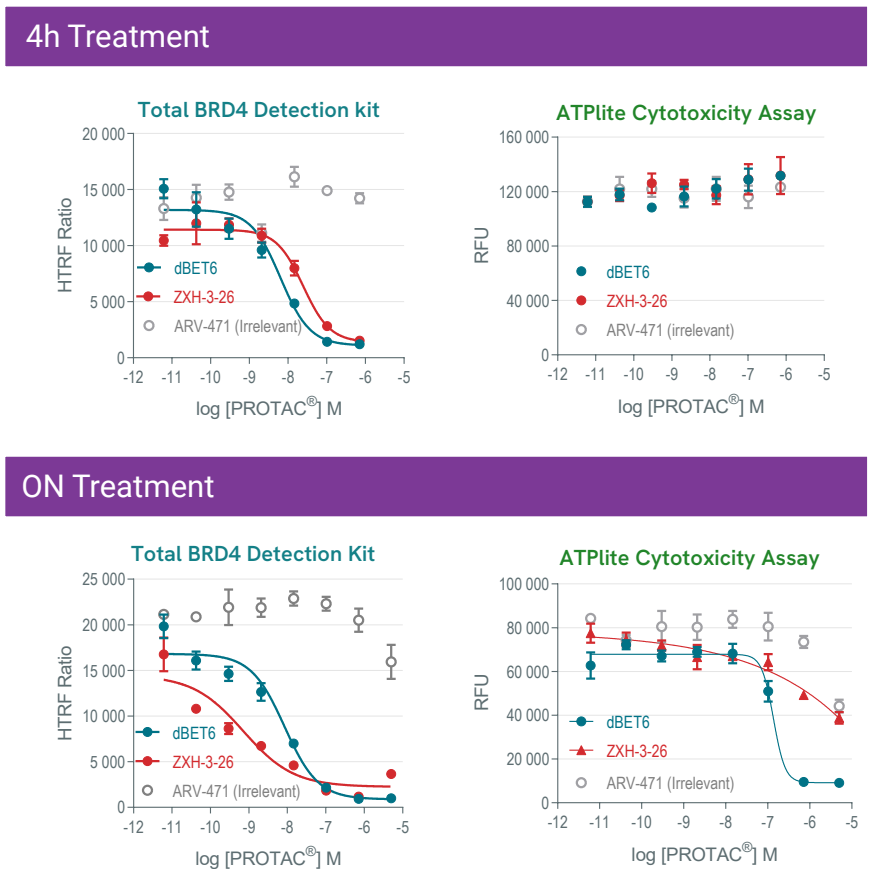


Figure 6: Kinetics of dBET6 and ZXH-3-26 PROTAC®s mediated degradation and cytotoxicity effects. HeLa cells were treated 4h or overnight with increasing concentrations of PROTAC®s. Experiments were carried out following instruction of each kit to monitor BRD4 expression level with HTRF total BRD4 kit, and Cytotoxicity with ATPlite kit.

PROTAC® induced BRD4 degradation is mediated by the Ubiquitin Proteasome System

PROTAC®s are specific degraders which exploit the Ubiquitin Proteasome System machinery to induce the degradation of targeted proteins. This mode of action can be illustrated by blocking the proteasome, thereby preventing PROTAC® induced POI degradation and leading to POI rescue mechanism. Here, we have explored the UPS-mediated BRD4 degradation upon dBRD6 (BET Family PROTAC®) and ZXH-3-26 (BRD4 selective PROTAC®) treatment.

The results represented in Figure 7 show that BRD4 degradation induced by dBTE6, and ZXH-3-26 is prevented is the presence of the proteasome inhibitor epoxomicin. Note that cell viability remains unchanged in the same conditions. These results nicely demonstrate that BRD4 degradation induced by dBET6 and ZXH-3-26 PROTAC®s is mediated by the proteasome.

Figure 7: PROTAC® induced BRD4 degradation through the Ubiquitin Proteasome System. HeLa cells were pre-treated 1h with the Epoxomicin proteasome inhibitor at 1 µM before dBET6 and ZXH-3-26 PROTAC®s treatment for an additional 4h (without Epoxomicin removal). After cell lysis step, HTRF Total BRD4 kit and ATPlite kits were used to quantify BRD4 expression level and Cytotoxicity effect respectively.

Differential regulation of c-Myc mediated by dBET6 and ZXH-3-26 induced BRD4 degradation

Investigating downstream biological responses is pivotal to deeply understand the mechanisms of actions which underlie therapeutic effects. Since BRD4 is involved in the control of c-Myc expression, the modulation of c-Myc level in response to dBET6 and ZXH-3-26 was assessed with HTRF c-Myc kit.

The results presented in Figure 8 show that both BRD4 targeting PROTAC® compounds result in c-Myc downregulation after 4h and overnight treatment. However, dBET6 exhibits a partial effect after 4h compared to overnight treatment. These results are consistent with the study of Bauer *et al.*[19] who reported c-Myc downregulation in dBET6 treated solid tumor cells. Interestingly, our results indicate that ZXH-3-26 is less efficient to trigger c-Myc decrease than dBET6. Indeed, c-Myc expression is not only controlled by BRD4 but also by other BET family members. Thus, dBET6 which is pan BET family degrader is likely to exert a stronger downstream response than ZXH-3-26 which is BRD4 selective.

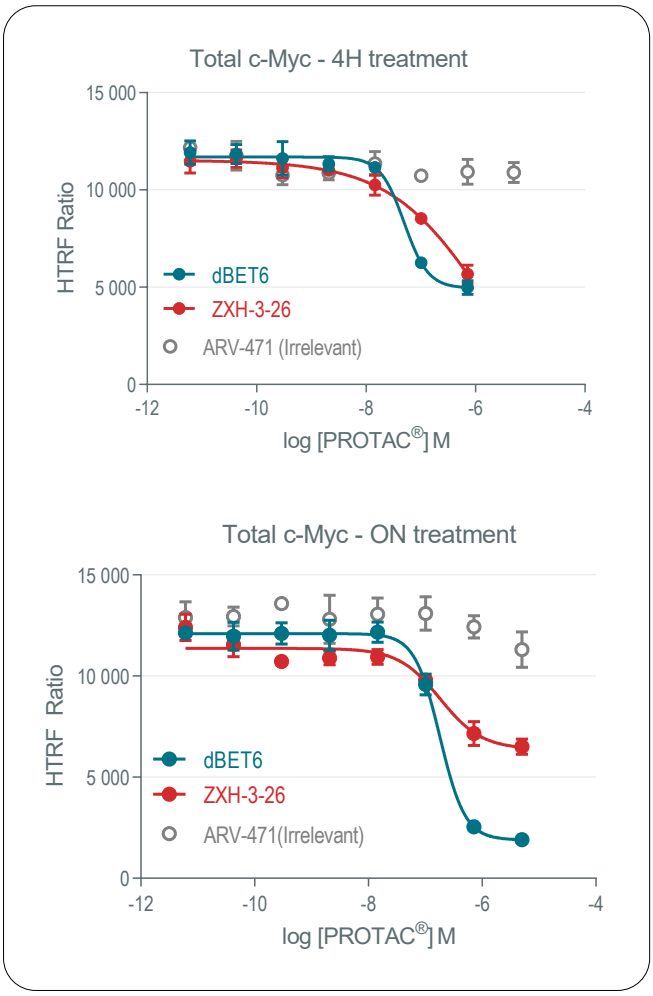


Figure 8: Modulation of c-Myc expression as a downstream BRD4 readout after PROTAC® treatment. HeLa cells were treated with increasing concentrations of dBET6 (pan BET family), ZXH-3-26 (selective BRD4 PROTAC®) as well as ARV-471 irrelevant PROTAC®) for 4h or overnight.

Conclusion

BRD4 is involved in gene transcription and is frequently dysregulated in many types of cancer. BRD4 is one of the most studied proteins in the Targeted Protein Degradation space where a number of PROTAC® molecules are being developed. Herein we describe a method enabling extensive characterization of PROTAC® molecules targeting BRD4 with Revvity's no-wash platform comprising HTRF biochemical and cell-based assays. Using a relevant panel of PROTAC®s, we determined key PROTAC®s features such as binding affinity, degradation efficiency and selectivity as well as mechanisms of action such as cytotoxicity, proteasome mediated degradation and downstream signaling events.

References

[1] : An *et al.*, EBioMedicine review, (2018) 36: 553–562.

[2] : Pettersson *et al.* Drug Discov. Today Technol.(2019) 31.

[3] : Eron *et al.* ACS Chem. Biol. (2021) 16 ; 2228–2243

[4] : Bai *et al.*, J. Biol. Chem. (2022) 298 (4) 101653.

[5] : Qin *et al.*, Cell Death and Disease (2020) 11:805.

[6] : Wang *et al.*, J Enzyme Inhib Med Chem. (2022) 37 (1): 1694–1703.

[7] : Lu *et al.*, Chem Biol. (2015) 22 (6): 755–763.

[8] : Eron *et al.*, ACS Chem. Biol. (2021) 16 : 2228–2243.

[9] : Girardini *et al.* Bioorganic & Medicinal Chemistry (2019) 27: 2466–2479

[10] : Troup *et al.*, Explor Target Antitumor Ther. (2020) 1: 273-312.

[11] : Chen *et al.*, Redox Biology (2023) 59 ; 102552.

[12] : Tokunaga *et al.* Scientific Reports (2018) 8:17131

[13] : Mori *et al.* Scientific Reports (2018) 8:1294

[14] : Imaide *et al.*, Nat Chem Biol.(2021) 01; 17(11): 1157–1167.

[15] : Winter *et al.*, Mol Cell. (2017) 67(1): 5–18.e19.

[16] : Checchini *et al.*, Frontiers in chemistry. (2021) 9, 672267.

[17] : Hu *et al.*, Eur.J.Med.Chem.(2022) ; (244) ; 114775.

[18] : Nowak *et al.*; Nat.Chem.Biol. (2018) ; 14(7) : 706 :714.

[19] : Bauer *et al.* ; Am.J.Cancer.Res. (2021) ; 11(2) : 530-545

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Appendix

Specificity and selectivity validation of the HTRF Total BRD kits

At first step, all HTRF Total BRD kits used in this study were evaluated for the specific and selective detection of the different BRD proteins. Gene silencing method was applied by using HAP1 knock-out cell lines or siRNAs.

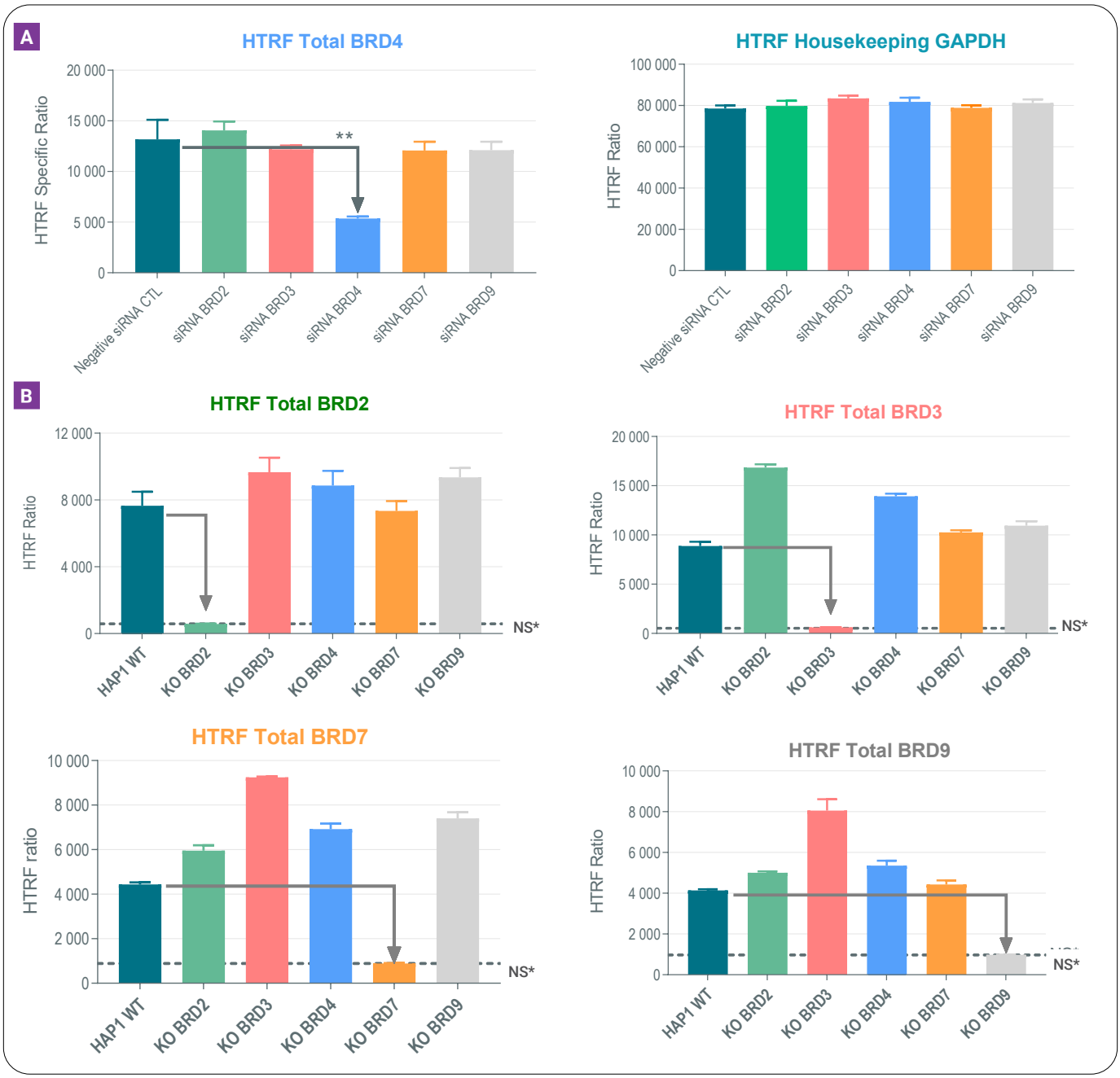

Figure 9: Specificity and selectivity of HTRF Total BRD assays. **A**. MCF7 cells at 25kcells/well were treated with various BRD specific SiRNAs (25 nM) for 24h. Lysates were transferred into a 384-w lv plate and detection of total proteins allowed using HTRF Total BRD4 kit in association with GAPDH housekeeping control. Signal clasps using BRD4 SiRNA with no impact of other BRD siRNAs demonstrate Total BRD4 kit specificity and selectivity **B**. HAP1 WT and HAP1 KO for BRD2, BRD3, BRD4, BRD7 and BRD9 (horizon discovery) were used in HTRF Total BRD2, BRD3, BRD7 and BRD9 kits to demonstrate their specificity and selectivity. *NS: not specific signal.

The results in Figure 9 clearly show that each HTRF Total BRD kit detects its corresponding protein specifically and selectively. Interestingly, it was noticed in some cases that silencing bromodomain proteins can lead to significant increase of other BRD protein members. For example, as shown for HTRF Total BRD3 detection kit, silencing BRD2 gene induces significant increase of BRD3 protein in comparison with basal values on HAP1 Wt. This is suggesting a compensatory cellular mechanism activation.

Cellular model selection

To ensure the possibility of ternary complex formation and thus PROTAC® correct characterization, E3 ligase and protein targets expression levels were quantified in various cancer cell lines to select the one properly expressing both partners. To assess degradation experiment with PROTAC®s, BRD4 protein and Cereblon E3 ligase expression levels were quantified using HTRF total BRD4 and HTRF total Cereblon detection kits. Furthermore, to address PROTAC® profiling, other BRD2, BRD3, BRD4, BRD7 and BRD9 protein expression levels were checked to select the most relevant cell line for the global study.

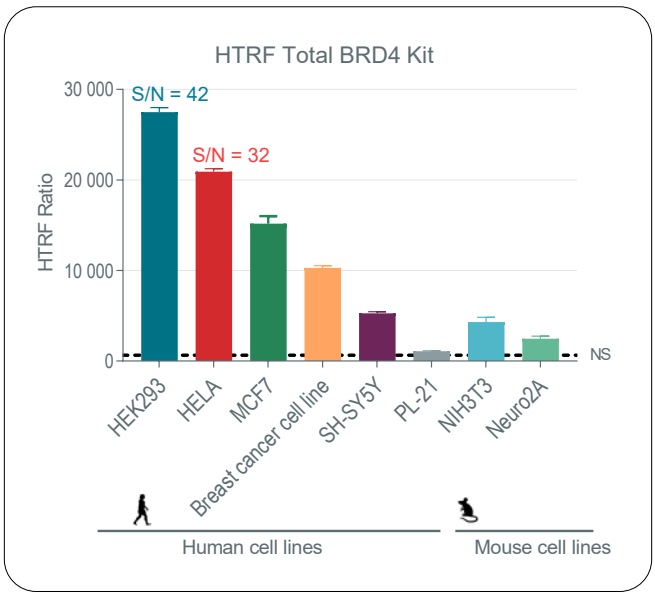

Figure 10: BRD4 protein expression level on various cell lines at a density of 100 kcells /well using HTRF Total BRD4 kit. *NS: not specific signal

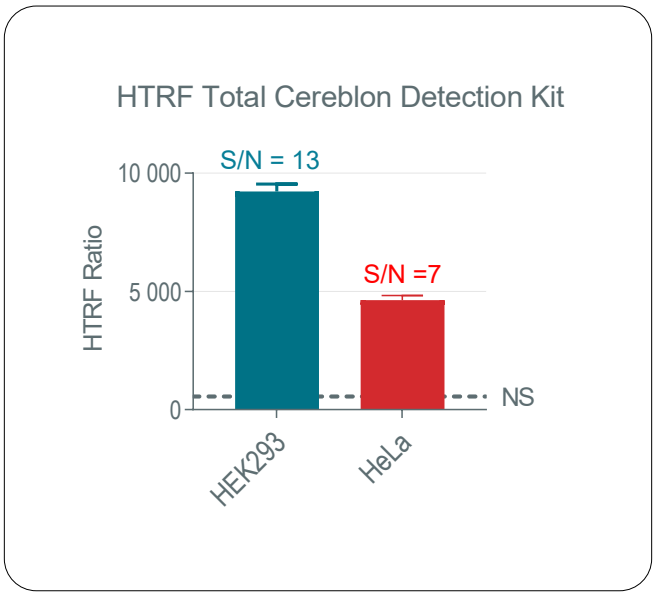

Figure 11: Cereblon E3 Ligase protein expression level on HEK293 and HeLa cells using HTRF Total Cereblon kit. *NS: not specific signal

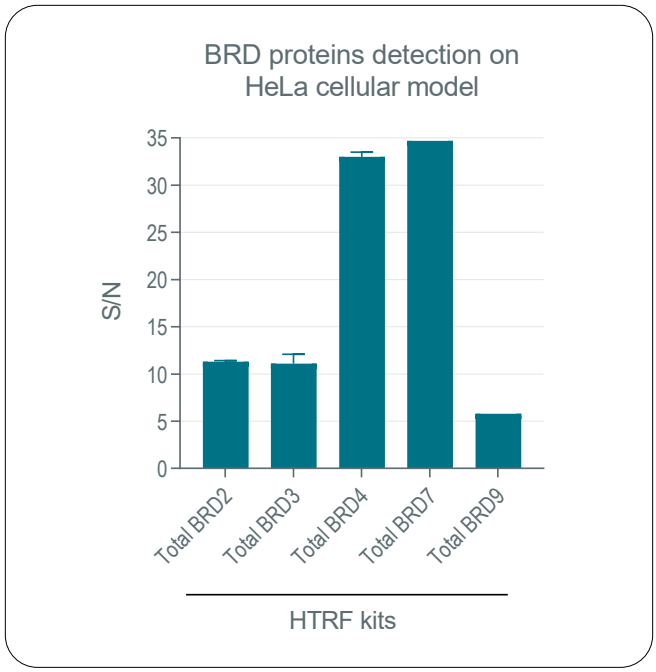

Figure 12: BRD protein expression on HeLa cells. BRD2, BRD3, BRD4, BRD7 and BRD9 proteins are well expressed on HeLa cellular model.

Data obtained with optimal cellular density within dynamic range of the kits are shown. BRD4 protein is well detected in many cell lines at different expression levels (Figure 10). HEK293 and HeLa, the most expressing BRD4 cell lines, are also well expressing Cereblon E3 Ligase (Figure 11). Other BRD2, BRD3, BRD7 and BRD9 expression at significant level was confirmed on HeLa cells as shown Figure 12. Thus, HeLa cervix cancer cell line, widely used for PROTAC® studies in cancer research, was selected for this BRD4 PROTAC®s characterization study.

Materials and reagents

	Reagent	Supplier	Part Number
Microplates	CulturPlate-96, Black opaque (Tissue Culture Treated)	Revvity	6005660/8/9
	AlphaPlate-384, Light gray detection plate	Revvity	6005350/9
	ProxiPlate-384 Plus, White 384-shallow well detection plate	Revvity	6008280/9
	HTRF 96-well low volume white detection plate	Revvity	66PL96005
HTRF kits	HTRF Cereblon Binding Kit	Revvity	64BDCRBNPEG/H
	HTRF VHL Binding Kit	Revvity	64BDVHLPEG/H
	HTRF Human Total BRD2 Detection Kit	Revvity	64BRD2TPEG/H
	HTRF Human Total BRD3 Detection Kit	Revvity	64BRD3TPEG/H
	HTRF Human Total BRD4 Detection Kit	Revvity	64BRD4TPEG/H
	HTRF Human Total BRD7 Detection Kit	Revvity	64BRD7TPEG/H
	HTRF Human Total BRD9 Detection Kit	Revvity	64BRD9TPEG/H
	HTRF GAPDH Housekeeping Cellular Kit	Revvity	64GAPDHPEG/H
	HTRF Human c-Myc Cell-based Kit	Revvity	63ADK053PEG/H
	HTRF Cereblon Cellular Kit	Revvity	64CRBNPEG/H
Luminescence cytotoxicity assay	ATPlite™ 1step Luminescence Assay Kit	Revvity	6016736/1/9
SiRNAs	ON-TARGETplus Human BRD2 (6046) siRNA	Revvity	L-004935-00-0005/0010/0020/0050
	ON-TARGETplus Human BRD3 (8019) siRNA	Revvity	L-004936-00-0005/0010/0020/0050
	ON-TARGETplus Human BRD4 (23476) siRNA	Revvity	L-004937-00-0005/0010/0020/0050
	ON-TARGETplus Human BRD7 (29117) siRNA	Revvity	L-020297-00-0005/0010/0020/0050
	ON-TARGETplus Human BRD9 (659807) siRNA	Revvity	L-014250-02-0005/0010/0020/0050
Cell lines	HAP1	Revvity	C631
	Human HAP1 Knockout Cell Lines - BRD2	Revvity	HZGHC000356c015
	Human HAP1 Knockout Cell Lines – BRD3	Revvity	HZGHC005081c002
	Human HAP1 Knockout Cell Lines - BRD4	Revvity	HZGHC000937c004
	Human HAP1 Knockout Cell Lines – BRD7	Revvity	HZGHC000923c010
	Human HAP1 Knockout Cell Lines – BRD9	Revvity	HZGHC000934c003
Compounds	SIM1	TOCRIS	7432
	dBET6	Selleckem	S8762
	MZ-1	TOCRIS	6154
	ARV-771	Selleckem	S8532
	dBET1	Medchemexpress	HY-101838
	MZP-54	Medchemexpress	HY-112376
	ZXH-3-26	Medchemexpress	HY-122826
	VZ185	TOCRIS	6936
	dBRD9	TOCRIS	6606
	ARV-471	Medchemexpress	HY-138642

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