# Beyond Degradation: Cell-based and Biophysical PROTAC Characterization in a CRO

James Chamberlain, Robert Workman, Patrick McIntyre, Rachel Doidge, Alison Gordon, Ryan Mordue[1], Kathy Dodgson and Gary Allenby

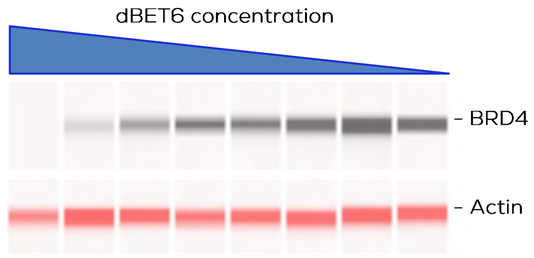
Charnwood Discovery, Charnwood Campus, Summerpool Road, Loughborough LE11 5RD

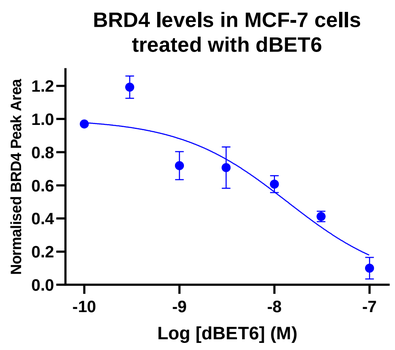
## Introduction

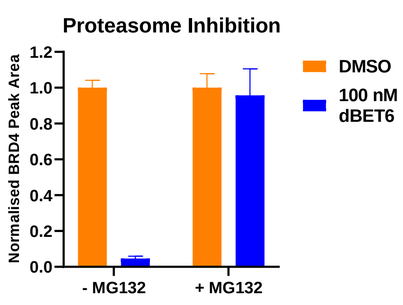
In the development of PROTACs it is vital to have a varied suite of assays that can inform the structure-activity relationship (SAR) and progress the design-make-test cycle. A number of PROTACs have previously been developed to target the well-studied protein BRD4 - a member of the BET (bromodomain and extra-terminal) family of proteins whose dysregulation is linked to human cancers. At Charnwood Discovery we have used cell-based and biophysical techniques to investigate - amongst others - the BRD4-targeting PROTAC **'dBET6'** to elucidate a binding mechanism, confirm proteasomal degradation and monitor its impact on cellular behaviour. Here we demonstrate a range of techniques that we might typically implement in a PROTAC development cascade.

## Monitoring PROTAC-induced protein degradation

Confirmation of target protein degradation is an essential element of any PROTAC development cascade. The data here were generated using the Jess™, a Simple Western™ automated Western blot system, to investigate BRD4 degradation induced by dBET6. The pseudo blot image below visualized BRD4 and actin (used as a loading control) in MCF-7 cell lysates produced after 24 hours culture in the presence of a range of dBET6 concentrations.

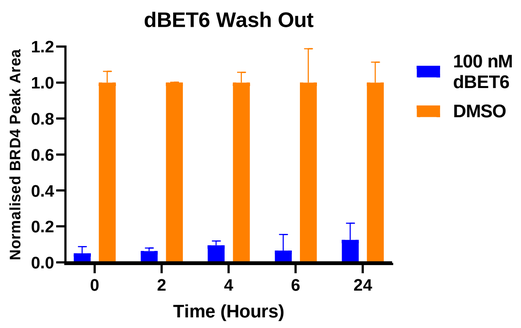



Treatment with dBET6 results in a concentration-dependent reduction in BRD4 signal, with an $IC_{50}$ of 13.7 nM. To confirm proteasomal degradation the proteosome inhibitor MG132 (1 μM) was included with 100 nM dBET6 over a 24 hour period. This successfully inhibited degradation of BRD4, but did not impact BRD4 levels in the vehicle-treated control.

We also performed a washout experiment to examine the timeframe for BRD4 levels to recover after 24 hours incubation with the PROTAC. Extracellular dBET6 was removed from MCF-7 cultures and lysates produced 2, 4, 6 and 24 hours post-washout.




Even 24 hours after dBET6 was removed from *in vitro* culture, BRD4 levels had not recovered to the levels observed in vehicle-treated controls.

As BRD4 is involved in regulating the transcription of a number of genes (c-Myc and Aurora kinase B for example), the downstream impacts of it's degradation could also be investigated using qPCR to monitor mRNA levels, or investigate these targets at the protein level using automated immunoblotting.

## Summary and Conclusion

Here we have used a range of techniques to probe the dBET6-mediated degradation of BRD4. **SPR** was used to characterize the kinetics of small molecule-protein interaction. **Automated immunoblotting** has confirmed proteosomal degradation of the target protein and shown the impact of PROTAC removal on protein levels. Phenotypic changes at the cellular level, investigated using **live-cell imaging**, has highlighted the difference in response between BRD4 degradation and inhibition.

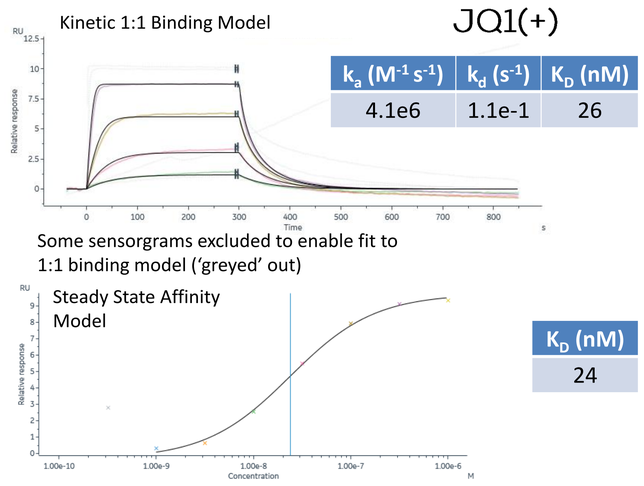
This set of orthogonal assays was used to probe BRD4 PROTAC activity, but they could be equally utilized in the development of PROTACs for a wide range of targets alongside other, more target-specific, bespoke assays.

## Characterization binding modes using SPR

SPR is a well-regarded biophysical technique that can equally be applied to initial primary screening of compound libraries as it can to detailed characterization of an individual molecular interaction. The data below shows such a characterization performed on our Biacore 8K instrument measuring the kinetics and affinity of a PROTAC molecule and the warhead compound it was derived from against BRD4.

In the case of the BRD4-targeting warhead JQ1(+) we can measure very high quality sensorgrams for its interaction with BRD4. At some of the higher concentrations of compound we see a deviation away from 1:1 binding and these sensorgrams have to be excluded to allow a good fit of the model to the data.

The subsequent kinetic fit ($K_D$ = 26 nM) shows good agreement with the affinity fit ($K_D$ = 24 nM).



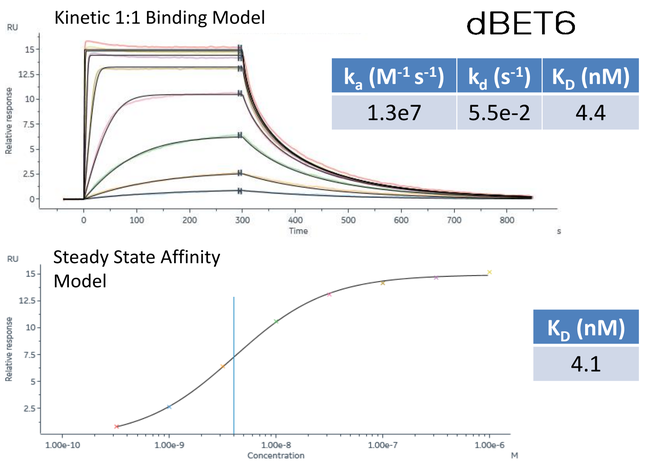

| $k_a$ ($M^{-1}$ $s^{-1}$) | $k_d$ ($s^{-1}$) | $K_D$ (nM) |
|---|---|---|
| 4.1e6 | 1.1e-1 | 26 |

Some sensorgrams excluded to enable fit to 1:1 binding model ('greyed' out)

| $K_D$ (nM) |
|---|
| 24 |

PROTAC molecule dBET6 is based on JQ1(+) – it uses this as its BRD4-targeting warhead. dBET6 shows an excellent fit to a 1:1 binding model at all concentrations – presumably some properties conferred on the whole PROTAC molecule from either the linker region or the Cereblon-targeting warhead have positively influenced the molecule's binding to BRD4 and general solubility-related properties.

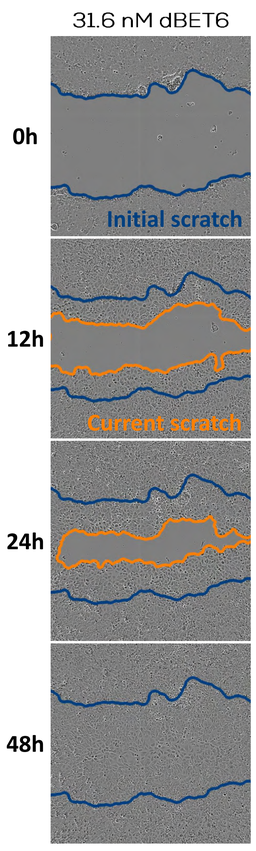
The subsequent kinetic fit ($K_D$ = 4.4 nM) shows excellent agreement with the affinity fit ($K_D$ = 4.1 nM).



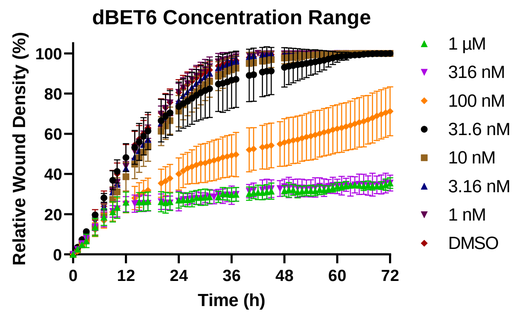

| $k_a$ ($M^{-1}$ $s^{-1}$) | $k_d$ ($s^{-1}$) | $K_D$ (nM) |
|---|---|---|
| 1.3e7 | 5.5e-2 | 4.4 |

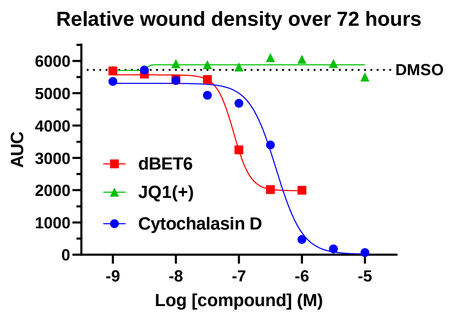
| $K_D$ (nM) |
|---|
| 4.1 |

## Scratch wound migration and cell health




The IncuCyte® SX5 live-cell kinetic imager allows us to monitor cell behaviour in real-time and understand the phenotypic results of PROTAC treatment. Here we use a scratch wound model to investigate the ability of MCF-7 cells to migrate when treated with serial dilutions of compounds. Cytochalasin D, an actin polymerization inhibitor, was used as a positive control to compare to dBET6 and JQ1(+). Image analysis is handled by trainable algorithms that identify the initial scratch wound and track wound closure over the course of the experiment to calculate a "relative wound density". This was measured over 72 hours and area under curve (AUC) analysis used to plot kinetic data, showing that dBET6, but not JQ1(+), inhibits cell migration into the scratch in a concentration-dependent manner. dBET6 is a more potent inhibitor of migration in MCF-7 cells than cytochalasin D (producing $IC_{50}$s of 83 and 391 nM respectively), but does not fully inhibit migration at high concentrations.




This system was also used to monitor MCF-7 health in the presence of BRD4-interacting compounds. MCF-7 confluency measured over 72 hours shows that dBET6 inhibits proliferation with an $IC_{50}$ of 54 nM, compared to JQ1(+) at 3 μM. Apoptosis induction was detected using a cell-permeable dye which a produces signal on caspase 3/7 activation.

This also showed that dBET6 ($EC_{50}$ 71 nM) is a more potent inducer of apoptosis than JQ1(+). These results highlight the difference in phenotypic response with target protein degradation (dBET6) compared to inhibition (JQ1(+)).