SPR-measured kinetics of PROTAC ternary complexes influence target degradation rate.

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ABSTRACT: Bifunctional degrader molecules, known as proteolysis-targeting chimeras (PROTACs), function by recruiting a target to an E3 ligase, via a target:PROTAC:ligase ternary complex. Despite the importance of this key intermediate species, no method to monitor ternary complex kinetics has been reported, and it remains to be addressed how this might impact on the kinetics of protein degradation. Here, we develop an SPR-based assay to quantify the stability of PROTAC-induced ternary complexes by measuring for the first time the kinetics of their formation and dissociation. We benchmark our assay using four PROTACs that target the bromodomains (BDs) of BET proteins Brd2, Brd3 and Brd4 to the E3 ligase VHL. We reveal marked differences in ternary complex off-rates for different PROTACs that form either cooperative or non-cooperative complexes. The positively cooperative degrader MZ1 forms comparatively stable and long-lived ternary complexes with either $Brd4^{BD2}$ or $Brd2^{BD2}$ and VHL. Equivalent complexes with $Brd3^{BD2}$ are destabilized due to a single amino acid difference (Glu/Gly swap) present in the bromodomain. We observe that this difference in ternary complex dissociative half-life correlates to a greater initial rate of intracellular degradation of Brd2 and Brd4 relative to Brd3. These findings establish a novel assay to measure the kinetics of PROTAC ternary complexes and elucidate the important kinetic parameters that drive effective target degradation.

PROTACs are bivalent molecules consisting of ligands for each of a target protein and an E3 ligase, joined *via* a linker.[1-2] PROTAC behaviour can be modelled by three-body binding equilibria.[3] Formation of a target:PROTAC:ligase ternary complex triggers proximity-dependent target protein ubiquitylation and degradation *via* the ubiquitin-proteasome-system.[2, 4] PROTAC drug discovery is rapidly advancing in both academia and industry, fuelled by both improvement in drug-like properties and broader recognition of mechanistic advantages of degradation over inhbition.[1, 5-6] PROTACs offer potential for improved selectivity beyond that of the constituent target ligand, by harnessing additional stabilising or destabilising *de novo* protein-protein or protein-linker interactions formed *via* the ternary complex.[2, 7-9] Developing new tools to understand cooperativity/avidity effects in PROTAC design is thus of significant interest. Although functional degraders can be generated in the absence of positive cooperativity,[8, 10] mounting evidence suggests enhancing cooperativity could be an effective strategy in PROTAC design. Cooperativity is expected to counter the 'hook effect' often exhibited by bifunctional molecules,[3] thereby widening the concentration window for PROTAC activity, and could enable use of weaker-affinity ligands.[11-12] Despite a growing literature of methods to measure thermodynamics of PROTACs at steady-state,[1-2, 8-10, 13-14] there is currently a dearth of methods to measure kinetics of formation and dissociation of PROTAC ternary complexes in real-time. We were thus interested in exploring surface plasmon resonance (SPR) as a suitable label-free technique to monitor the kinetics of PROTAC-induced ternary complexes, the required intermediate species in the mechanism.[4] Herein we develop the first SPR-based assay to quantitatively measure the kinetics of complex formation and dissociation, which we use to characterise the lifetime of ternary complexes composed of bromodomain-containing target proteins, PROTACs, and the von Hippel-Lindau E3 ligase (VHL).

SPR has previously been utilised to characterise three-body binding systems (including complexes composed of protein, DNA and small-molecules),[15-18] which can be experimentally involving due to the complex nature of the binding equilibria.[3] We sought a general-purpose and conceptually-simple assay format to study PROTACs ternary complexes. Importantly, we recognised for bivalent molecules that the 'hook effect' would preclude use of saturating concentrations of PROTAC in the running buffer. We reasoned that by immobilising the E3 ligase, a single sensor surface might be utilised to measure diverse PROTAC/target combinations. To improve uniform presentation on the chip surface, we designed a VHL:ElonginB:ElonginC (VCB) construct harboring an AviTag sequence C-terminal to ElonginB for site-specific biotinylation (hereafter 'biotin-VHL').[19-20]

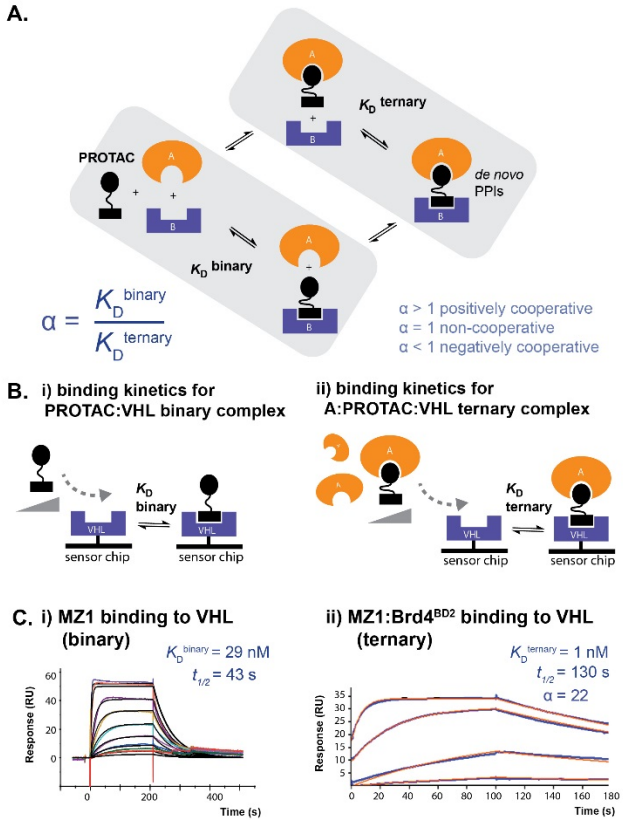
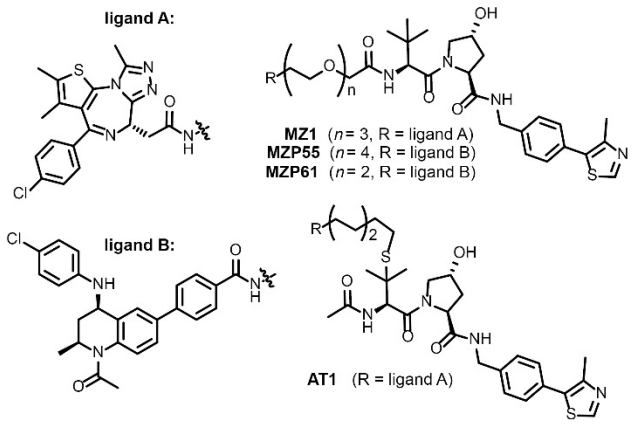


Figure 1. Schematic of SPR approach (A-B) and representative binding data (C).

Using a Biacore T200 SPR instrument and streptavidin-immobilised biotin-VHL, we measured the kinetics and affinity of VHL binding for a concentration series of either PROTAC alone (to form binary complex with VHL, K_D^{binary}) or PROTAC pre-incubated with saturating concentrations of target protein (to form ternary complex with VHL, $K_D^{ternary}$) (Figure 1). Experiments were performed in either multi-cycle (binary) or single-cycle (ternary) format without regeneration, to ensure a maximally stable surface and reduce experimental run-times for PROTAC:BD complexes exhibiting slow dissociation kinetics.[21] Doubly-referenced replicate data were fitted globally (over multiple surface densities where applicable) to a 1:1 Langmuir binding model incorporating a parameter for mass transfer effects, to determine kinetic constants (k_{on}, k_{off}) from which dissociation constants were calculated ($K_D = k_{off} / k_{on}$). Cooperativity (α) was calculated as the ratio $K_D^{binary}/K_D^{ternary}$.

To benchmark the assay, we utilised the PROTAC MZ1, which forms a highly cooperative ternary complex with VHL and Brd4^{BD2} that we have previously characterised both biophysically and structurally.[2, 7, 10] In addition to MZ1, we selected three other PROTACs with ITC-measured affinities for ternary complex formation with VHL/BET bromodomains (Chart 1).[2, 10] Combined, these encompass a range of both binary binding affinities and ternary complex cooperativities (both positive and negative). This set includes MZ1 and AT1, two positively-cooperative PROTACs based on a triazolodiazepine BET inhibitor JQ1 (Ligand A, Chart 1), as well as MZP55 and MZP61, two negatively-cooperative PROTACs based on a more potent tetrahydroisoquinolone BET inhibitor I-BET726 (ligand B, Chart 1).

Chart 1. PROTACs utilised in this study.



SPR binding studies were performed with MZ1, AT1, MZP55 and MZP61 alone (binary) or in complex with Brd4^{BD2} as a representative BET bromodomain (ternary), and binding data compared to the ITC-obtained values (Table 1, Supplementary Table S1, Figure S1).[2, 10] For the panel of PROTACs evaluated, the measured K_D values for binary and ternary complexes, as well as the calculated values for the both cooperativity and change in complex stability (ΔΔG), were remarkably comparable using either ITC or SPR (Table 1). For MZP61 and MZP55 some nonspecific effects were observed for binary binding to VHL (Supplementary Figure S1); in the case of MZP61 these effects were sufficiently pronounced as to preclude accurate kinetic fitting, so steady state fitting was used to measure K_D^{binary}.

Table 1: Binding of PROTACs or PROTAC:Brd4^{BD2} complexes to immobilized VHL (SPR) and comparison to ITC data.

		SPR (VHL)[a]				ITC (VHL)[2, 10]		
PROTAC	**+ target**	**K_D (nM)**	**$t_{1/2}$ (s)**	**α**	**ΔΔG[b] (kcal/mol)**	**K_D (nM)**	**α**	**ΔΔG[b] (kcal/mol)**
MZ1	binary	29	43	-	-	66[d]	-	-
	Brd4^{BD2}	1	130	22	–1.8	3.7[d]	17.8[d]	–1.7
AT1	binary	110	17	-	-	335[d]	-	-
	Brd4^{BD2}	24	-	4.7	–0.9	46[d]	7.3[d]	–1.2
MZP55	binary	69	48	-	-	109[e]	-	-
	Brd4^{BD2}	185	1	0.4	+0.6	183[e]	0.6[e]	+0.3
MZP61	binary	104[c]	-	-	-	116[e]	-	-
	Brd4^{BD2}	465	1	0.2	+0.9	781[e]	0.1[e]	+1.1

[a] Refer to Supplementary Table S1 for full details.
[b] Difference in Gibbs free energy of binding for ternary complex relative to binary.
[c] Fitting by steady state affinity. [d] Literature value (ref. [2]). [e] Literature value (ref. [10]).

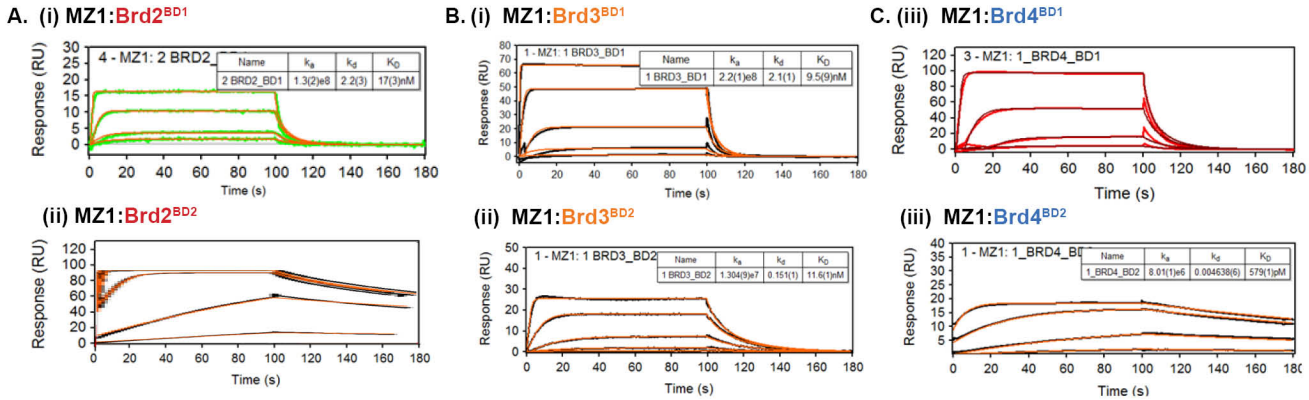


Figure 2. SPR sensorgrams for MZ1:BD complexes binding to immobilized VHL.

Relative to the binary equilibria, the VHL:MZ1:Brd4^{BD2} ternary complex displayed both a faster k_{on} and slower k_{off}, leading to the tighter $K_D^{ternary}$ and significant positive cooperativity ($\alpha \approx 20$) (Figure 1C, Table 1, Supplementary Table S1). As anticipated, the VHL:AT1:Brd4^{BD2} ternary complex also exhibited positive cooperativity ($\alpha \approx 5$). In stark contrast, ternary complexes formed by either of MZP55 and MZP61 with Brd4^{BD2} had very fast dissociation kinetics (>80-fold faster than the VHL:MZ1:Brd4^{BD2} complex) reflecting overall negative cooperativity, with the most negatively-cooperative ternary complex being formed by MZP61 (Supplementary Table S1, Figure S1). Whilst we have shown that both the ITC and SPR approaches are complimentary and yield similar values, the notable advantages of our SPR method are increased throughput and yielding kinetic information, including estimates of the lifetime of the ternary complex. This information is invaluable to better understand PROTAC function and ternary complex stability in an enzymatic context,[4] analogously to how quantification of inhibitor residence time has helped to understand pharmacological function in certain occupancy-driven small-molecule contexts.[22]

MZ1 has been shown to degrade Brd4 more potently than Brd2 or Brd3 (despite near equipotent binding of the constituent warhead ligand to all BET bromodomains),[7, 10, 23] resulting from the high thermodynamic stability of the VCB:MZ1:Brd4^{BD2} complex.[2] We sought to quantify the overall kinetics of VHL:PROTAC:BD ternary complexes for the different BET bromodomains (Figure 2, Supplementary Table S2, Figures S2, S3). Ternary complexes consisting of VHL, MZ1 and the first bromodomain (BD1) of either Brd2, Brd3, or Brd4 all displayed very fast dissociation kinetics ($t_{1/2} < 1$ sec), resulting overall in either low positive cooperativity or no cooperativity ($\alpha \approx 1$). In the case of the second bromodomains (BD2), we were struck by the significantly longer ternary half-life of the VCB:MZ1:BD ternary complexes with Brd2^{BD2} and Brd4^{BD2} ($t_{1/2} \approx 70$ and 130 sec respectively) relative to the more short-lived ternary complex with Brd3^{BD2} ($t_{1/2} \approx 6$ sec).

Overlay of crystal structures of either Brd2^{BD2} (PDB: 3ONI) or Brd3^{BD2} (PDB: 3S92) in complex with JQ1 and the VCB:MZ1:Brd4^{BD2} structure (PDB: 5T35),[2] suggested that ternary complex formation could be influenced by a single amino acid residue difference (Glu^{344} within the ZA loop of Brd3^{BD2}, which corresponds to Gly^{382} and Gly^{286} in Brd2^{BD2} and Brd4^{BD2}) (Figure 3A). In an equivalent VCB:MZ1:Brd3^{BD2} complex, the side-chain of Glu^{344} would induce severe steric clash with the VHL:MZ1 portion of the complex, leading to destabilisation. We therefore generated reciprocal point mutant swaps and measured ternary complex formation by SPR with MZ1 or AT1. In all cases, the resulting SPR binding profiles reflected the effect predicted for the point mutation. The G-to-E point mutation in Brd4^{BD2} or Brd2^{BD2} shortened the ternary complex half-life, decreasing cooperativity and complex stability; whilst the reverse point mutation in Brd3^{BD2} extended the ternary half-life to resemble the profile for Brd4^{BD2} and correspondingly increased cooperativity and stability (Figure 3B, Supplementary Table S2, Figure S4). As a cross-validation, we evaluated these complexes in a competitive fluorescence polarization (FP) assay; measuring VHL binding of PROTAC or a PROTAC:BD binary complex, *via* displacement of a fluorescent HIF-1α peptide probe (Supplementary Table S2, Figure S5). Good correlation was observed between SPR-fitted dissociation constants (K_D) and FP-derived inhibition constants (K_I) (Figure 4A). Cooperativity values were also comparable using either approach (Supplementary Table S2). Together, these results underscore the robustness of our SPR approach, and further support the conclusion that the described VCB:MZ1:Brd4^{BD2} structure (PDB: 5T35)[2] reflects the predominant (long-lived) species present in solution.

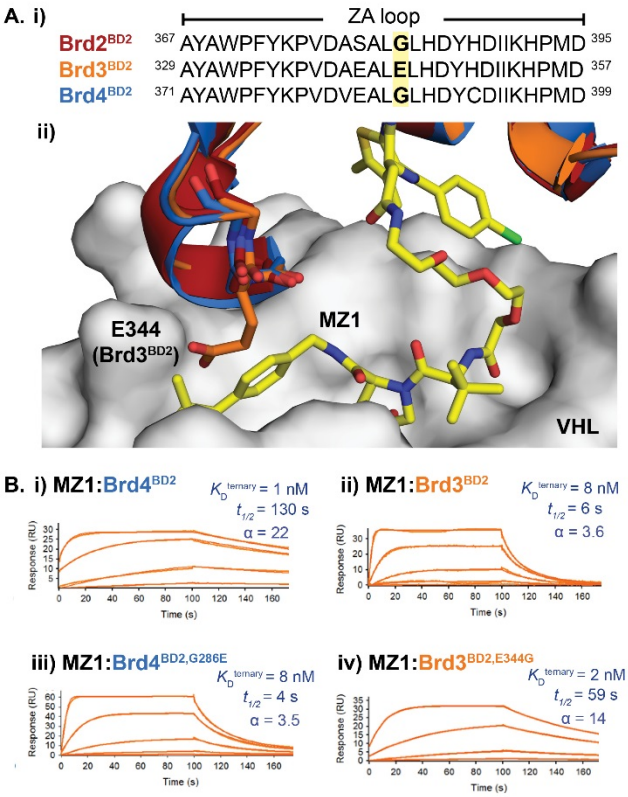


Figure 3. A. Overlay of Brd2^{BD2} (PDB: 3ONI) and Brd3^{BD2} (PDB: 3S92) with the crystal structure of the VCB:MZ1:Brd4^{BD2} ternary complex (PDB: 5T35) suggests the VHL:MZ1:Brd3^{BD2} ternary complex could be destabilized by steric clash with VHL:MZ1 of a single amino acid within the ZA loop. B. Reciprocal exchange of this single Gly/Glu residue in Brd4^{BD2} (i, iii) or Brd3^{BD2} (ii, iv) yields a corresponding swap of the kinetic profile in the resulting VHL:MZ1:BD ternary complex SPR sensorgram.

These SPR data illustrate kinetically a mechanistic difference between different PROTAC 'architypes'.[10] On the one hand, MZP55 and MZP61 are PROTACs with high binary target affinity (for Brd4), but low or negative cooperativity, thus likely forming highly-populated binary complexes but a very transient ternary complex. In contrast, MZ1 and AT1 exhibit weaker binary target affinity (for Brd4), but this is compensated for in the case of Brd4^{BD} by significant positive cooperativity (to form stable ternary complexes). This latter case is predicted to fit a 'rapid equilibrium' kinetics model, where a rate-limiting ubiquitination step is dependent on the concentration of PROTAC-induced ternary complex.[4] In this regime, an extension in ternary complex stability, and hence lifetime, would be expected to increase rates of target protein ubiquitination and degradation, particularly at early time points prior to countervailing factors such as protein resynthesis or feedback mechanisms.

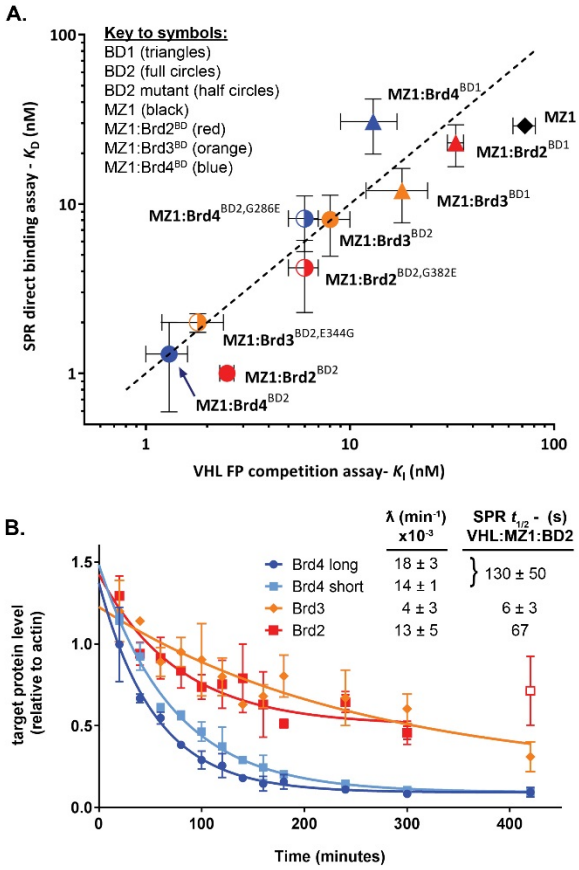


Figure 4. A. Correlation between binary (MZ1) and ternary (MZ1:BD complex) binding to VHL *via* SPR or FP (mean ± SD). B. Initial degradation profile for BET proteins in HEK293 cells in response to MZ1 treatment (333 nM), with initial degradation rates (λ) estimated from data fitting (mean ± SEM, N = 3) and SPR ternary half-lives for corresponding VHL:MZ1:BD2 complexes (mean ± SD for N=2).

We wished to examine this possibility in a cellular context, in light of the greater stability of ternary complexes of MZ1 with $Brd2^{BD2}$ or $Brd4^{BD2}$ and VHL, relative to the equivalent complex with $Brd3^{BD2}$. Time-course studies were performed to measure initial rate of degradation of Brd4, Brd3, or Brd2 in response to treatment of HEK293 cells with MZ1 (Figure 4B). We observed rapid degradation of Brd2 and both isoforms of Brd4, whilst degradation of Brd3 was significantly slower (Figure 4B, Supplementary Table S3). Recent results by an independent group, published whilst this manuscript was under preparation, strongly support our conclusion.[23] Consistent with our data, Riching *et. al.* observed more rapid initial rates of ubiquitination and degradation of Brd2 and Brd4 than Brd3 in response to MZ1 treatment.[23] Together, these observations strongly suggest a mechanistic link between the relative half-life of a given target-PROTAC-E3 ligase ternary complex and initial rates of target degradation, which drives a faster and more profound target depletion in cells.

In conclusion, we demonstrate a simple and robust SPR-based method to quantify for the first time the stability of target-PROTAC-ligase ternary complexes by measuring the kinetics of their formation and dissociation. We demonstrate that our surface-based SPR method yields values for affinity, cooperativity (α) and complex stability comparable to ITC in solution,[2, 10] with increased throughput and yielding additional kinetic information not achievable using other assays described to date. We show by SPR that a single residue can impart significant changes in cooperativity, stability and dissociative half-lives of ternary complexes formed with different but highly conserved target proteins. Lastly, we observe that these kinetic differences of ternary complexes correlate to relative initial rates of target degradation. Together, these findings establish a new assay for PROTAC ternary complex kinetics and illuminate on ternary complex stability and dissociative half-lives as key optimization parameters for PROTAC design and discovery campaigns. We anticipate that our SPR kinetic assay will become an established tool to drive PROTAC development and to further elucidate dynamic processes governing their mode of action. Beyond PROTACs, this assay could be applied more broadly to study three-body binding equilibria induced by other classes of heterobivalent molecules.[24]

ASSOCIATED CONTENT

Supporting Information. Supplementary results (Tables S1–S3 and Figures S1–S5); supplementary materials and methods sections; supplementary references.

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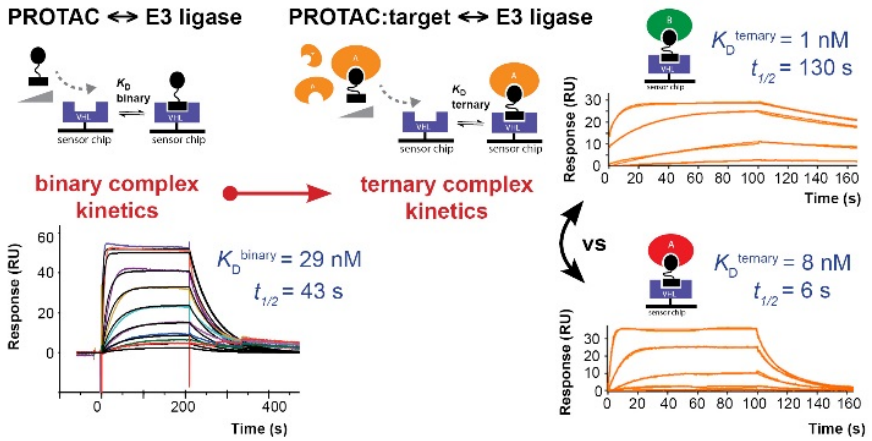
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