A Bespoke Screening Platform to Study Mono(ADP-Ribosylation)

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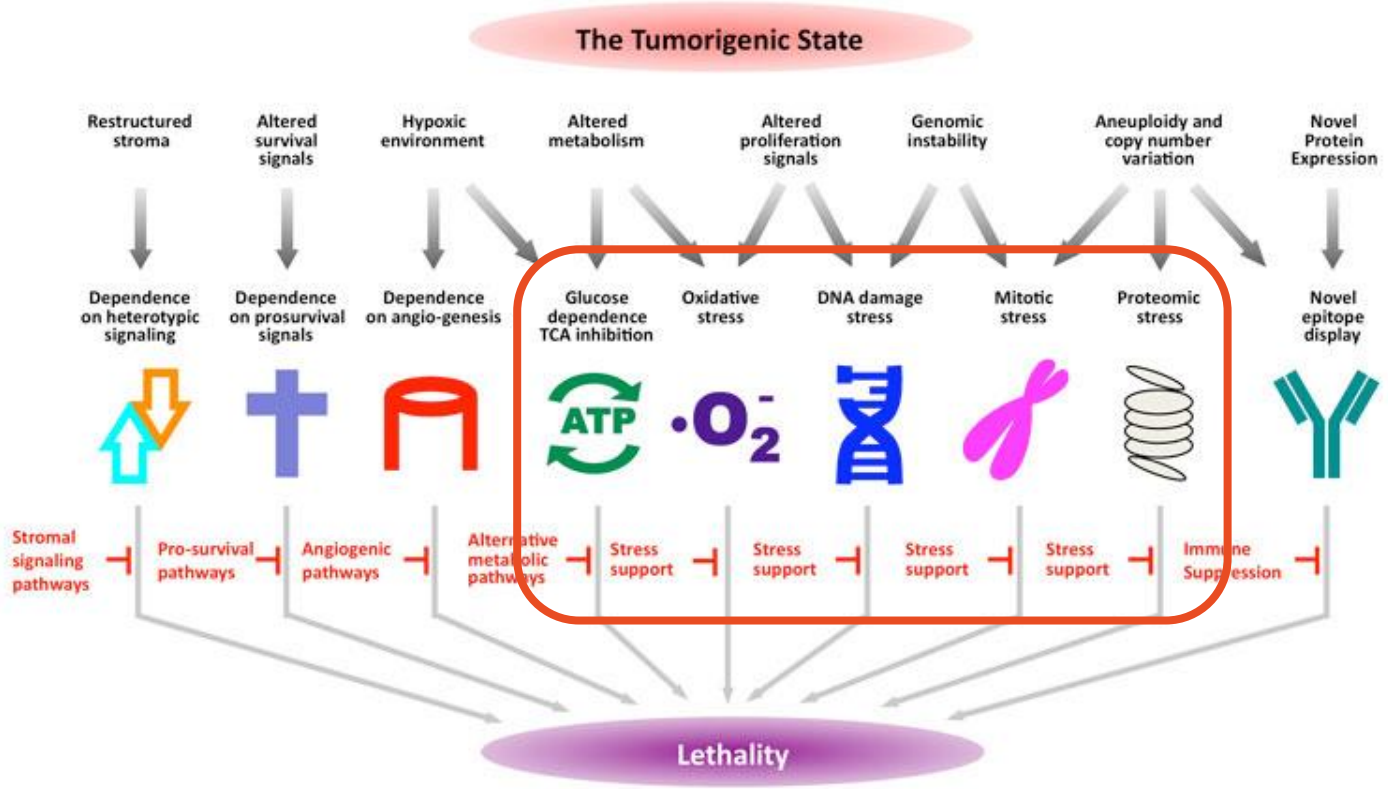
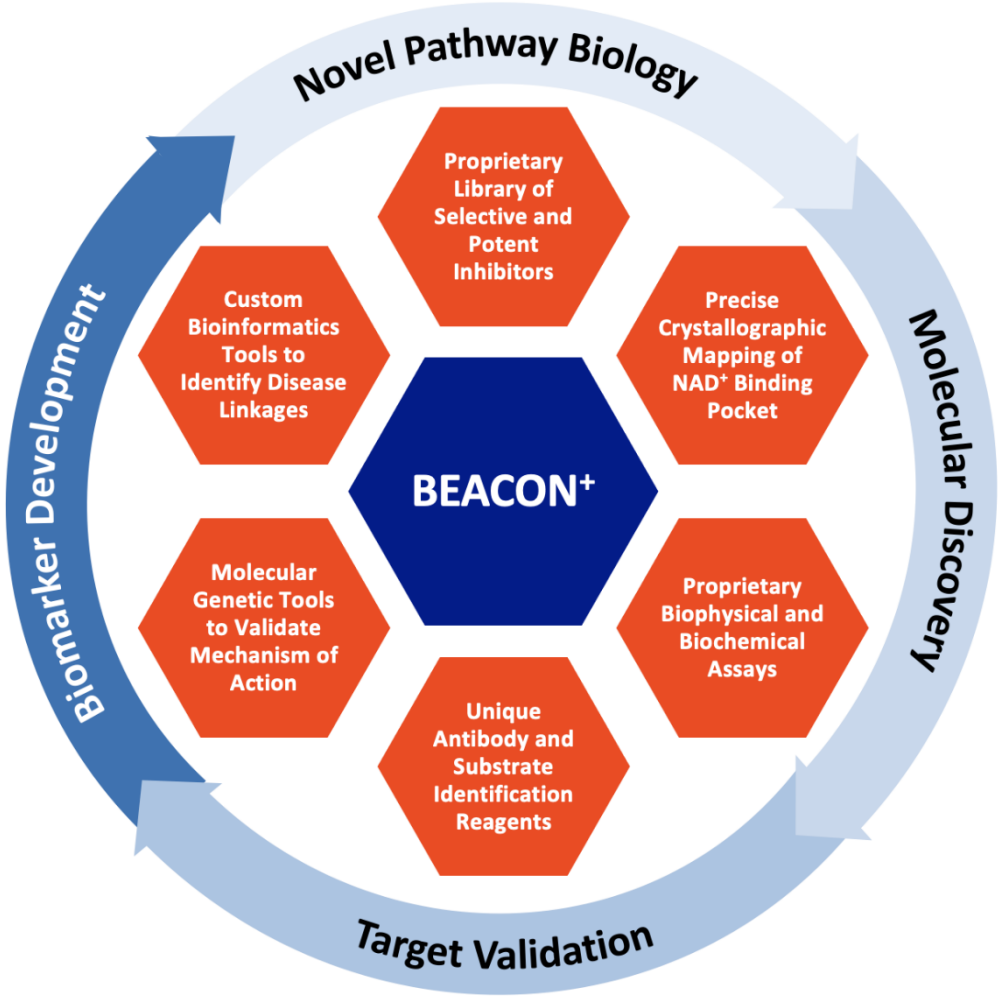
Blocking Cancer's Fundamental Stress Response to Develop New Therapies

- The "tumorigenic state" creates multiple types of stress and cancers depend on overcoming stress to survive
- Blocking stress support pathways represents a new approach to treat cancer
- NAD^+-using enzymes, e.g., **PARPs,** have evolved to regulate stress pathways



Luo et al, *Cell* (2009)

Proprietary BEACON$^+$ Platform: Unlocking the Biochemical Roles of NAD$^+$-Utilizing Enzymes for the Treatment of Cancer



Blocking the **E**nzyme **A**ctivity **C**omponent **O**f **N**AD$^+$

- **Proprietary collection of biochemical tools and technologies** to elucidate NAD$^+$-utilizing enzyme biology
- **Proprietary small molecule library** from which to design and develop selective and potent NAD$^+$-utilizing enzyme inhibitors
- **Broad library of crystal structures** to precisely map the NAD$^+$ binding pocket
- **Custom bioinformatics analyses** to identify novel therapeutic targets and their linkage to cancer
- **Sophisticated molecular genetic tools** to validate drug mechanism of action

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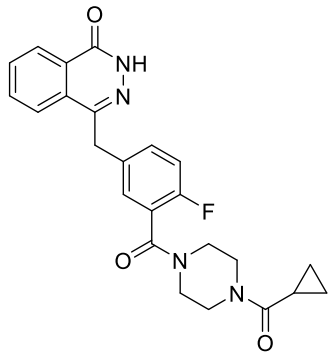
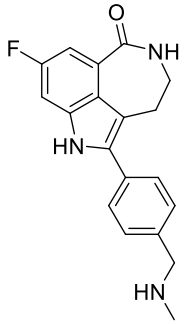
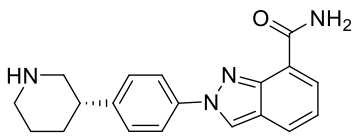
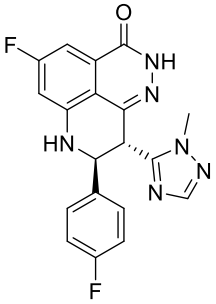
Common “PARP” Misconception

“PARP” Inhibitor =



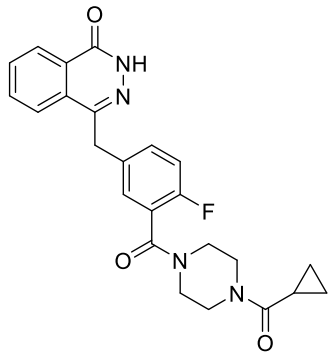


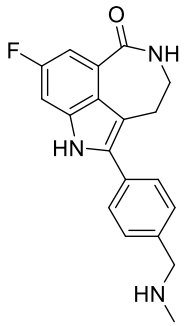


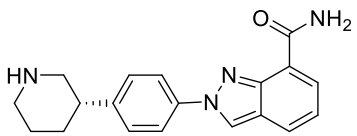


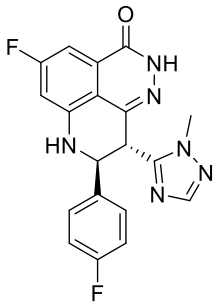
Setting the Record Straight on “PARP” Inhibitors

“PARP1/2” Inhibitor =

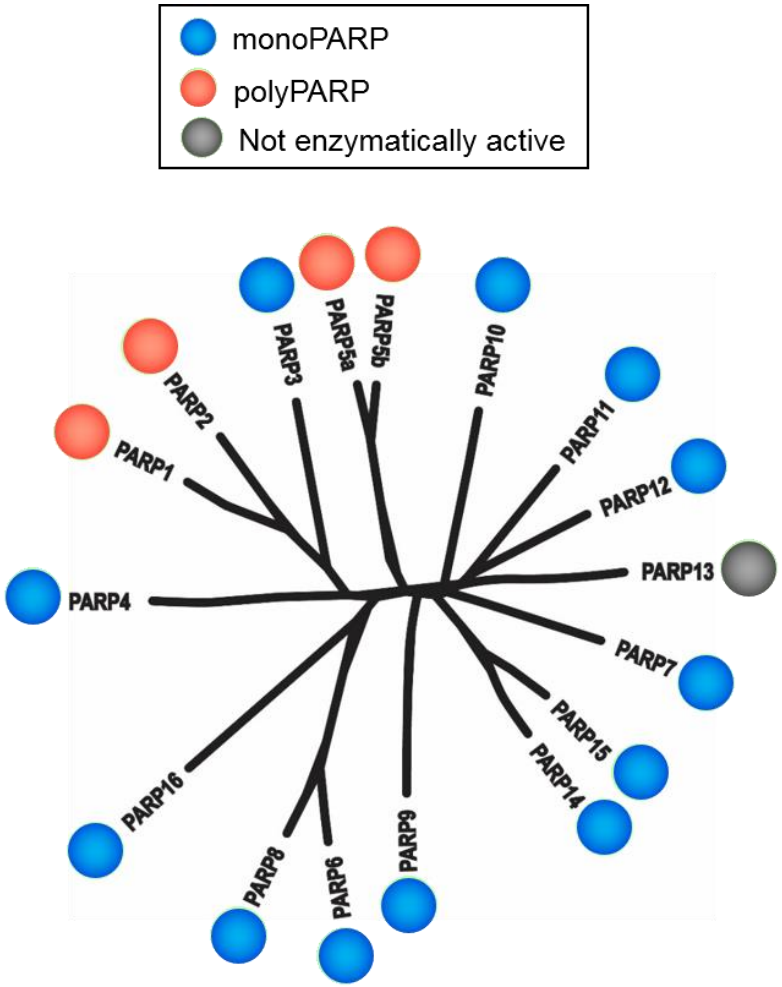


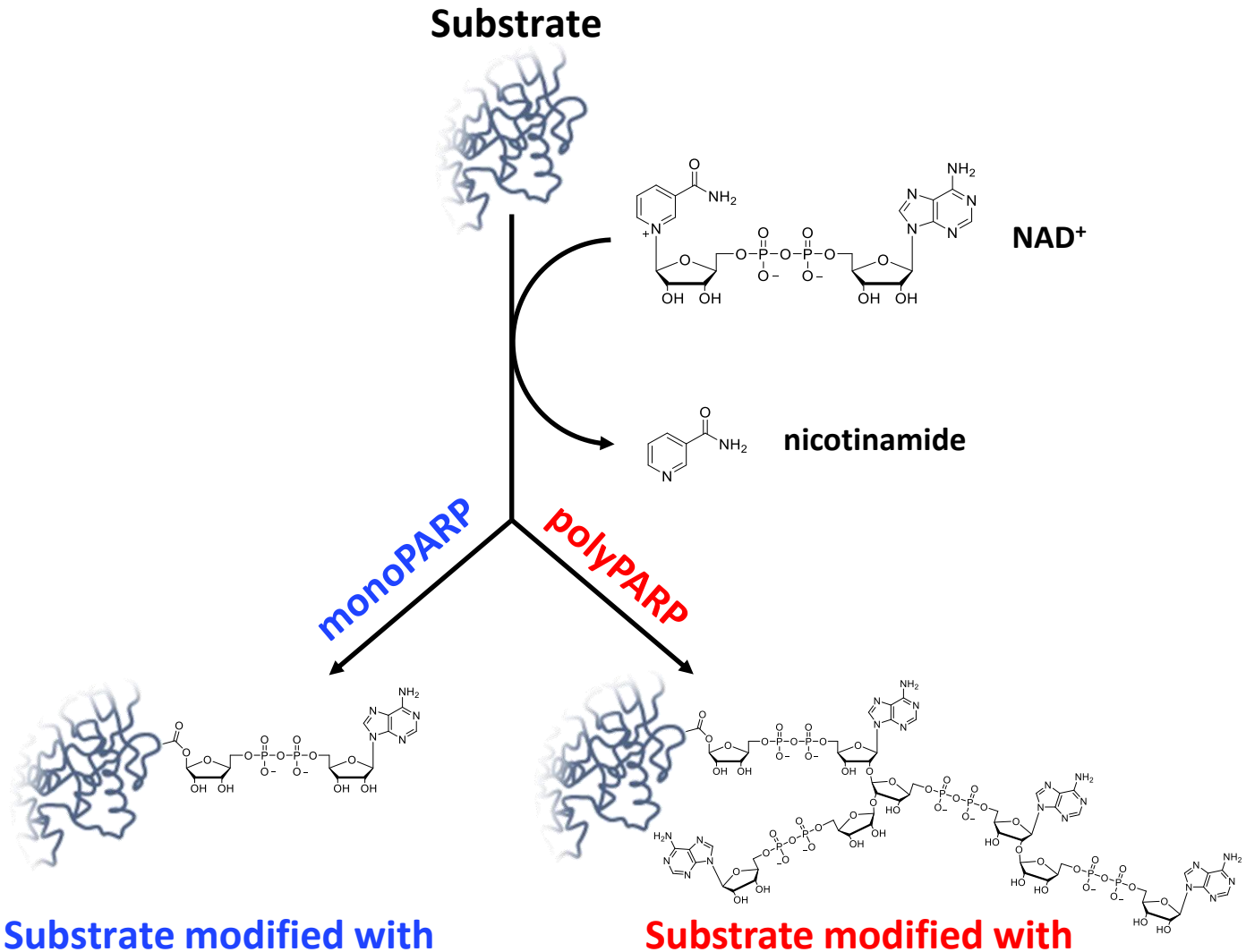




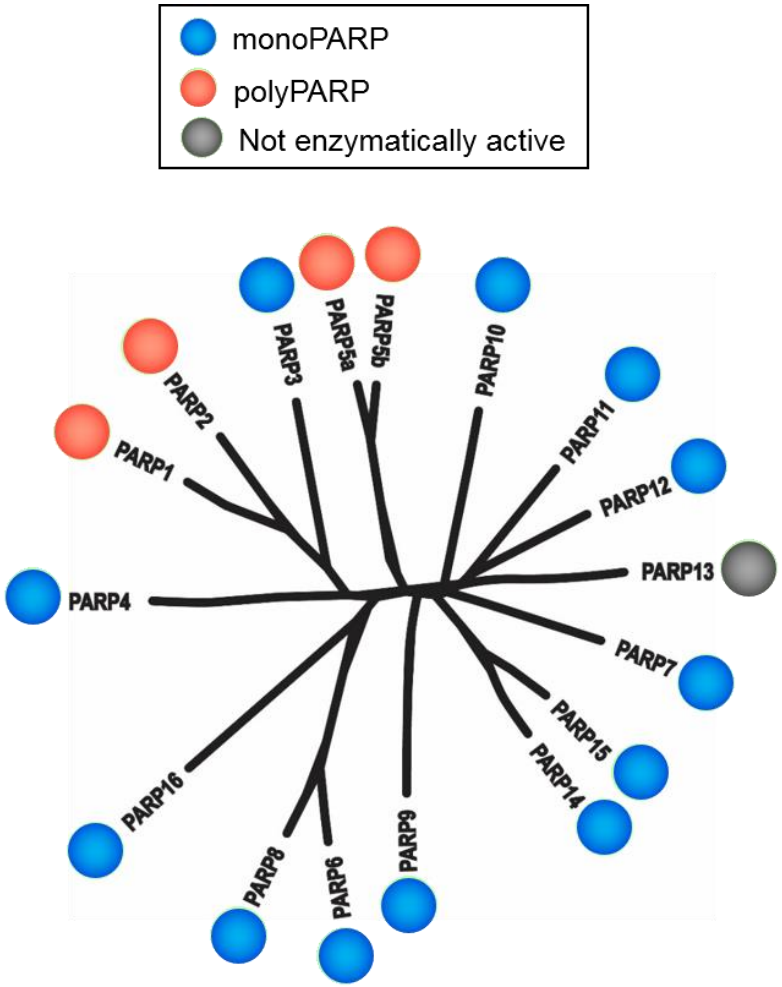


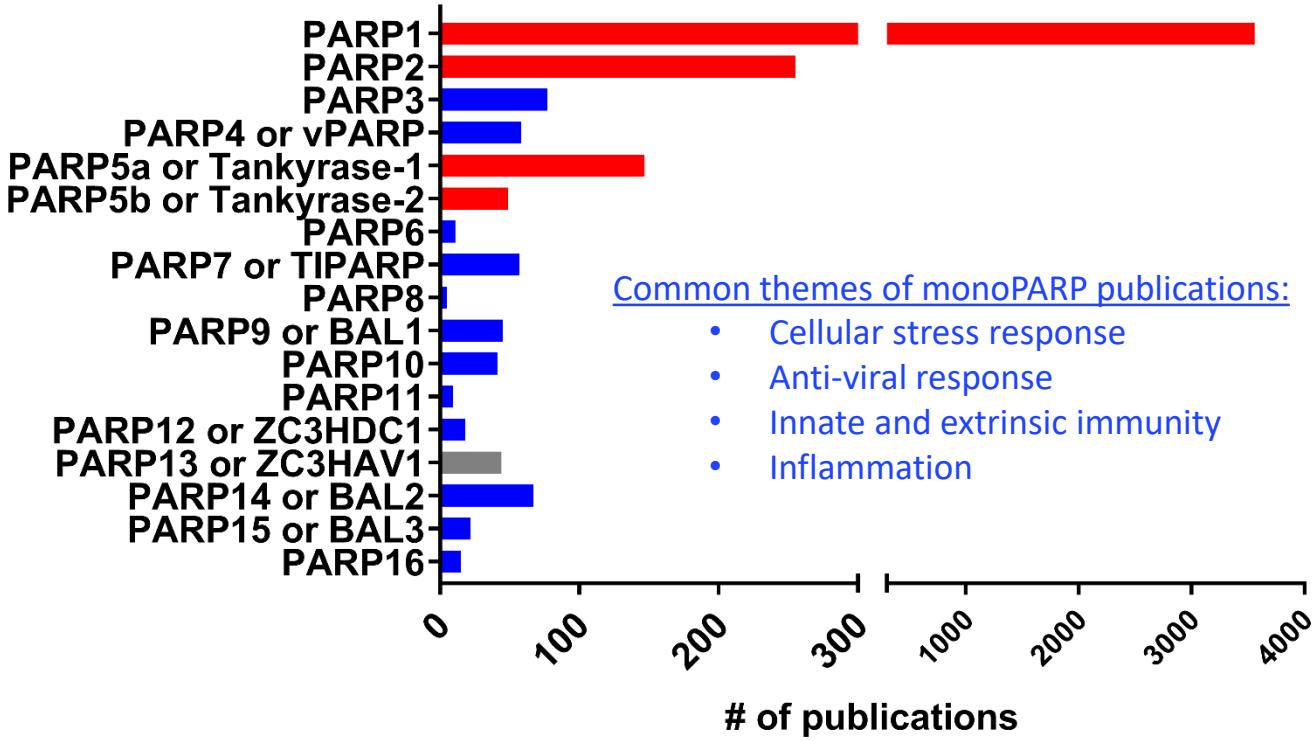
The PARP Enzyme Family is Sub-Divided Based on the Type of ADP-Ribosylation Performed





MonoPARPs Are an Underexplored Enzyme Class





Common themes of monoPARP publications:

- Cellular stress response
- Anti-viral response
- Innate and extrinsic immunity
- Inflammation

Potent and selective inhibitors would facilitate studies on role of monoPARPs in human diseases

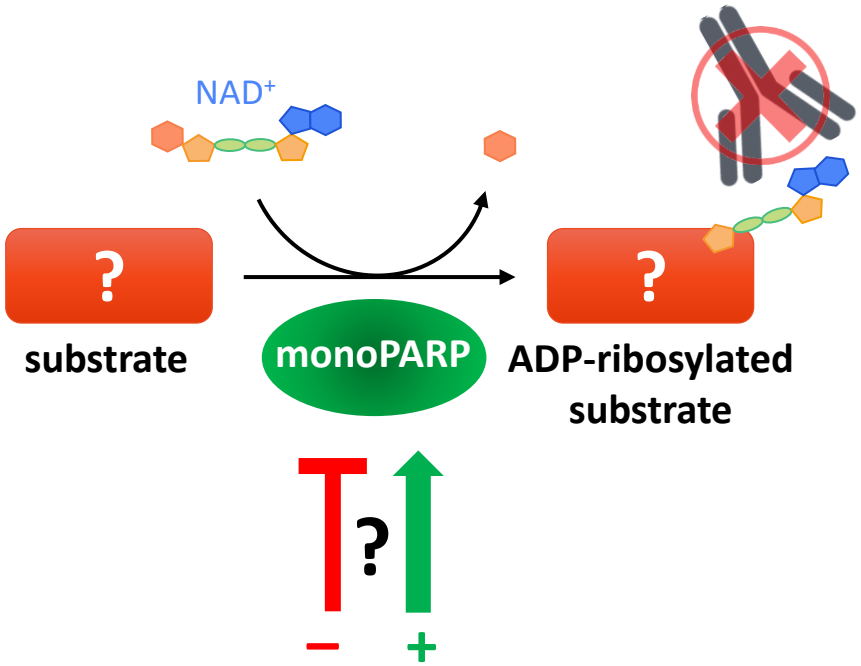
→ **Screening assays needed**

Wigle et al., *SLAS Discovery* (2019)

Challenges Associated with Developing Biochemical & Cell-Based Assays to Screen MonoPARP Enzymes

Lack of validated substrates for in vitro enzyme assays

- No X-ray or NMR structures of monoPARPs bound to substrates exist in PDB
- Reported recombinant protein substrates mixed with enzyme are not modified to detectable levels
- Reported self-modification is virtually undetectable



No selective anti-MAR antibodies for assay development

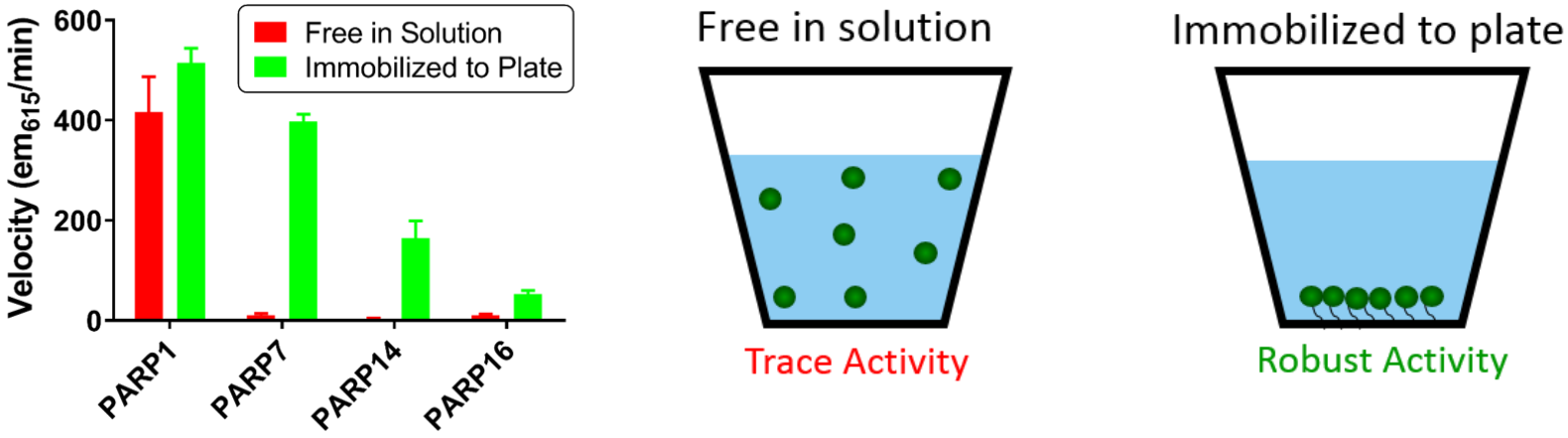
- Unclear what are best antigens and how to make them
- Unclear if antigens are stable in animals
- MAR-binding protein domains ("MAR readers") have modest affinity for MAR and context-dependent binding

Unclear how monoPARPs are activated

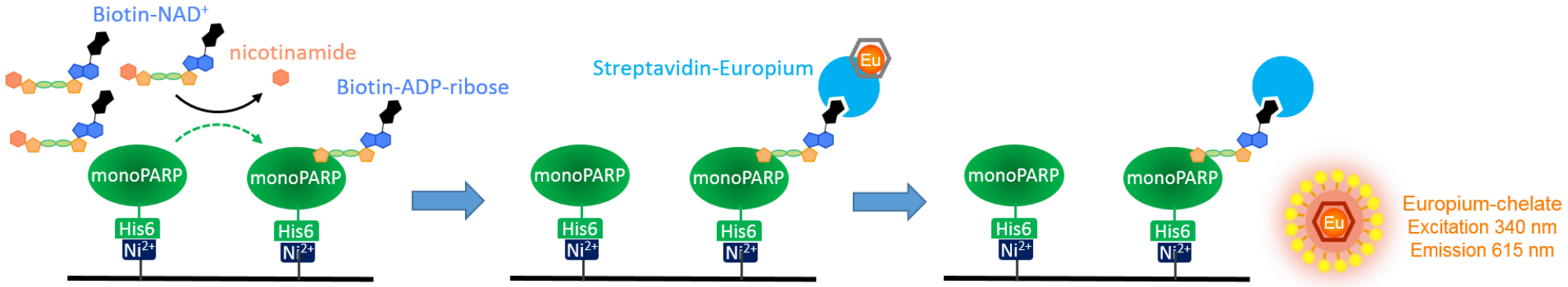
- Cellular stress linked to activation but mechanisms unclear

Strategy for First Generation Biochemical MonoPARP Screening Platform: Forced Self-Modification of Immobilized Enzymes

Immobilization overcomes weak K_M for self-modification



Dissociation Enhanced Lanthanide Fluorescence Immunoassay (DELFIA) of Immobilized MonoPARPs

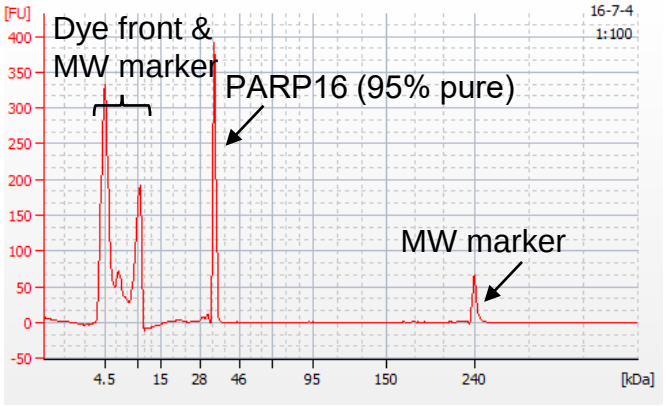


1. Immobilize His6-enzyme to Ni-NTA plate
2. Add biotin-NAD+
3. Wash out unbound biotin-NAD+
4. Add streptavidin-Europium
5. Wash out unbound streptavidin-Europium
6. Detect Europium

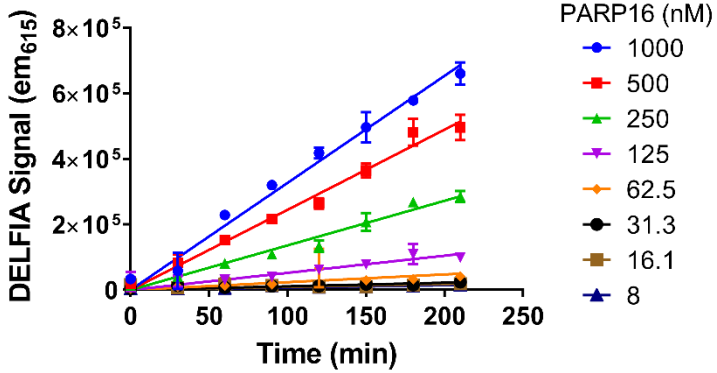
Wigle et al., *SLAS Discovery* (2019)

Example of DELFIA Assay Development for PARP16 Self-Modification

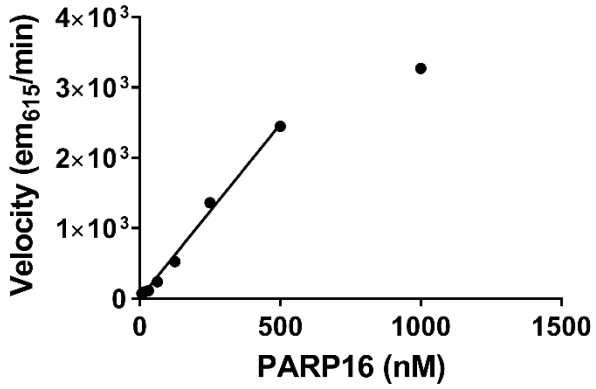
Produced pure protein



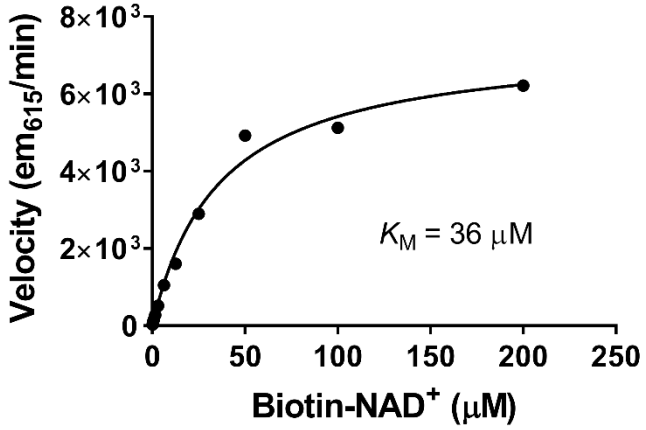
Product formation linearity vs. time



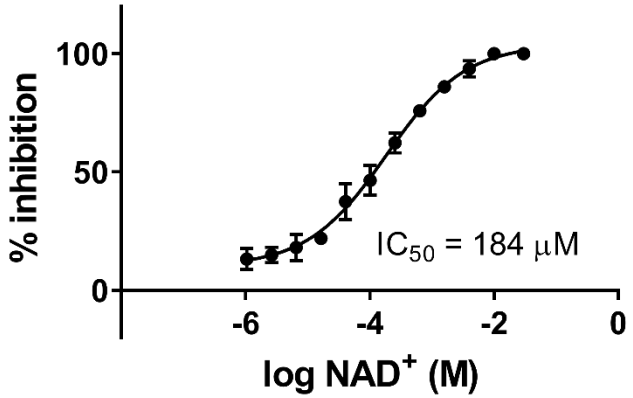
Velocity vs. [enzyme]



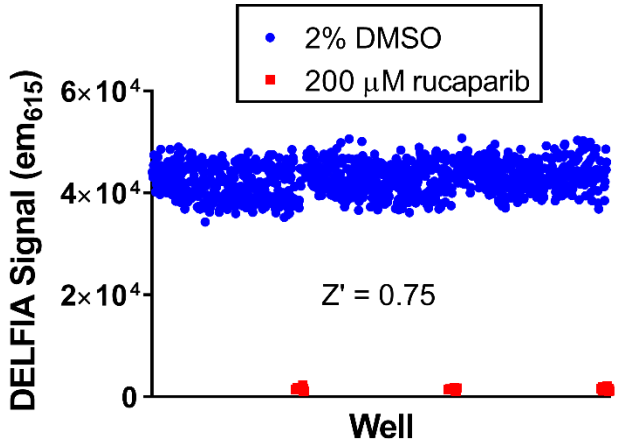
Biotin-NAD$^+$ K_M^{app}



IC_{50} of unlabeled NAD$^+$

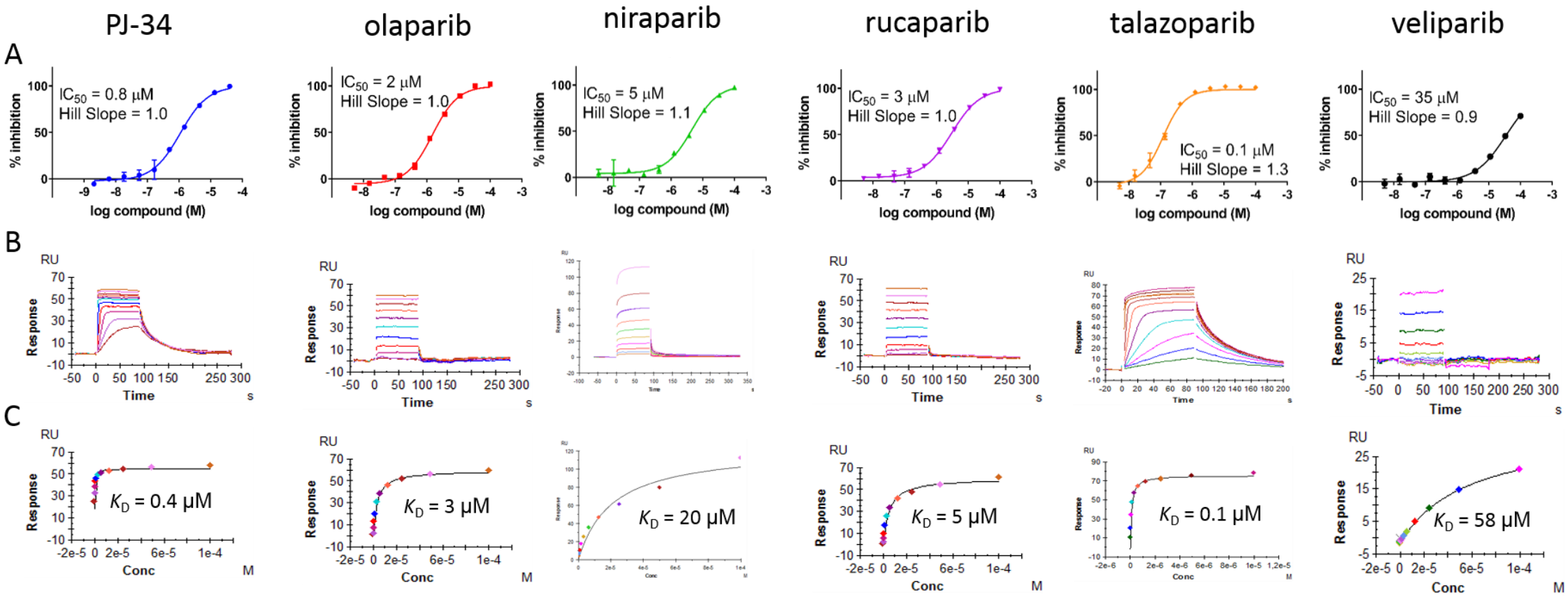


Automation & uniformity



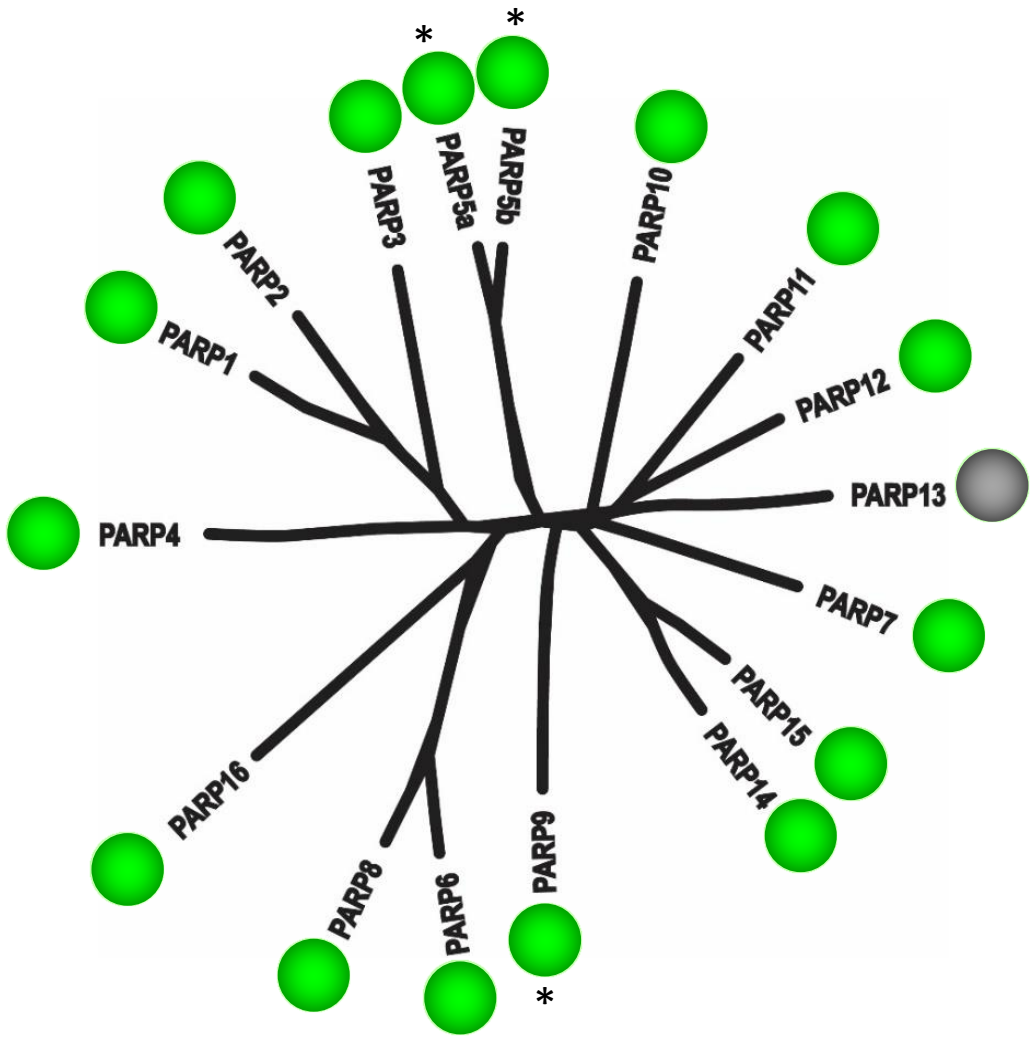
Wigle et al., *SLAS Discovery* (2019)

SPR Shows PARP16 NAD$^+$-Competitive Ligand Binding Affinity Correlates to Enzyme Inhibition



Wigle et al., *SLAS Discovery* (2019)

Self-Modification DELFIA Assays Are a Scalable Approach to Family-Wide PARP Assay Development & Screening

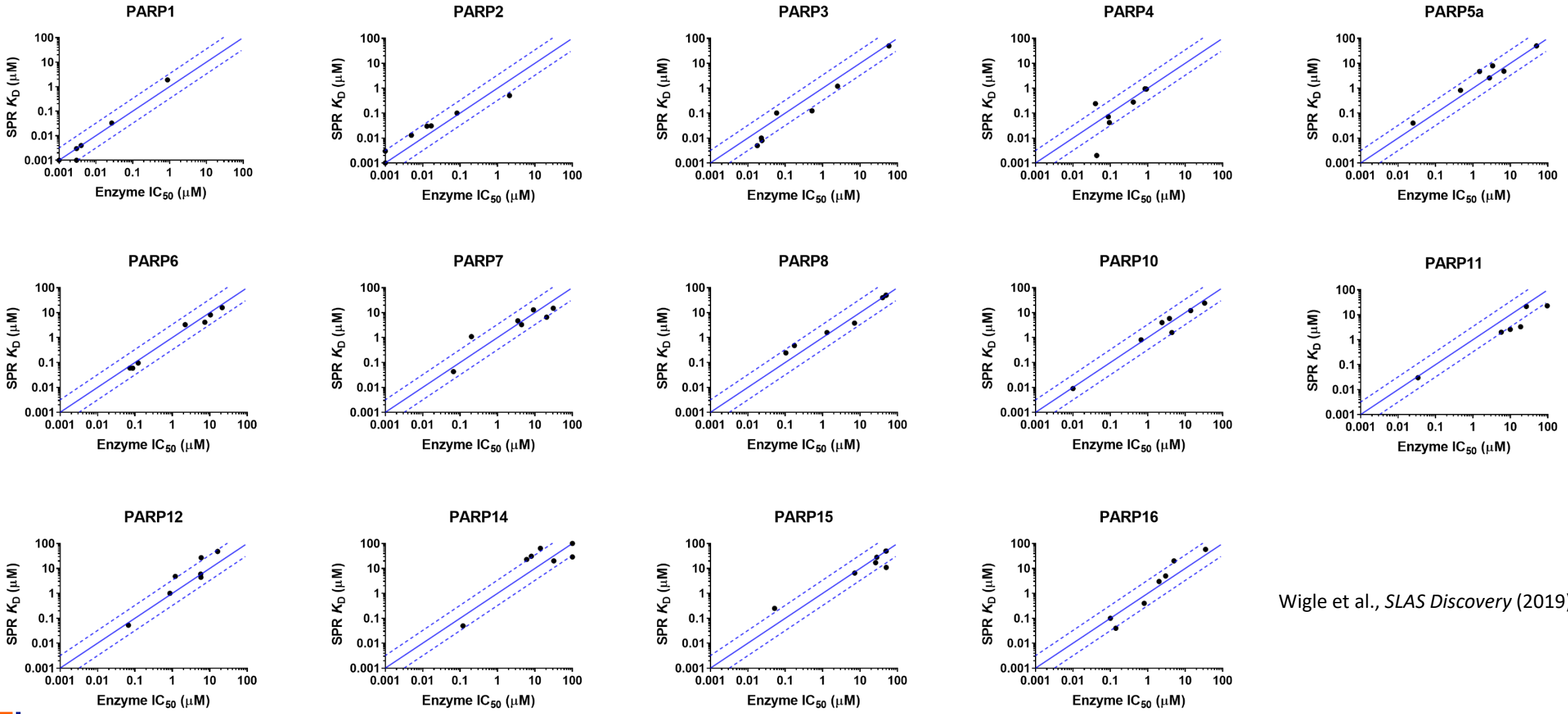


= DELFIA assay developed

= not enzymatically active

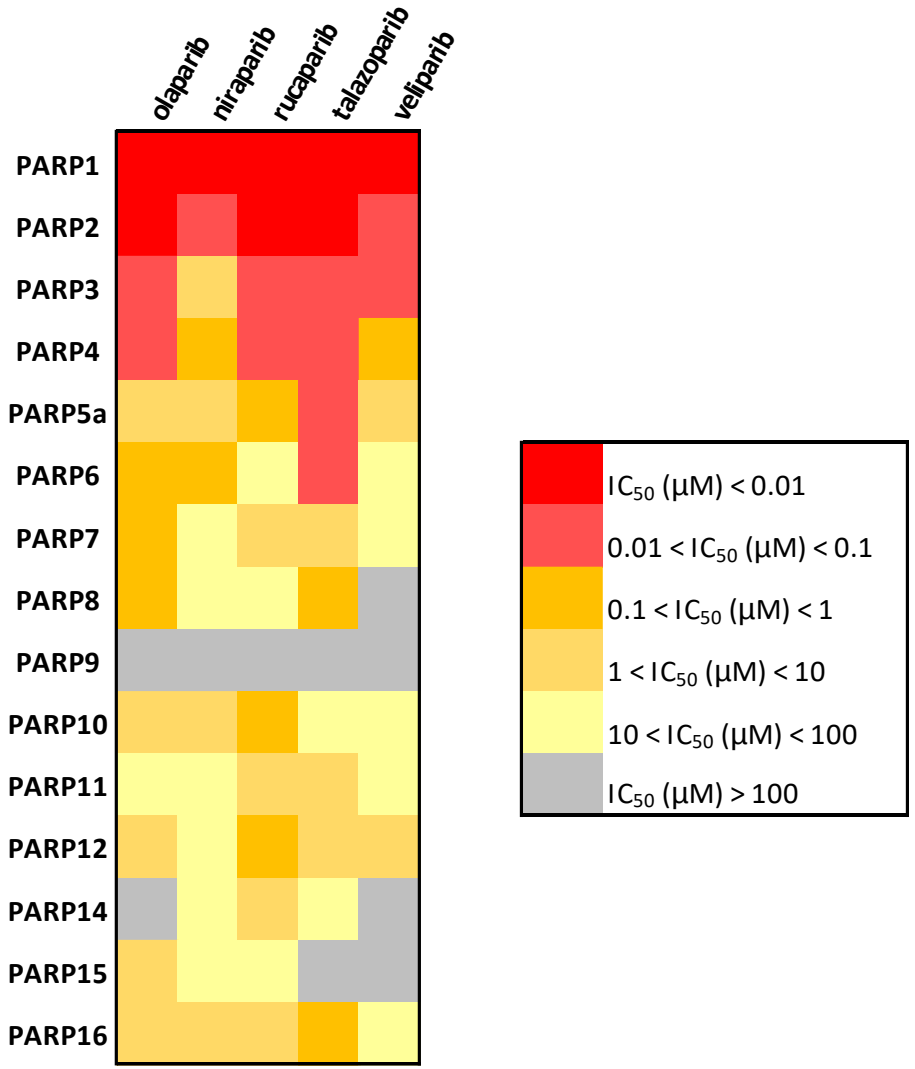
* = assay uses a substrate (not self-modification)

SPR Shows Ligand Binding Correlates Well to Enzyme Inhibition Across the Entire PARP Family



Wigle et al., *SLAS Discovery* (2019)

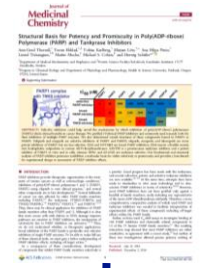
In Vitro Self-Modification Assays Reveal PARP1 and PARP2 Inhibitors Are Not Very Potent Against MonoPARPs



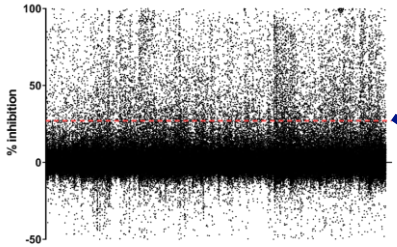
Wigle et al., *SLAS Discovery* (2019)

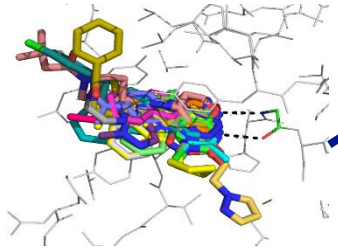
Cross Screening Panel Used to Determine Selectivity of Novel MonoPARP Starting Points

Literature polyPARP inhibitors



HTS of 500,000 compounds vs. PARP14





SPR fragment screen identifies 19 fragments bound to PARP16 (confirmed by X-ray)

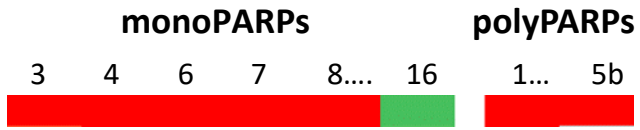
Medicinal Chemistry

Cross Screening Panel

- PARP1
- PARP2
- PARP3
- PARP4
- PARP5a
- PARP16

Every compound is screened against the panel of PARPs to understand the determinants of selectivity and potency

Selective Inhibitor



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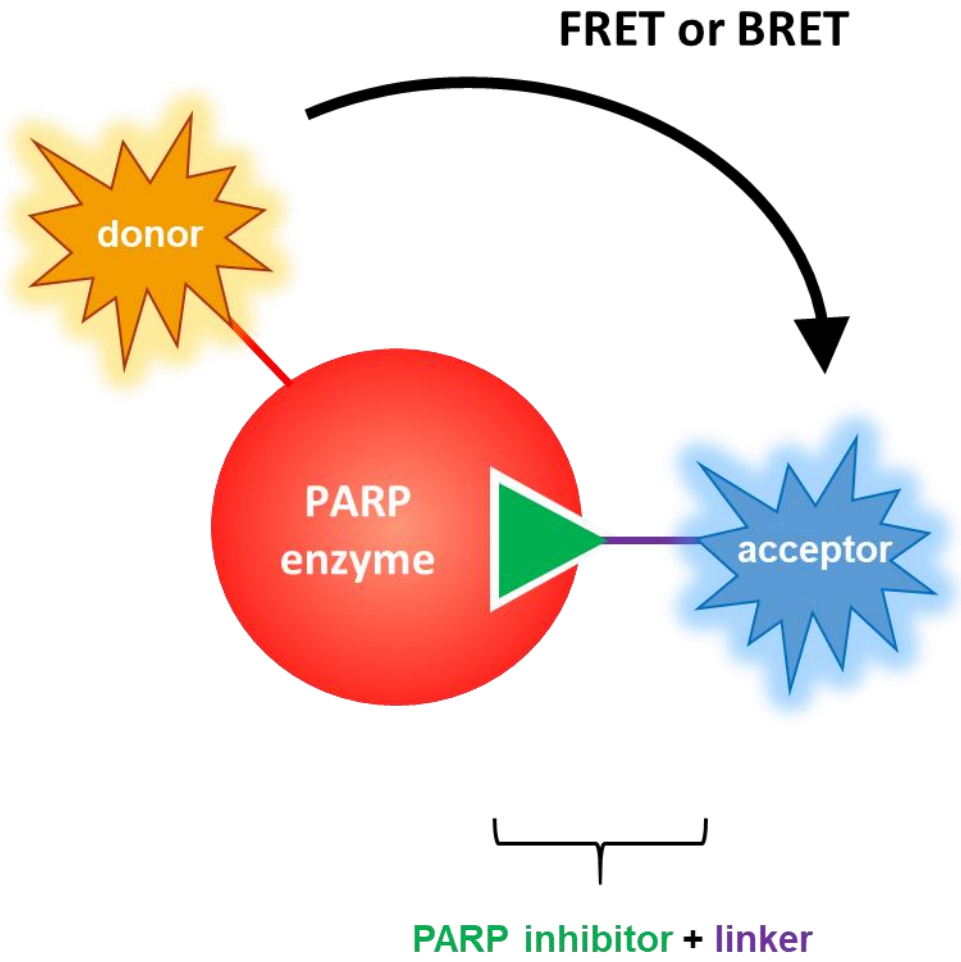
A Limitation of Self-Modification Format: High Amounts of Enzyme Needed Restrict Resolution of Potency

PARP Assay	Enzyme concentration (µM)	Length of Assay (min)
PARP1	0.002	60
PARP2	0.002	120
PARP3	0.0025	120
PARP4	**0.075**	180
PARP5a	0.01	120
PARP6	0.003	180
PARP7	**0.075**	240
PARP8	**0.05**	180
PARP9	0.008	180
PARP10	0.015	180
PARP11	0.008	180
PARP12	0.015	180
PARP14	**0.05**	180
PARP15	0.001	1440
PARP16	**0.15**	180

$$Max\ IC_{50}\ measurable = \frac{[enzyme]}{2}$$

- Some self-modification assays use high amounts of enzyme, limiting the ability to resolve potent inhibitors
- More sensitive in vitro assays are needed
- Cellular assays needed to characterize inhibitors of increasing potency

Investigating Active Site Probe Displacement to Generate More Sensitive Assays

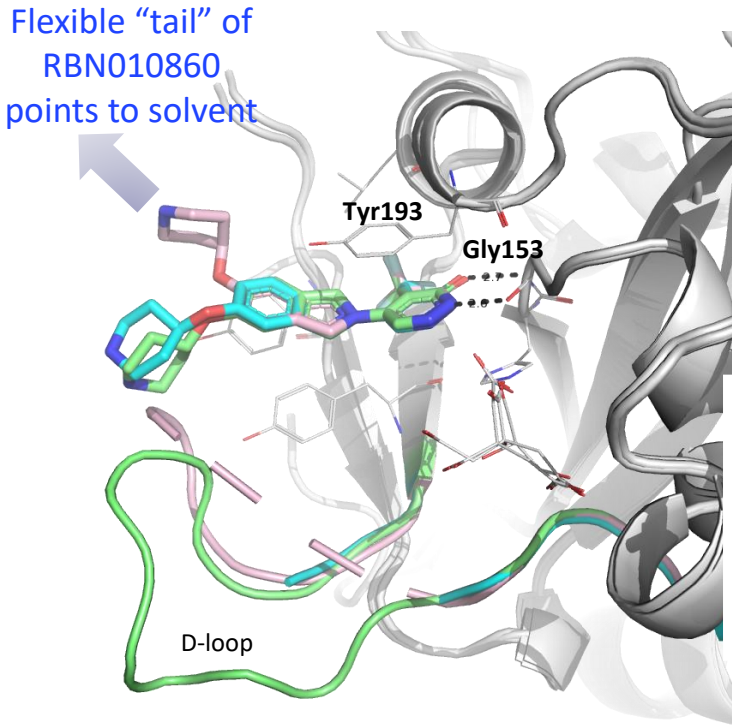


Active Site Probes Designed to Enable Orthogonal Assay Development

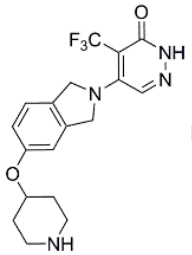
Design of potent monoPARP probe ligands

SPR assays show that probes retain high affinity to most PARPs

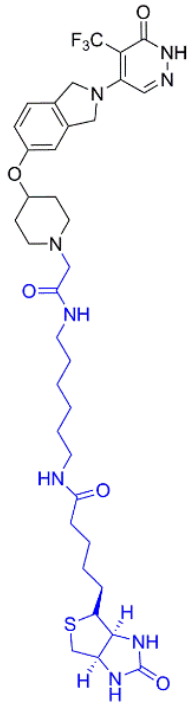
RBN010860/PARP16 (2.1 Å)



RBN010860
Parent

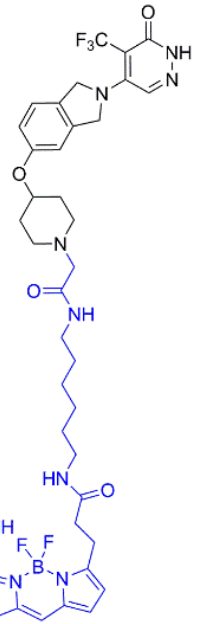


RBN011147
in vitro TR-FRET probe

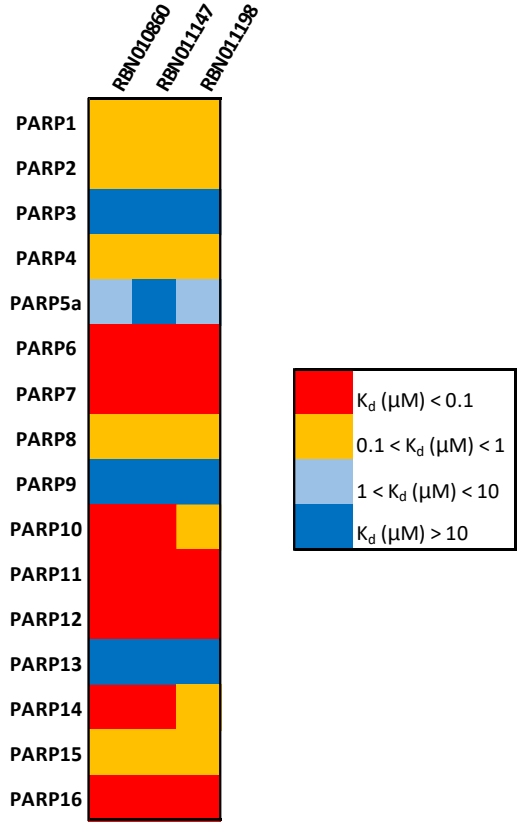


linker + biotin

RBN011198
cellular NanoBRET probe

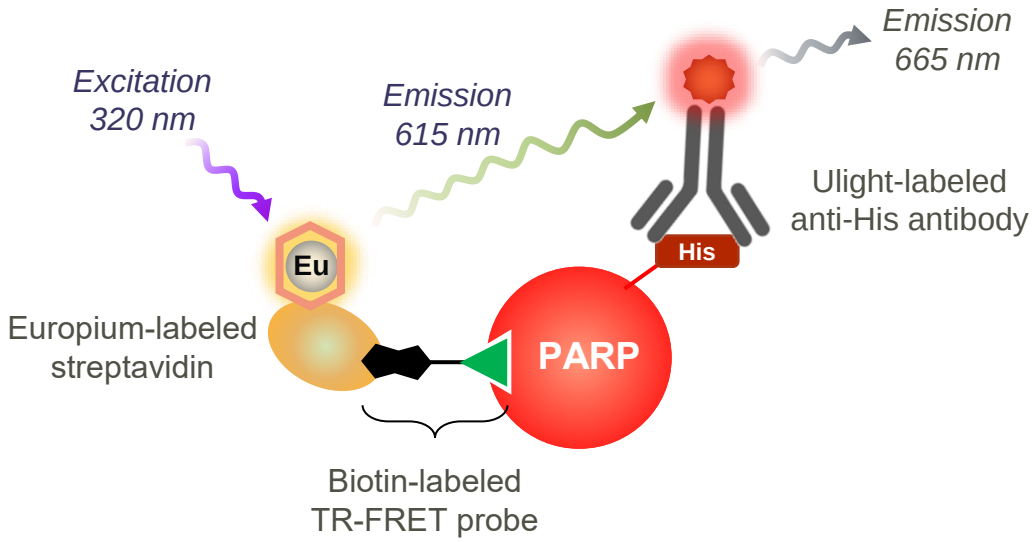


linker + SE590

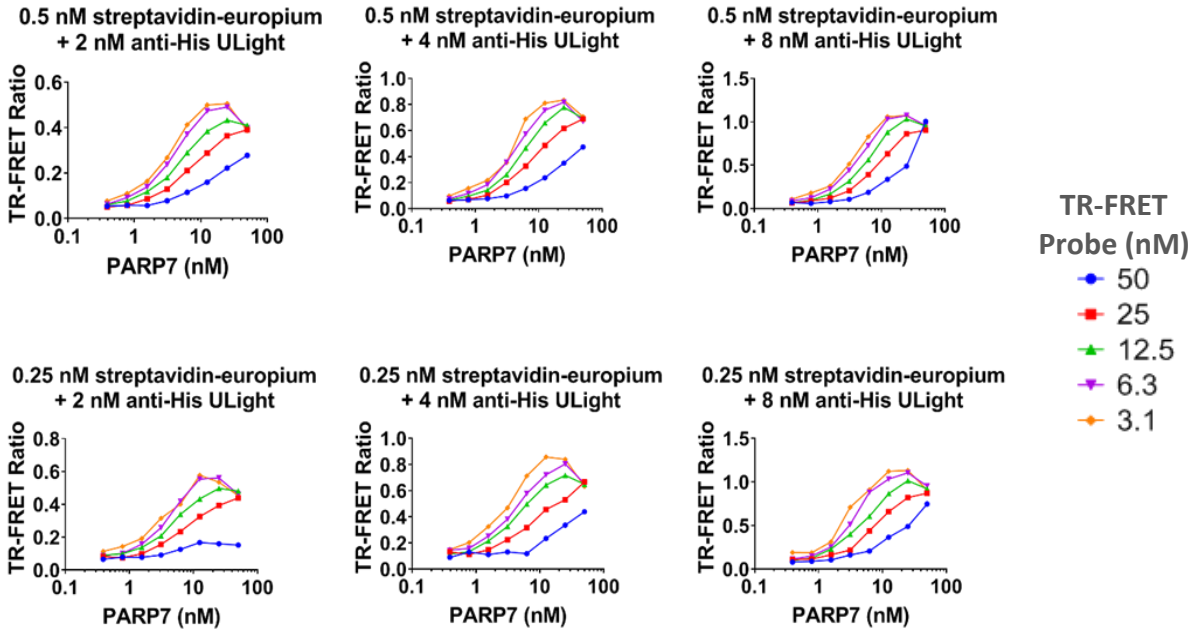


Wigle et al., *Cell Chemical Biology* (2020)

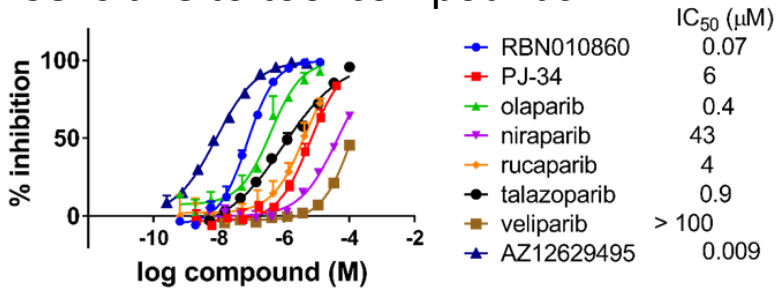
Development of a Sensitive PARP7 In Vitro TR-FRET Probe Displacement Assay



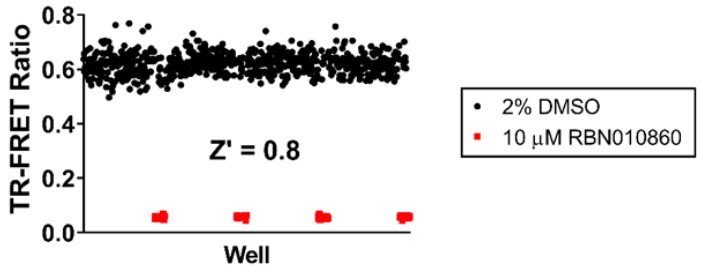
Simultaneous Titration of All Components



Sensitive to tool compounds

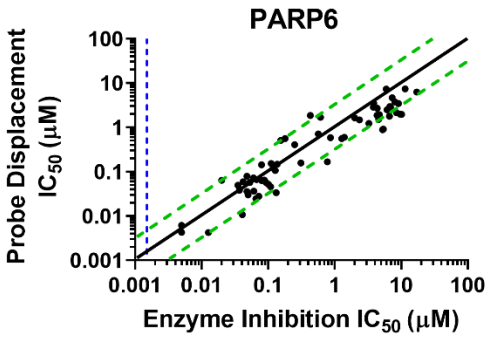


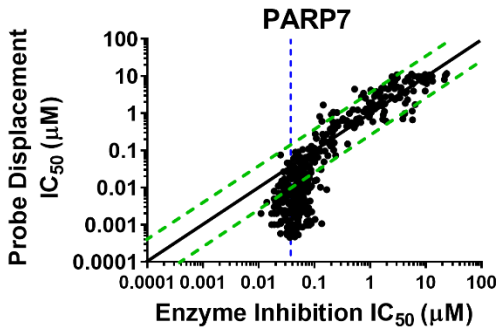
Automated assay is robust

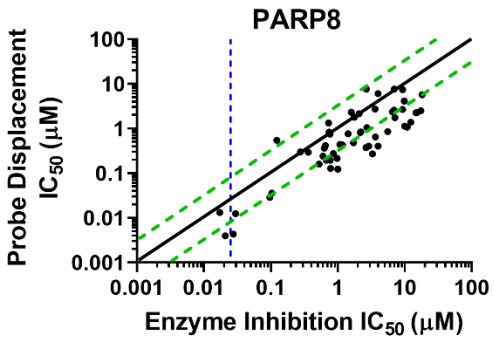


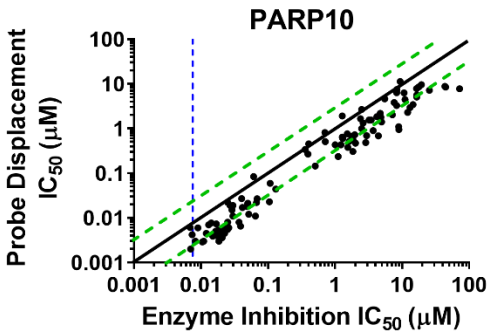
Wigle et al., *Cell Chemical Biology* (2020)

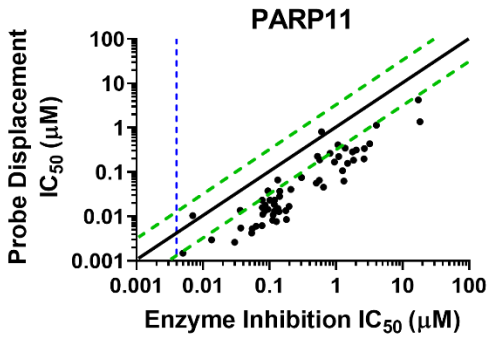
In Vitro TR-FRET Probe Displacement Assays Correlate to In Vitro Enzyme Inhibition Assays and Improve Potency Limit for Several PARPs

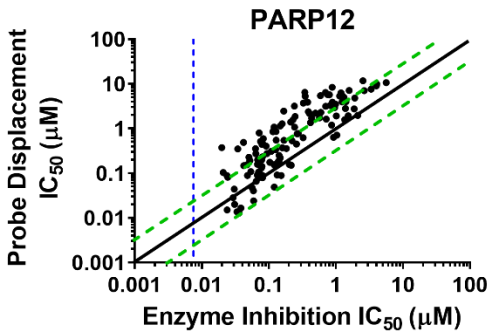


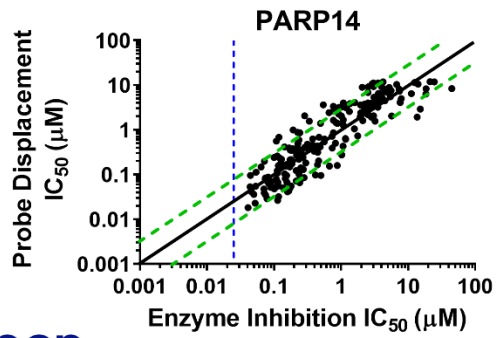


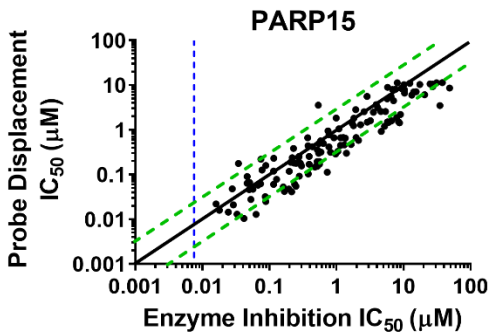


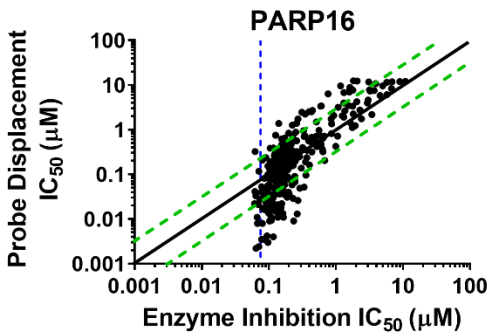










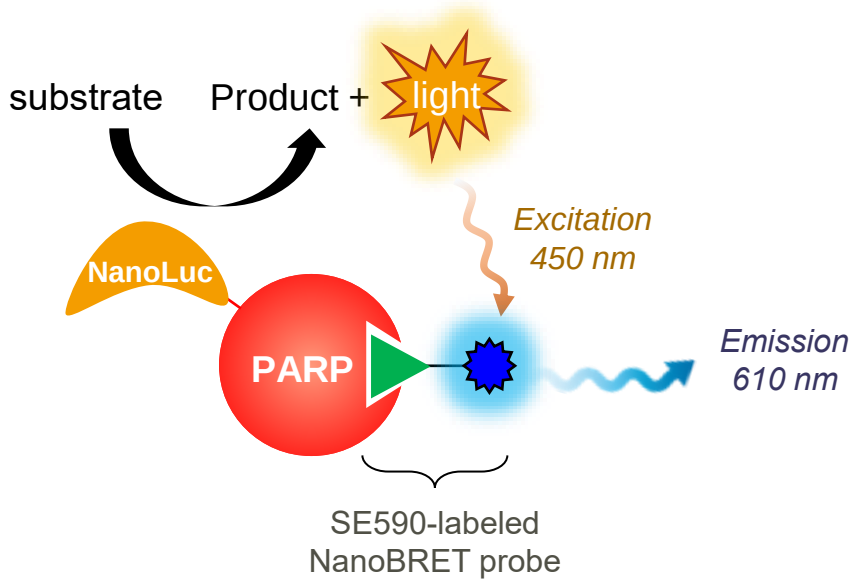


For some monoPARPs, probe displacement assay extends the range of measurable potency by 1 – 2 orders of magnitude over self-modification assay

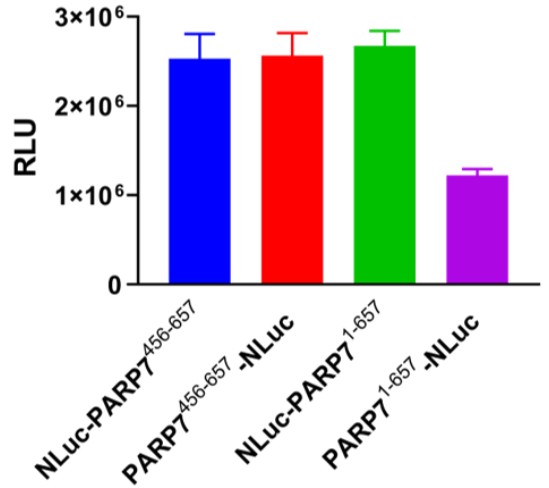
= theoretical potency limit of assay

Wigle et al., *Cell Chemical Biology* (2020)

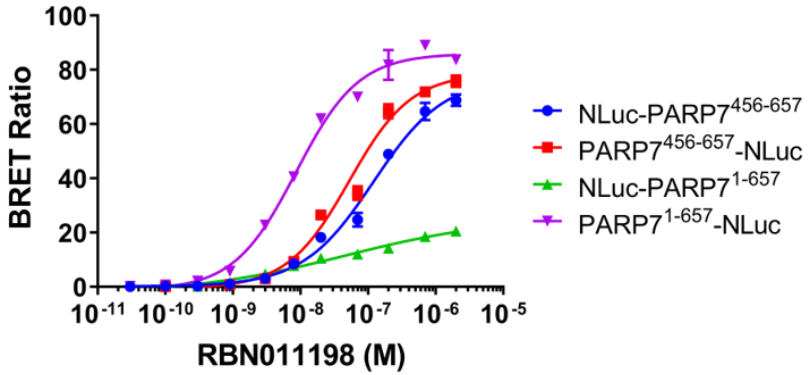
Development of NanoBRET Assays to Measure Cellular Target Engagement for PARP7



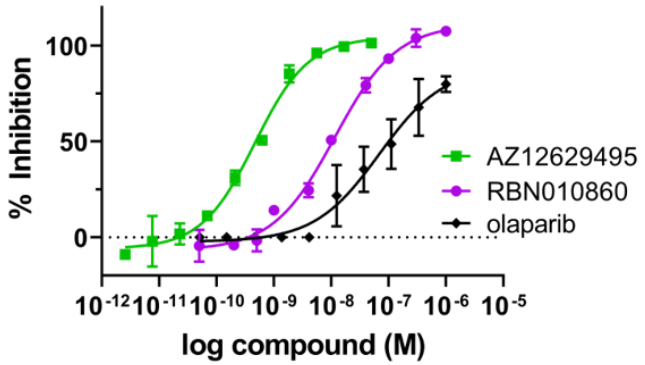
Testing expression of different constructs



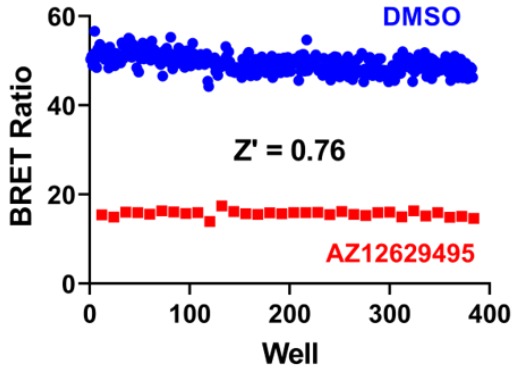
Probe titration vs. all constructs



Inhibition using tool compounds

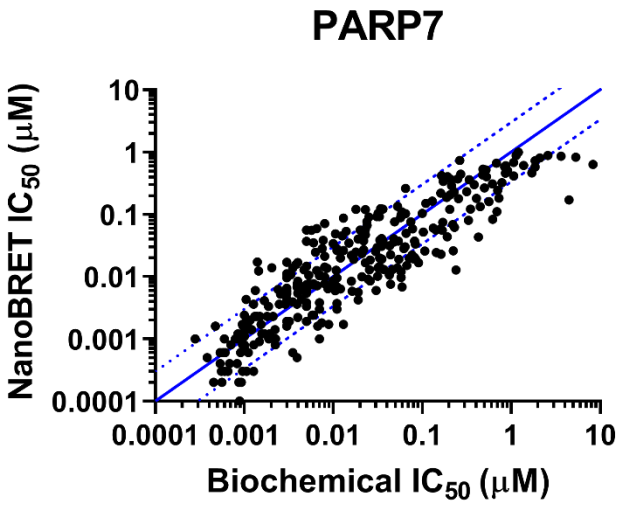


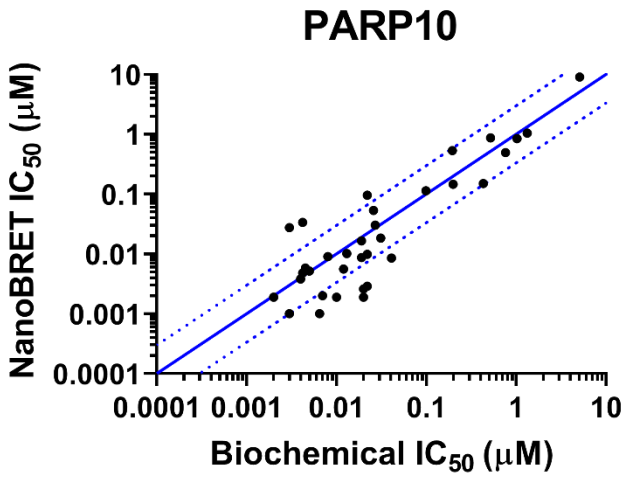
Automation & uniformity

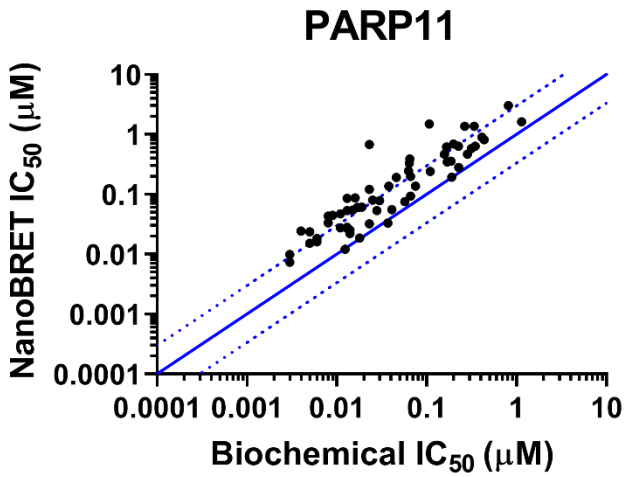


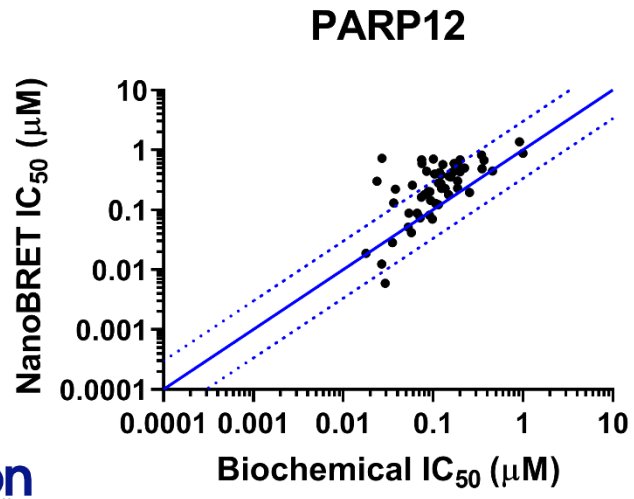
Wigle et al., *Cell Chemical Biology* (2020)

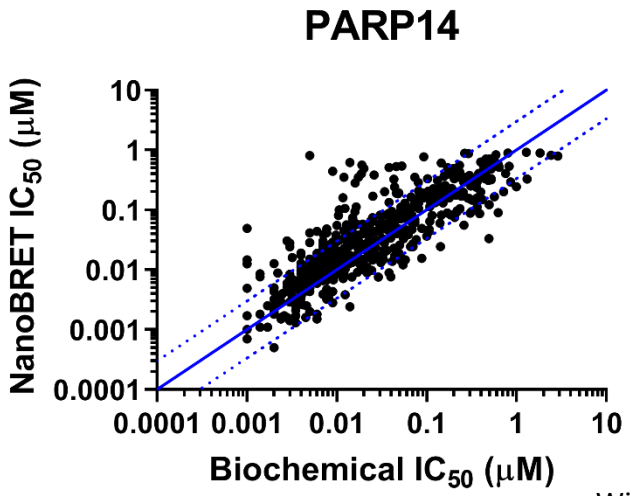
Correlation of NanoBRET to Biochemical Assays Across Multiple MonoPARPs

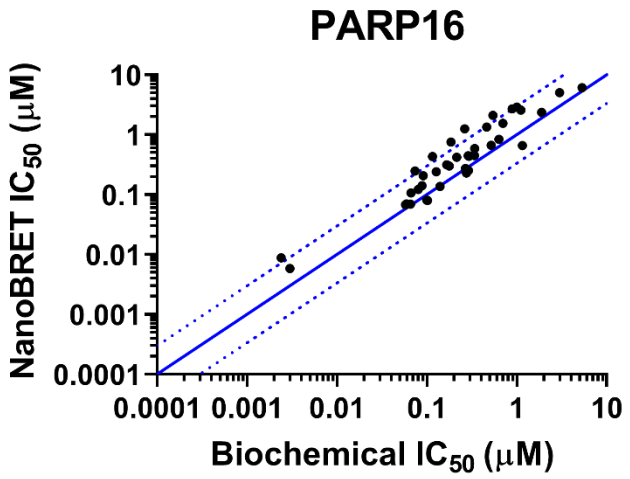








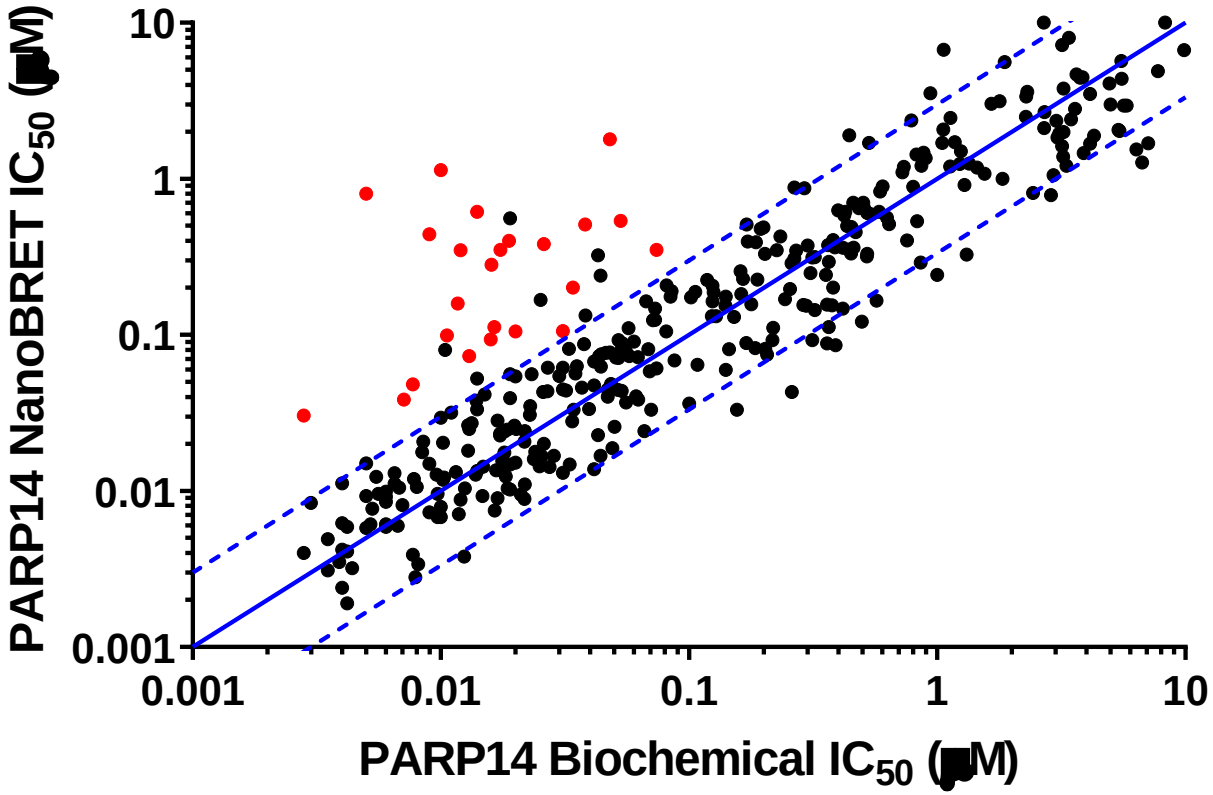




Wigle et al., *Cell Chemical Biology* (2020)

Less Potent Outliers in PARP14 NanoBRET Have Low Permeability

Compounds highlighted with red have low permeability measured by MDCK-MDR1 assay



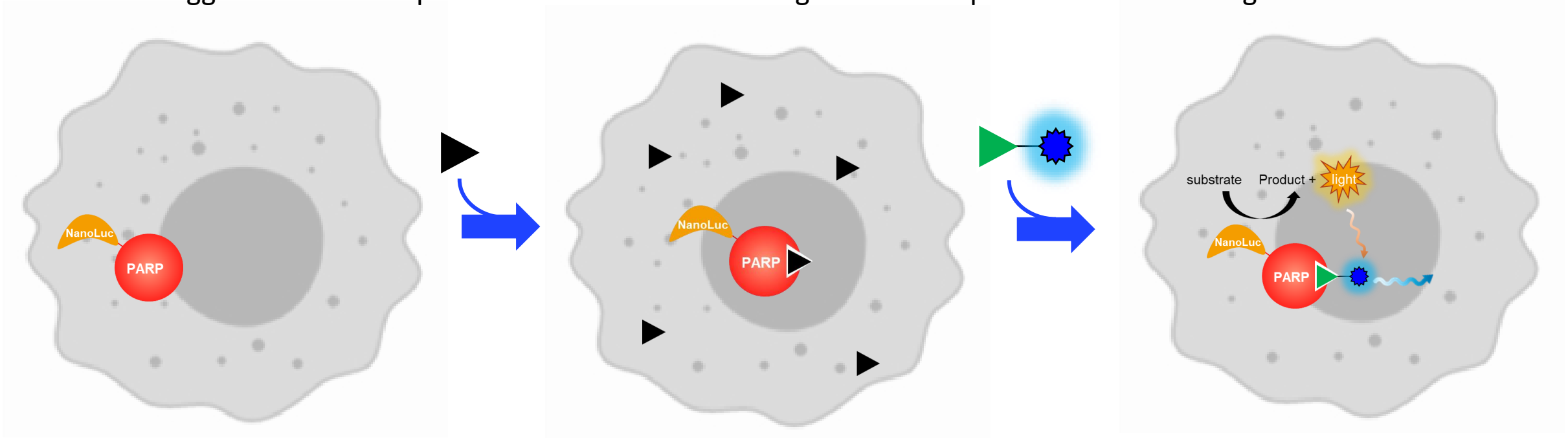
Wigle et al., *Cell Chemical Biology* (2020)

NanoBRET Can Be Used to Measure Inhibitor Residence Time in Cells

Overexpress NanoLuc-tagged PARP

Add excess PARP inhibitor @ 10X IC_{50} and equilibrate to saturate all binding sites

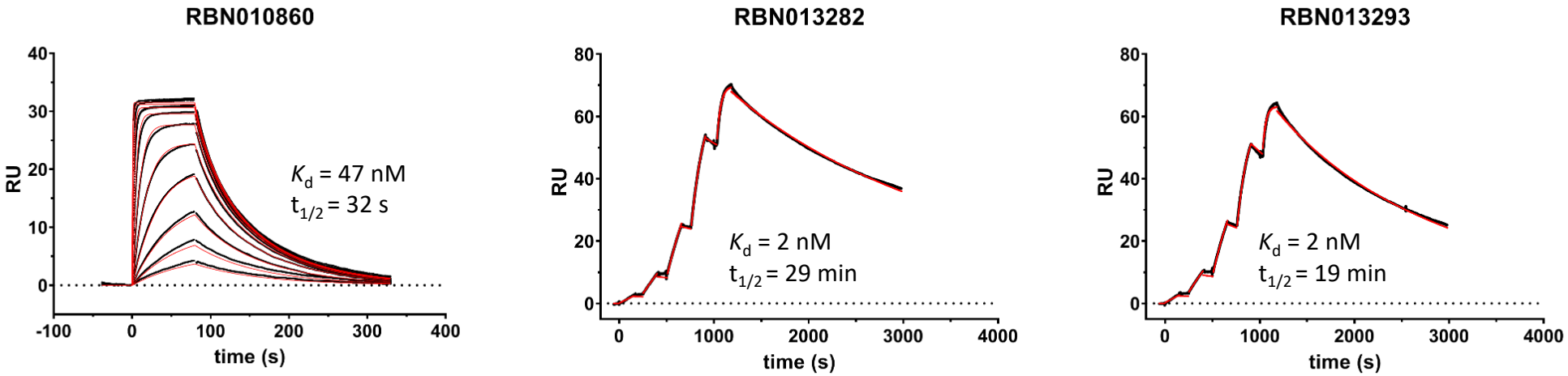
Wash out unbound inhibitor then add NanoBRET probe and measure signal increase in real-time



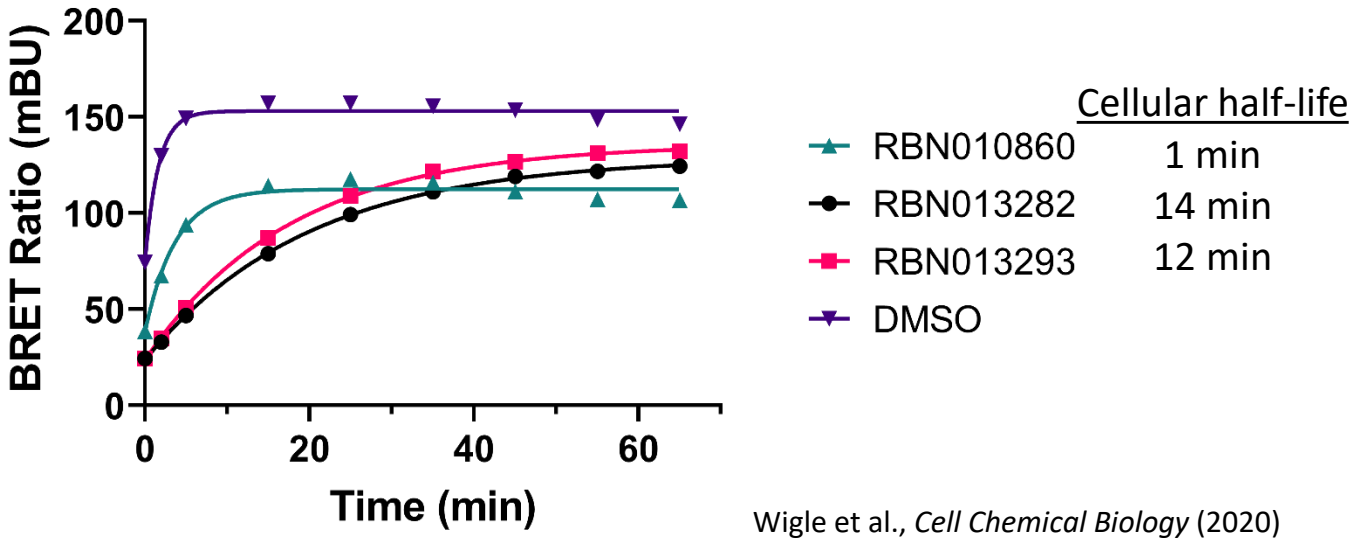
Wigle et al., *Cell Chemical Biology* (2020)

Cellular Residence Time Analysis by NanoBRET Gives Similar Results to SPR for Moderately Slow-Off PARP14 Inhibitors

SPR



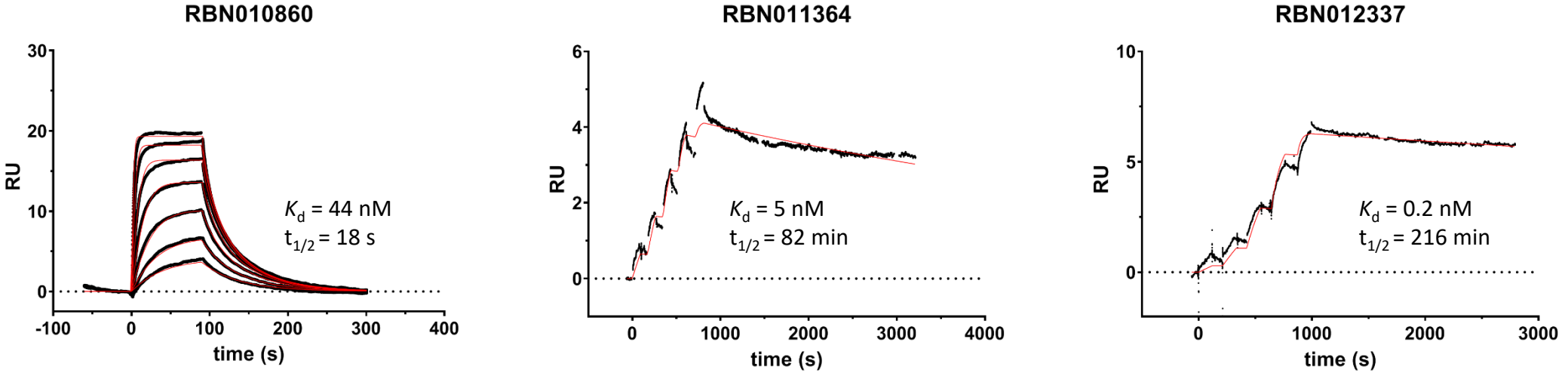
NanoBRET



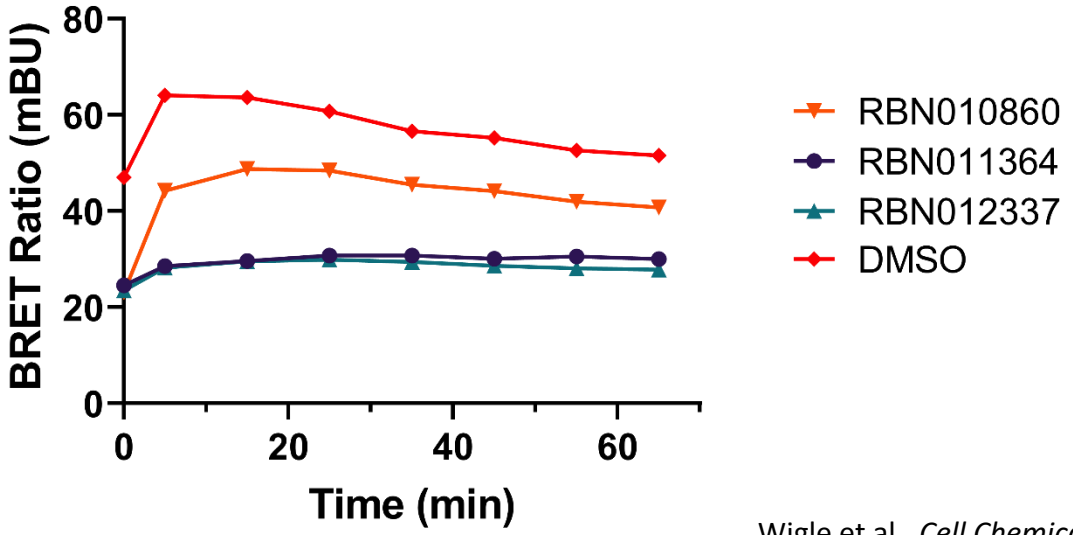
Wigle et al., *Cell Chemical Biology* (2020)

Cellular Residence Time Analysis by NanoBRET Gives Similar Results to SPR for Very Slow-Off PARP7 Inhibitors

SPR



NanoBRET

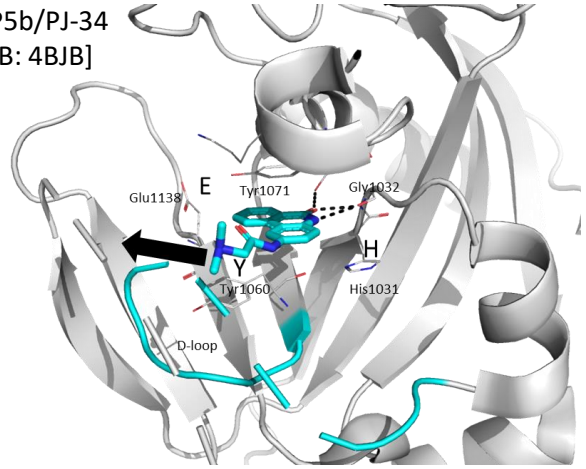


Wigle et al., *Cell Chemical Biology* (2020)

NanoBRET Probe for polyPARP Enzymes

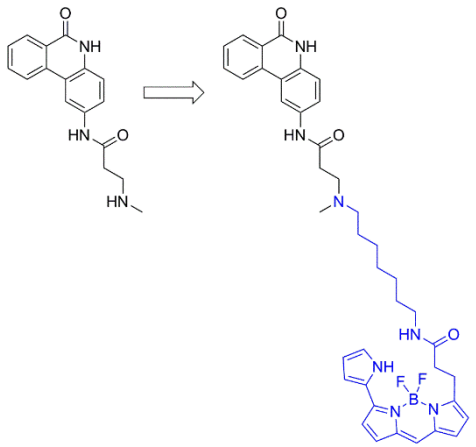
Designed a potent PARP1/3 probe

PARP5b/PJ-34
[PDB: 4BJB]

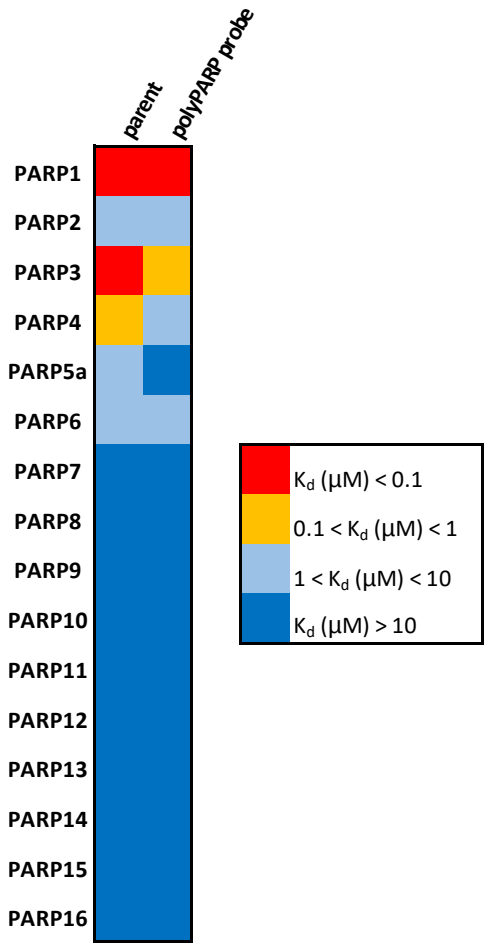


RBN011829
Parent

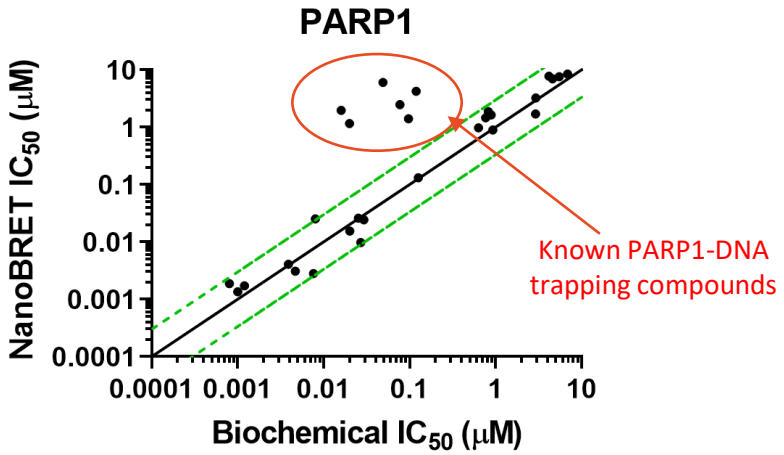
RBN012148
cellular NanoBRET probe

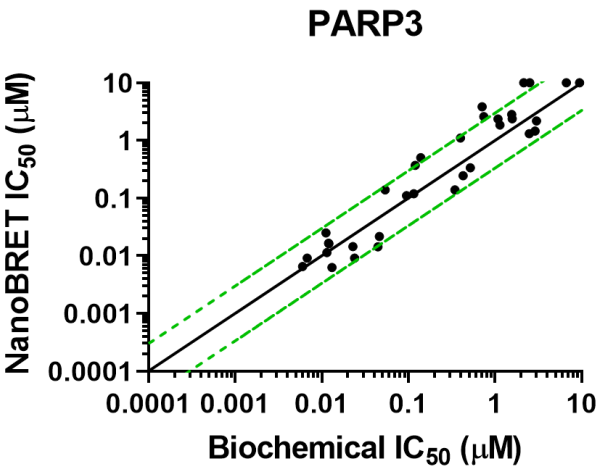


SPR confirms probe binds to PARP1 and PARP3



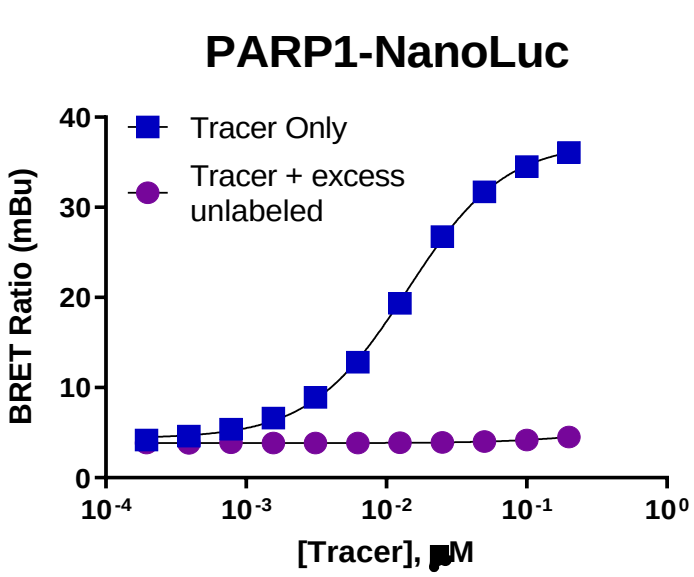
Comparison of NanoBRET & enzyme inhibition assay

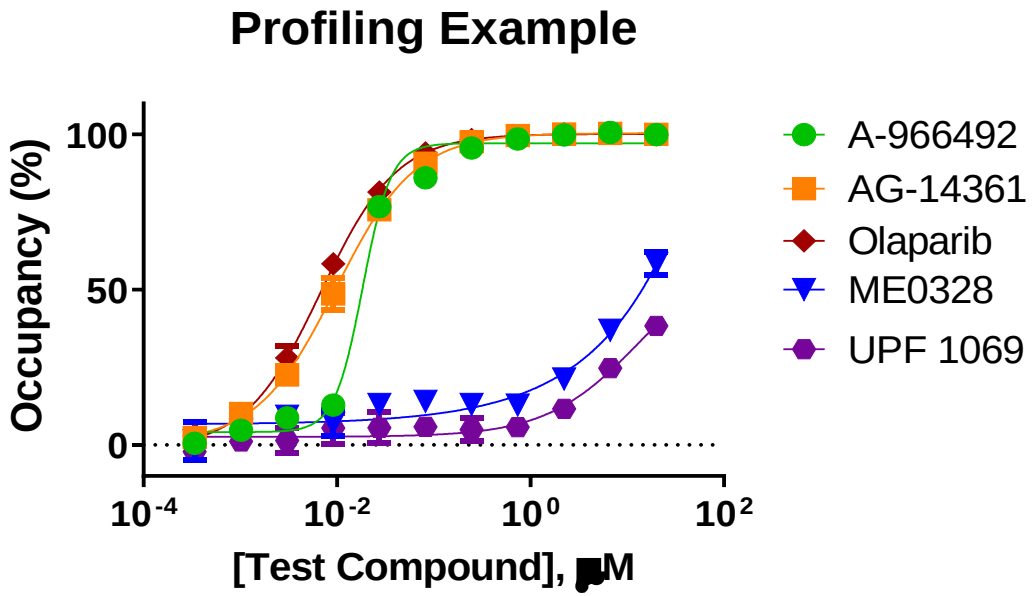




Wigle et al., *Cell Chemical Biology* (2020)

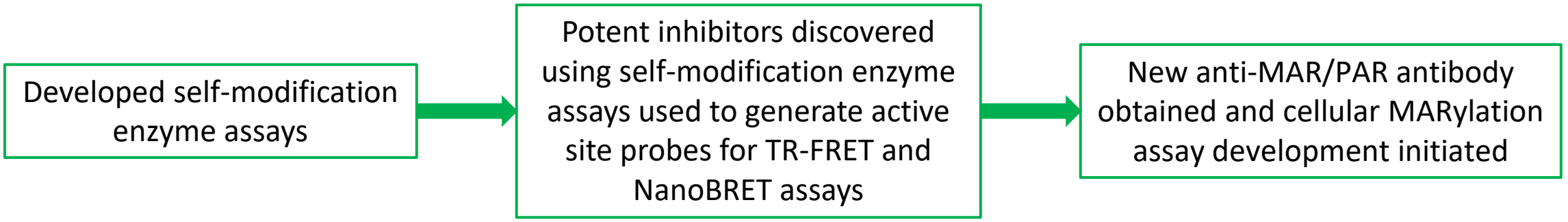
Off-The-Shelf NanoBRET Probes for PARP1 Also Available





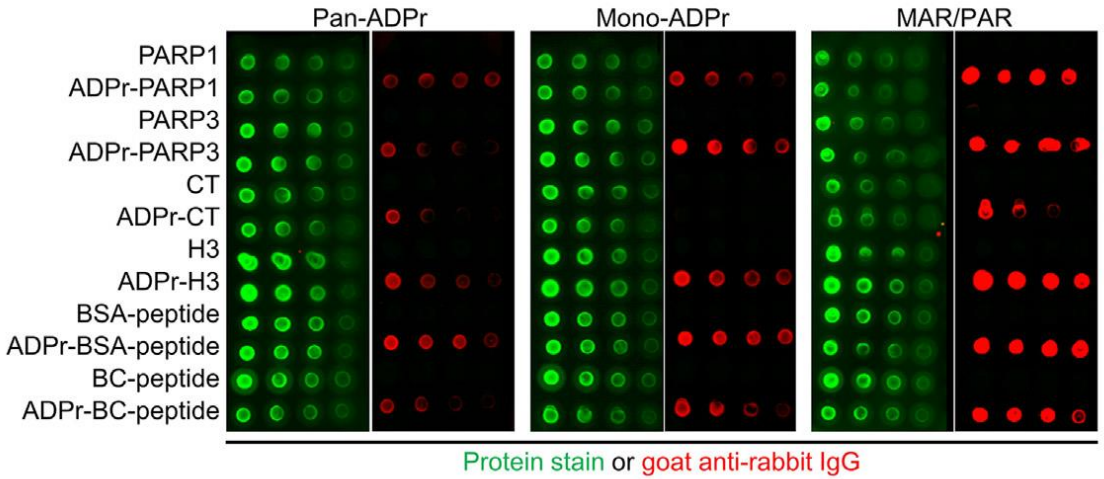
Promega

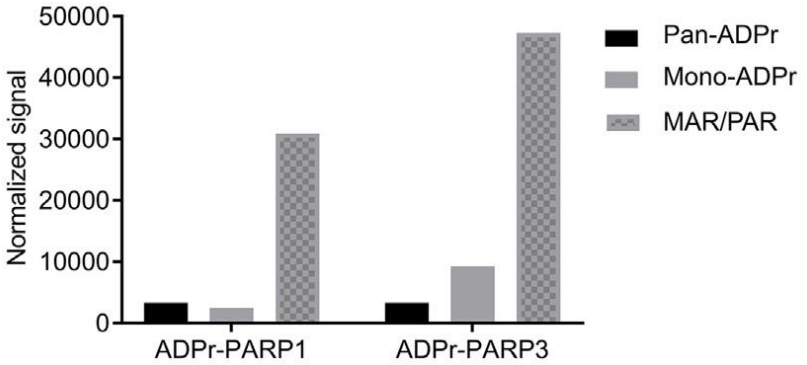
Measuring monoPARP Enzyme Inhibition in Cells: The Next Frontier



Characterization of a Novel Antibody that Binds to MAR & PAR

MAR/PAR antibody gives more robust signals than protein-based reagents in spot blot

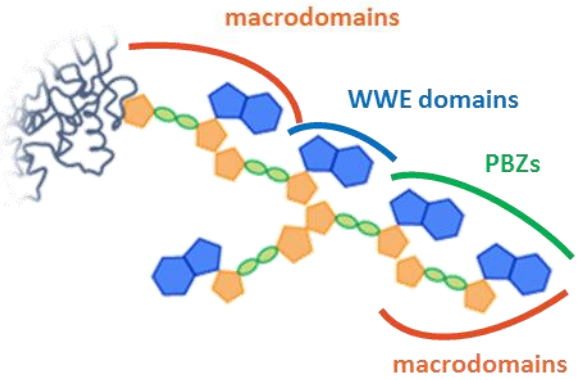




Pan-ADPr
- Macrodomain of Af1521 from the archaebacteria *Archaeoglobus fulgidus* fused to rabbit IgG
- Binds MAR and PAR

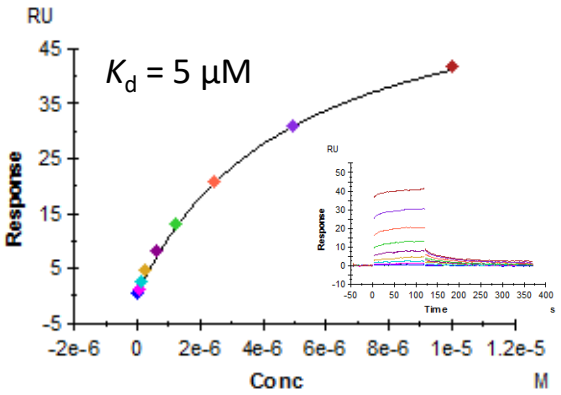
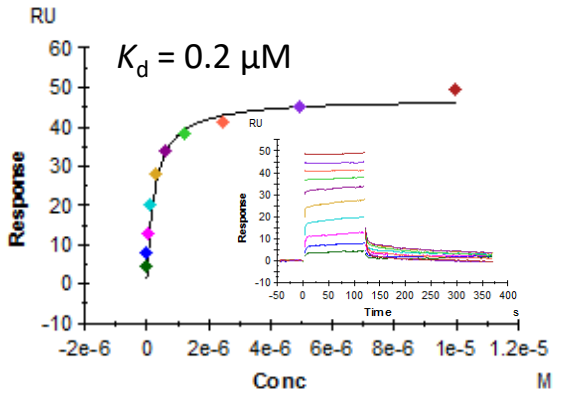
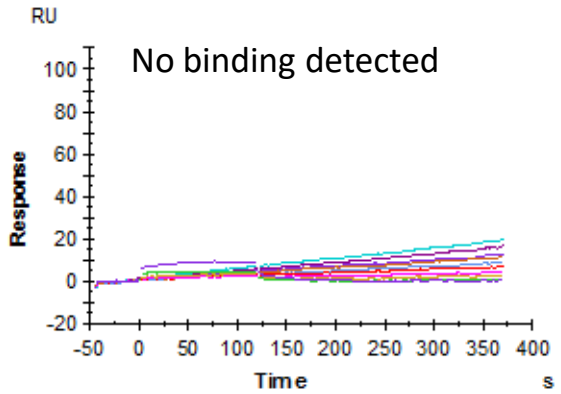
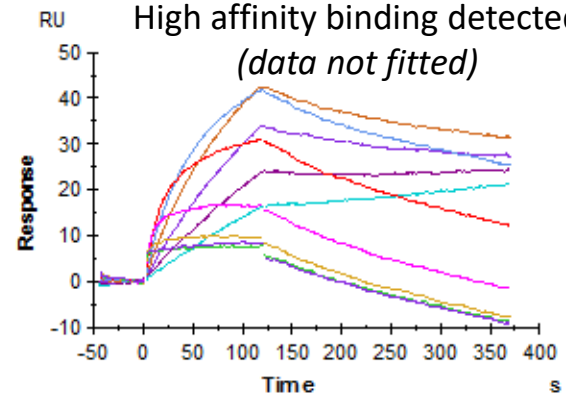
Mono-ADPr
- Macrodomain of human PARP14 fused to rabbit IgG
- Binds MAR only

MAR/PAR
- Rabbit IgG antibody
- Binds MAR and PAR
- Not available until 2018



Lu et al., *Biochemical Pharmacology* (2019)

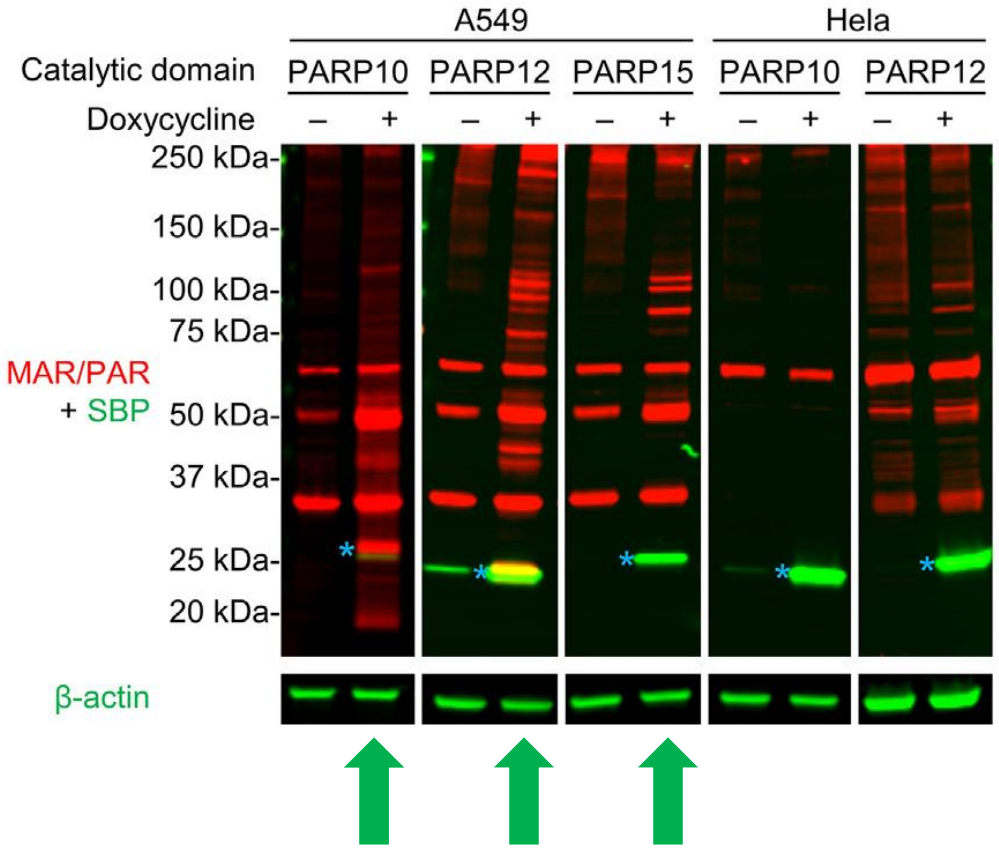
MAR/PAR Antibody Binds MARylated Substrates with Higher Affinity than MAR "Reader" Protein Domains

	MAR "Reader" Protein Reagent	MAR/PAR antibody
MARylated peptide		
MARylated BSA		

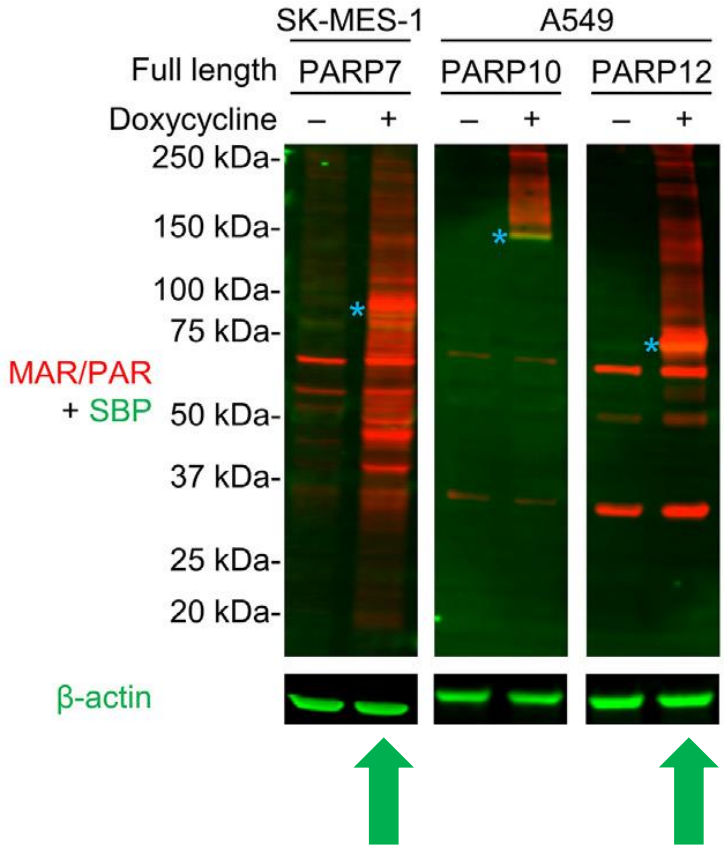
- MAR/PAR antibody binds with higher affinity in SPR assay
- Evidence of context-dependent binding for MAR "reader" protein reagent

Overexpression of MonoPARPs Leads to Differential MARylation Banding Patterns on MAR/PAR Western Blot

Overexpression of catalytic domains



Overexpression of full-length PARPs



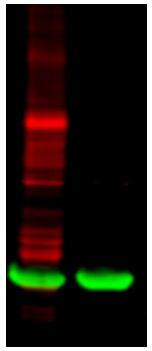
- Not all PARPs lead to MAR changes under these conditions
- Cell-line and construct dependencies observed

Lu et al., *Biochemical Pharmacology* (2019)

MAR/PAR Antibody Enables Multiple High-Throughput Methods of Detecting Inhibition of PARP7 Enzymatic Activity in Cells

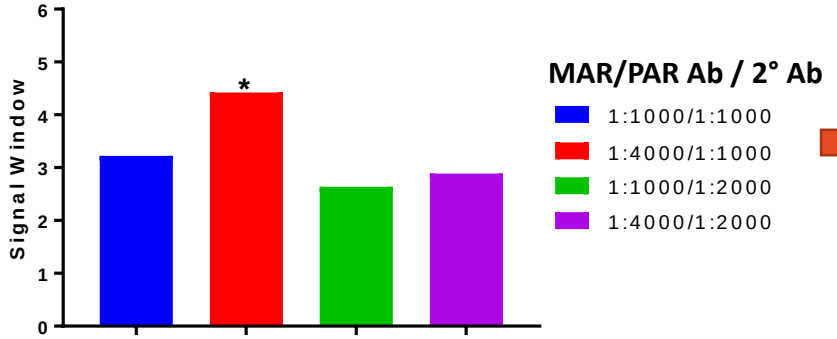
Increase in MAR observed after PARP7 stable overexpression by Western

PARP7i - +

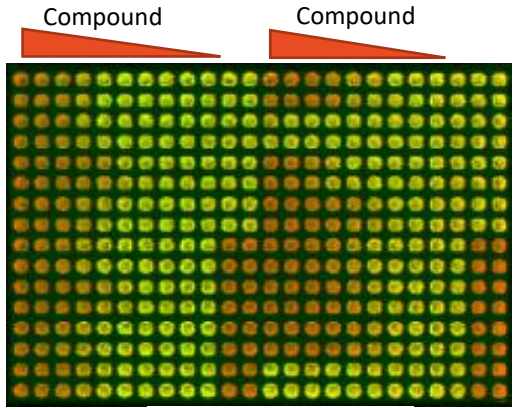


MAR Ab + anti-β-actin

Conversion to In-Cell Western: Optimization of Signal Window

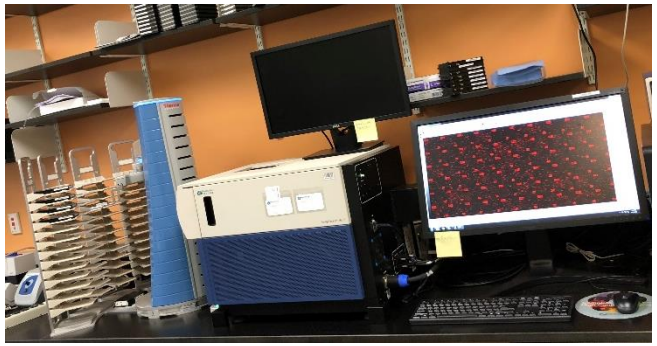


Compound Dose Response by ICW Format

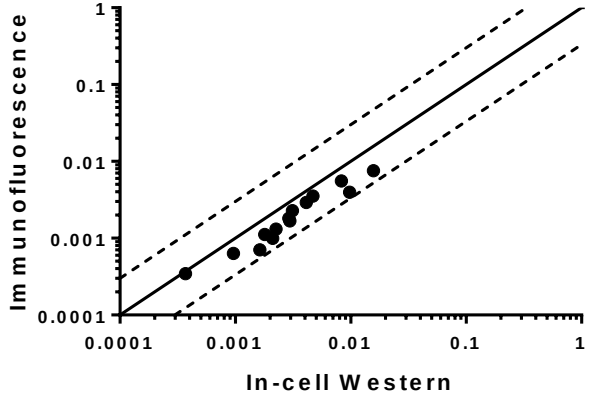


MAR/PAR -- DNA

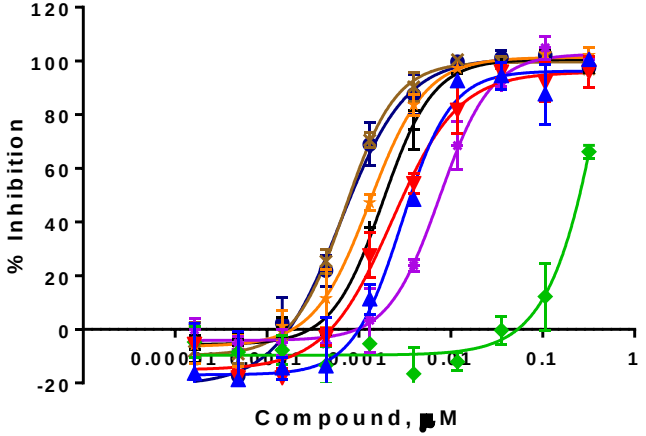
Conversion to High Content Microscope



Conversion from ICW to Immunofluorescence (IF) on High Content Microscope

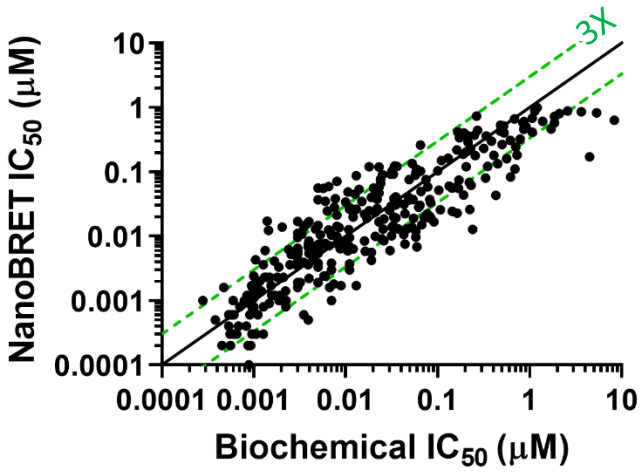


Robust Measurement of MAR Inhibition by IF

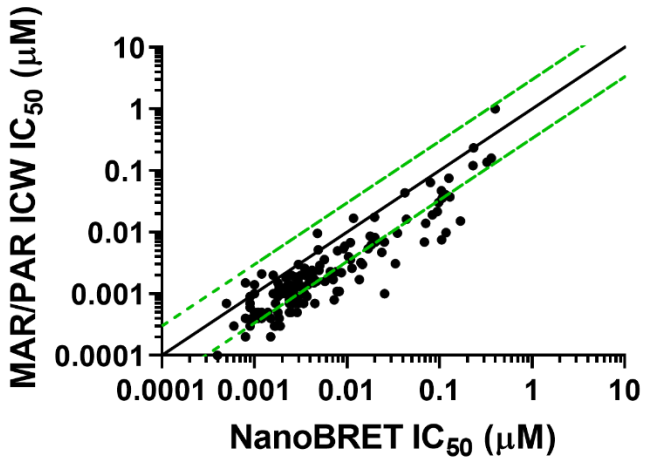


PARP7 NanoBRET Assay Correlates with Cellular Enzyme Inhibition and Phenotypic Screening Funnel Assays

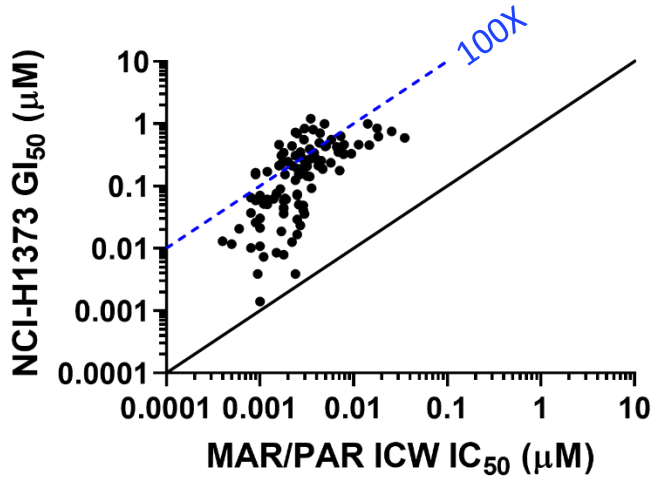
NanoBRET vs. Biochemical



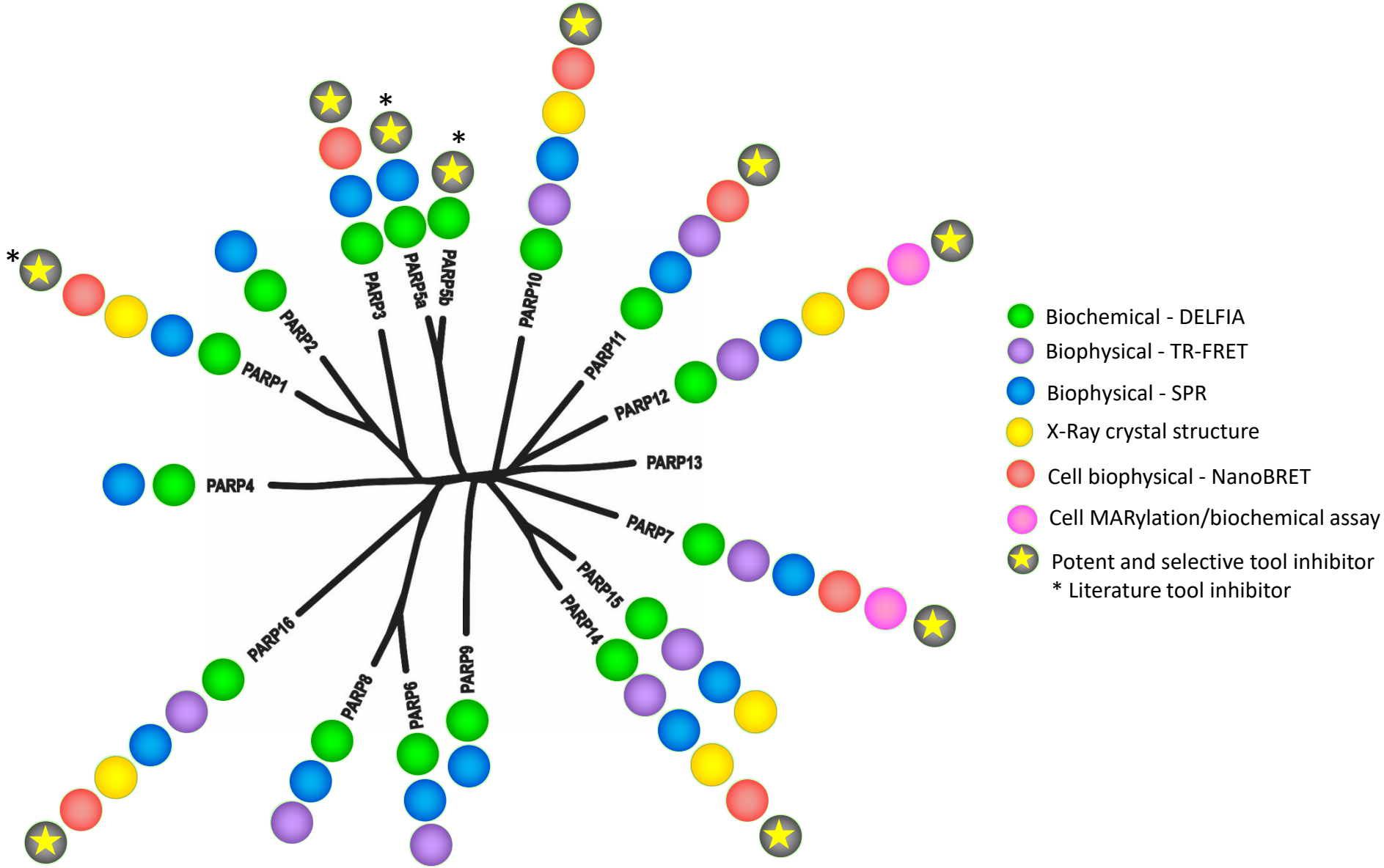
Cellular Enzyme Inhibition vs. NanoBRET



Phenotypic vs. Cellular Enzyme Inhibition



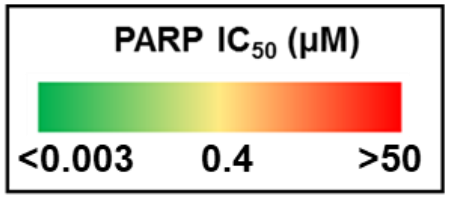
BEACON+ Platform Generates Suite of Screening Assays for PARP Family



BEACON+ Platform Generates Selective Inhibitors Across the Entire PARP Family

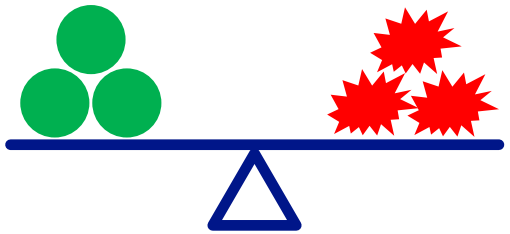
Target PARP	IC_{50} (μM)	PARP 1	PARP 2	PARP 3	PARP 4	PARP 5b	PARP 6	PARP 7	PARP 8	PARP 9	PARP 10	PARP 11	PARP 12	PARP 14	PARP 15	PARP 16
PARP1	0.001															
PARP3	<0.001															
PARP5b	0.003															
PARP7	<0.003															
PARP10	0.008															
PARP11	0.03															
PARP12	<0.008															
PARP14	<0.003															
PARP16	0.1															
monoPARPs	Pan inhib															

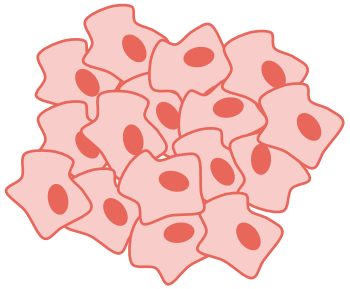
- PARP1 inhibitors do not inhibit monoPARPs
- No potent and selective monoPARP inhibitors existed in the literature prior to Ribon
- Ribon has developed multiple selective monoPARP inhibitors



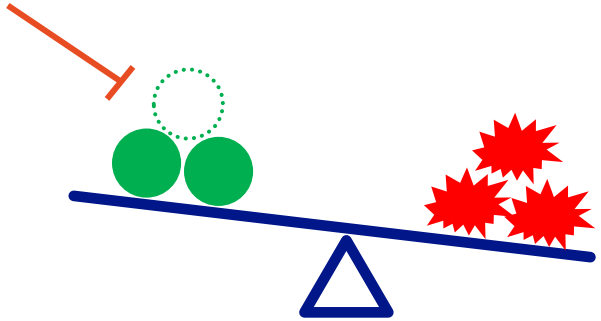
ribon therapeutics

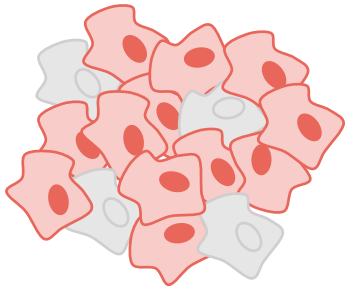
Targeting Stress Support Pathways: Activating Both Tumor Intrinsic Killing and Activation of the Immune System





Cancer cells ( ) must balance cellular stresses ( ) with stress support pathways ( ) in order to proliferate.





Inhibiting ( ) stress support pathways initiates a tumor-intrinsic effect on proliferation…

… leading to extrinsic activation of the immune system ( ), adding stress and enhancing tumor killing ( ).

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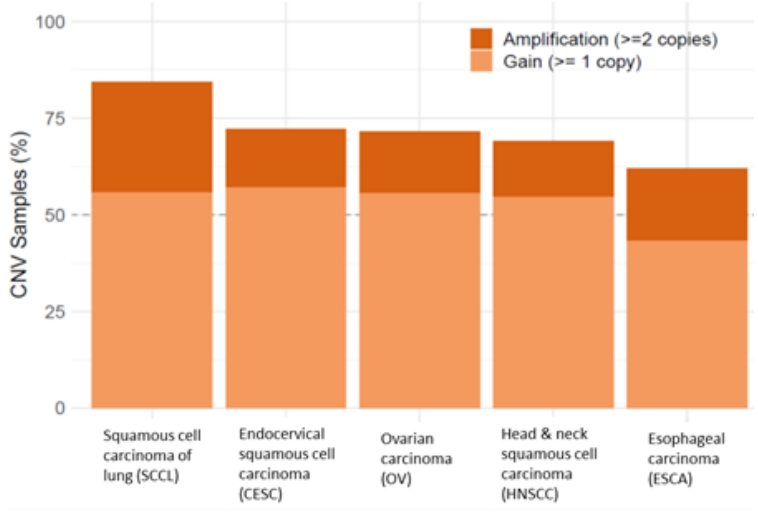
RBN-2397, a Small Molecule Inhibitor of PARP7: Eliminates Stress Support in Tumors and Activates Anti-Tumor Immune Response

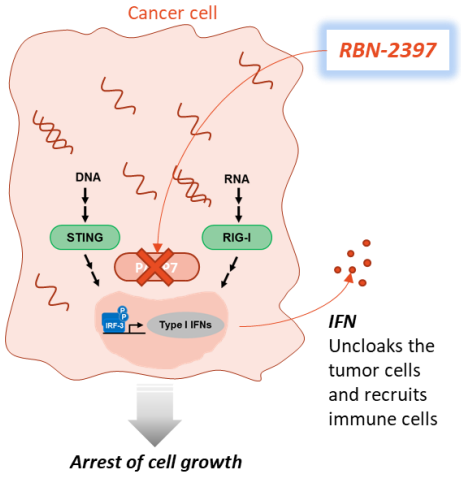
- PARP7 is a stress-induced protein and is amplified in multiple tumor types including squamous carcinoma of the lung and head and neck cancer
- RBN-2397 is a potent and selective inhibitor of PARP7 and causes complete regressions and anti-tumor immunity in preclinical tumor models by restoring nucleic acid sensing and induction of interferon signaling
- RBN-2397 is in a phase 1 clinical trial in cancer patients and is well-tolerated with preliminary evidence of clinical activity

PARP7 is genetically amplified in several cancer indication (TCGA)

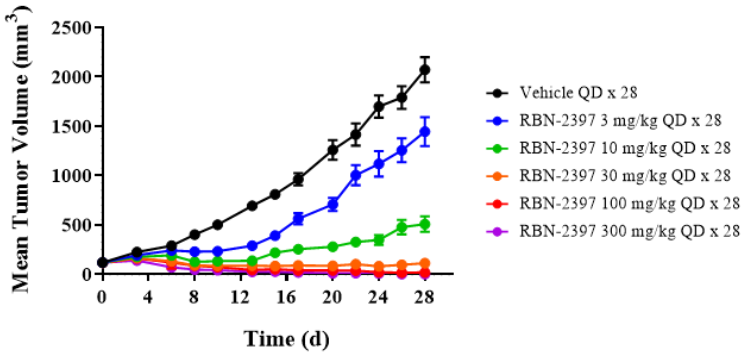


Source: TCGA

RBN-2397 inhibits PARP7 and restores nucleic acid sensing and Type I interferon expression



RBN-2397 causes dose-dependent complete regressions in preclinical tumor models



NCI-H1373 human lung cancer xenograft

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Summary

- BEACON^{+} Platform contains suite of de novo biochemical and biophysical assays for monoPARP enzymes that do not rely on knowledge of the substrates for each enzyme; assays correlate well with each other
  - Self-modification enzyme assays of immobilized protein detected by DELFIA
  - SPR assays
  - NAD^{+}-competitive active site probes
    - Detected in vitro by TR-FRET
    - Detected in cells by NanoBRET

- Newly available MAR/PAR antibody enabled observation of changes in global MARylation detected by in-cell Western and immunofluorescence when monoPARP enzymes were overexpressed
  - Assay does not require knowledge of substrates
  - Correlates with phenotypic effect as shown with PARP7 inhibitors in NCI-H1373 cells

- Screening platform used to develop tool compounds for multiple monoPARP enzymes, including PARP7 inhibitor (RBN-2397) in phase 1 clinical trial in oncology

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