Hold-and-Kill: Decoding RIPTAC Mechanism of Action and Novel Target Pair Validation Strategies

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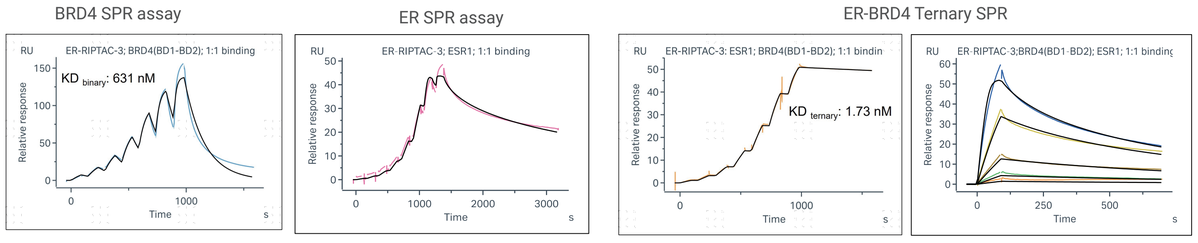
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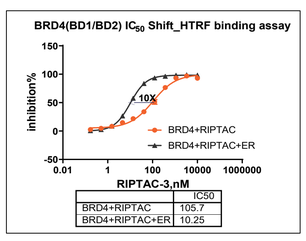
Abstract

Regulated Induced Proximity Targeting Chimeras (RIPTACs) represent a novel therapeutic modality designed to elicit a "Hold-and-Kill" mechanism by cross-linking a tumor-specific target with an essential effector protein (e.g., BRD4), leading to robust cell death. However, validating novel RIPTAC pairs requires rigorous multi-dimensional evaluation. Here, we present a systematic validation platform for new RIPTAC and TCIP pairs. Our strategy initiates with AI-driven (AIDD) structural predictions of ternary protein-protein interactions. At the protein level, biophysical and biochemical assays, including SPR and HTRF, are deployed to quantify binding kinetics and ternary cooperativity. Moving to the cellular context, we utilize NanoBRET, AlphaLISA, and rigorously controlled inducible expression systems to confirm target-dependent induced proximity in live cells. Functionally, extensive cell panel assays demonstrate a strong correlation between biomarker expression (e.g., ESR1 nTPM) and RIPTAC-mediated cytotoxicity, successfully overcoming resistance mechanisms seen with traditional degraders like Fulvestrant. Finally, transcriptomic and biomarker analyses elucidate the distinct mechanism of action, highlighting severe transcriptional stress and unique cell death pathways. Furthermore, we demonstrate the platform's versatility by expanding to novel targets, including p53-Y220C complexes. This integrated workflow streamlines the discovery, optimization, and mechanistic validation of next-generation induced-proximity therapeutics.

I | Ternary PPI Validation from Protein Level - Biophysical & Biochemical (In Vitro)



Cooperativity assay



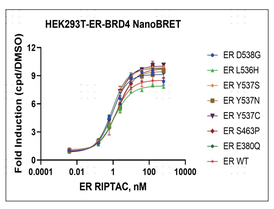
Compound	BRD4 SPR KD (binary)	ESR1-BRD4 SPR KD (BRD4 immobilized) (Ternary)	Cooperativity (SPR)	ESR1 SPR KD (binary)	ESR1-BRD4 SPR KD (ESR1 immobilized) (Ternary)	Cooperativity (SPR)
ER-RIPTAC-3	631 nM	1.73 nM	365 x	11.4 nM	2.4nM	4.8x

Compound	HTRF IC_{50} w/o ER (nM)	HTRF IC_{50} w/ ER (nM)	Cooperativity (HTRF)
ER-RIPTAC-3	105.7	10.25	10 x

Figure 1. Cell-free protein binding assays using Surface Plasmon Resonance (SPR) to determine kinetic parameters (ka, kd, KD) and Homogeneous Time-Resolved Fluorescence (HTRF) to evaluate biochemical cooperativity during ternary assembly.

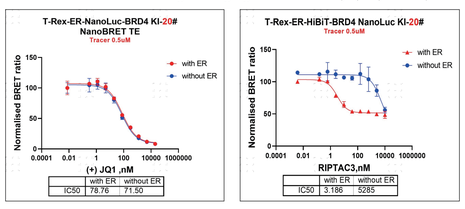
II: Ternary PPI validation in cellular level: NanoBRET, ELISA and AlphaLISA

RIPTAC could induce ER mutants interaction with BRD4

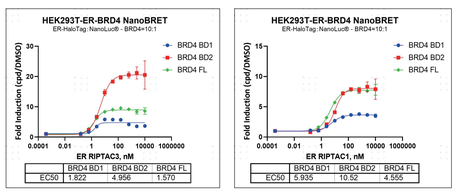


ESR1 status	EC50
ESR1 D538G	0.91
ESR1 L536H	1.1
ESR1 Y537S	1.1
ESR1 Y537N	1.6
ESR1 Y537C	1.1
ESR1 S463P	1.8
ESR1 E380Q	0.89
ESR1 WT	1.2

BRD4 NanoBRET target engagement assay (TE) assay



RIPTAC molecule binding to BD2 is stronger than BD1 domain



RIPTAC induced ER&BRD4 interaction in AlphaLISA Ternary assay

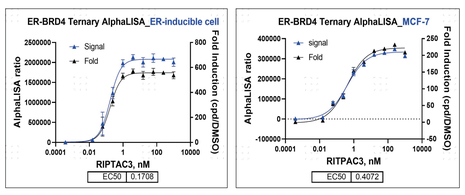
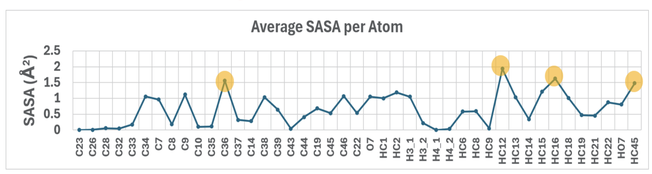
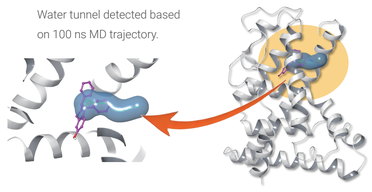
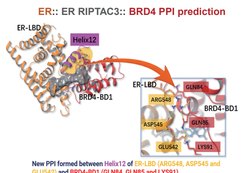


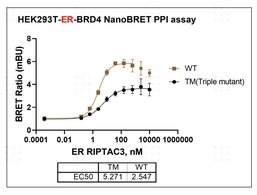
Figure 2. Quantitative intracellular ternary detection: NanoBRET ternary formation across mutants, BRD4 NanoBRET target engagement, and orthogonal ELISA / AlphaLISA confirmation in T-Rex inducible systems titrating ER levels.

III: AIDD model prediction and validation for new RIPTAC target pair





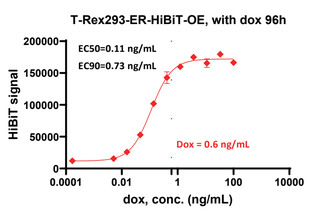


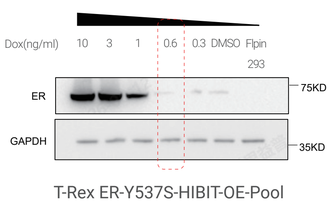


TM	WT
EC50	5.271	2.547

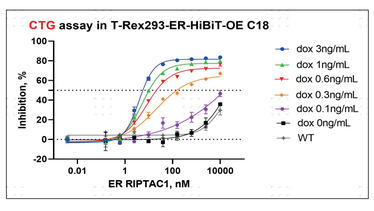
Figure 3. Utilization of AI-driven drug design (AIDD) to predict the structural conformation and protein-protein interaction (PPI) interfaces of the ternary complex comprising the target protein, the RIPTAC molecule, and the effector protein (e.g., BRD4).

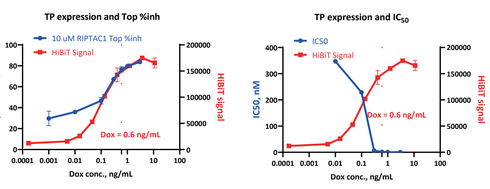
IV: Hold-and-kill correlation test

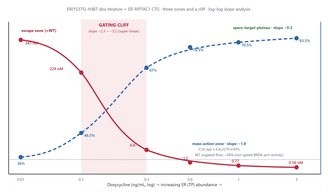




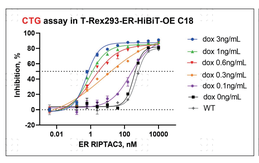
T-Rex ER-Y537S-HIBIT-OE-Pool

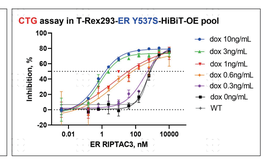






Dox conc., ng/mL	Top %inh		IC_{50}, nM
3	83	84	0.56
1	79	80	0.77
0.6	75	78	1.3
0.3	67	67	6.6
0.1	49	44	229
0.01	36	36	347





Dox (ng/mL)	ER WT	ER Y537S	ER D538G
10	-	0.7	-
3	0.6	0.8	0.4
1	0.8	4.6	0.7
0.5/ 0.6	1.3	6.8	61
0.3	6.6	341	279
0.1	229	-	406
0 (uninduced)	347	491	572
Parental WT	522	519	-
IC_{50} shift (induced -> none)	~620 x	~740 x	>1500 x

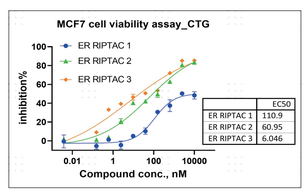
Figure 4. Inducible system to validate cell killing, check therapeutic window in vitro with and without tumor protein

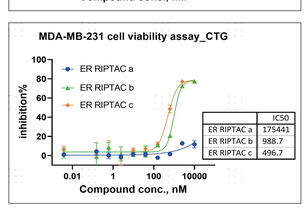
Summary

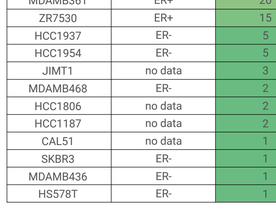
ICE Biosciences' RIPTAC platform integrates biochemical screening, computational docking, and multi-level cellular and in vivo assays to connect target engagement with therapeutic efficacy. By combining ternary complex prediction, kinetic modeling, and validation across biochemical, cellu lar, and tumor tissue contexts, the platform establishes mechanistic links between ER expression and selective cancer cell inhibition. Supported by our extensive expertise in targeted protein degradation (TPD), the modular design enables rapid migration to new target pairs. This flexibility, to gether with translational profiling and safety evaluation, empowers domestic and international partners to accelerate first-in-class drug discovery programs and expand the induced proximity landscape with clinically meaningful insights.

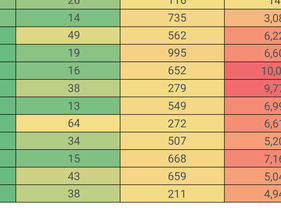
V | Cell Panel Pilot Test to Confirm RIPTAC Activity

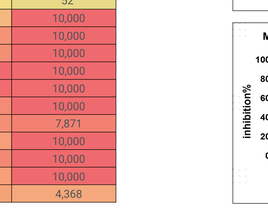
Cell line	ER status	ESR1 nTPM	BRD4 nTPM	RIPTAC3	Vepdegestrant	Fulvestrant
HCC1500	ER+	111	22	59	6	2
MDAMB134VI	ER+	109	27	12	8	3
HCC1428	ER+	81	16	39	25	5
T47D	ER+	49	16	20	155	88
T47D	ER+	49	16	22	150	64
MCF-7	ER+	49	18	13	1,623	21
ZR751	ER+	33	21	25	1,825	10,000
BT474	ER+	21	37	503	1,453	10,000
MDAMB361	ER+	20	26	116	148	52
ZR7530	ER+	15	14	735	3,089	10,000
HCC1937	ER-	5	49	562	6,224	10,000
HCC1954	ER-	5	19	995	6,608	10,000
JIMT1	no data	3	16	652	10,000	10,000
MDAMB468	ER-	2	38	279	9,777	10,000
HCC1806	no data	2	13	549	6,991	10,000
HCC1187	no data	2	64	272	6,616	7,871
CAL51	no data	1	34	507	5,204	10,000
SKBR3	ER-	1	15	668	7,160	10,000
MDAMB436	ER-	1	43	659	5,046	10,000
HS578T	ER-	1	38	211	4,944	4,368



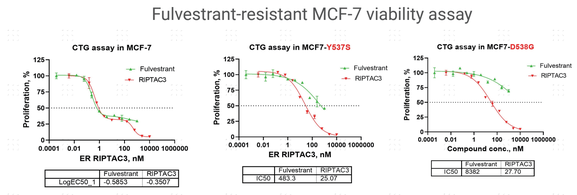








Fulvestrant-resistant MCF-7 viability assay



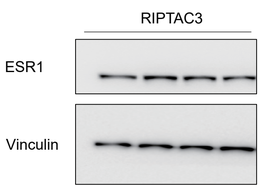
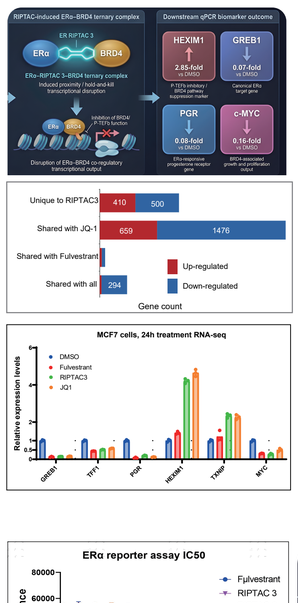
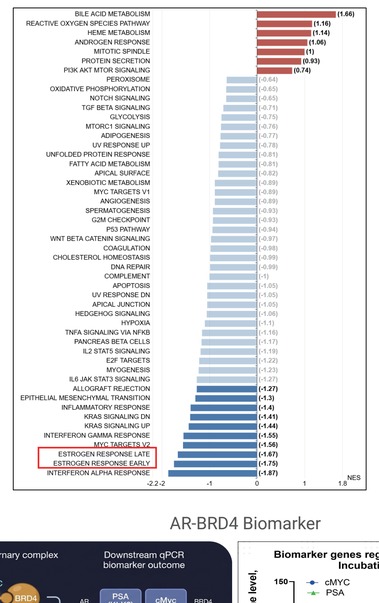
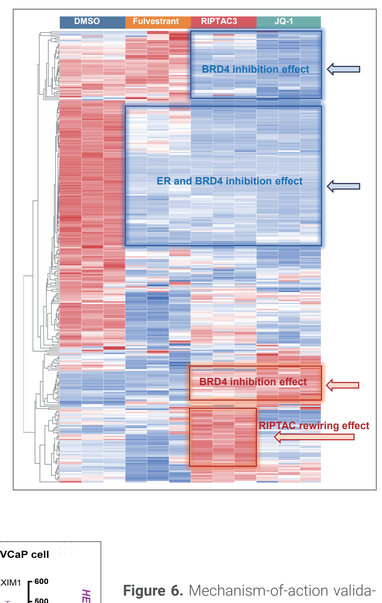


Figure 5. Profiling the IC50 of RIPTAC3 against traditional degraders (Fulvestrant, Vepdegestrant) across an extensive cancer cell line panel, correlating drug sensitivity with target transcriptional abundance (e.g., ESR1 nTPM).

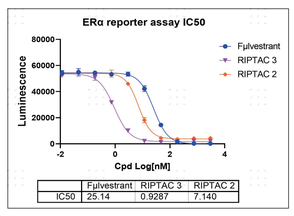
II: Ternary PPI validation in cellular level: NanoBRET, ELISA and AlphaLISA



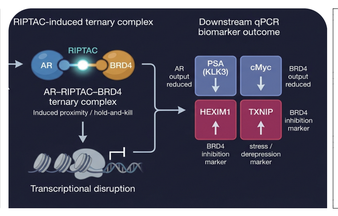


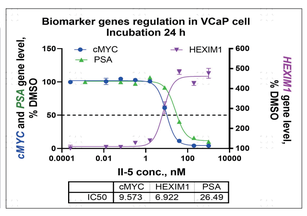


AR-BRD4 Biomarker



	Fulvestrant	RIPTAC 3	RIPTAC 2
IC50	25.14	0.9287	7.140



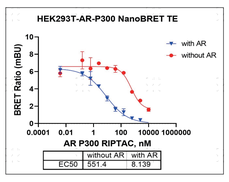


	cMYC	HEXIM1	PSA
IC50	9.573	6.922	26.49

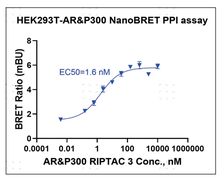
Figure 6. Mechanism-of-action validation for AR/ER RIPTACs. RIPTAC-induced ERα–BRD4/AR–BRD4 ternary complexes suppressed ER/AR reporter activity and downstream PD markers.

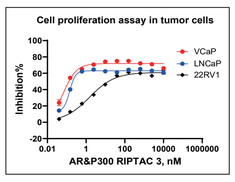
VII: Induced Proximity – RIPTAC and TCIP new pairs

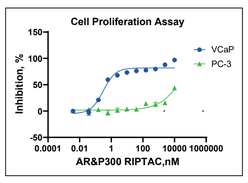
AR-P300



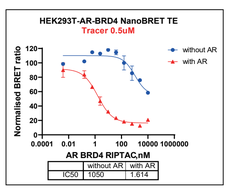
	without AR	with AR
EC50	551.4	8.139



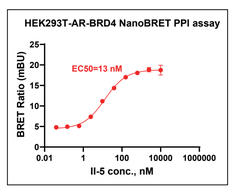


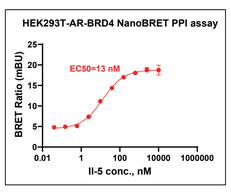


AR-BRD4



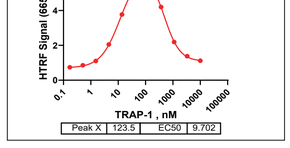
	without AR	with AR
IC50	1050	1.614





EC_{50} or IC_{50}, nM	AR&P300 RIPTAC 3	AR&P300 RIPTAC	AR-BRD4 Il-5
NanoBRET Ternary	1.6	3.9	13
VCaP	0.07	0.3	12
LnCaP	0.14		15
22RV1	1.53		377
PC-3		19547	479

p53-Y220C-BRD4 ternary complex formation assay



Peak X	123.5	EC50	9.702

EC50	11.53

EP NanoBRET TE assay

	with TP53 Y220C	without TP53 Y220C
IC50	110.2	204.6

p53-Y220C inducible cell Viability assay

	10 ng/mL Dox	0 ng/mL Dox	WT
EC50	1540	1771	2323

BCL6 -BRD4 ternary complex formation assay

Peak X	123.5	EC50	28.58

Peak X	41.15	EC50	14.57

	BRD4-BCL6 RIPTAC	BRD4-BCL6 TCIP1
EC50	2.802	0.8881

Cell Viability assay

	IC50
BRD4-BCL6 RIPTAC	2.974
BRD4-BCL6 TCIP1	11.85

Figure 7. Application of the established validation cascade to novel, highly challenging targets, such as mutant p53 (e.g., p53-Y220C-BRD4), P300 (e.g., AR-P300), BCL6 (e.g., BCL6-BRD4) using HTRF, NanoBRET and CTG.

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