

Supplementary Information

Antibody-Drug Nanoparticle Induces Synergistic Treatment

Efficacies in HER2 Positive Breast Cancer Cells

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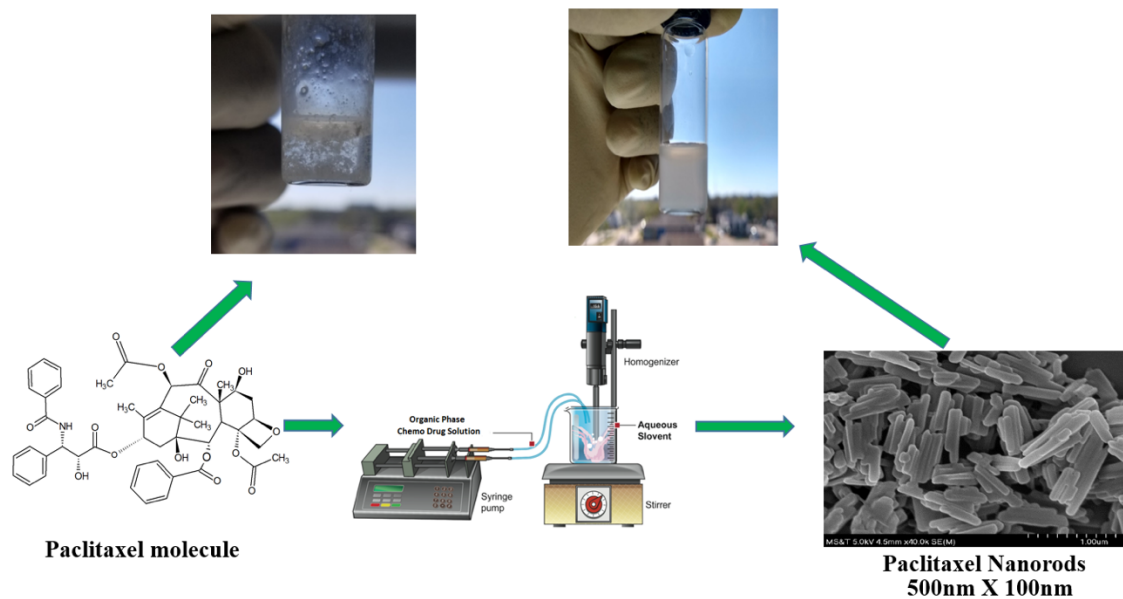
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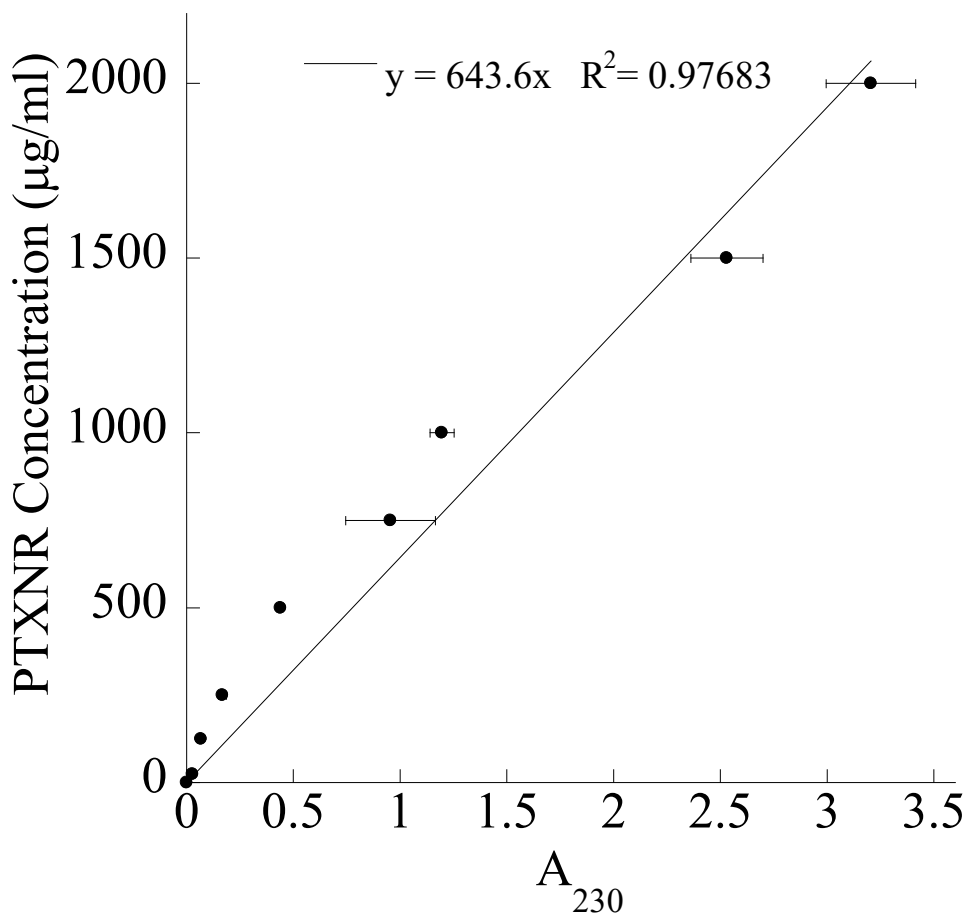
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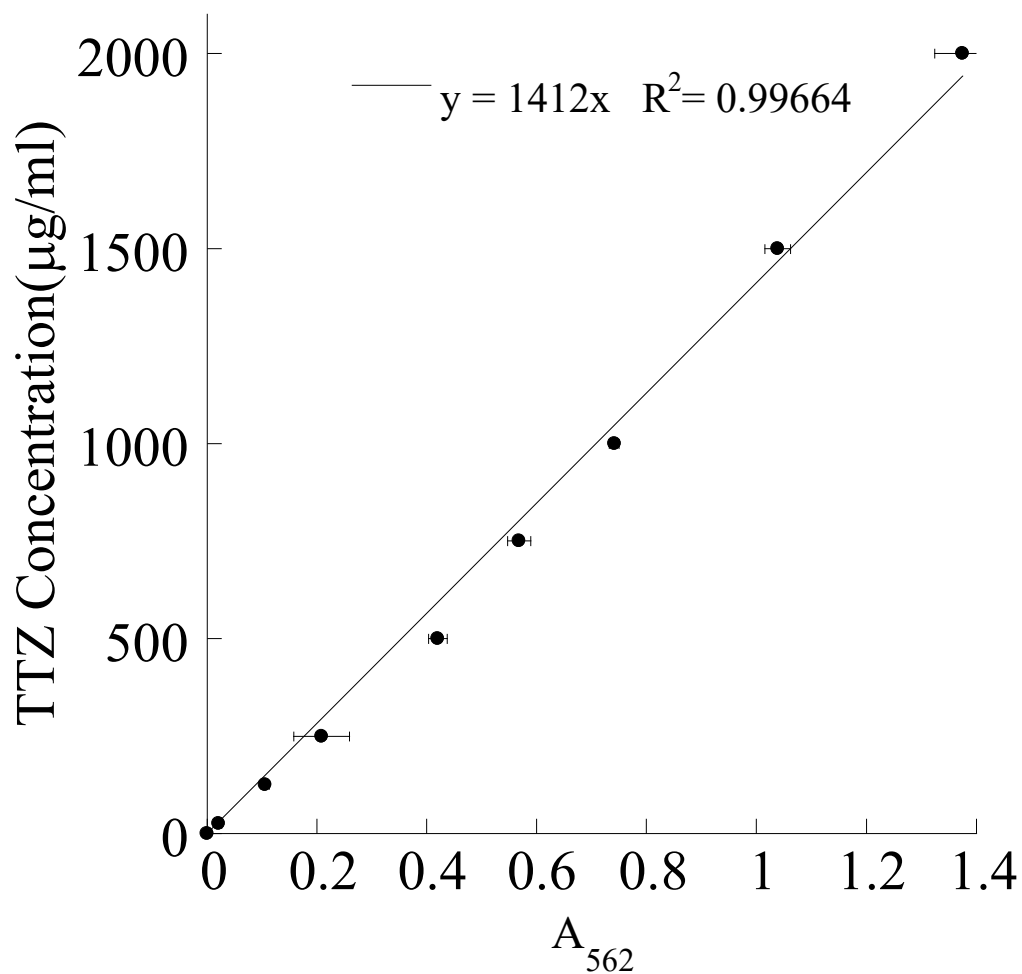
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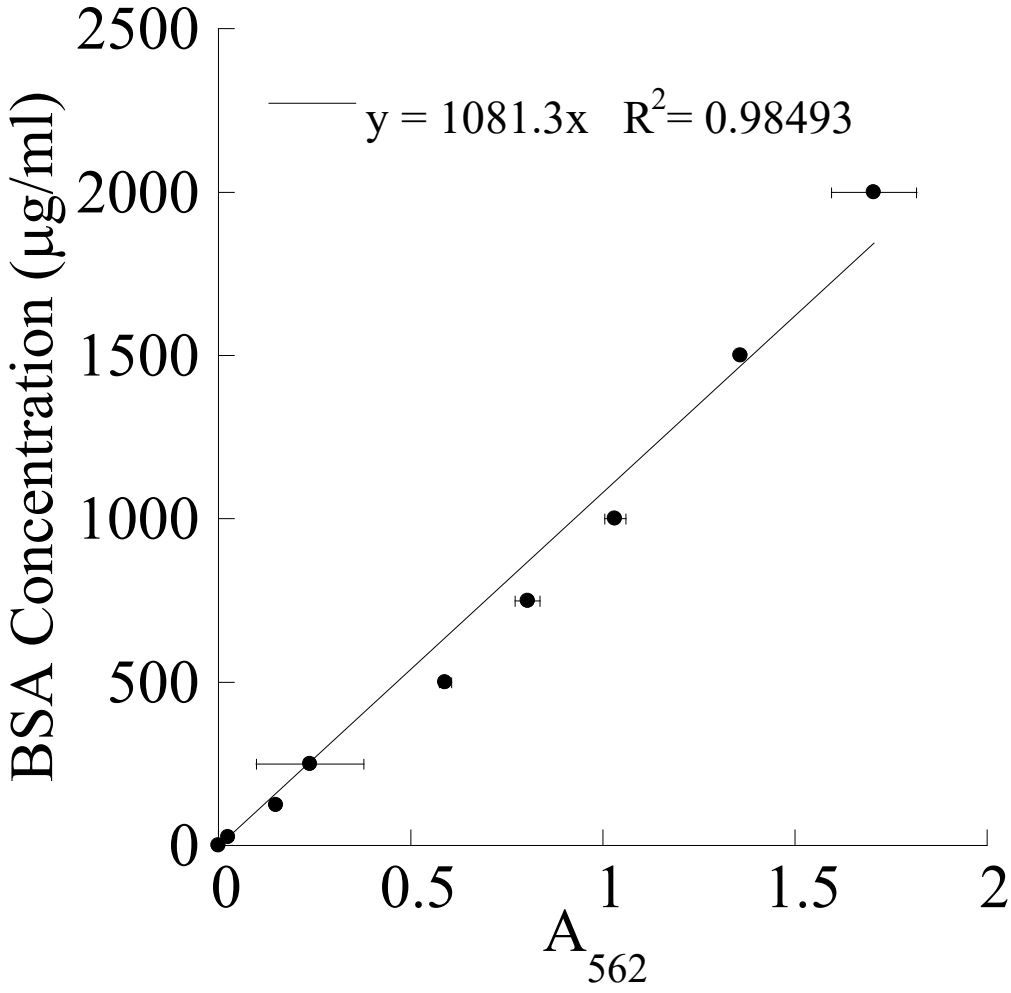
SI Figure 1: Synthesis process of PTXNRs. PTXNRs were synthesized using the nanoprecipitation method. Paclitaxel molecules show white floccular aggregation in water, while PTXNRs display complete dispersion in water after the synthesis process.



SI Figure 2: Calibration curve of PTX for quantification of the amount of PTXNRs in PTXNR-TTZ particles after conjugation with TTZ. The calibration curve was obtained using PTXNRs in PBS at 230 nm absorbance values.

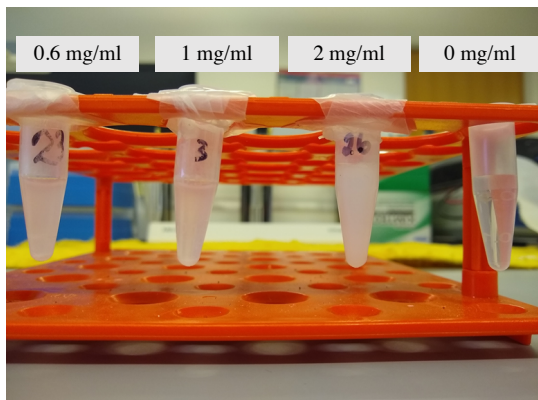


SI Figure 3: Calibration curve of Trastuzumab (TTZ) monoclonal antibody for quantification of unconjugated TTZ after conjugation with Paclitaxel nanorods (PTXNRs). The calibration curve was obtained using a BCA protein quantification assay at 562 nm absorbance values.

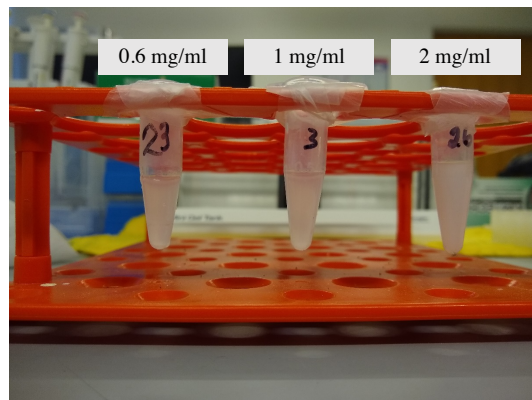


SI Figure 4: Calibration curve of BSA protein for quantification of protein in cell lysates for Western blot experiments. The calibration curve was obtained using a BCA protein quantification assay at 562 nm absorbance values.

PTXNR-TTZ in DI Water



PTXNR-TTZ in PBS



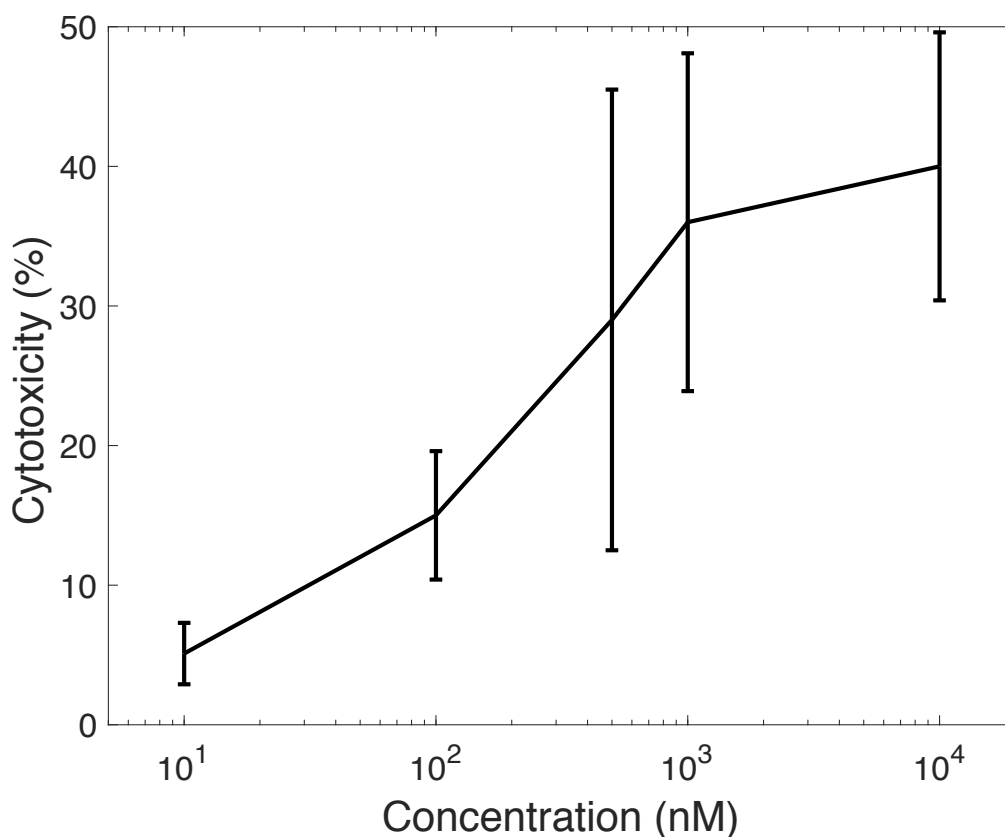
SI Figure 5: Colloidal suspensions of PTXNR-TTZ in DI water and PBS at 0.6, 1, and 2 mg/ml concentrations.

SI Table 1. Stability analysis of PTXNR and PTXNR-TTZ as a measure of zeta potential value, ζ (mV)

Sample	Zeta potential, ζ (mV)	
	DI water	PBS
PTXNR	-32.6± 4.82	-13.4± 2.81
PTXNR-TTZ	-17.1±3.83	-9.5± 0.02

SI Table 2: Analysis of TTZ conjugation on the surface of PTXNRs

Initial PTX amount (mg)	% NR formation	Initial PTXNR-CDI amount (mg)	Initial TTZ amount (mg)	% TTZ conjugation efficiency	Amount of PTXNR in 1 mg of PTXNR-TTZ (mg)	Amount of TTZ in 1 mg of PTXNR-TTZ (mg)	PTXNR : TTZ (w/w) (molar ratio)
30	64.98±2.37	1.0	0.10	95.24±1.40	0.8±0.02	0.2±0.02	4.0 ± 0.53 (681.67 ± 90.23)



SI Figure 6: PTXNR-TTZ showed non-specific cytotoxicity in MCF-10A normal cells *in vitro*.

SI Table 3: Cytotoxicity Analysis

BT-474

Treatment	vs. Treatment	<i>p</i> -value				
		10 nM	100 nM	500 nM	1000 nM	10000 nM
PTXNR-TTZ	PTXNR	0.8206	0.0296*	0.0004*	0.0007*	<.0001*
PTXNR-TTZ	TTZ	0.9579	0.0003*	<.0001*	<.0001*	<.0001*
PTXNR-TTZ	PTX solution	0.2244	0.3757	0.0003*	0.0002*	<.0001*
PTXNR-TTZ	PTX solution+ TTZ	0.9677	0.0273*	<.0001*	0.1745	0.0019*
PTX solution+ TTZ	PTXNR	0.7809	<.0001*	0.6475	0.0188*	0.0743
PTX solution+ TTZ	TTZ	0.9898	<.0001*	0.1194	<.0001*	<.0001*
PTX solution+ TTZ	PTX solution	0.2241	0.1499	0.7523	0.0055*	0.0014*
PTXNR	TTZ	0.7712	0.0207*	0.0562	<.0001*	0.0031*
PTXNR	PTX solution	0.1495	0.0020*	0.8910	0.5452	0.0873
PTX solution	TTZ	0.2280	<.0001*	0.0743	0.0006*	0.1651

MDA-MB-231

Treatment	vs. Treatment	<i>p</i> -value				
		10 nM	100 nM	500 nM	1000 nM	10000 nM
PTXNR-TTZ	PTXNR	0.1170	0.0002*	0.0186*	0.0246*	0.0775
PTXNR-TTZ	TTZ	0.0750	<.0001*	<.0001*	<.0001*	0.0002*
PTXNR-TTZ	PTX solution	0.2051	0.7412	0.4026	0.3234	0.8855
PTXNR-TTZ	PTX solution+ TTZ	0.9123	<.0001*	0.0031*	<.0001*	<.0001*
PTX solution+ TTZ	PTXNR	0.1282	<.0001*	<.0001*	<.0001*	<.0001*
PTX solution+ TTZ	TTZ	0.0811	<.0001*	<.0001*	<.0001*	<.0001*
PTX solution+ TTZ	PTX solution	0.1562	<.0001*	0.0005*	0.0009*	<.0001*
PTXNR	TTZ	0.8651	<.0001*	<.0001*	<.0001*	0.0152*
PTXNR	PTX solution	0.0070*	0.0004*	0.1434	0.0032*	0.1228
PTX solution	TTZ	0.0070*	<.0001*	<.0001*	<.0001*	0.0004*

SK-BR-3

Treatment	vs. Treatment	<i>p</i> -value				
		10 nM	100 nM	500 nM	1000 nM	10000 nM
PTXNR-TTZ	PTXNR	<.0001*	0.8554	0.6319	0.2213	0.0920
PTXNR-TTZ	TTZ	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*
PTXNR-TTZ	PTX solution	0.5424	0.0941	0.5570	0.1245	<.0001*
PTXNR-TTZ	PTX solution+ TTZ	0.8684	0.6750	0.5804	0.0776	0.0011*
PTX solution+ TTZ	PTXNR	<.0001*	0.7713	0.9276	0.4897	0.0286*
PTX solution+ TTZ	TTZ	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*
PTX solution+ TTZ	PTX solution	0.3447	0.1213	0.9661	0.8003	0.2079
PTXNR	TTZ	0.9758	<.0001*	<.0001*	<.0001*	<.0001*
PTXNR	PTX solution	<.0001*	0.0680	0.8939	0.6738	0.0011*
PTX solution	TTZ	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*

SI Table 4: Apoptosis Assay (Annexin-V-FITC/PI)

BT-474

Treatment	vs. Treatment	<i>p</i> -value											
		Early apoptotic (Annexin-V-FITC)				Non-apoptotic dead (PI)				Late apoptotic dead (Annexin-V-FITC/PI)			
		0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
PTXN R-TTZ	Untreated	0.6536	0.0257*	0.0017*	<.0001*	0.5569	<.0001*	0.0003*	0.6488	0.0831	0.1321	0.0005*	0.0003*

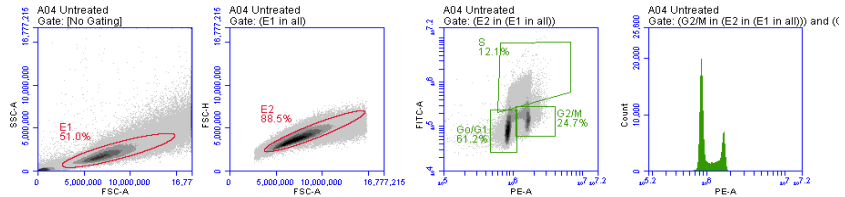
MDA-MB-231

Treatment	vs. Treatment	<i>p</i> -value											
		Early apoptotic (Annexin-V-FITC)				Non-apoptotic dead (PI)				Late apoptotic dead (Annexin-V-FITC/PI)			
		0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
PTXN R-TTZ	Untreated	0.6624	0.4486	0.0082*	0.0407*	0.0037*	0.0008*	0.0002*	0.0144*	0.0064*	0.0104*	<.0001*	0.0429*

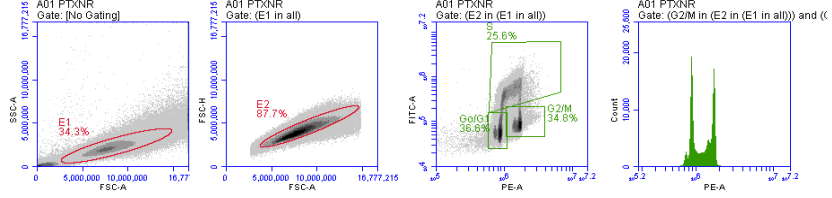
SK-BR-3

Treatment	vs. Treatment	<i>p</i> -value											
		Early apoptotic (Annexin-V-FITC)				Non-apoptotic dead (PI)				Late apoptotic dead (Annexin-V-FITC/PI)			
		0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
PTXN R-TTZ	Untreated	-	0.1871	0.2947	0.3135	0.4910	0.0553	0.0295*	0.5938	0.1957	0.0964	0.0002*	0.0003*

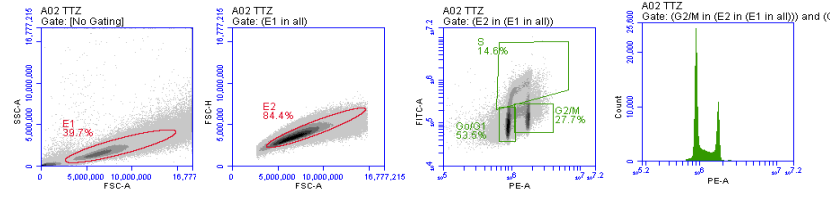
(a) Untreated



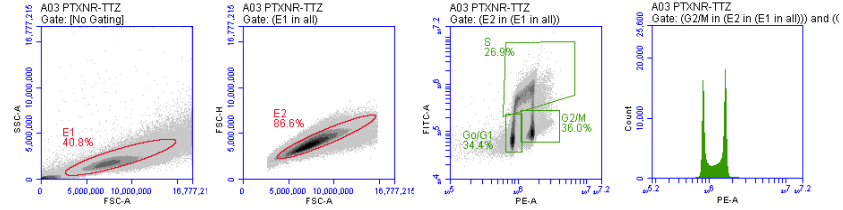
(b) PTXNR



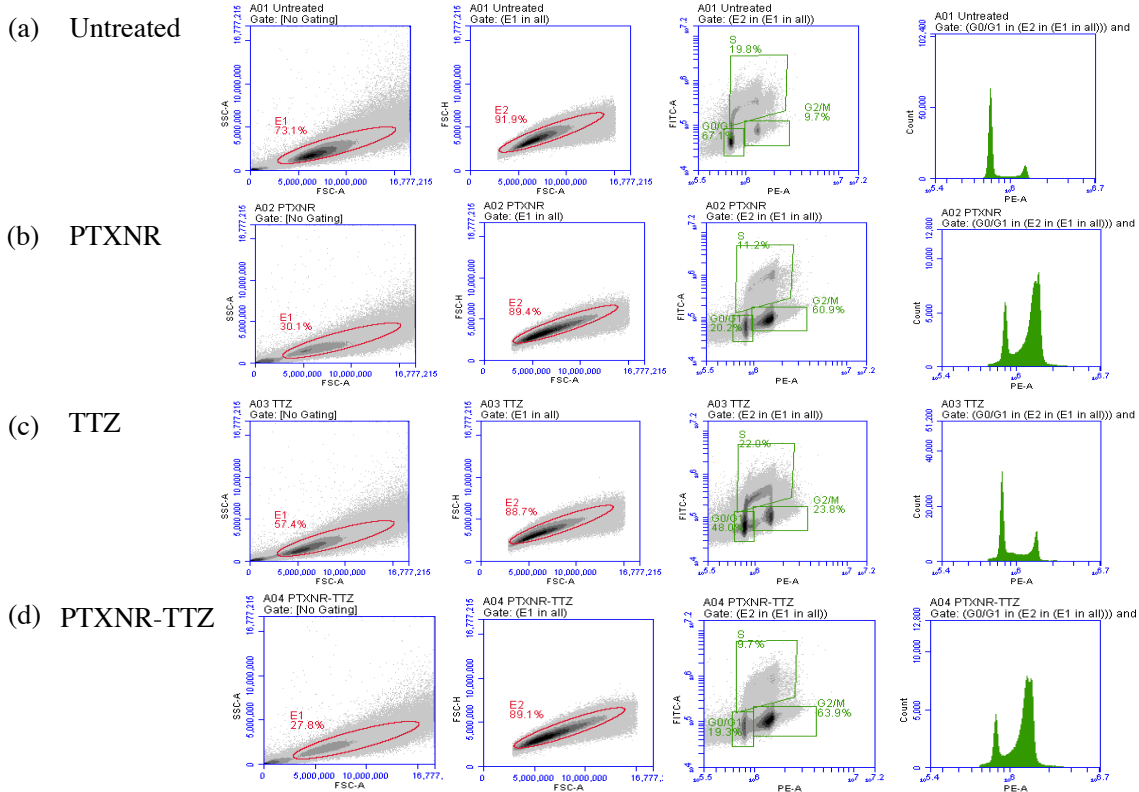
(c) TTZ



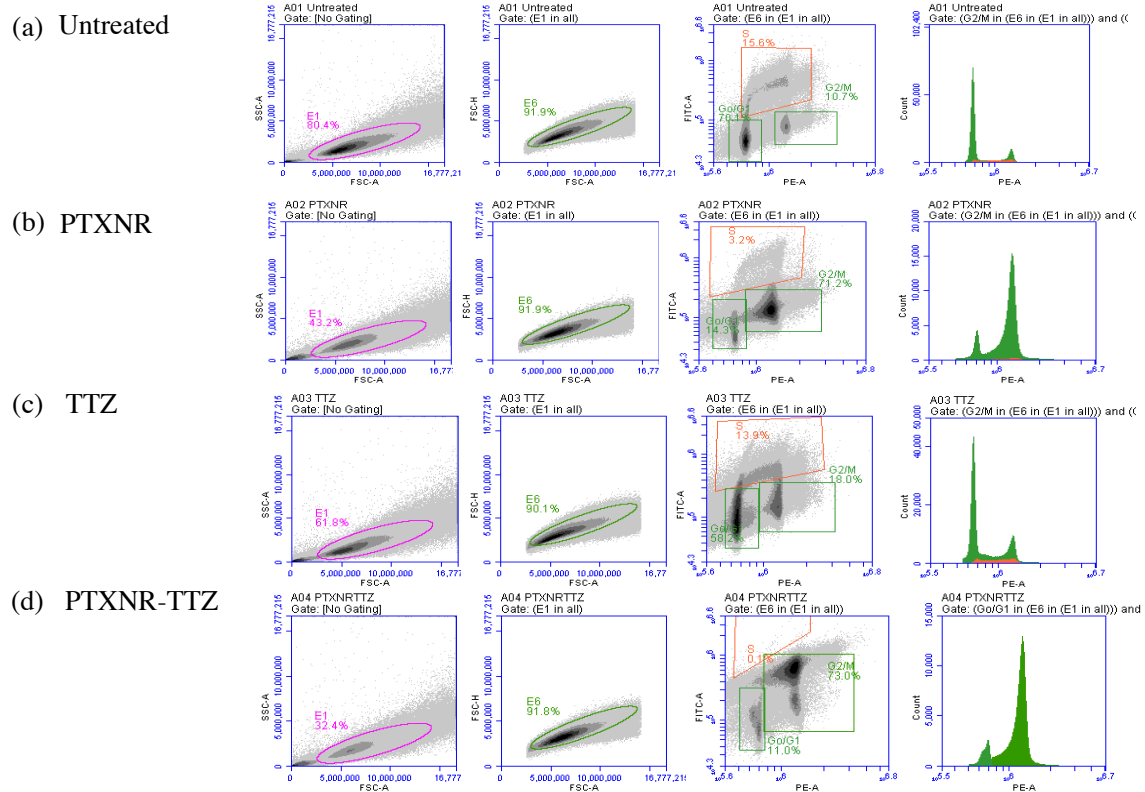
(d) PTXNR-TTZ



SI Figure 7: Flow cytometry data for cell cycle analysis of (a) untreated, (b) PTXNR, (c) TTZ, and (d) PTXNR-TTZ treated BT-474 cells after 24 h. The side scatter (SSC-A) versus forward scatter (FSC-A) gating is used to identify BT-474 cells as gated by E1. The forward scatter height (FSC-H) versus forward scatter area (FSC-A) density plots exclude doublet cells for accurate counting as shown by E2 gating. The plots showing FITC-A and PE-A represent live cells and their stained DNA, respectively, and gated for DNA analysis using Count versus PE-A plots. The Count versus PE-A plots were analyzed using the flow cytometry software to calculate the percentage arrests in G0/G1 (first sharp peak), S (flattened areas), and G2/M (second sharp peak) phases.



SI Figure 8: Flow cytometry data for cell cycle analysis of (a) untreated, (b) PTXNR, (c) TTZ, and (d) PTXNR-TTZ treated BT-474 cells after 48 h. The histogram of relative DNA content in Count *versus* PE-A plots shows a shift in peaks from G0/G1 to G2/M cells in PTXNR and PTXNR-TTZ treated cells compared to untreated and TTZ treated cells.



SI Figure 9: Flow cytometry data for cell cycle analysis of (a) untreated, (b) PTXNR, (c) TTZ, and (d) PTXNR-TTZ treated BT-474 cells after 72h. By the end of the incubation periods, >80% of cells were arrested in G2/M phases after PTXNR and PTXNR-TTZ treatments, while untreated and TTZ treated cells showed most cells in G0/G1.

SI Table 5: Cell Cycle Analysis

BT-474

Treatment	vs. Treatment	<i>p</i> -value								
		24 h			48 h			72 h		
		G0/G1	S	G2/M	G0/G1	S	G2/M	G0/G1	S	G2/M
PTXNR-TTZ	Untreated	0.0551	0.5392	0.0382*	0.0029*	0.2282	0.0017*	0.0005*	0.0021*	0.0007*
PTXNR-TTZ	PTXNR	0.8429	0.8978	0.9780	0.8852	0.6973	0.7662	0.6049	0.1159	0.3776
PTXNR-TTZ	TTZ	0.2262	0.6231	0.3015	0.0245*	0.4362	0.0139*	0.0014*	0.0051*	0.0018*
PTXNR	Untreated	0.0689	0.6218	0.0393*	0.0027*	0.3725	0.0020*	0.0006*	0.0072*	0.0010*
PTXNR	TTZ	0.2902	0.7131	0.3121	0.0214*	0.6787	0.0181*	0.0018*	0.0238*	0.0032*
TTZ	Untreated	0.2786	0.8962	0.1364	0.0408*	0.6070	0.0286*	0.0646	0.2088	0.0858

SI Table 6: Western Blot Analysis

BT-474

Treatment	vs. Treatment	<i>p</i> -value					
		XIAP	Actin	Cleaved Caspase - 9 (p10)	Caspase 3	Cleaved Caspase -3	Cyt- C
PTXNR-TTZ	Untreated	0.0094*	<.0001*	0.0235*	0.0150*	0.0494*	0.0391*
PTXNR-TTZ	PTXNR	0.3926	0.0721	0.3251	0.4507	0.7168	0.7549
PTXNR-TTZ	TTZ	0.0243*	<.0001*	0.0693	0.0099*	0.4133	0.5425
PTXNR	Untreated	0.0375*	<.0001*	0.0094*	0.0622	0.0301*	0.0548
PTXNR	TTZ	0.0989	<.0001*	0.0230*	0.0417*	0.6332	0.7576
TTZ	Untreated	0.5503	0.6209	0.3330	0.8262	0.0163*	0.0779

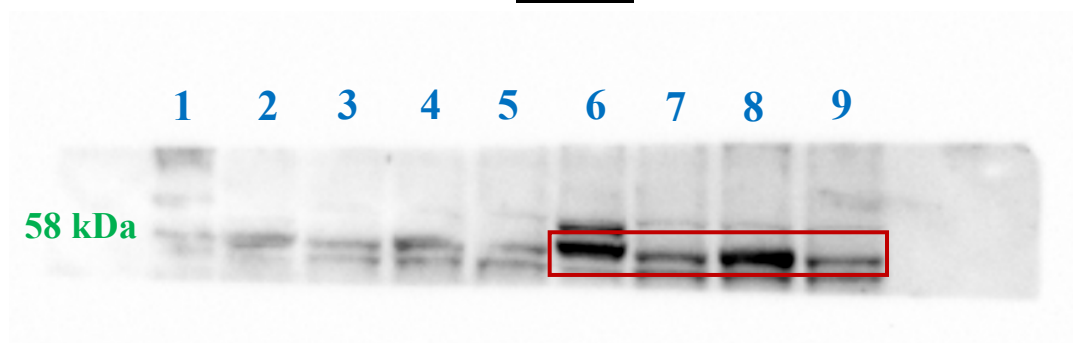
MDA-MB-231

Treatment	vs. Treatment	<i>p</i> -value					
		XIAP	Actin	Cleaved Caspase - 9 (p10)	Caspase 3	Cleaved Caspase -3	Cyt- C
PTXNR-TTZ	Untreated	0.5631	0.0028*	0.7904	0.4751	-	0.0160*
PTXNR-TTZ	PTXNR	0.1208	0.0009*	0.5113	0.3466	-	0.3400
PTXNR-TTZ	TTZ	0.2954	0.0046*	0.3973	0.4565	-	0.7792
PTXNR	Untreated	0.2525	0.0919	0.6854	0.8126	-	0.0429*
PTXNR	TTZ	0.4880	0.0393*	0.8317	0.1058	-	0.4777
TTZ	Untreated	0.5973	0.4643	0.5437	0.1578	-	0.0207*

Full Western Blot Images of BT-474 as shown in Figure 7(a)

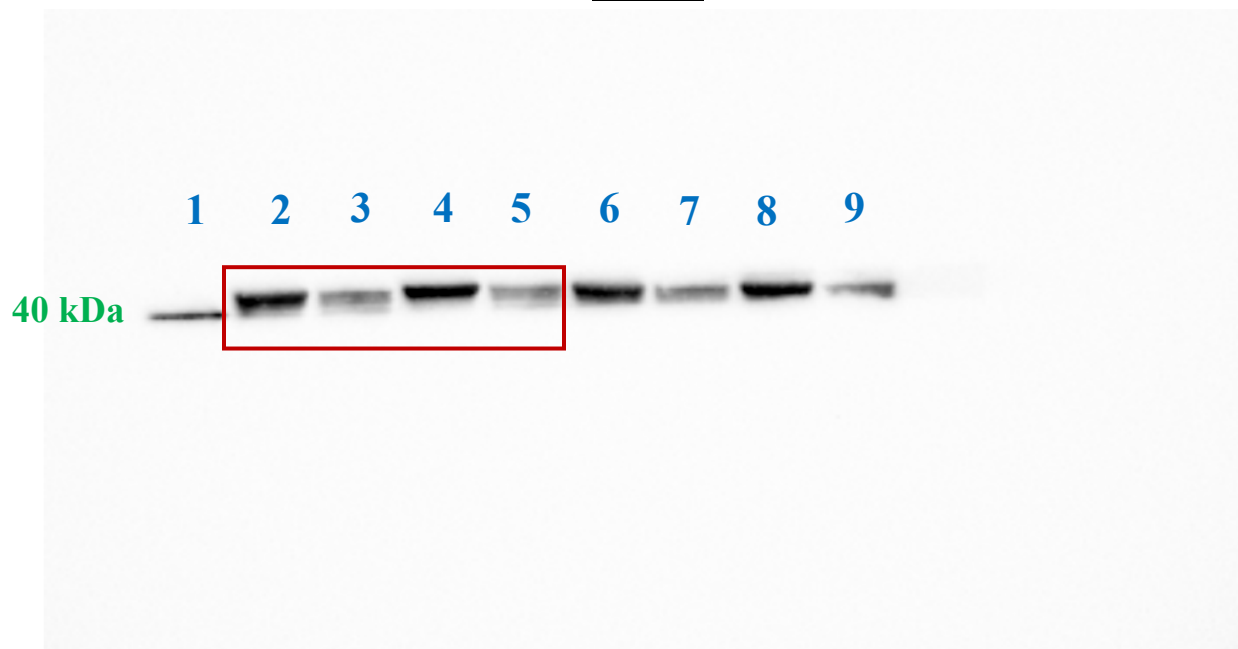
SI Figure 10 (a): A single full-length Western gel blot was used for probing multiple protein expressions in BT-474 cells. The blot was cut into multiple strips following the protein transfer as revealed in **Figure 7(a)**. Here, the strip containing approximately 40-80 kDa region was imaged.

XIAP



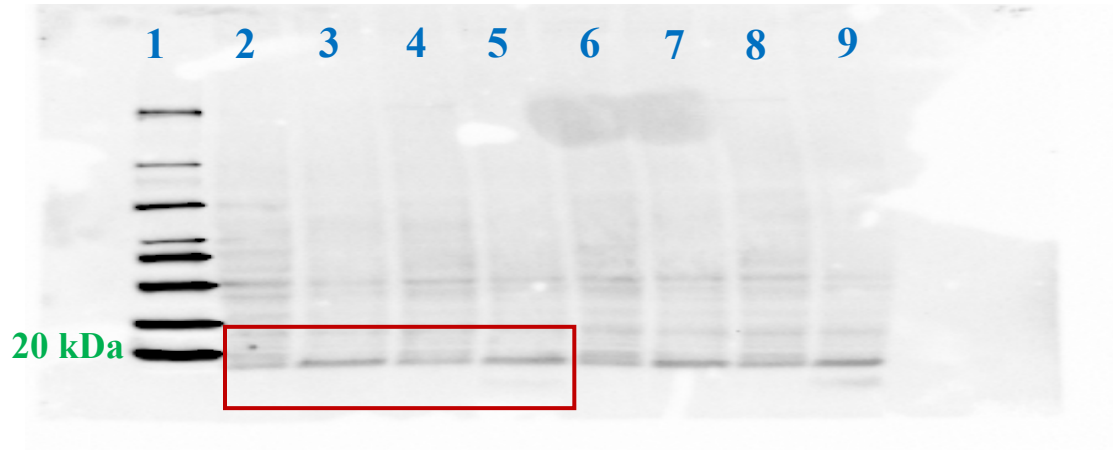
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Actin



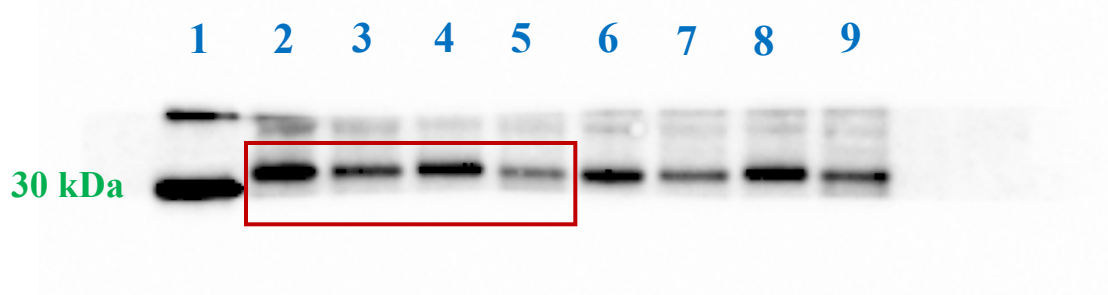
1, Protein ladder; 2, Untreated; 3, PTXNR; 4, TTZ; 5, PTXNR-TTZ; 6, Untreated; 7, PTXNR; 8, TTZ; 9, PTXNR-TTZ

Fully Cleaved Caspase-9



1, Protein ladder; 2, Untreated; 3, PTXNR; 4, TTX; 5, PTXNR-TTX; 6, Untreated; 7, PTXNR; 8, TTX; 9, PTXNR-TTX

Caspase-3



1, Protein ladder; 2, Untreated; 3, PTXNR; 4, TTX; 5, PTXNR-TTX; 6, Untreated; 7, PTXNR; 8, TTX; 9, PTXNR-TTX. Note: The blot was cut into multiple strips following the protein transfer.

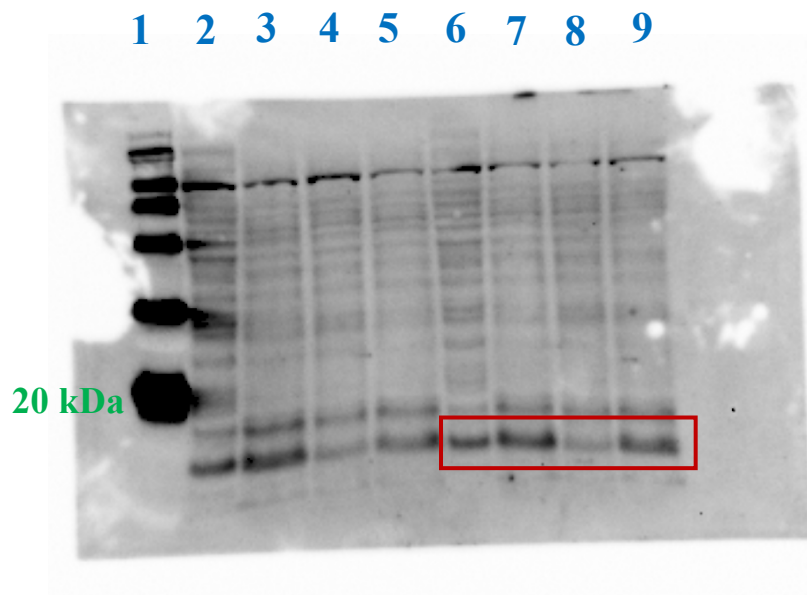
A single full-length blot was used for probing multiple protein expression. Here, the strip containing 30-40 kDa region was imaged

Cleaved Caspase-3



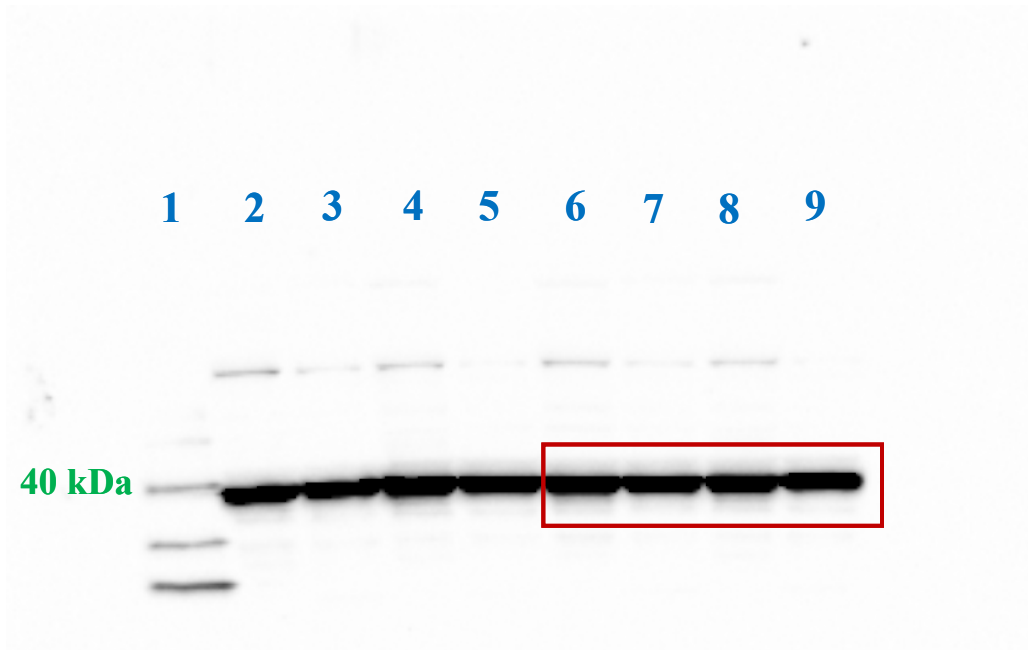
1, Protein ladder; 2, Untreated; 3, PTXNR; 4, TTZ; 5, PTXNR-TTZ; 6, Untreated; 7, PTXNR; 8, TTZ; 9, PTXNR-TTZ

Cytochrome-C



1, Protein ladder; 2, Untreated; 3, PTXNR; 4, TTZ; 5, PTXNR-TTZ; 6, Untreated; 7, PTXNR; 8, TTZ; 9, PTXNR-TTZ

GAPDH

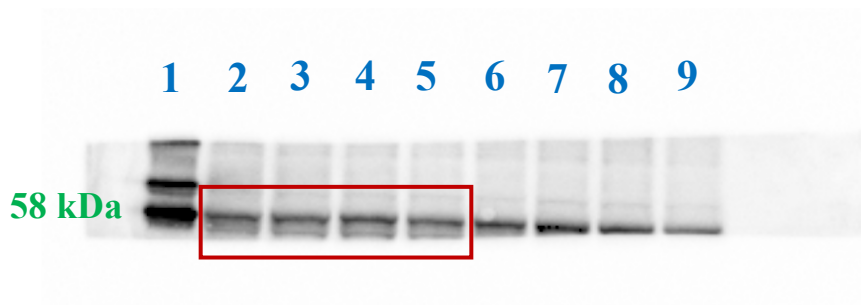


1, Protein ladder; 2, Untreated; 3, PTXNR; 4, TTZ; 5, PTXNR-TTZ; 6, Untreated; 7, PTXNR; 8, TTZ; 9, PTXNR-TTZ

Full Western Blot Images of MDA-MB-231 as shown in Figure 7(b)

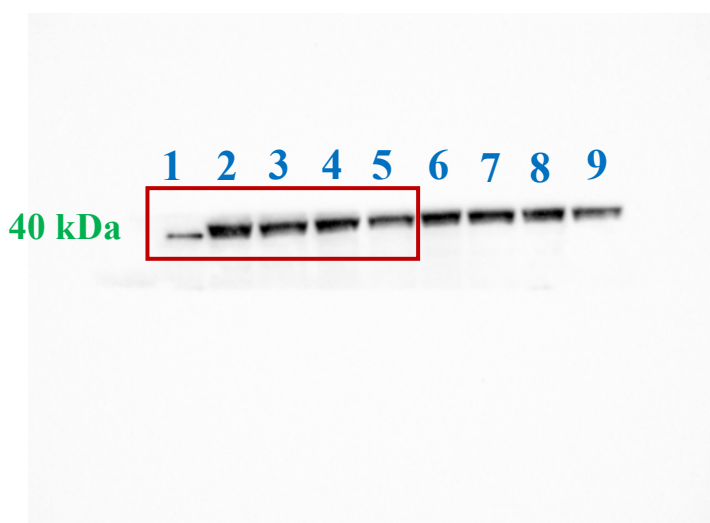
SI Figure 10 (b): A single full-length blot was used for probing multiple protein expressions in MDA-MB-231 cells. The blot was cut into multiple strips following the protein transfer. Here, the strip containing 40-80 kDa region was imaged as revealed in **Figure 7(b)**.

XIAP



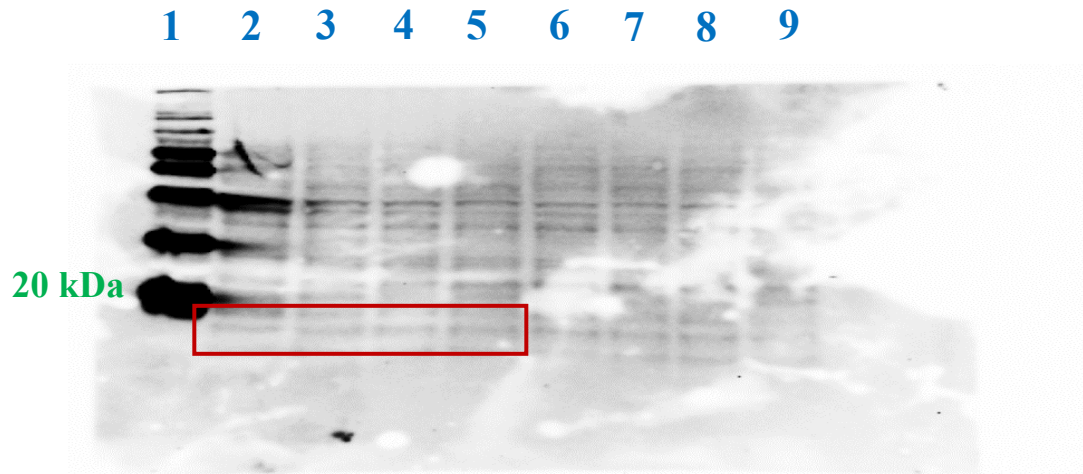
1, Protein ladder; 2, Untreated; 3, PTXNR; 4, TTZ; 5, PTXNR-TTZ; 6, Untreated; 7, PTXNR; 8, TTZ; 9, PTXNR-TTZ

Actin



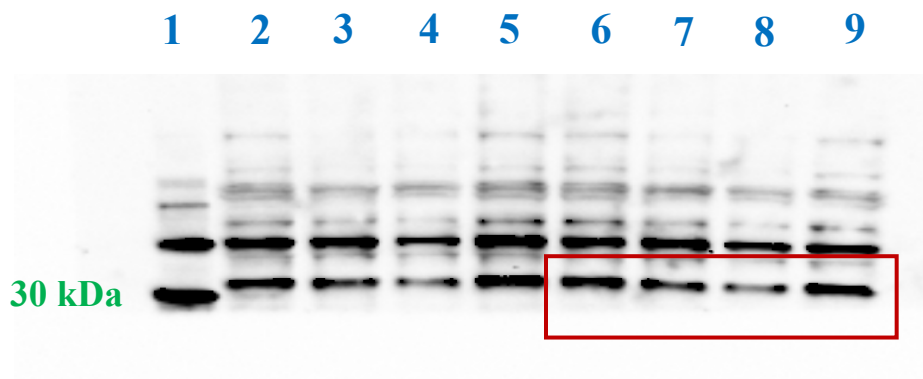
1, Protein ladder; 2, Untreated; 3, PTXNR; 4, TTZ; 5, PTXNR-TTZ; 6, Untreated; 7, PTXNR; 8, TTZ; 9, PTXNR-TTZ

Fully Cleaved Caspase-9



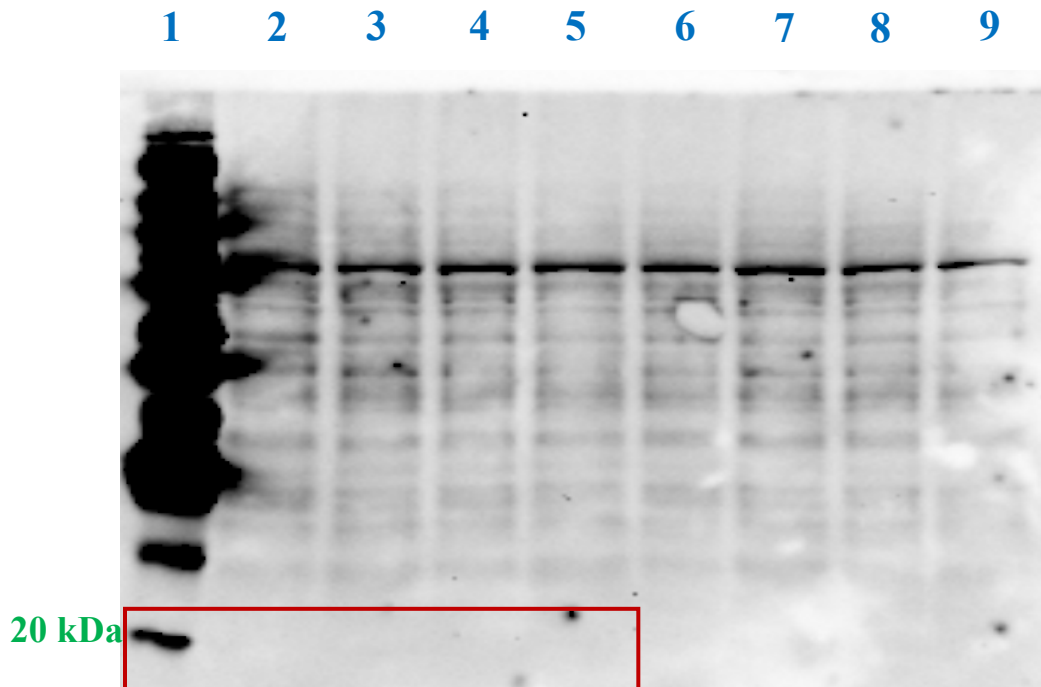
1, Protein ladder; 2, Untreated; 3, PTXNR; 4, TTZ; 5, PTXNR-TTZ; 6, Untreated; 7, PTXNR; 8, TTZ; 9, PTXNR-TTZ

Caspase-3



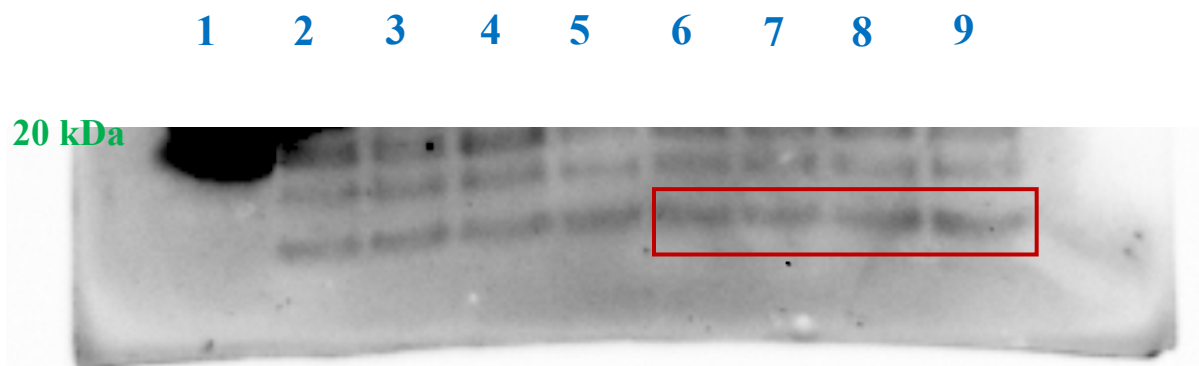
1, Protein ladder; 2, Untreated; 3, PTXNR; 4, TTZ; 5, PTXNR-TTZ; 6, Untreated; 7, PTXNR; 8, TTZ; 9, PTXNR-TTZ

Cleaved Caspase-3



1, Protein ladder; 2, Untreated; 3, PTXNR; 4, TTZ; 5, PTXNR-TTZ; 6, Untreated; 7, PTXNR; 8, TTZ; 9, PTXNR-TTZ

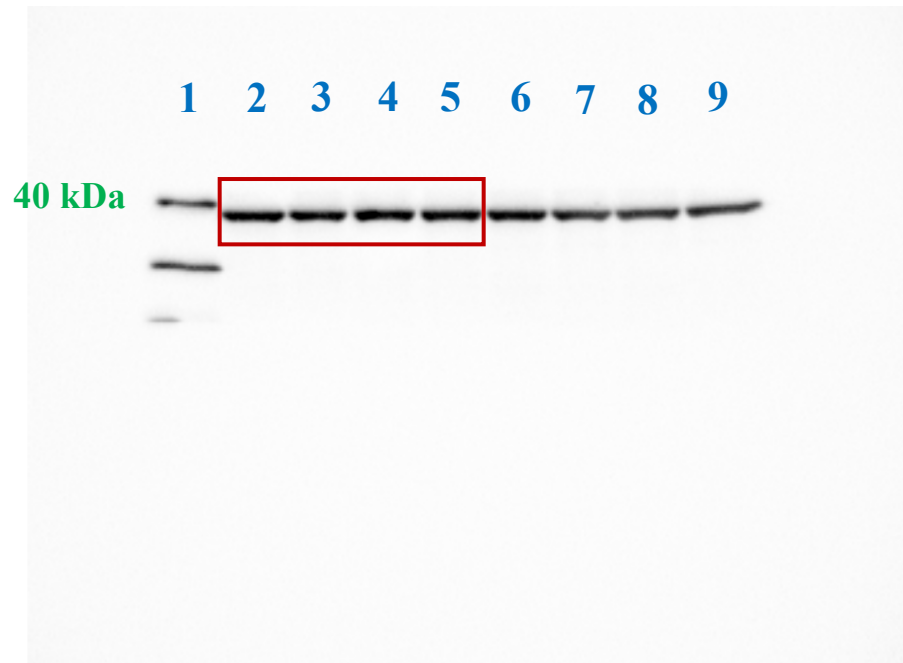
Cytochrome-C



1, Protein ladder; 2, Untreated; 3, PTXNR; 4, TTZ; 5, PTXNR-TTZ; 6, Untreated; 7, PTXNR; 8, TTZ; 9, PTXNR-TTZ. Note: The blot was cut into multiple strips following the protein transfer.

A single full-length blot was used for probing multiple protein expression. Here, a narrow strip around >20 kDa region was imaged

GAPDH



1, Protein ladder; 2, Untreated; 3, PTXNR; 4, TTZ; 5, PTXNR-TTZ; 6, Untreated; 7, PTXNR; 8, TTZ; 9, PTXNR-TTZ