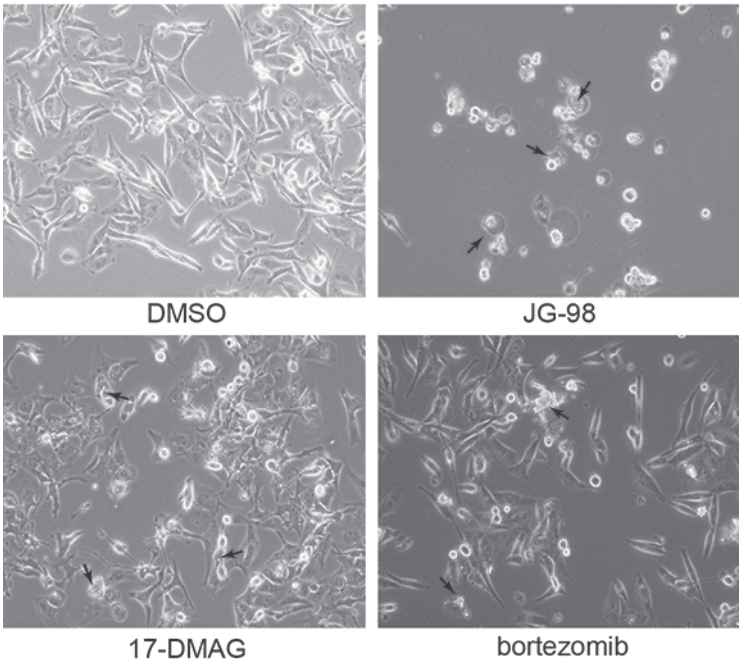
(A) Proteostasis inhibitors induce apoptosis in MDA-MB-231 cells



(B) JG-98 treated MDA-MB-231 cells proceed through an apoptotic pathway, as judged by flow cytometry

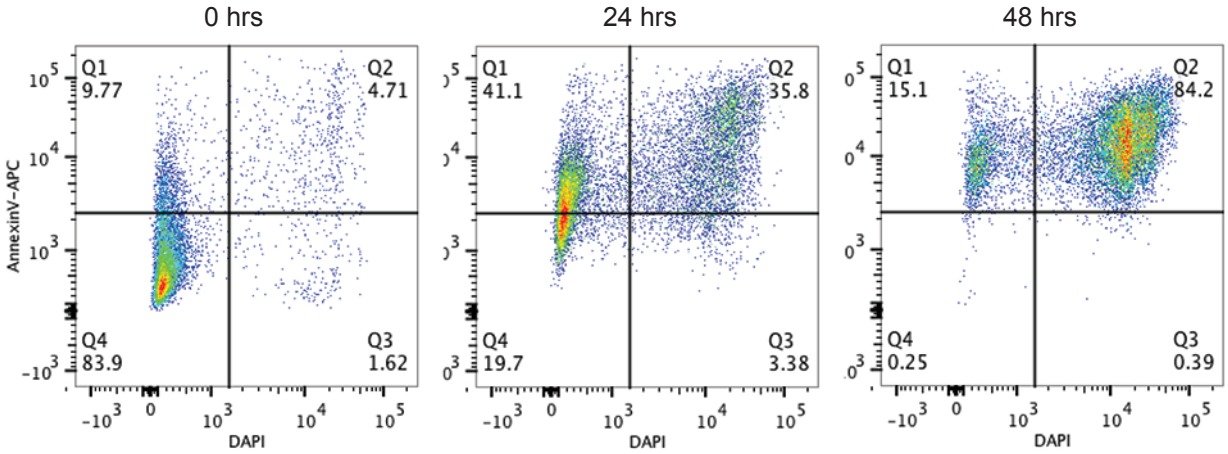
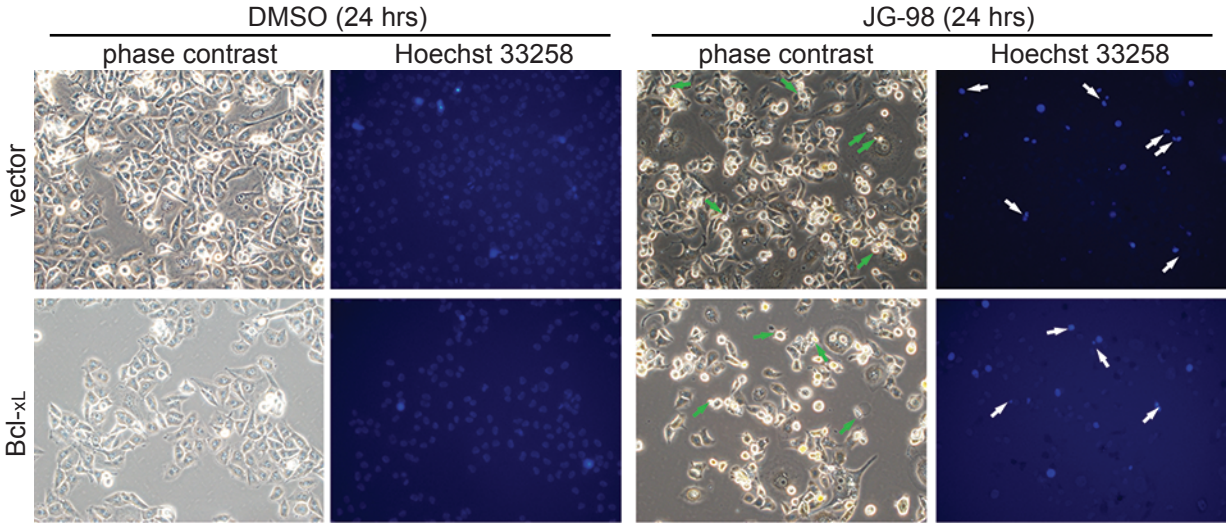


Figure S1. JG-98 Induces Apoptosis in MDA-MB-231 cells. (A) Phase contrast microscopy of MDA-MB-231 cells treated with JG-98 (10 μM), 17-DMAG (10 μM), or bortezomib (40 nM) for 24 hours. Apoptotic cells are indicated by black arrows. (B) Flow cytometry of MDA-MB-231 cells treated with JG-98 (10 μM) for the indicated times and labeled with annexin V and DAPI. Results are representative of two independent experiments performed in duplicate.

(A) MDA-MB-231 cells overexpressing Bcl-xL still undergo apoptosis when treated with JG-98



(B) JG98 treatment does increase cytoplasmic COX IV

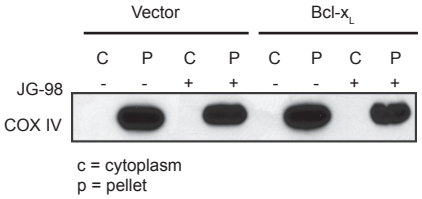


Figure S2. JG-98 Activates the Mitochondrial Death and Necroptosis Pathways. (A) Fluorescence microscopy of MDA-MB-231 cells over-expressing Bcl-xL treated with JG-98 (10 μM). Features are consistent with apoptosis (green and white arrows). Cells were treated for 24 hours with either DMSO or compound. (B) JG-98 (10 μM) does not cause a non-specific release of COX IV into the cytoplasm, suggesting that mitochondrial integrity is maintained. MDA-MB-231 cells treated for 24 hrs. Results are representative of duplicates.

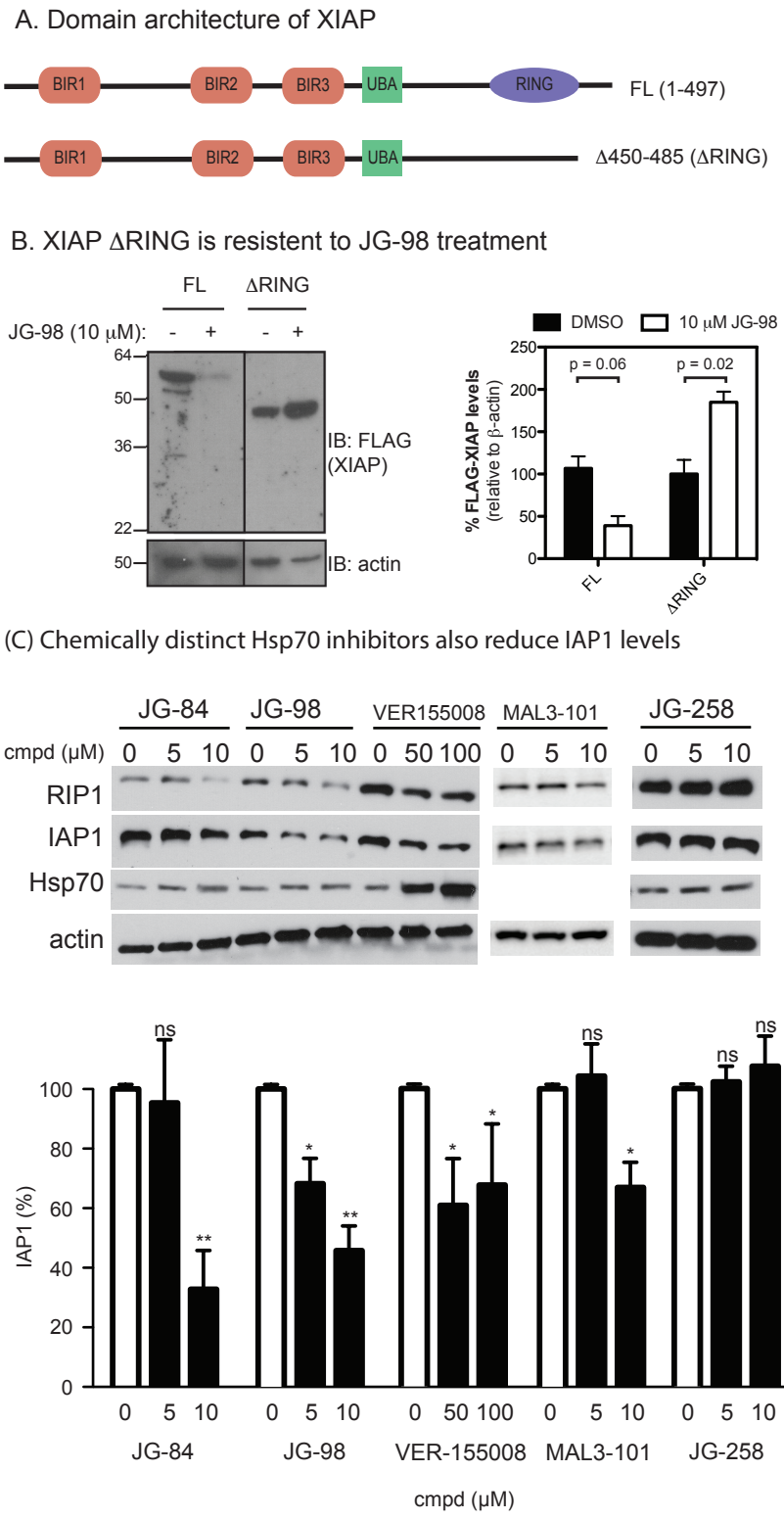
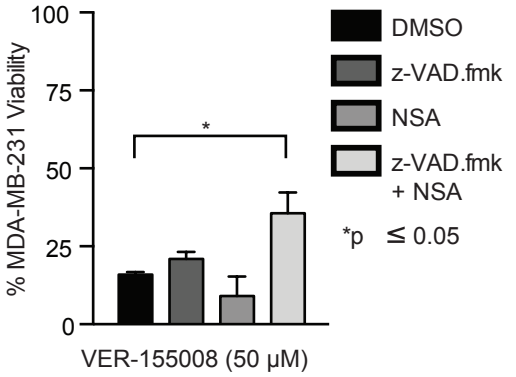


Figure S3. Mechanism of IAP turnover by JG-98 and other Hsp70 inhibitors. (A) Representation of XIAP clones. (B) Treatment of MDA-MB-231 cells with JG-98 (10 μM, 12 hrs) reduces XIAP levels, but deletion of the RING domain is protective. Results of three independent experiments were quantified and averaged. Error bars represent SEM. (C) MDA-MB-231 cells were treated with the indicated compound for 12 hours. JG-84 is an active analog of JG-98, while JG-258 is structurally related but inactive (see Fig 1a). VER-155008 and MAL3-101 are structurally unrelated Hsp70 inhibitors (see Fig 1a). The Western blot is representative and MAL3-101 and JG-258 results were from a different gel. Bar graphs show the average of at least three independent experiments. Error bars represent SEM.

(A) MDA-MB-231 cells require z-VAD-fmk and NSA treatment to protect from VER-155008



(B) Co-treatment with z-VAD-fmk and NSA is required to protect from JG-98 in most cancer cells tested

		JG-98 EC_{50} (μM; fold change)				
Tissue	Cell line	DMSO	z-VAD.fmk		z-VAD.fmk + NSA	
Breast	MDA-MB-231	1.8 ± 0.5	1.6 ± 0.2	(1.0)ns	4.3 ± 0.6	(2.3)**
	MCF-7	1.1 ± 0.2	1.3 ± 0.2	(1.0)ns	2.8 ± 0.6	(1.4)*
	SK-BR-3	2.0 ± 0.3	2.2 ± 0.3	(1.0)ns	2.9 ± 0.5	(1.3)*
	T-47D	9.7 ± 1.0	10 ± 1.0	(1.1)ns	8.6 ± 1.8	(1.1)ns
Leukemia	Jurkat	31 ± 7	34 ± 8	(1.1)ns	56 ± 8	(1.8)*
Cervix	HeLa	5.0 ± 0.6	14 ± 5	(2.0)*	15 ± 3	(2.3)*
Lung	A549	34 ± 7	32 ± 8	(1.0)ns	13 ± 2	(0.7)*
Colon	HT-29	12 ± 2	11 ± 1	(1.0)ns	1.7 ± 0.3	(0.1)**

ns = not significant
*p ≤ 0.05
**p ≤ 0.01

Figure S4. Hsp70 inhibitors activate both apoptotic and necroptotic pathways, but the predominant routes of cell death depends on cell type. (A) VER-155008 (50 μM) induced cell death in MDA-MB-231 cells, which was partially blocked by the combination of z-VAD-fmk and NSA, but not either compound individually. Results are the average of at least three independent experiments performed in tripicate and the error bars represent SEM. (B) Cell lines derived from a variety of tissue origins were treated with JG-98, JG-98 + z-VAD.fmk, or JG-98 + z-VAD.fmk and NSA in combinations. z-VAD.fmk alone partially protected HeLa cells, but had no effect on the other lines. Combinations of z-VAD.fmk and NSA protected against cell death in most the cells, but had no effect in T47-D, A549 or HT-29 cells. Results are the average of at least three experiments performed in triplicate each. Error bars are SEM.

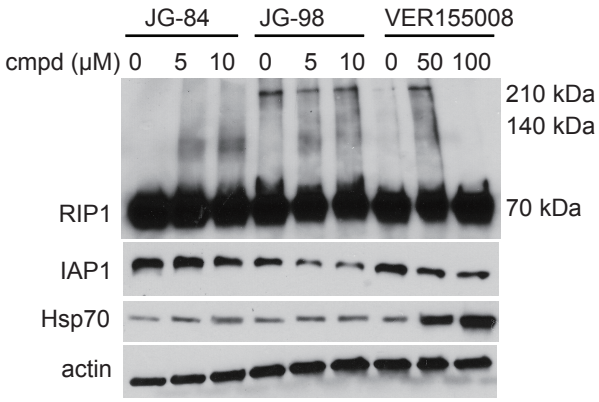


Figure S5. Other Hsp70 inhibitors also relieve suppression of RIP1 oligomerization.MDA-MB-231 cells were treated for 12 hours at the indicated concentrations. Results are representative of experiments performed in triplicate.