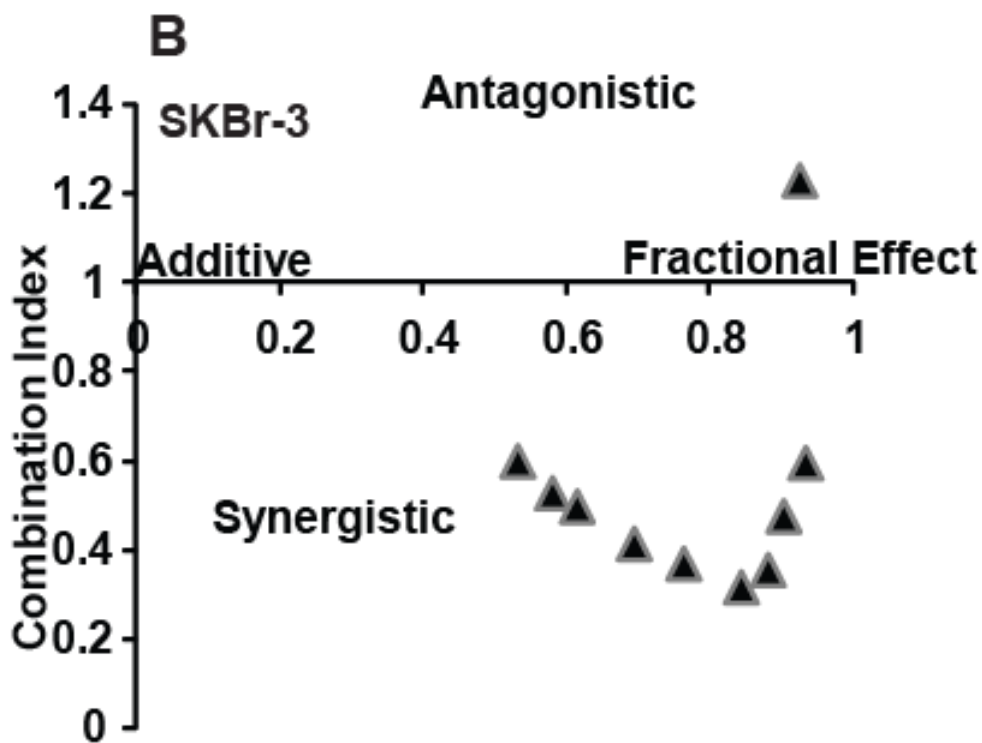
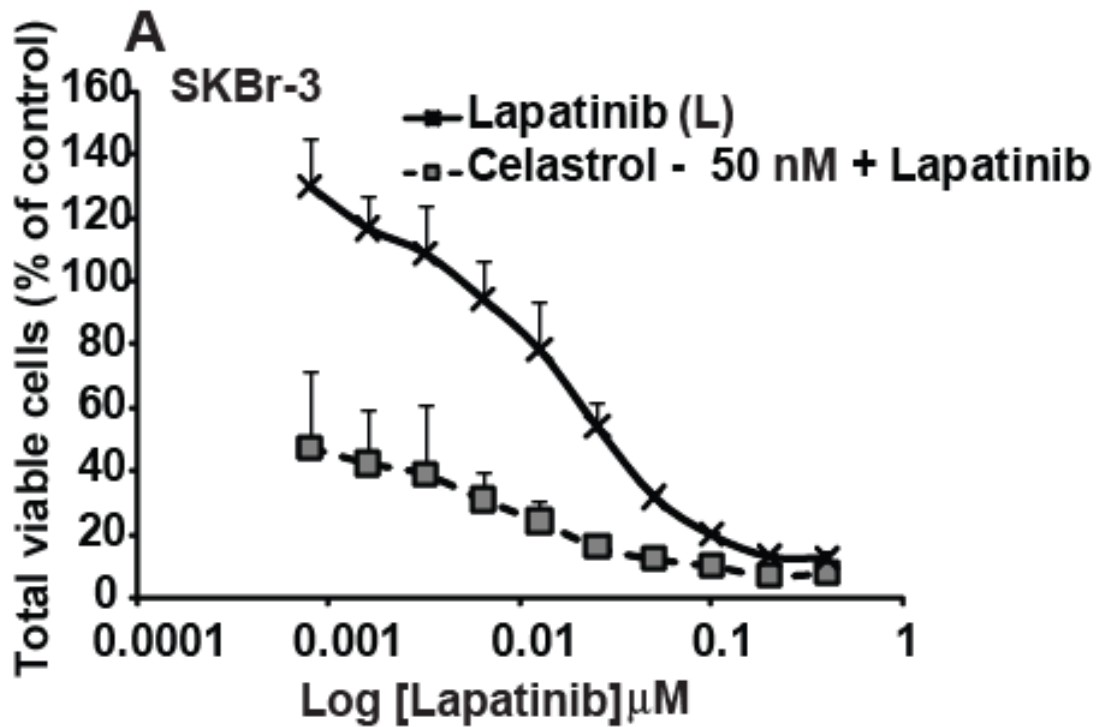


Supplementary Information

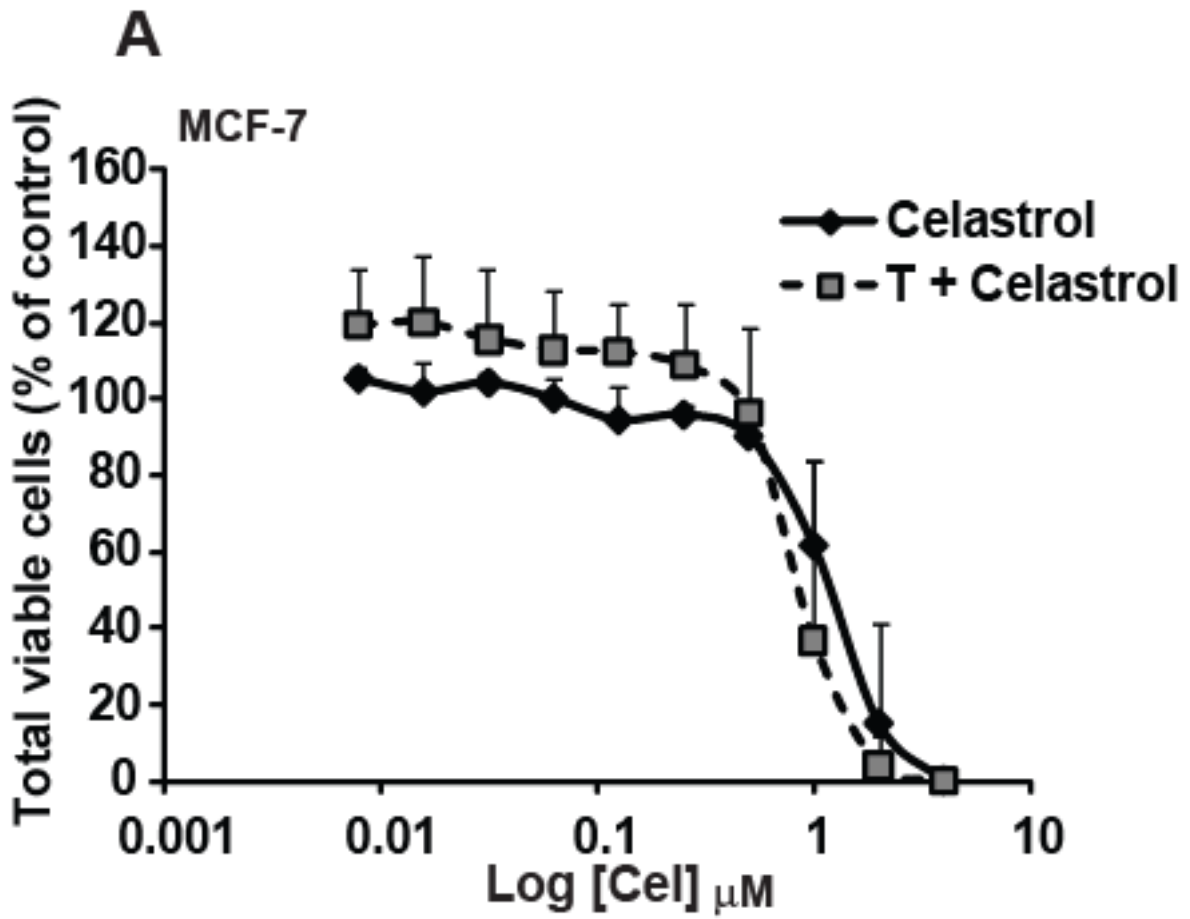
Title: Anticancer activity of Celastrol in combination with ErbB2-targeted therapeutics for treatment of ErbB2-overexpressing Breast Cancers.

Authors: Srikumar M. Raja^{1,4,§}, Robert J. Clubb^{1,4,5}, Cesar Ortega-Cava^{1,4}, Stetson H. Williams¹, Tameka A. Bailey¹, Lei Duan^{1,4,5}, Xiangshan Zhao^{3,4}, Alagarasamy L. Reddi^{4,5}, Abijah M. Nyong¹, Amarnath Natarajan¹, Vimla Band^{1,3,4} and Hamid Band^{1,2,3,4,§}

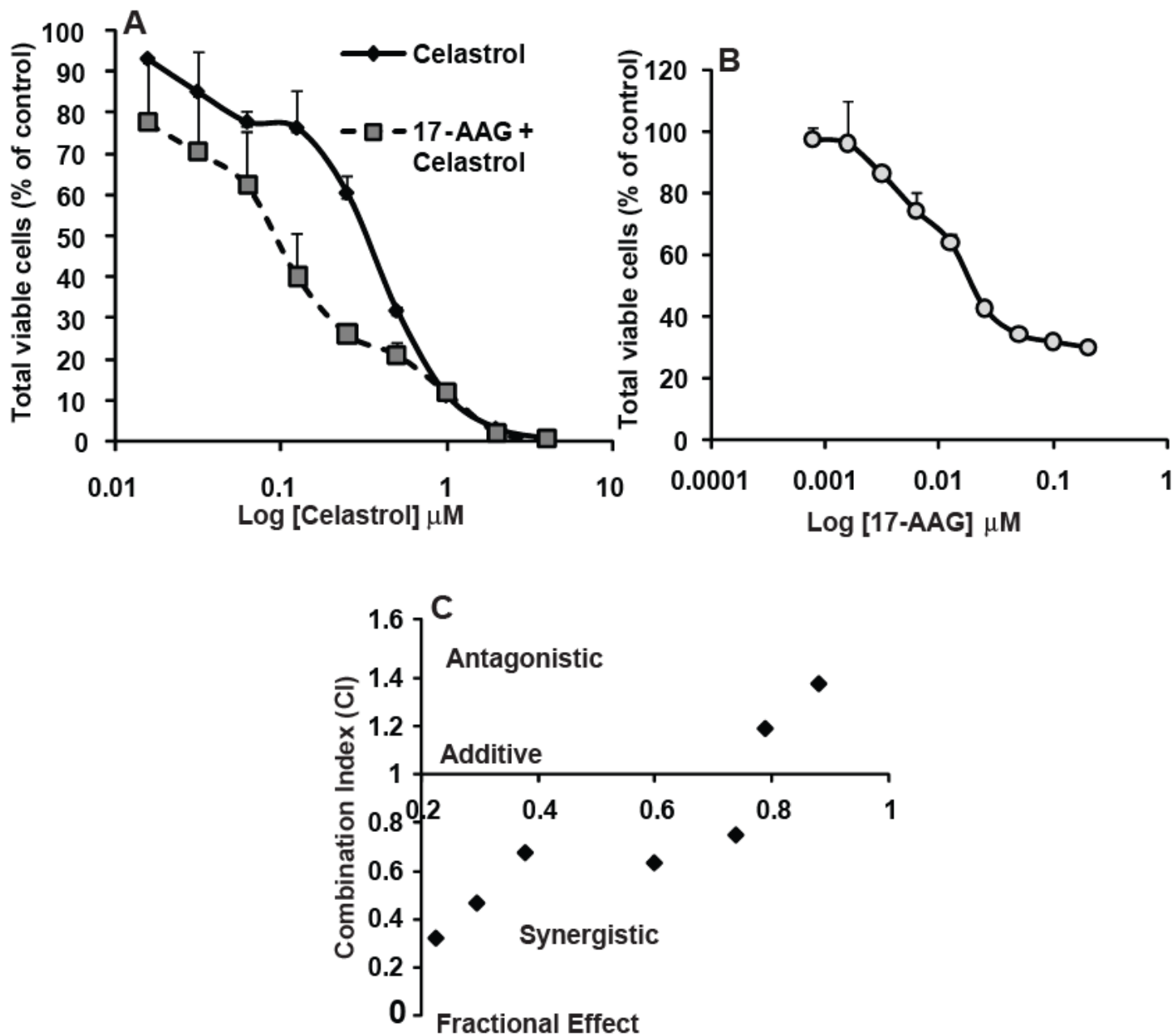
Affiliations: ¹Eppley Institute for Research in Cancer and Allied Diseases; ²Departments of Biochemistry & Molecular Biology, Pathology & Microbiology and Pharmacology & Neuroscience, and ³Department of Genetics, Cell Biology and Anatomy, College of Medicine, University of Nebraska Medical Center, Omaha, NE 68198-5950.



Supplementary Fig. 1. *Celastrol plus Lapatinib is synergistic in killing ErbB2-overexpressing SKBr-3 cells:* A. SKBr-3 cells were treated with Celastrol or Lapatinib alone or a combination of fixed concentration of Celastrol (at 50 nM) and varying concentrations of Lapatinib. The viable cells at the end of treatment were assessed using the MTT dye assay and data represented as % of vehicle control. The IC_{50} for Lapatinib alone was 25.5 ± 2.1 nM, whereas it was 0.74 ± 0.1 nM when combined with Celastrol at 50 nM. The surviving fraction with Celastrol alone at 50 nM was $69.8 \pm 1.7\%$ of vehicle control. B. Chou-Talalay analysis was performed as detailed in the methods section.

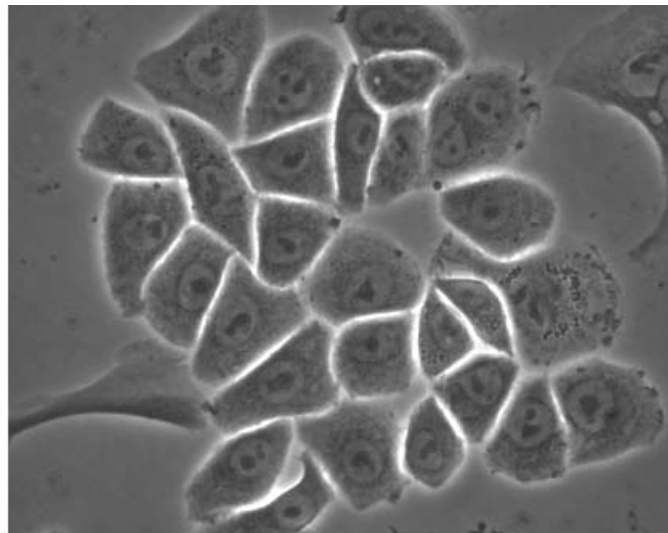


Supplementary Fig. 2: *Lack of synergism between Celastrol and Trastuzumab in ErbB2-low MCF-7 cells:* MCF-7 cells were treated with single agents or their combination, as detailed in the legend to Fig. 3. Viable cells at the end of treatment were assessed using the MTT dye assay and data represented as % of vehicle control.



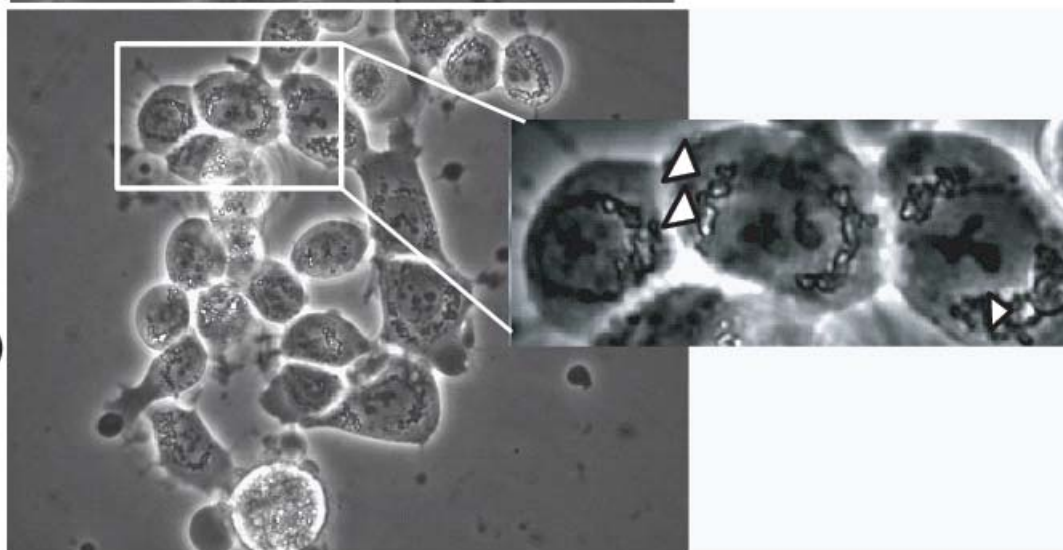
Supplementary Fig. 3: Mild synergism between Celastrol and 17-AAG against ErbB2-overexpressing SKBr-3 cell line: Treatments were performed as in legend to Fig. 3. For Celastrol plus 17-AAG combination, both Celastrol and 17-AAG were serially diluted 2-fold at a fixed ratio of 20:1. Total viable cells were assessed using the MTT dye assay after 7 days. The IC_{50} for Celastrol was $0.18 \pm 0.08 \mu\text{M}$ as a single drug versus $0.078 \pm 0.08 \mu\text{M}$ when combined with 17-AAG. Chou-Talalay analysis is shown in the bottom panel. Combination index (CI) < 1 indicates synergy; CI \sim 1 indicates additive effects; CI > 1 indicates antagonism.

SKBr-3

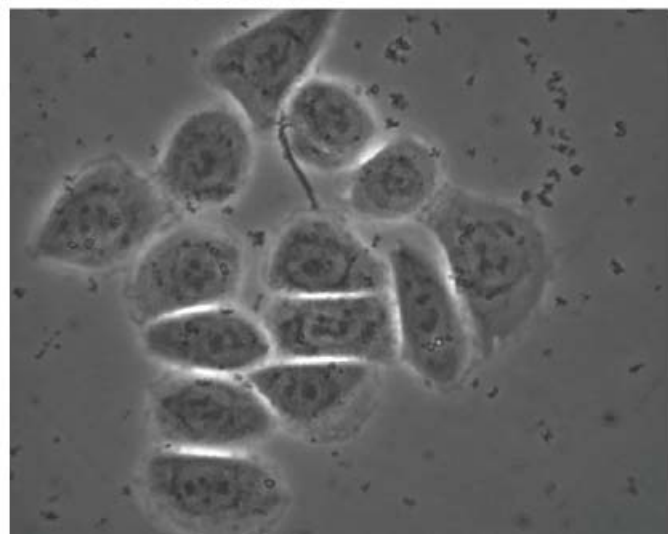


DMSO

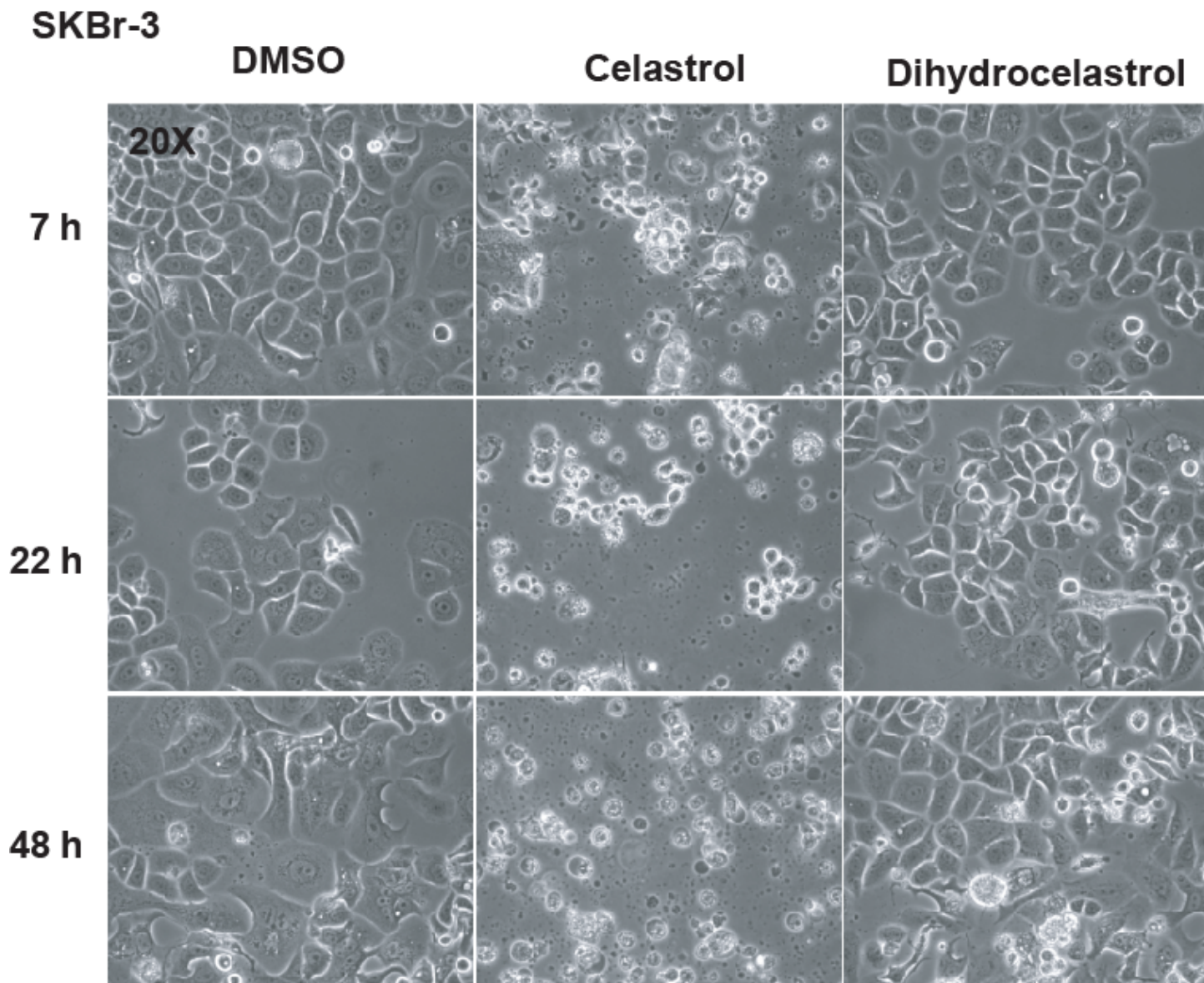
Celastrol
(4 μ M; 4h)



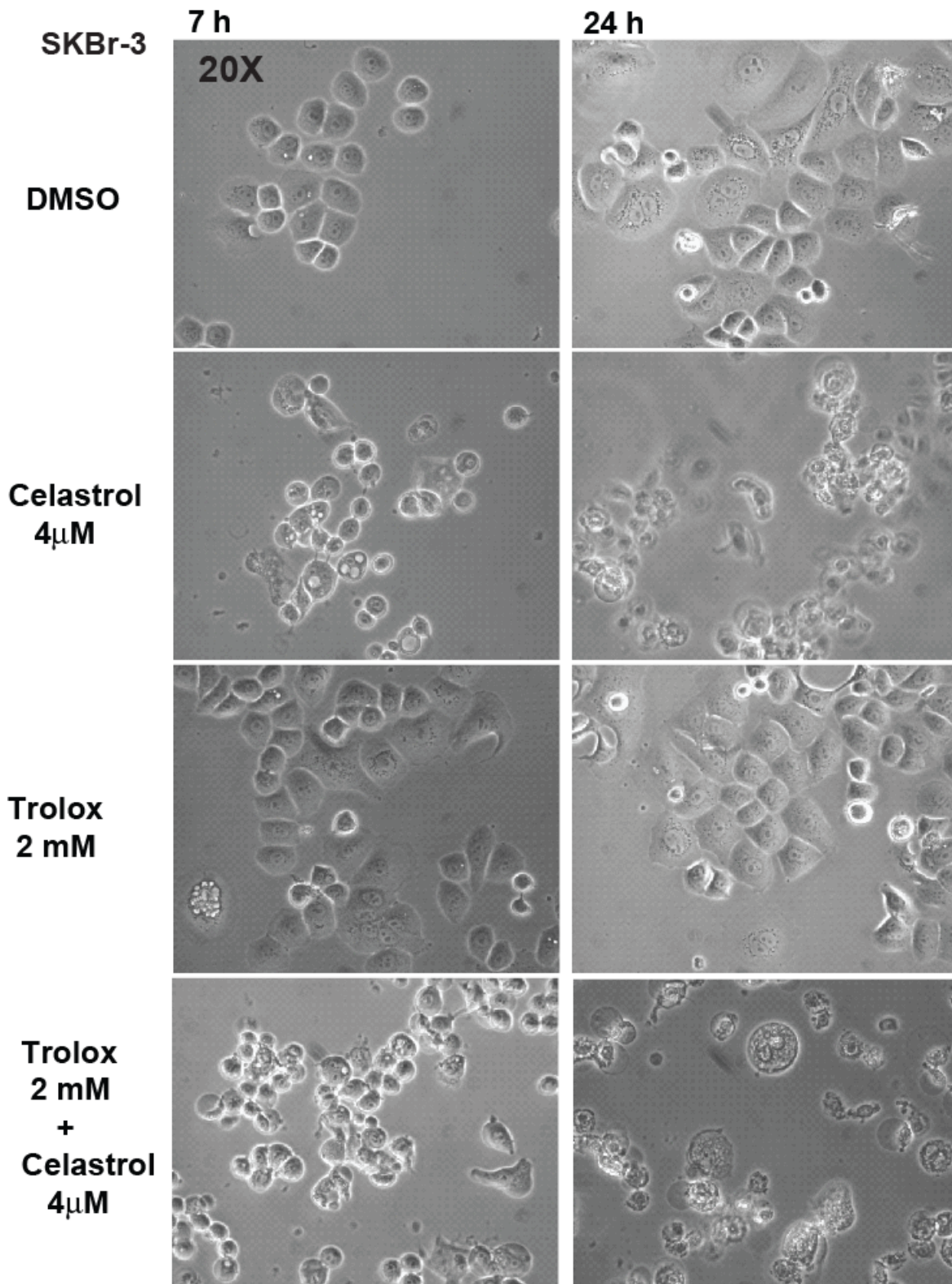
Celastrol
+ DTT
(4 μ M; 4h)



Supplementary Fig. 4: *The 'Michael Acceptor' functionality is important for the bioactivity of Celastrol:* Effect of reduction of the α,β -unsaturated carbonyl group by DTT on cell-death in SKBr-3 cells induced by Celastrol; Cells were incubated with the indicated concentrations of untreated or DTT-treated Celastrol for the indicated time. The changes cell morphology was followed by bright field microscope using a 40 X objective. The inset shows induction of vacuolation by Celastrol but not DTT-treated Celastrol.



Supplementary Fig. 5: *The 'Michael Acceptor' functionality is important for the bioactivity of Celastrol.* Effect of reduction of the α,β -unsaturated carbonyl group on cell-death in SKBr-3 cells induced by Celastrol; Cells were left untreated or incubated with the 4 μ M Celastrol or Dihydrocelastrol for the indicated time. The changes cell morphology was followed by bright field microscope using a 20 X objective.



Supplementary Fig. 6 *Effect of anti-oxidant Trolox on Celastrol induced cell death.* SKBr-3 cells were treated with the indicated concentrations of Celastrol with or without 500-fold excess of Trolox. The cell morphology was monitored at the indicated time periods by a bright field microscope using a 20X magnification objective. Shown here is the effect of anti-oxidants Trolox on the cytotoxicity of Celastrol.

Supplementary Table 1: List of antibodies used for this study.

Antibody	Species	Company	Description	Dilution	Application
Anti-ErbB2	Rabbit polyclonal	Santacruz biotech	Neu/C18	1/1000 (0.2µg/ml)	Western-blotting; Immunoprecipitation
Anti-ErbB2	Goat polyclonal	R&D Systems	AF1129	1/1000 (0.2µg/ml)	Immunofluorescence
Anti-Hsc70	Mouse monoclonal	Santacruz	B-6	1/1000 (0.2 µg/ml)	Western-blotting
Anti-β-Actin	Mouse monoclonal Ascites	Sigma	AC-15	1/1000	Western-blotting
Anti-Ubiquitin	Mouse monoclonal	Covance	P4D1	1/1000 (1 µg/ml)	Western-blotting
Anti-LAMP-1	Mouse monoclonal Ascites	Developmental Hybridoma	H4A3	1/1000	Immunofluorescence
Anti-phosphotyrosine	Mouse monoclonal Ascites	Gift from Dr. Brian Drucker	4G10	1/1000	Western-blotting
Anti-p-AKT1/2/3 (Ser 473)	Rabbit polyclonal	Santacruz	SC-7985-R	1/1000 (0.2 µg/ml)	Western-blotting
Anti-PARP	Mouse monoclonal	BD Pharmingen	556494	1/500	Western-blotting