---------- **SUPPLEMENTAL MATERIAL** ----------

A tumor cell-selective inhibitor of mitogen-activated protein kinase phosphatases sensitizes breast cancer cells to lymphokine-activated killer cell activity

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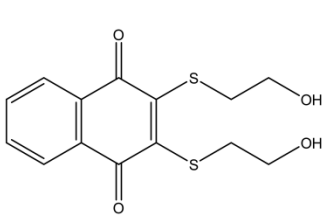
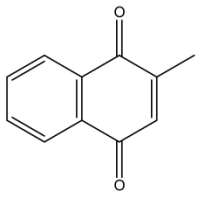
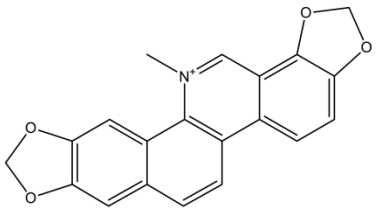
Supplemental Figure 1. Structures of compounds used in this study. The study comprises comparative evaluations of three previously described DUSP inhibitors (NSC95397; sanguinarine, (*E*)-2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1*H*-inden-1-one (BCI), its newly discovered, non-toxic analog (BCI-215), and menadione (vitamin K3) as a positive control for hepatotoxicity). MAPK inhibitors used for pathway evaluation SCH772984, SB203580, SP600125 and JNK-IN-08 were from commercial sources.



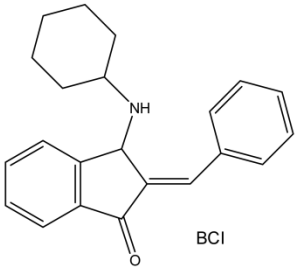
NSC95397



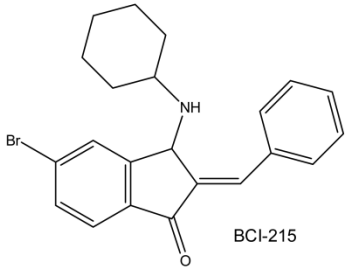
menadione



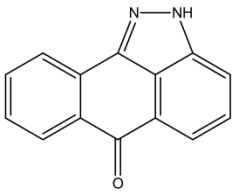
sanguinarine



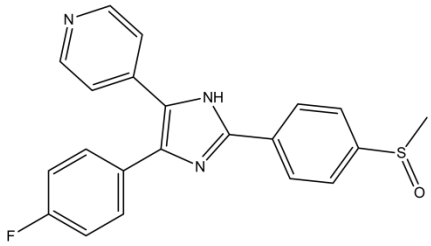
BCI



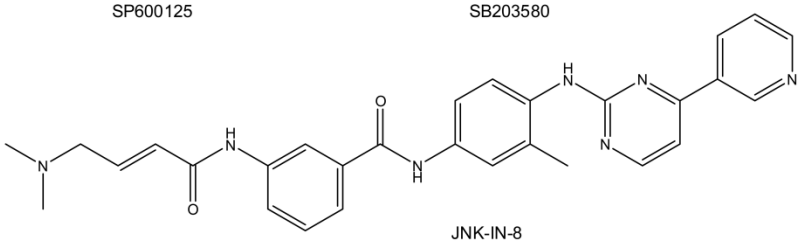
BCI-215



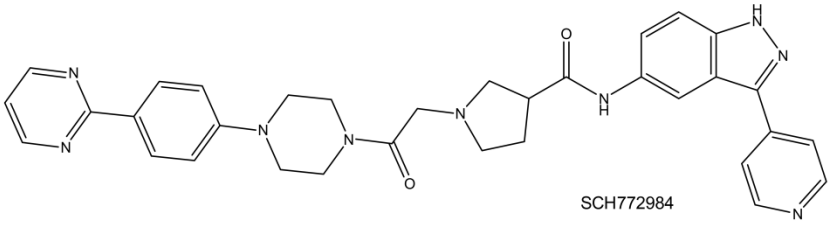
SP600125



SB203580

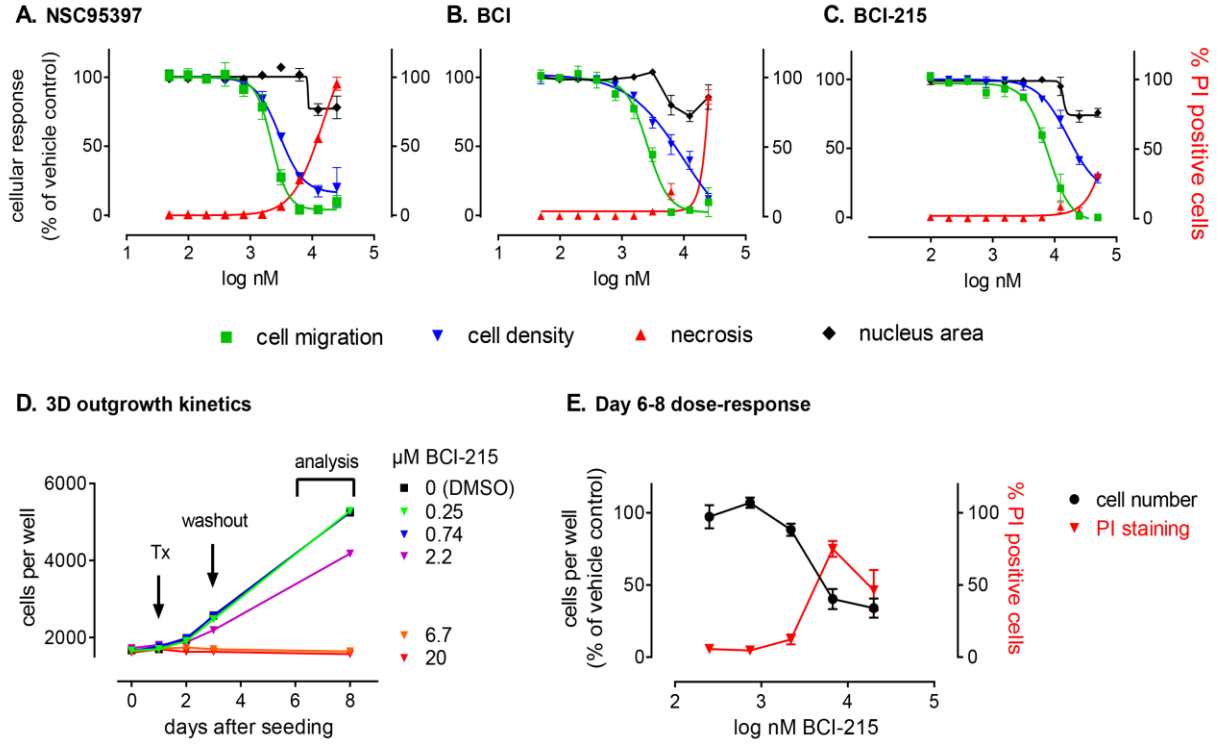


JNK-IN-8



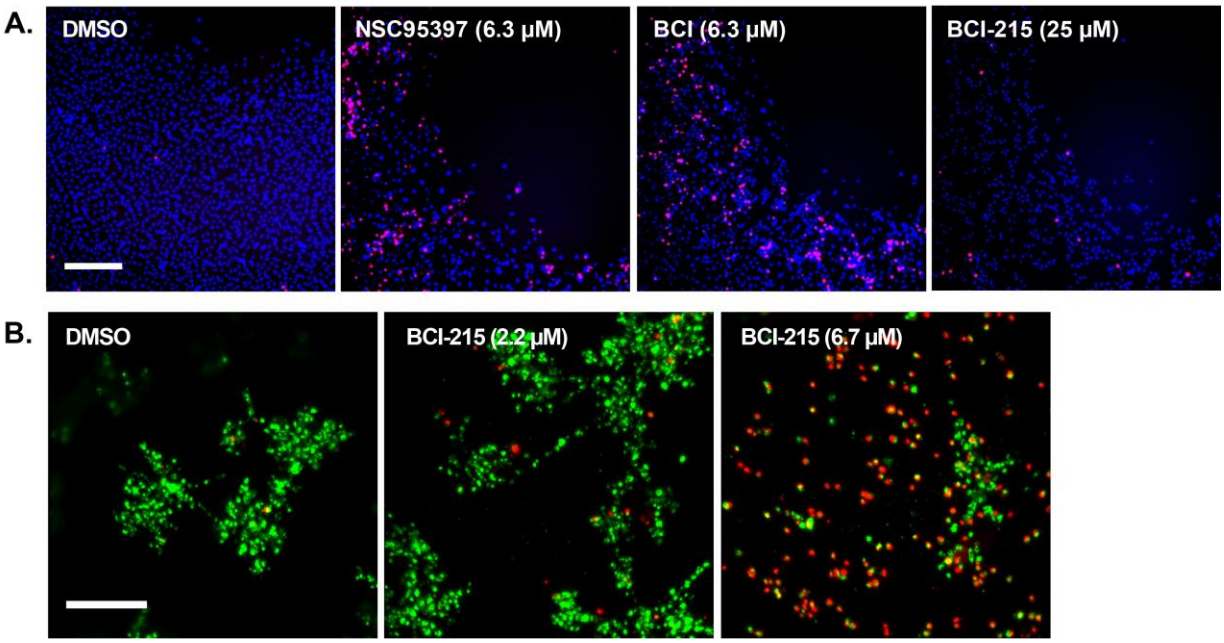
SCH772984

Supplemental Figure 2



Supplemental Figure 2. BCI-215 inhibits motility, survival, and metastatic outgrowth of human breast cancer cells. (A-C) MDA-MB-231 cells were plated in the wells of an Oris™ Pro 384 cell migration plate, stained with PI and Hoechst 33342 48 h thereafter, and analyzed by high-content analysis for cells that had migrated into the exclusion zone (cell migration), cell loss (cell density), necrosis (% PI positive cells), and nuclear shrinkage (nucleus area). Each data point is the mean of four technical replicates ± SEM from a single experiment that has been repeated four times. All agents inhibited cancer cell migration and caused cell loss with IC50s between 7-15 µM. BCI-215 showed no signs of necrosis at antimigratory and cytotoxic concentrations. **(D)** MDA-MB-231 cells carrying a mitochondrial-targeted, GFP-labeled cytochrome C biosensor were seeded on a layer of matrigel and treated with BCI-215 the next day (Tx). After two days of exposure, drug was washed out and cells allowed to grow for an additional three to five days. Z-stacks were acquired at the indicated time points and cell numbers calculated from maximum projection images. At the end of the study (day 6-8), cells were incubated with PI and the percentage of PI positive cells determined. **(E)** BCI-215 inhibits colony formation and causes pronounced secondary cell lysis in the six-day colony formation assay. Data are the averages ± SEM of three independent experiments, each performed in triplicate.

Supplemental Figure 3. Fluorescence micrographs of propidium iodide stained MDA-MB-231 cells in 2D and 3D.



A. Short-term toxicity and motility inhibition on collagen-coated plastic. MDA-MB-231 cells (15,000/well) were plated in the wells of an Oris™ Pro 384 cell migration plate and stained with PI and Hoechst 33342 48 h thereafter. Images show the bottom left quarter of an entire microwell, acquired on the ArrayScan II at 5X, and demonstrate closure of the cell exclusion zone (bare area in the upper right hand corner), cell density (Hoechst stained nuclei in blue), and PI positive cells (red). Scale bar, 300 µm. **B. Toxicity in matrigel six days after treatment with BCI-215.** MDA-MB-231 cells (2000/well) transduced with a biosensor consisting of EGFP with a mitochondrial targeting sequence derived from cytochrome-C oxidase subunit VIII were plated on a cushion of matrigel and treated with vehicle or BCI-215. After two days, medium was replaced and cells allowed to recover for 4 days. Images show GFP/PI overlays of collapsed Z-stacks (20 planes, 5 µm) acquired at 20X magnification on the ImageXpress Ultra. Scale bar, 200 µm.

Supplemental Table 1. Quantification of multiparametric evaluation of cellular toxicity, caspase-3 activation, and ERK phosphorylation.

compound	parameter	IC50 (μM)	SE	95% CI	n
BCI	Nuclear condensation	12.85	1.24	8.261 to 20.00	5
BCI-215	Nuclear condensation	12.77	1.21	8.633 to 18.89	5
BCI	ERK phosphorylation	8.59	1.18	6.137 to 12.03	5
BCI-215	ERK phosphorylation	15.37	1.21	10.35 to 22.81	5
BCI	Caspase-3 cleavage	9.17	1.22	6.019 to 13.97	4
BCI-215	Caspase-3 cleavage	7.33	1.25	4.609 to 11.66	4

Images of pERK stained cells were acquired on the ArrayScan II and analyzed by the Target Activation Bioapplication as described in Materials and Methods. Data are the averages of the indicated numbers of independent experiments, each performed in quadruplicate. For quantification of phospho-ERK and cleaved caspase-3, each well was background corrected by subtracting mean phospho-ERK or cleaved caspase-3 intensities from wells that had received secondary antibody only. IC50, standard error, and 95% confidence intervals were calculated by two way ANOVA with Bonferroni correction in GraphPad Prism.

SUPPLEMENTARY MATERIALS AND METHODS

HCA of cell motility and cytotoxicity was performed essentially as described (1). MDA-MB-231 cells (15,000/well) were plated in collagen-coated Oris™ Pro 384-well microplates (Platypus Technologies cat # PRO384CMACC5) containing a chemical exclusion zone that dissolves upon cell seeding. Two hours after plating, medium was removed, and cells treated with ten-point, two-fold concentration gradients of test agents. Forty-eight hours after treatment, cells were stained with 10 µg/ml Hoechst 33342 and 1 µg/ml PI in HBSS for 15 min at 37°C. Plates were washed once with PBS and scanned live on the ArrayScan II using a 5X objective. To capture cells that had entered into the exclusion zone, a single field was acquired in the center of the well and nuclei therein enumerated. To assess changes in cell loss, nuclear size, and necrotic cell death, a second scan was performed that captured one field at the edge of the well (see (1) for more detail). Parameters exported and plotted were SelectedObjectCountPerValidField (cell density), MEAN_ObjectAreaCh1 (nucleus size), and %RESPONDER_MeanAvgIntenCh2 (percent PI positive cells based on based on a threshold set with vehicle treated cells).

Colony formation in three dimensional matrigel culture. MDA-MB-231 cells (2000/well) transduced with a biosensor consisting of EGFP with a mitochondrial targeting sequence derived from cytochrome-C oxidase subunit VIII (2) were trypsinized, resuspended in RPMI1640 containing 2% FBS and 2% matrigel, and seeded in 384 well microplates on a 15 µl cushion of undiluted matrigel. After 24 hours, cells were treated with various concentrations of BCI-215 or vehicle (0.2% DMSO). After two days, drug was washed out and cells allowed to expand for an additional three to five days. At the end of the study, medium was replaced with HBSS containing 4 µg/ml PI for 1 hour, and plates scanned live on an ImageXpress Ultra HCS reader, acquiring z-stacks (4X objective, 20 planes, 50 µm) in the green and red

channels. Cell numbers were quantified from maximum projection images using the Multiwavelength Cell Scoring application.

REFERENCES

1. Joy ME, Vollmer LL, Hulkower K, Stern AM, Peterson CK, Boltz RC, et al. A high-content, multiplexed screen in human breast cancer cells identifies profilin-1 inducers with anti-migratory activities. PLoS One. 2014;9:e88350.

2. Senutovitch N, Vernetti L, Boltz R, DeBiasio R, Gough A, Taylor DL. Fluorescent protein biosensors applied to microphysiological systems. Exp Biol Med (Maywood). 2015;240:795-808.