Supplemental Figures

Supplemental Figure 1: A) The spheroid derived cells were derived for 7- 14 days and then disassociated and plated into 1536 well assay plates. Adherent cells were also seeded at the same time in media containing FBS. Both cells types were treated with the MIPE library using a 1536-well head pintool. After 48 hours, changes in cell proliferation are measured using Cell TiterGlo^{TM} and luciferase readout was captured on a ViewLux. Bright field 10X images of the initial spheres and the seeded spheres and adherent cells are shown and were acquired using a Nixon TS100 Eclipse. B) Dose response curves for the positive controls salinomycin and the protease inhibitor bortezomib were found to significantly inhibit the growth of the spheres from both cell types. The treated cells were normalized to DMSO treated wells to generate the % viability. The IC_{50} for salinomycin in PANC1 spheres was 13 uM and in the LNCaP spheres was 9 uM. For bortezomib the I IC_{50} in the PANC1 spheres was 8 nM and in the LNCaP spheres was 23 nM. C) Plate statistics from adherent LNCaP cells treated with DMSO (column 1-2 and 4-48) and 16 point dose response curves for bortezomib (column 3) and salinomycin (column 4) demonstrated very clean plates with only an 8% coefficent of variation (CV) and the average Z' for salinomycin and bortezomib was 0.8. D) Similar results were found in the LNCaP spheres demonstrating an average Z' for bortezomib at 0.76 and for salinomycin 0.82, yet a higher CV at 18%. The red dashed line in C and D demonstrates the cut-off used for active hits which is three standard deviations from the mean DMSO treated wells for the spheroid derived cells.

Supplemental Figure 2: Venn Diagrams demonstrating overlapping active compounds determined using ≤30% viability as the cut-off between the different cell lines (LnCAP and PANC1) and growth conditions (sphere and adherent). Overall, only 2 unique compounds were active in both cell lines and growth conditions using % viability as the data analysis technique.

Supplemental Figure 3: Venn Diagrams demonstrating overlapping active compounds determined using the curve class response (CRC) system between the different cell lines (LnCAP and PANC1) and growth conditions (Sphere and Adherent). Overall only 5 unique compounds were active in both cell lines and growth conditions using the CRC analysis technique.

Supplemental Figure 4 : A) PANC1 spheres or B) LNCaP spheres treated with CMP1 for 48 hours and then assayed cell proliferation using CellTiter Glo®. The red line indicates the predicted dose response curve used to generate Hill Slope values for IC_{50}s. Percent viability compared to DMSO control treated cells appears on the Y-axis and the log molar concentration on the X-axis. C) Dose response curves of bortezomib with CRCs and predicted IC_{50}s from MIPE plate screen for cell proliferation in adherent LNCaP (black), LNCaP spheres (red), adherent PANC1 (blue) and PANC1 spheres (gray). Percent viability compared to DMSO control treated cells appears on the Y-axis and the log molar concentration on the X-axis.

Supplemental Figure 5: Dose response with predicted IC_{50s} and CRCs of gemcitabine treated cells from MIPE plate screen in adherent LNCaP (black), LNCaP spheres (red), adherent PANC1 (blue) and PANC1 spheres (gray). Percent inhibition compared to DMSO control treated cells appears on the Y-axis and the log molar concentration on the X-axis.

Supplemental Tables

Supplemental Table 1: Assay Protocol for 1536-well cell proliferation assay.

Supplemental Table 2: Correlation Matrices for the data set using curve response classes (CRCs).

Supplemental Table 3: Comparison in compound overlap identified by % viability and CRCs which hit in both cell lines LnCAP and PANC1 and in growth conditions, spheres and adherent. Only 2 compounds were identified by each independent analysis method, and only 1 compound, CMP2, overlapped between the two.

Supplemental Methods

High Throughput Screen: Assay robustness was calculated as follows: *Z'* factor = 1 − 3(SD_{total} + SD_{basal})/($mean_{total}$ − $mean_{basal}$), where SD_{total} is the standard deviation of DMSO-treated wells, SD_{basal} is the standard deviation of bortezomib or salinomycin-treated wells [21], $mean_{total}$ is the mean of DMSO-treated wells, and $mean_{basal}$ is the mean of Bortezomb or salinomycin-treated wells, and CV = SD_{total}/$mean_{basal}$, expressed as a percentage.

MIPE Compound Library: The library contains compounds which are in all stages of development including pre-clinical, Phase I-III, approved and some discontinued compounds. A major component of this library are known drugs with a long history of clinical use and recently disclosed clinical compounds targeting novel cellular targets relevant to the cancer types to be screened, including receptor tyrosine kinase inhibitors. Importantly, we have given significant consideration to the role that polypharmacology will play in a molecule's activity profile. For instance, the ultimate cellular phenotype associated with DNA alkylating agents is dominated by the primary pharmacology and is not likely to be different based upon the potential polypharmacology of these agents and, as such, only one DNA alkylating agent is included in the collection.

Sphere Formation Assays: Using 1000 cells per mL in 50 mL SCM+KO+ITS in non-adherent T75 flasks coated with Hydrogel (Corning Life Sciences, Chemlsford, MA, USA). The pancreatic spheres were generated for 14 days and then harvested by spinning at 1500 rpms for 5 minutes. LNCaP spheres were generated using 1000 cells per mL in 50 mL SCM+KO+ITS in regular tissue culture treated T75 flasks for 7 days and then harvested by spinning at 1500 rpms for 5 minutes. The sphere pellets were then

trypsinized with 10 mL of 0.25% trypsin per T75 for 10 minutes at 37 ˚C with gentle shaking every 3 minutes. The trypsin was then inactivated with SCM, spun and further washed with 1X PBS. The disassociated cells were resuspended in SCM+KO+ITS, passed through a 40 µm filter and counted.

Curve Response Class Classification from Dose Response HTS: Normalized data as described above was fitted to a 4-parameter dose response curves using a custom grid-based algorithm to generate curve response class (CRC) values for each compound [21]. The resultant inhibition/activation curves were then classified using a heuristic curve classification scheme, allowing us to rapidly prioritize high quality curves from lower to poor quality ones. Briefly, a curve (and hence a compound) was classified as 1.1 if it exhibited well defined upper and lower asymptotes, with a good fit to the observed data points (R^2 >= 0.9) and an efficacy greater than 80%. A class 2.1 curve is similar to a 1.1 curve, but exhibits only one well defined asymptote. A curve that exhibited poorer efficacy (between 30% and 80%) was classified as a 1.2 or a 2.2 if it had two asymptotes or one asymptote respectively. A curve class 4 is inactive. Negative curve classes demonstrate inhibitory responses and positives demonstrate a stimulatory response relative to control compounds used. Additional curve classifications which are grouped as inconclusive; Curve class 1.3 refers to those will full efficacy and a complete curve, yet demonstrating a poor fit. Furthermore, curve classes of 1.4 are those demonstrating a partial efficacy and a complete curve, yet demonstrating a poor fit. A class 3 curve refers to one that was poorly fit or only exhibited activity at the highest concentration (and thus represents inconclusive activity),. Curve class 5 is assigned to those compounds that cannot be fit. The CRCS were generated using the NCGC Virtual Client.

Matrigel Invasion Assay: The PANC1 cell line was tested with CMP1 identified from the proliferation screen, a compound with similar function CMP1-S, as well as the positive controls. A total of 70,000 cells were seeded in the top chamber in 500 µL DMEM and the drug was added to 750 µL SCM contained in the bottom chamber. The cells were incubated using cell culture conditions for 24 hours and then

stained using the Diff Quick staining kit according to the manufacturer's instructions (Dade Bearing, Inc., Newark, DE, USA). Brightfield 10X images were acquired using a Nixon TS100 Eclipse.

4T1.2 Nanog GFP Cells: A murine Nanog promoter reporter plasmid for lentiviral expression was originally purchased from System Biosciences (Mountain View, CA). Directional restriction cloning was used to generate the new plasmid vectors. Briefly NotI and AflIII restriction enzymes were used to excise a 4.7 kb DNA fragment containing the murine Nanog promoter, destabilized copepod GFP and Zeocin resistance genes from lentivirus expression vectors. The insert was then subcloned into the Sleeping Beauty (pT2HB) multiple cloning site to create the murine Nanog promoter reporter. Orientation and sequence fidelity were confirmed by dye terminator sequencing (NIH Intramural Sequencing Center). pT2HB was kindly provided by Perry B. Hackett (Univ. MN). Sleeping Beauty reporter and the hyperactive transposase HSB2 plasmids were co-delivered to tumor cells by nucleofection (Lonza, Switzerland). HSB2 was supplied by Xin Chen (UC-SF). Transfected cells were grown in the absence of selection for 7-10 days before isolation of GFP-expressing cells by flow cytometric cell sorting. A threshold for reporter GFP expression was set on un-transduced parental cells and viability determined by 7-AAD (Becton Dickinson) exclusion.

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High Content Imaging Nanog-GFP Assay: An assay to measure Nanog-GFP levels in 4T1.2 cells was conducted in using the ArrayScan VTI (Thermo Fisher). Sterile, tissue culture treated 1536-well black clear bottom tissue plates (catalog number 19324, Aurora Biotech-Nexus Biosystems, Poway, CA, USA). A total of 200 cells per well in 5 µL of RMPI+ 5% FBS were seeded using a Multidrop Combi Reagent

dispenser with a small tip cassette . The cells were allowed to adhere overnight at 37 ˚C, with 5% CO_2 and 95% relative humidity, and then 23 nL of the compound solutions in DMSO were transferred using a Kalypsys pintool. The protease inhibitor bortezomib and antibiotic salinomycin were used as positive controls for cell death (final concentration 9 μM). The plates were then covered with stainless steel Kalypsys lids and placed into incubator at 37˚C, with 5% CO_2 and 95% relative humidity for 48 hours before fixing with 4% para- formaldehyde and stained with 1 μM Hoechst 33342 in PBS containing 1% Triton-X 100 for 30 minutes at room temperature. One image per well was acquired with 20X magnification using the ArrayScan VTI (Thermo Fisher). The Compartmental Analysis Bio Application was used for analysis of viability and mean nuclear GFP content per cell with segmentation applied per cell. The percentage of viable cells (% activity) was calculated by comparing DMSO treated cells in the nuclear channel and mean nuclear Nanog GFP signal per cell was also normalized to the DMSO treated cells to calculate a % GFP signal.

Supplemental Figure 1

A

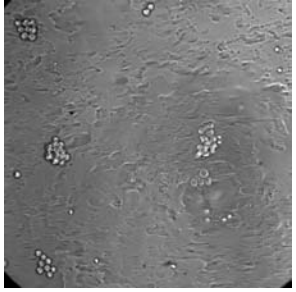
CSC Spheroid Isolation

7-14 days

Dissociation of spheroids/ Clonal seeding into assay plate

Addition of controls and MIPE to both

Spheres



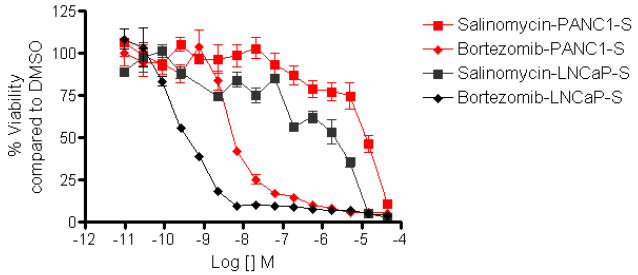
2 days

Measure Cell viability (CellTiterGlo™)

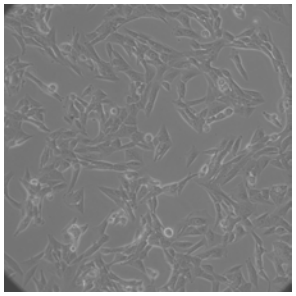
SCM+KO+ITS

SCM+KO+ITS

B



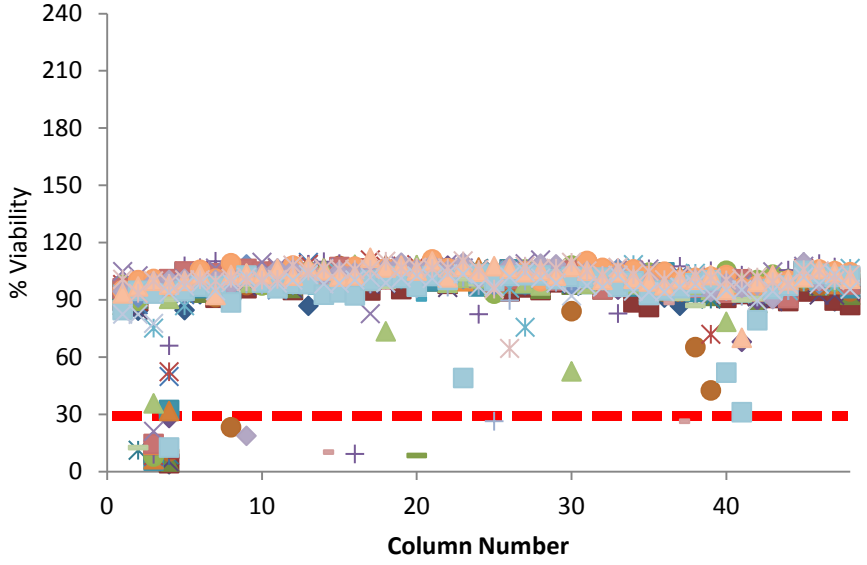
Cell Seeding



DMEM or RPMI+5%FBS

Adherent

C



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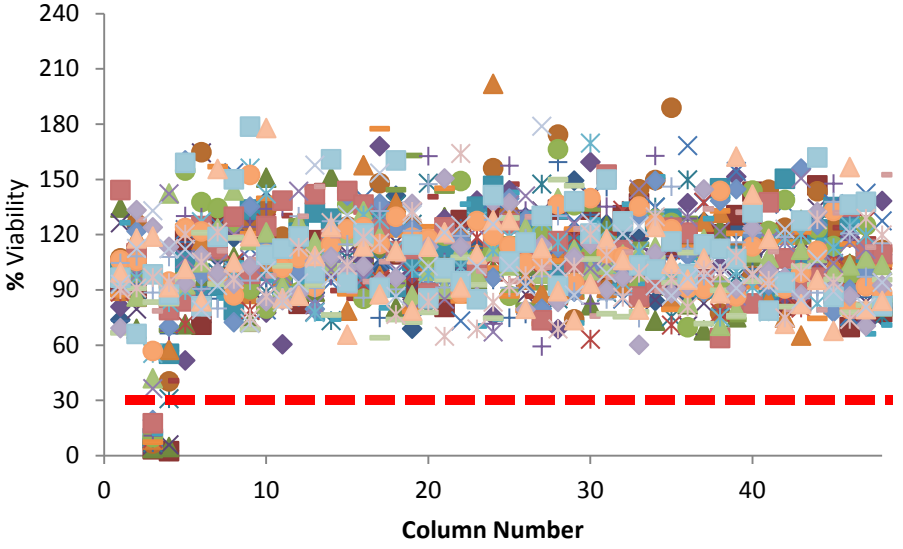
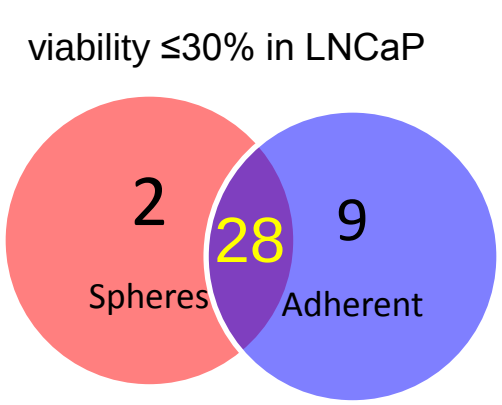
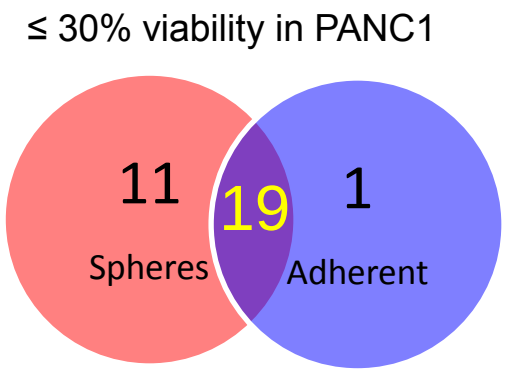
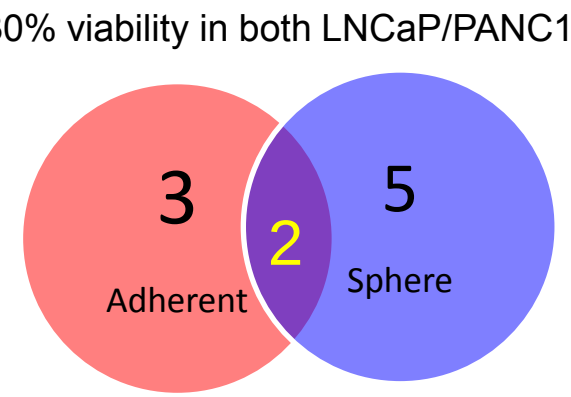
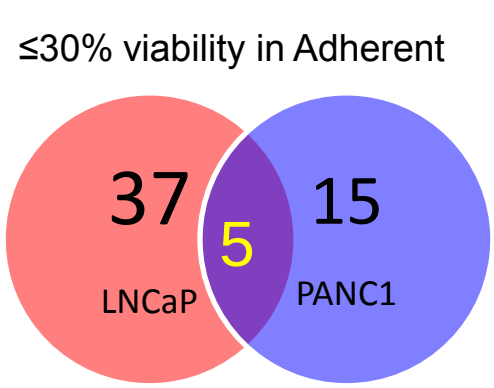


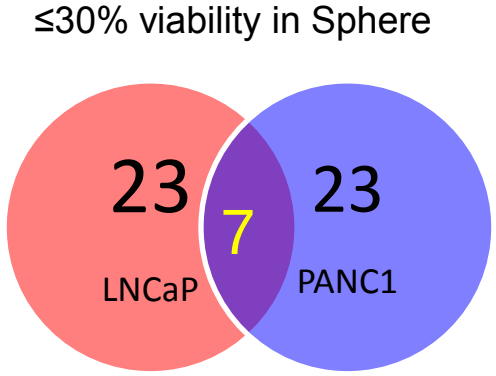
Supplemental Figure 2

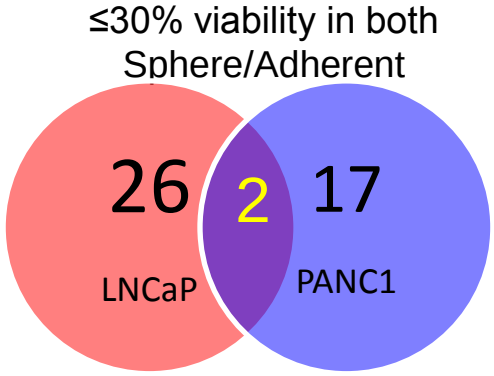




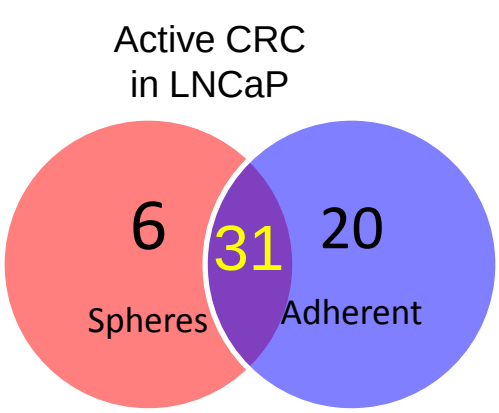


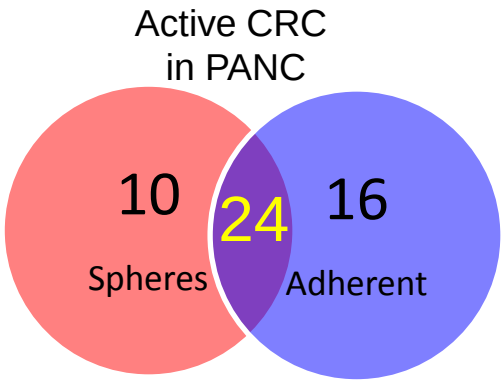


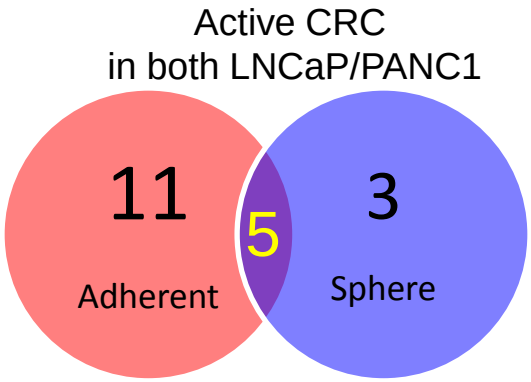


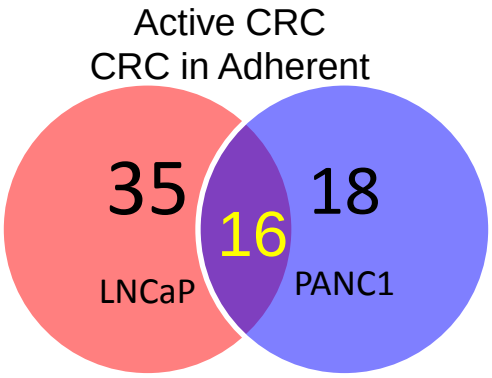


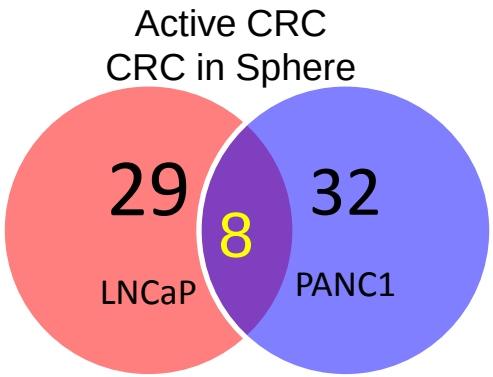
Supplemental Figure 3

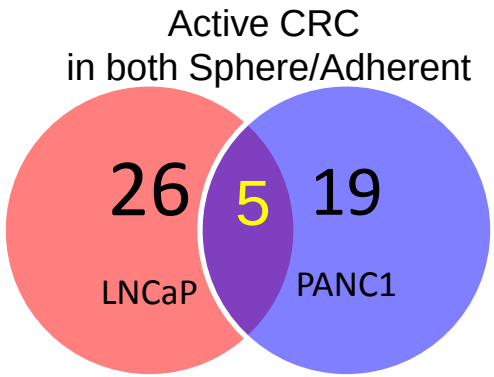




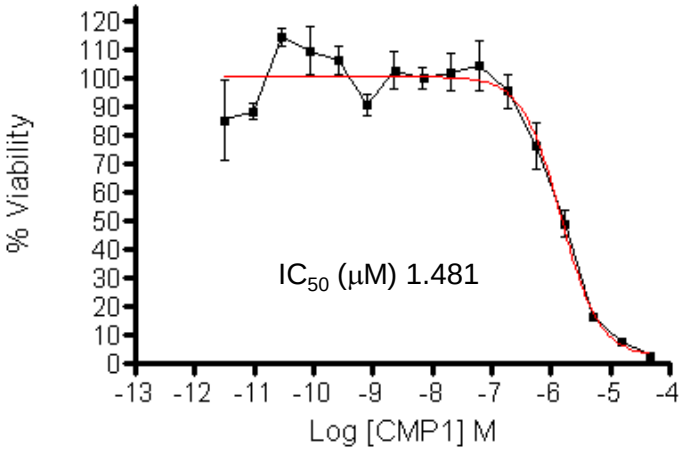








A



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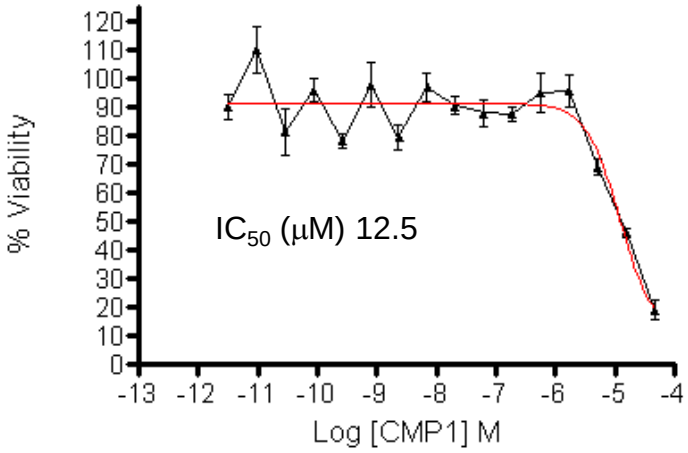

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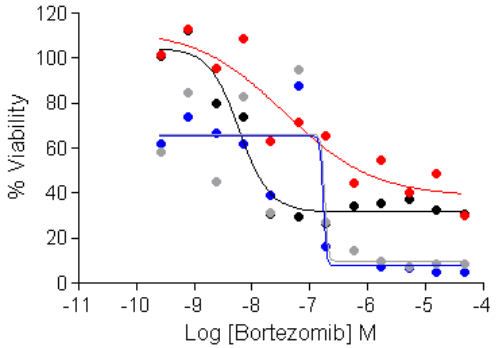
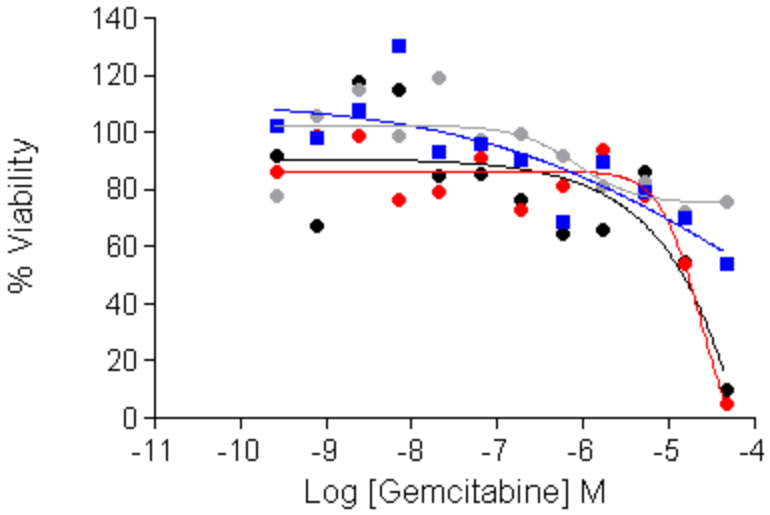


Cell Line	CRC	IC_{50} (µM)
LNCaP A	-1.2	0.007
LNCaP S	-1.1	0.04
PANC1 A	-1.4	0.03
PANC1 S	-1.4	0.01

Supplemental Figure 4



Cell Line	CRC	IC_{50} (μM)
LNCaP A	-2.4	45.6
LNCaP S	-2.1	40.7
PANC1 A	-2.4	1
PANC1 S	-1.4	45.6

Supplemental Figure 5

Supplemental Table 1

Step	Parameter	Value	Descrption
1	Reagent	5 uL	Dispense 200 cells/well in 5 uL SCM+KO+ITS or DMEM or RPMI+FBS solid bottom high base white greiner using multidrop
2a	Positive controls	23 nL	9.2 uM Bortezomib or Salinomycin
2b	Compound Library	23 nL	MIPE Library
3	Incubate	48 hours	Incubate 48 hr (37C, 5% CO2, 95% Relative Humidity with low evaporation lids)
4	Reagent	3 uL	CellTiter Glow reagent w/ FRD
5	Time	30 minutes	Incubate at room temperature
6	Spin	10 seconds	1000 rpms to remove bubbles
7	Dectector	10 seconds	View Lux

Supplemental Table 2

1. concordance between sphere layers in the two cell lines

	LNCaP					
PANC1	-1.1	-1.2	-2.1	-2.2	4	Inconclusive
-1.1	1	0	0	0	4	5
-1.2	0	0	2	1	4	3
-2.1	1	0	1	1	5	2
-2.2	0	1	0	0	5	4
4	2	5	2	1	8	6
Inconclusive	2	4	8	5	16	13

2. concordance between adherent layers in the two cell lines

	LNCaP					
PANC1	-1.1	-1.2	-2.1	-2.2	4	Inconclusive
-1.1	1	2	1	1	1	0
-1.2	1	0	2	2	3	2
-2.1	0	0	2	0	1	3
-2.2	1	1	1	1	5	3
4	1	4	2	5	3	9
Inconclusive	4	9	7	3	10	21

3. Concordance between sphere and adherent layer in PANC-1

	Sphere					
Adherent	-1.1	-1.2	-2.1	-2.2	4	Inconclusive
-1.1	6	0	0	0	0	0
-1.2	0	5	0	0	2	3
-2.1	1	0	4	0	0	1
-2.2	0	2	3	3	1	3
4	0	2	0	1	13	8
Inconclusive	3	1	3	6	8	33

4. Concordance between sphere and adherent layer in LNCaP

	Sphere					
Adherent	-1.1	-1.2	-2.1	-2.2	4	Inconclusive
-1.1	2	2	3	0	0	1
-1.2	2	4	0	4	2	4
-2.1	2	1	8	0	1	3
-2.2	0	1	1	1	3	6
4	0	0	0	0	18	5
Inconclusive	0	2	1	3	18	14

Supplemental Table 3

	% Viability	CRC
CMP1	√	-
CMP2	√	√
CMP3	-	√