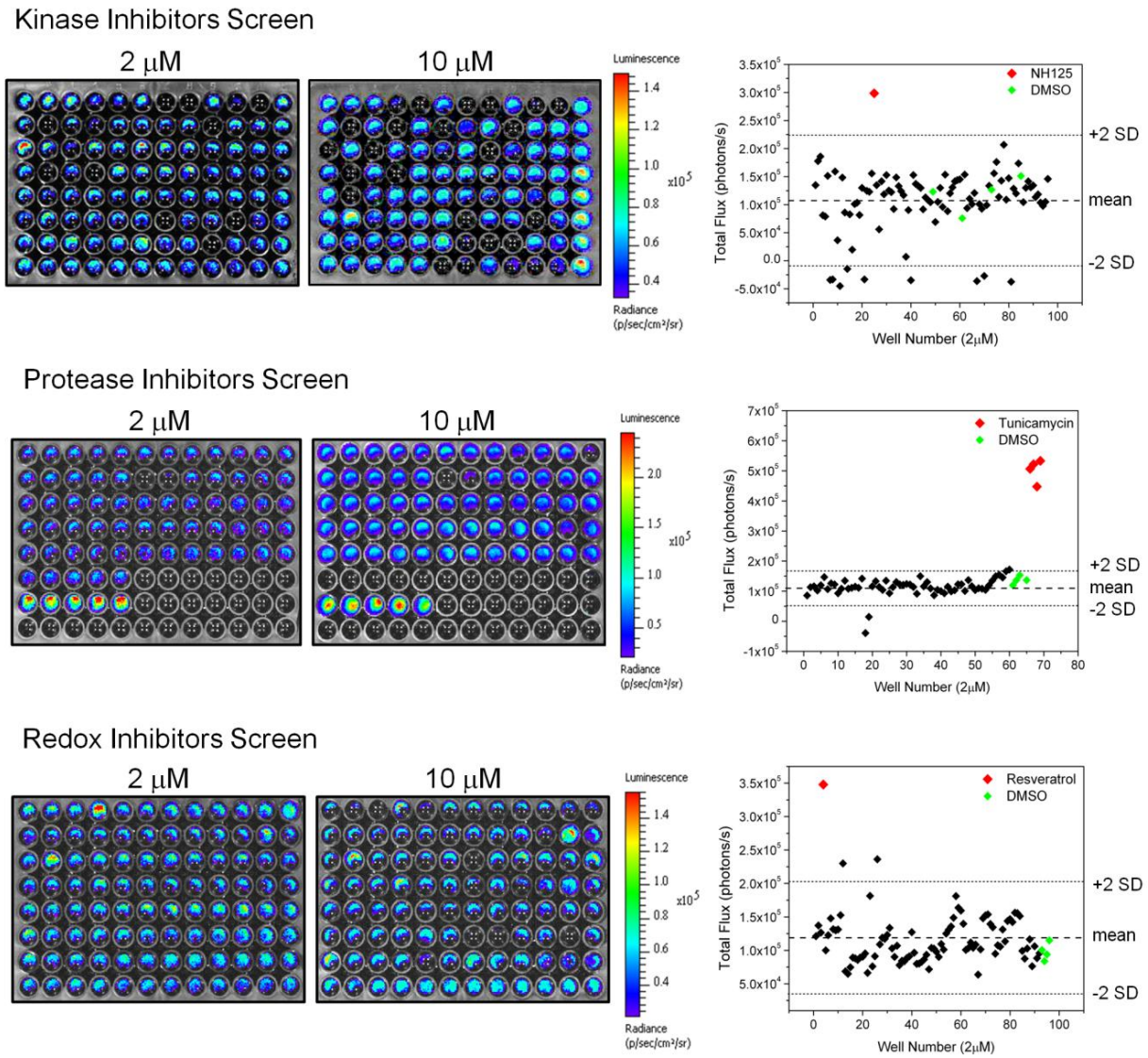
Supplementary Figures

Legend Figure S1:

Raw bioluminescence data obtained from screens of kinase, protease, and redox inhibitors. U251 DR5 Luciferase cells were treated with compounds from three commercially available kinase, protease, and redox inhibitor libraries for twenty-four hours at 2 and 10 μM. Cells were also treated with 1% DMSO (negative control) and 1 μg/mL Tunicamycin (positive control). Positive hits were defined as compounds that produced bioluminescent readings that were greater than two standard deviations above their respective plate means. Based upon these criteria, NH125, HBED (metal chelator), Piceatannol (stilbene phenolic antioxidant), and Resveratrol were identified as positive hits. Only NH125 was identified as a true positive as Resveratrol, Piceatannol, and HBED all produced luminescence in manner that was not consistent with integrated stress signaling (data not shown). Bioluminescent signals less than two standard deviations below the plate mean were due to compounds causing a decrease in cell viability. These compounds include: the (R) and (S) isomers of CR8, a Cdc7/Cdk9 Inhibitor, Keratinocyte Differentiation Inducer (casein kinase II Inhibitor), a Cdk2/9 Inhibitor, 5-Iodotubercidin (adenosine kinase inhibitor), PI 3-Kα Inhibitor VIII, UCN-01 (staurosporine analogue that inhibits protein kinase c), Ro-31-8220 (inhibitor of protein kinase c), Gliotoxin (proteosome inhibitor), and MG-132 (proteosome inhibitor)

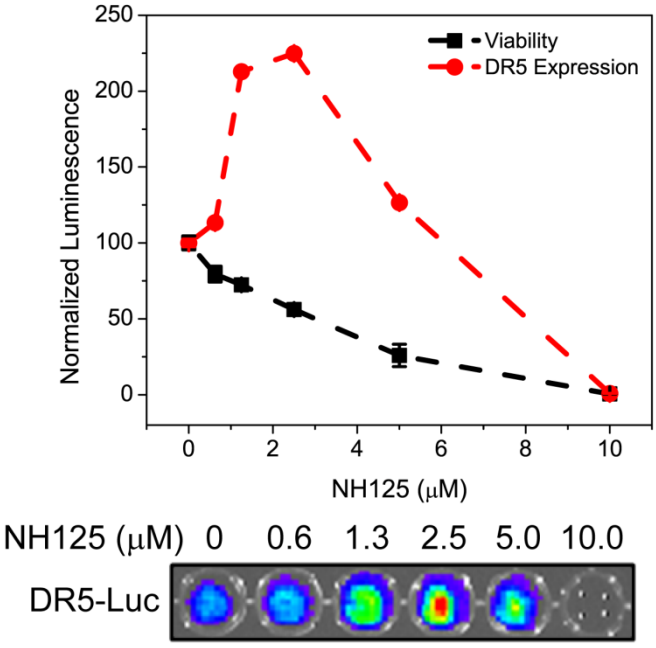
Figure S1:


Kinase Inhibitors Screen
2 μM
10 μM
Luminescence
Radiance (p/sec/cm²/sr)
NH125
DMSO
+2 SD
mean
-2 SD
Total Flux (photons/s)
Well Number (2μM)
Protease Inhibitors Screen
2 μM
10 μM
Luminescence
Radiance (p/sec/cm²/sr)
Tunicamycin
DMSO
+2 SD
mean
-2 SD
Total Flux (photons/s)
Well Number (2μM)
Redox Inhibitors Screen
2 μM
10 μM
Luminescence
Radiance (p/sec/cm²/sr)
Resveratrol
DMSO
+2 SD
mean
-2 SD
Total Flux (photons/s)
Well Number (2μM)

Legend Figure S2:

Bioluminescent and viability data from U251 DR5 Luciferase cells treated with an NH125 dilution series. Each data point represents the mean and standard deviation of biological triplicates normalized to vehicle (0.1% DMSO) treated controls. Data is plotted as individual points, and connected via a dotted line to guide interpretation of dose dependent changes in DR5 expression or viability. A representative bio-luminescent image of NH125 treated U251 DR5 Luciferase cells emphasizes that luminescence increases in a dose dependent manner. Luminescent values decrease at 5 and 10 μM due to corresponding decreases in cell viability.

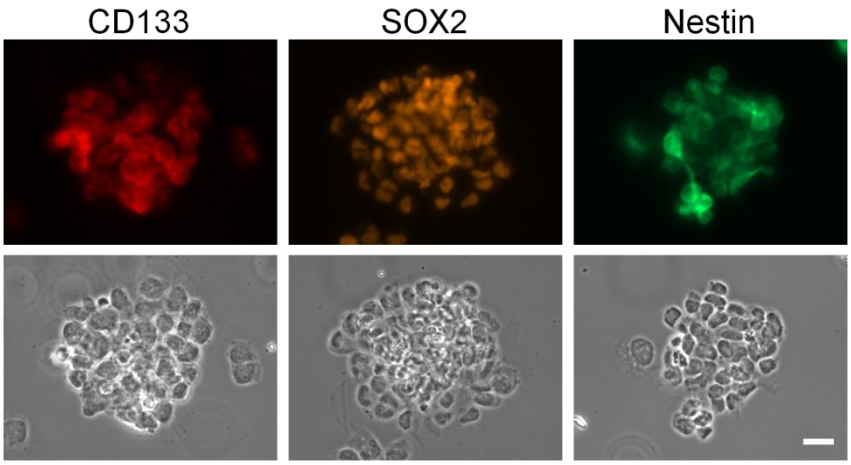
Figure S2:



Legend Figure S3:

Representative images of T4213 neurospheres taken under 40X magnification demonstrate that T4213 enrich for CD133 (red) SOX2 (orange) and Nestin (green) (scale bar = 25 microns).

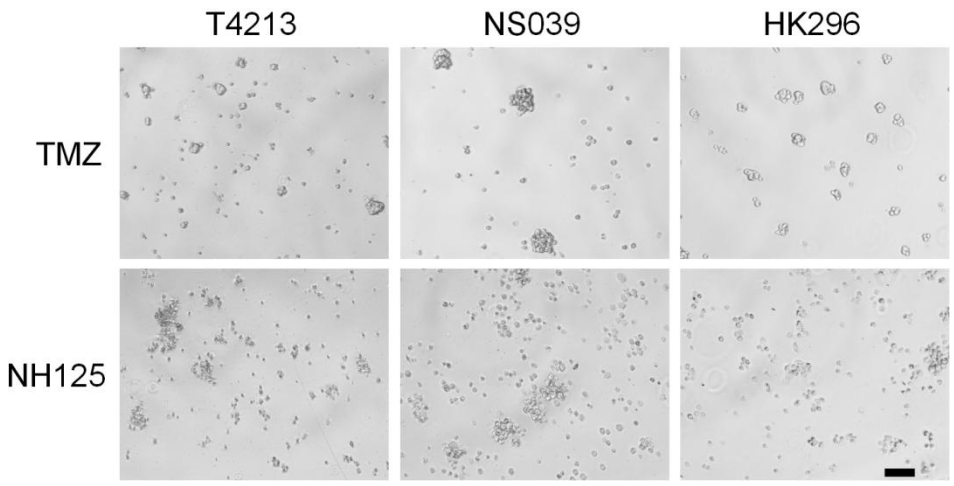
Figure S3:



Legend Figure S4:

Representative phase contrast images of TMZ, and NH125 treated GSC taken under 10X magnification (scale bar = 100 microns). The top row of images represent T4213, NS039, and HK296 cells treated with 1 mM TMZ for twenty-four hours. The bottom row images represent T4213, NS039, and HK296 cells treated with 5 μM NH125 for twenty-four hours.

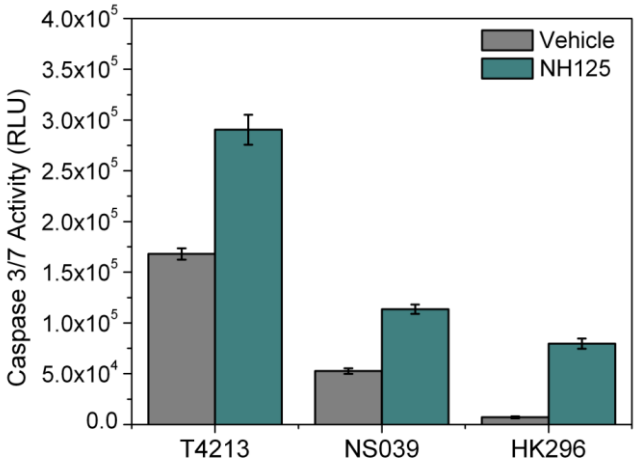
Figure S4:



Legend Figure S5:

Caspase 3/7 activity increases in NH125 treated GSC. GSC were incubated with either 0 μM NH125 (0.1% DMSO) or 5 μM NH125 for twenty-four hours followed by addition of Caspase 3/7 Glo. Caspase activity is increased in all NH125 treated GSC when compared to their vehicle treated counterparts. Data is plotted as means and standard deviations (n = 7).

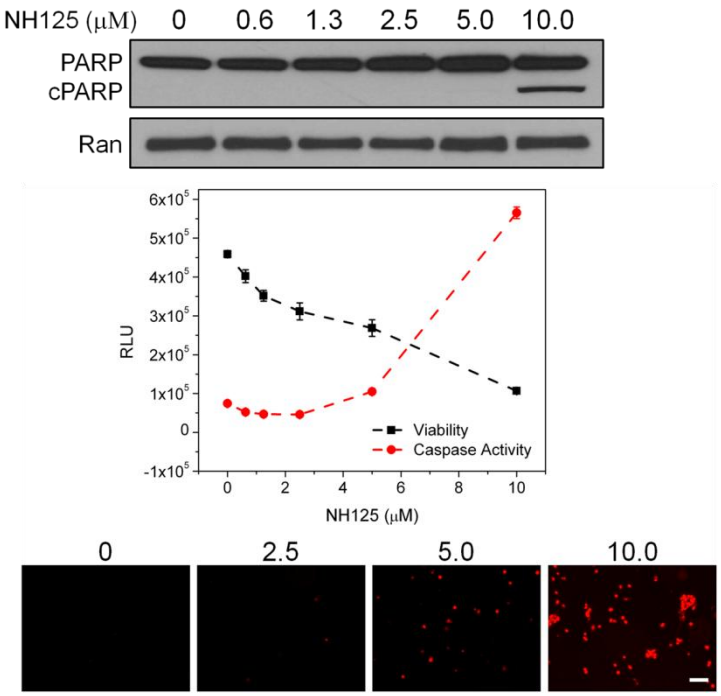
Figure S5:



Legend Figure S6:

NH125 leads to a dose dependent increase in markers of apoptosis. Incubation of U251 cells with increasing concentrations of NH125 leads to PARP cleavage at 10 μM. Caspase 3/7 activity increases as cell viability decreases in U251 cells incubated with increasing concentrations of NH125 for twenty-four hours. Each data point represents the mean and standard deviation from biological triplicates. Data is plotted as individual points and connected via a dotted line to guide interpretation of dose dependent changes in caspase and cell viability. Representative images taken under 10X magnification following treatment with either 0 (0.1% DMSO), 2.5, 5.0, and 10 μM NH125 reveal an increase in the number of propidium iodide stained cells (scale bar = 100 microns).

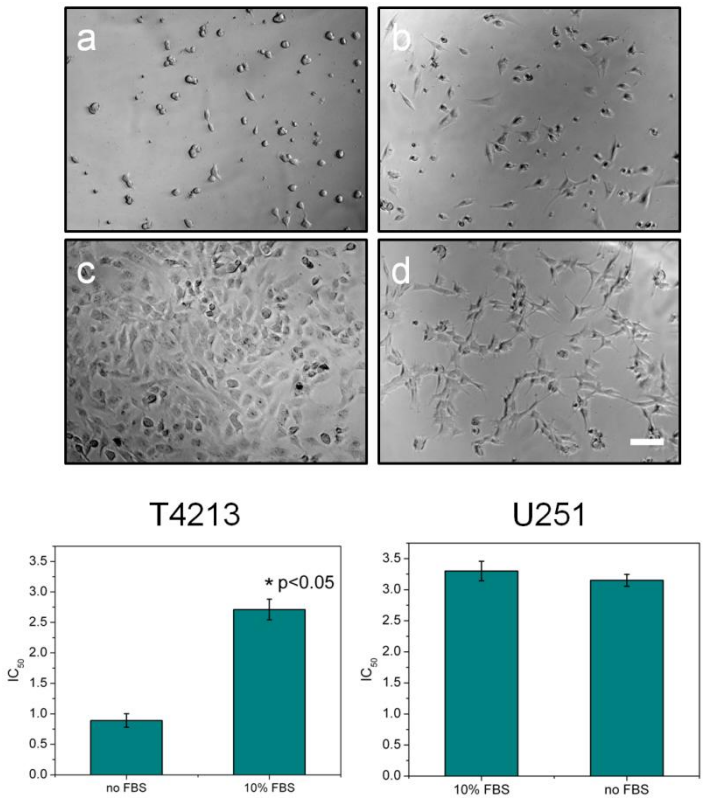
Figure S6:



Legend Figure S7:

GSC that acquire a differentiated morphology are less sensitive to NH125. Representative phase contrast images taken at 10X magnification reveal morphologic changes following addition and removal of serum from growth media (scale bar = 100 microns). (a) T4213 GSC grown in the absence of serum begin forming neurospheres at forty-eight hours. (b) T4213 grown in the presence of 10% FBS begin to acquire morphological features similar to U251 cells grown in the presence of serum (c) at forty-eight hours. (d) U251 cells grown in the absence of serum for forty-eight hours maintain their morphologic features, but have a slower growth rate. Addition of an NH125 dilution series to T4213 grown in the presence of serum for forty-eight hours results in a three-fold increase in the IC_{50} value when compared to T4213 treated in the absence of serum ($p < 0.05$). However, addition of an NH125 dilution series to U251 grown in the absence of serum for forty-eight hours results in minimal change of the IC_{50} when compared to U251 treated in the presence of serum. Each data point (bar) represents the means and standard deviation from three independent experiments.

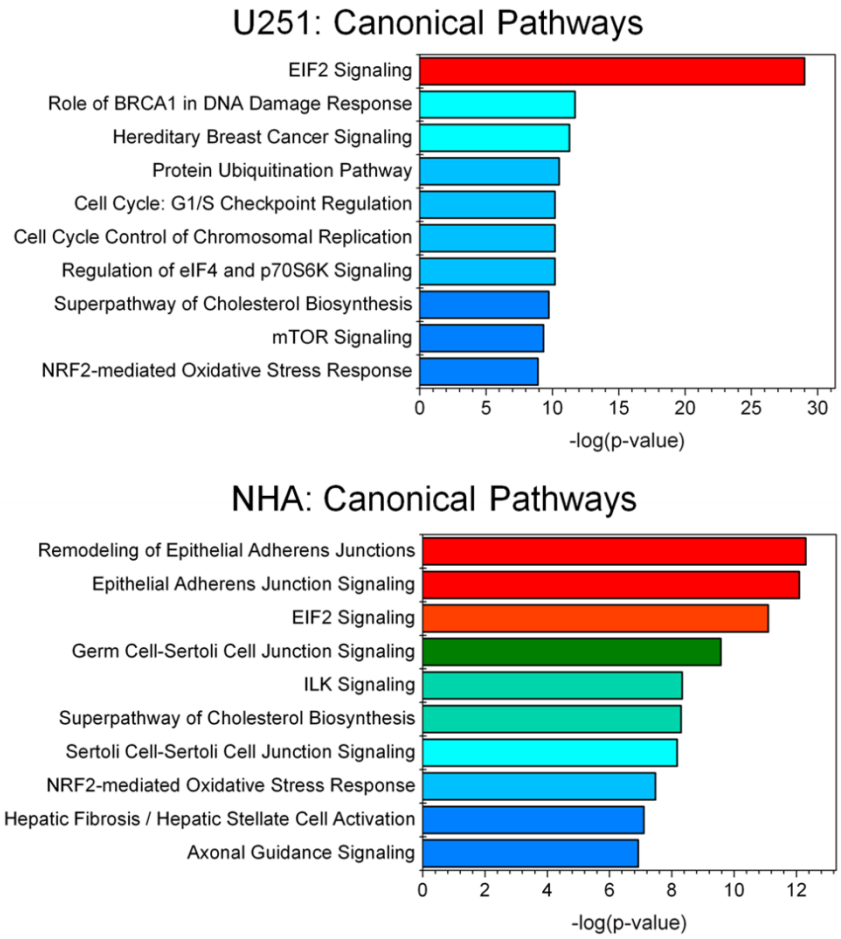
Figure S7:



Legend Figure S8:

The ten most significant canonical pathways from analysis of the transcriptional data of NH125 treated U251 and NHA. Significance is calculated using the Fischer's exact test to determine gene sets that closely match pathways from Ingenuity's Knowledge Base. A red-green-blue heatmap is used to emphasize the most significant canonical pathways.

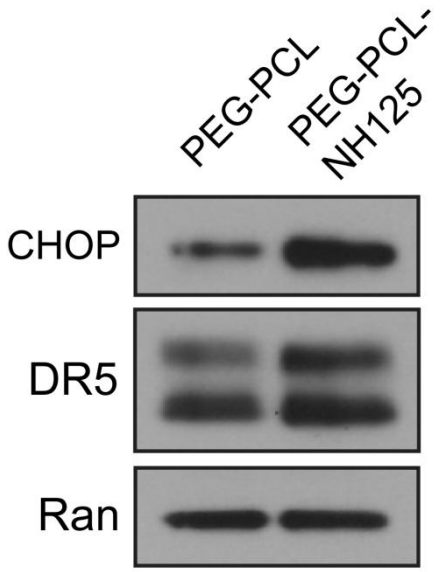
Figure S8:



Legend Figure S9:

PEG-PCL-NH125 treatment of U251 leads to an increase in CHOP and DR5 expression in vitro. U251 were treated with either 2.5 μg/mL of PEG-PCL or 2.5 μg/mL of PEG-PCL-NH125 for twenty-four hours. Lysate demonstrated an increase in CHOP and DR5 expression following twenty-four hour incubation with PEG-PCL-NH125 when compared to PEG-PCL.

Figure S9:



Supplementary Tables:

Table S1:

CHOP WT and KO TRAIL Synergy

NH125 (μM)	CHOP	TRAIL (ng/mL)	Combination Index
0.63	WT	25	0.29
1.25	WT	25	0.31
2.50	WT	25	0.32
5.00	WT	25	0.30
0.63	KO	25	1.00
1.25	KO	25	1.26
2.50	KO	25	1.61
5.00	KO	25	1.14

Table S1 Caption:

Knockout of CHOP leads to an abrogation of NH125 mediated TRAIL synergy. Calculation of the combination index following the addition of 25 ng/mL of TRAIL to NH125 treated U251 reveals the presence of a synergistic interaction (CI < 1.0). Addition of 25 ng/mL of TRAIL to NH125 treated U251 CHOP knockout cells (red shading) leads to combination indices equal to or greater than 1.0, indicating a loss of synergy. Data is presented from single replicate of experiments that were repeated twice.

Table S2:

IC_{50} Values

Cell Line	IC_{50} (μM)	Adj R^2
T4213	0.89 ± 0.11	0.99
NS039	1.95 ± 0.35	0.99
HK296	2.51 ± 0.41	0.97
U251	3.30 ± 0.16	0.99
Ovcar3	4.68 ± 0.34	0.97
A549	6.06 ± 0.23	0.99
GL261	6.20 ± 0.40	0.97
U87-MG	7.15 ± 0.16	0.98
H1299	11.02 ± 0.43	0.99

Table S2 Caption:

IC_{50} values from a panel of NH125 treated cells. IC_{50} values were calculated by fitting normalized viability data to a sigmoid dose response curve. Mean and standard deviations were calculated from three independent experiments. Adjusted R-squared values were also calculated from three independent experiments. All three GSC cell lines (red shading) demonstrate increased sensitivity to NH125 when compared to other malignant cell lines.

Table S3:

Low Dose TRAIL

Cell	NH125 (μM)	TRAIL (ng/mL)	CI
T4213	1.25	5.0	0.41
T4213	2.50	5.0	0.50
T4213	5.00	5.0	0.46
NS039	1.25	5.0	0.82
NS039	2.50	5.0	0.40
NS039	5.00	5.0	0.29
U251	1.25	5.0	0.92
U251	2.50	5.0	0.75
U251	5.00	5.0	0.78

Table S3 Caption:

Addition of low dose TRAIL leads to increased synergy in glioma stem cells. NH125 treated GSC (red shading) exhibit lower combination indices following addition of 5.0 ng/mL of TRAIL. This synergistic interaction decreases following the addition of 5.0 ng/mL TRAIL to NH125 treated U251. Data is presented from single replicate of experiments that were repeated twice.