Supporting Information for

Reactive Oxygen Species Synergize to Potently and Selectively Induce Cancer Cell Death

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1. General Information and Materials

Reagents

DNQ, IB-DNQ, and NHI-Glc-2 were synthesized as described.[1, 2] Hydrogen Peroxide (H_2O_2), *tert*-butyl hydroperoxide (TBHP), 2-Methoxyestradiol (2-ME), Embelin, Menadione, Sulforhodamine B, Trichloroacetic acid, Dicoumarol (DIC), ES936, *N*-Acetyl Cysteine (NAC), and deferoxamine (DFO) were purchased from Sigma Aldrich. Hoechst 33342, MitoSox Red, Carboxy-H_2DCFDA, Mitotracker Green FM, and 2-NBDG were purchased from Molecular Probes (Thermo Fisher Scientific). Elesclomol and Dihydroethidium (DHE) was purchased from Cayman Chemical Company. Rabbit monoclonal anti-LDHA (#3582), Mouse monoclonal anti-NQO1 (#3187), and Rabbit monoclonal anti-β-Actin (#4970) were purchased from Cell Signaling. Rabbit monoclonal anti-GLUT1 (#ab115730) and Goat anti-Rabbit IgG H&L (Alexa Fluor 488 conjugated, #ab150077) were purchased from Abcam.

Cell Lines and cell culture

A549, H460, H1993, and H1299 cell lines were obtained from ATCC and grown in RPMI 1640 medium with 10 % fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. IMR90 cell lines were obtained from ATCC and grown in EMEM with 10 % fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. MDA-MB-231 cells were obtained from the Boothman Lab (UT Southwestern) and grown in RPMI1640 medium with 10 % fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin and 1 µg/mL puromycin to select for transfected cells. Cells were cultured at 37 ℃ in a 5 % CO_2 – 95% air humidified atmosphere.

2. Experimental procedures

Cytotoxicity Assays

Cells were seeded on 96 well plates at the density of 1×10^4 cells per well. The cells were continuously treated with various concentrations of compounds, DNQ and/or NHI-Glc-2, for 48 h. When investigating the effect of NQO1 inhibitor (dicoumarol or ES936) or antioxidant NAC, cells were pretreated for 1h with 25 µM dicoumarol, 100 nM ES936, or 5 mM NAC before addition of DNQ and/or NHI-Glc-2. Then viability was assessed using the sulforhodamine B (SRB) assay.[3] Cells were fixed with 10% (w/v) trichloroacetic acid at 4 ℃ for overnight and then stained with 0.057% (w/v) SRB in 1% (v/v) acetic acid at room temperature for 30 min. The dye was solubilized in 10 mM Tis base (pH 10.5) and absorption at 510 nm was measured using a SpectraMax Plus 384 (Molecular Devices). Percent death was calculated by subtracting background from all wells and setting 0% death to DMSO-treated controls. All data are averages of at least three independent replicates.

Calculation of Combination Index

Combination Index has been calculated with a software named CompuSyn (ComboSyn, Inc., free download available upon registration: http://www.combosyn.com/register.html)

Western Blot Analysis

Cells were trypsinized and harvested at 70─90% confluency. After centrifugation and washing, the cells were lysed with RIPA buffer (with protease inhibitor) on ice for 30 min. Supernatant was collected after centrifugation. Protein concentration was determined by the Pierce BCA assay (Thermo Fisher Scientific). Protein content in all samples were normalized. Whole cell lysate (10 µg) was denatured and seperated by 10 % SDS-PAGE gel (Mini-PROTEAN TGX precast gel, Bio-Rad) electrophoresis at 120 V for 1 h. Proteins were transferred onto nitrocellulose membrane (45 V for 2 h). The non-specific interaction was blocked in 5% (w/v) BSA TBST for 1 h at RT. The membranes were blotted with anti-LDHA (1:2000 in 5 % BSA TBST), anti-NQO1 (1:2000 in 5 % BSA TBST), and anti-β-actin (1:1000 in 5% BSA TBST)

overnight at 4 ℃. The bound primary antibodies were detected using HRP conjugated secondary antibodies (1:5000 in TBST) for 90 min at RT and visualized by Pierce ECL autoradiography (Thermo Fisher Scientific).

ROS imaging

A549 cells were seeded on 35 mm cover glass bottom dish with 20 mm micro-well cover glass (Cellvis) at a density of 1×10^5 cells per dish. When cells reached 70% confluence, cells were treated with 250 nM DNQ and/or 15 μM NHI-Glc-2 for 1 h at 37 ℃ in a 95% air / 5 % CO_2 atmosphere. When determining the effect of ES936, cells were pretreated for 1 h with 100 nM ES396 and then co-treated with ES936 and compounds for 1 h. After washing the cells with HBSS, the cells were probed with ROS indicators according to the manufacturer's protocol. Briefly, the cells were treated with 25 μM carboxy-H_2DCFDA for 50 min. 5 μM MitoSox Red and 1 μg mL^{-1} Hoechst 33342 were added and co-incubated with carboxy-H_2DCFDA for 10 min. After gentle washing with HBSS, cellular fluorescence was observed using a LSM 700 confocal microscope (Carl Zeiss). When investigating the effect of elesclomol and menadione on ROS generation, cells were treated with 500 nM DNQ, 500 nM elesclomol, or 100 μM menadione for 1 h. After washing, the cells were probed with 25 μM carboxy-H_2DCFDA for 60 min or 5 μM DHE for 30 min. 1 μg mL^{-1} Hoechst 33342 was added 30 min before washing and co-incubated with carboxy-H_2DCFDA or DHE for 30 min.

Fluorescence image analysis

All fluorescence images taken with LSM 700 confocal microscope (Carl Zeiss) were analyzed using LSM Image Browser and Image J.

ROS measurement using flow cytometry

A549 cells were seeded on 6 well plates at a density of 2×10^5 cells per well. When cells reached 70─80 % confluence, cells were treated with DNQ and/or NHI-Glc-2 for indicated time. After washing with HBSS, the cells were treated with 5 μM MitoSox Red for 10 min or 10 μM carboxy-H_2DCFDA for 30 min. Then the cells were washed gently, trypsinized, and harvested. Cellular fluorescence was analyzed using BD LSR II flow cytometry analyzer (BD Biosciences).

GLUT1 immunofluorescence imaging

Cells were seeded on 35 mm cover glass bottom dish with 20 mm micro-well cover glass (Cellvis) at a density of 1×10^5 cells per dish. When cells reached 70% confluence, cells were treated with 250 nM DNQ for 4h. After washing, the cells were fixed with 4 % paraformaldehyde for 15 min at RT. Non-specific interaction was blocked with 5 % (w/v) BSA PBST for 30 min at RT. The cells were incubated with anti-GLUT1 (1:400 in 1 % (w/v) BSA PBST) overnight at 4℃. GLUT1 expression was visualized with Alexa Fluor 488 conjugated secondary antibody (1:500 in 1 % BSA PBST, 1h, RT). The cells were treated with 1 μg mL^{-1} Hoechst 33342 for 5 min at RT and gently washed with ice-cold PBS three times. Fluorescence was observed using a LSM 700 confocal microscope.

2-NBDG uptake measurement using flow cytometry

A549 cells were seeded on 6 well plates at a density of 2×10^5 cells per well. When cells reached 70─80 % confluence, cells were pretreated with or without 100 nM ES936 and then co-treated with 0─100 nM DNQ for 2 h. After washing with HBSS, the cells were treated with 50 μM 2-NBDG for 30 min. Then the cells were washed gently, trypsinized, and harvested. Cellular fluorescence was analyzed using BD LSR II flow cytometry analyzer (BD Bioscience).[4]

Cellular uptake of NHI-Glc-2 measurement

A549 cells were seeded on 6 well plates at a density of 2×10^5 cells per well. When cells reached 70─80 % confluence, cells were treated with 250 nM DNQ for 4h. After washing with PBS, the cells were treated with 20 μM NHI-Glc-2 for 30 min. Then the cells were washed gently, trypsinized, harvested, and lysed

with RIPA buffer. Protein concentration was determined by the BCA assay (Pierce) and total protein content for all samples were normalized. Concentration of NHI-Glc-2 in lysate was analyzed using reverse phase HPLC (Shimadzu Corporation).

Colony forming assay

Cells were seeded on 6 well plates at a density of 100 cells per well and allowed to attach overnight. Cells were then treated with IB-DNQ (30 nM) and/or NHI-Glc-2 (5 μM) simultaneously or at intervals of 6 or 24 h between IB-DNQ and NHI-Glc-2. The treatment was proceeded every other day for 3 or 4 times. Fresh media without the compounds were added after the final treatments and the cells were allowed to incubate for 4 days. The cells were fixed and stained with dH_2O containing 6.6 % (v/v) glyceraldehyde and 0.5 % (w/v) crystal violet.[5]

MTD of DNQ, NHI-Glc-2, and DNQ + NHI-Glc-2

The protocol was approved by the IACUC at the University of Illinois at Urbana-Champaign (Protocol Number: 15063). In these studies, 10- to 12- week-old female C57BL/6 mice were purchased from Charles River were used. The maximum tolerate dose of single compound was determined first. DNQ or NHI-Glc-2 were formulated as slurries in HPβCD containing water. DNQ or NHI-Glc-2 were given by oral gavage for 5 consecutive days and monitored for signs of toxicity. MTD was the highest dosage with acceptable toxicity (e.g. < 20% weight loss). The MTDs of DNQ (20 mg kg^{-1}) and NHI-Glc-2 (200 mg kg^{-1}) were used for determining the combined MTD. Beginning with each MTD, DNQ (20 mg kg^{-1}) and NHI-Glc-2 (200 mg kg^{-1}) were formulated simultaneously and given by daily oral gavage. 15 mg kg^{-1} DNQ + 150 mg kg^{-1} NHI-Glc-2 (oral gavage, every other day) was established and determined to be the combined MTD.

Pharmacokinetic assessment of IB-DNQ

The protocol was approved by the IACUC at the University of Illinois at Urbana-Champaign (Protocol Number: 15063). In these studies, 10- to 12- week-old female C57BL/6 mice were purchased from Charles River were used. IB-DNQ were formulated in HPβCD containing water. Mice were treated with IB-DNQ (11 mg kg^{-1}) via *i.v.* and oral gavage with three mice per time point (10, 20, 30, 40, 60, 120, 240, 480, and 1440 min). At specific time points, mice were sacrificed and blood was collected, centrifuged and the plasma was frozen at −80℃ until analysis. The proteins in a 50 μL aliquot of plasma were precipitated by the addition of 50 μL acetonitrile and the sample was centrifuged to remove the protein. Plasma concentrations of IB-DNQ were determined by reverse phase HPLC (Shimadzu Corporation). PK parameters were determined using GraphPad Prism Version 5.00 for Windows.

Pharmacokinetic assessment of NHI-Glc-2

The protocol was approved by the IACUC at the University of Illinois at Urbana-Champaign (Protocol Number: 15063). In these studies, 10- to 12- week-old female C57BL/6 mice were purchased from Charles River were used. NHI-Glc-2 was formulated as slurries in HPβCD containing water. Mice were treated with NHI-Glc-2 (200 mg kg^{-1}) via oral gavage with three mice per time point (30, 60, 120, 240, 480, and 1440 min). At specific time points, mice were sacrificed and blood was collected, centrifuged and the serum was frozen at −80℃ until analysis. The proteins in a 50 μL aliquot of serum were precipitated by the addition of 50 μL of acetonitrile and the sample was centrifuged to remove the protein. Serum concentrations of NHI-Glc-2 were determined by reverse phase HPLC (Shimadzu Corporation).

H460 surgical intervention metastasis model

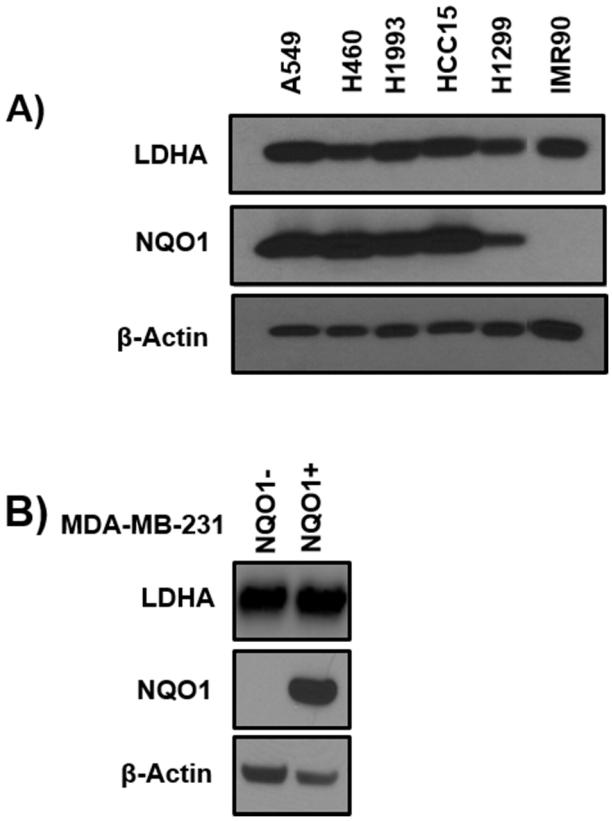
The protocol was approved by the IACUC at the Beth Israel Deaconess Mediacal Center. 1 million H460 cells were prepared in HBSS and injected subcutaneously in the mid-dorsum of SCID mice. The tumor was resected when the size was reached at 2 cm^3 and the mice were randomized into four treatment groups: vehicle, IB-DNQ alone, NHI-Glc-2 alone, and IB-DNQ + NHI-Glc-2. The dosing was started on the same day. IB-DNQ (15 mg kg^{-1}) and/or NHI-Glc-2 (125 mg kg^{-1}) was given via oral gavage for twice a week for

11 weeks. Kaplan-Meier survival curve was drawn to show survival of mice. When mice were dead, the lungs were excised, weighed, and submitted for the counting of metastasis.

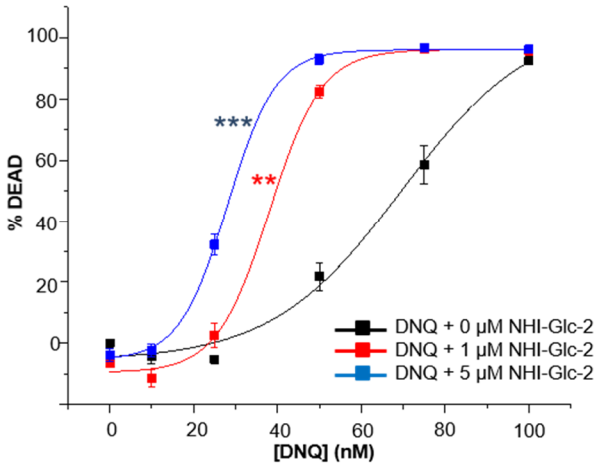
Statistical Analysis

All statistical analysis was performed using an unpaired, two-tailed student's t test with p values < 0.05 were considered statistically significant. For Survival analysis, Kaplan-Meier Log Rank Survival Test was performed using Origin Pro 9 (Northampton).

3. Supporting Figures

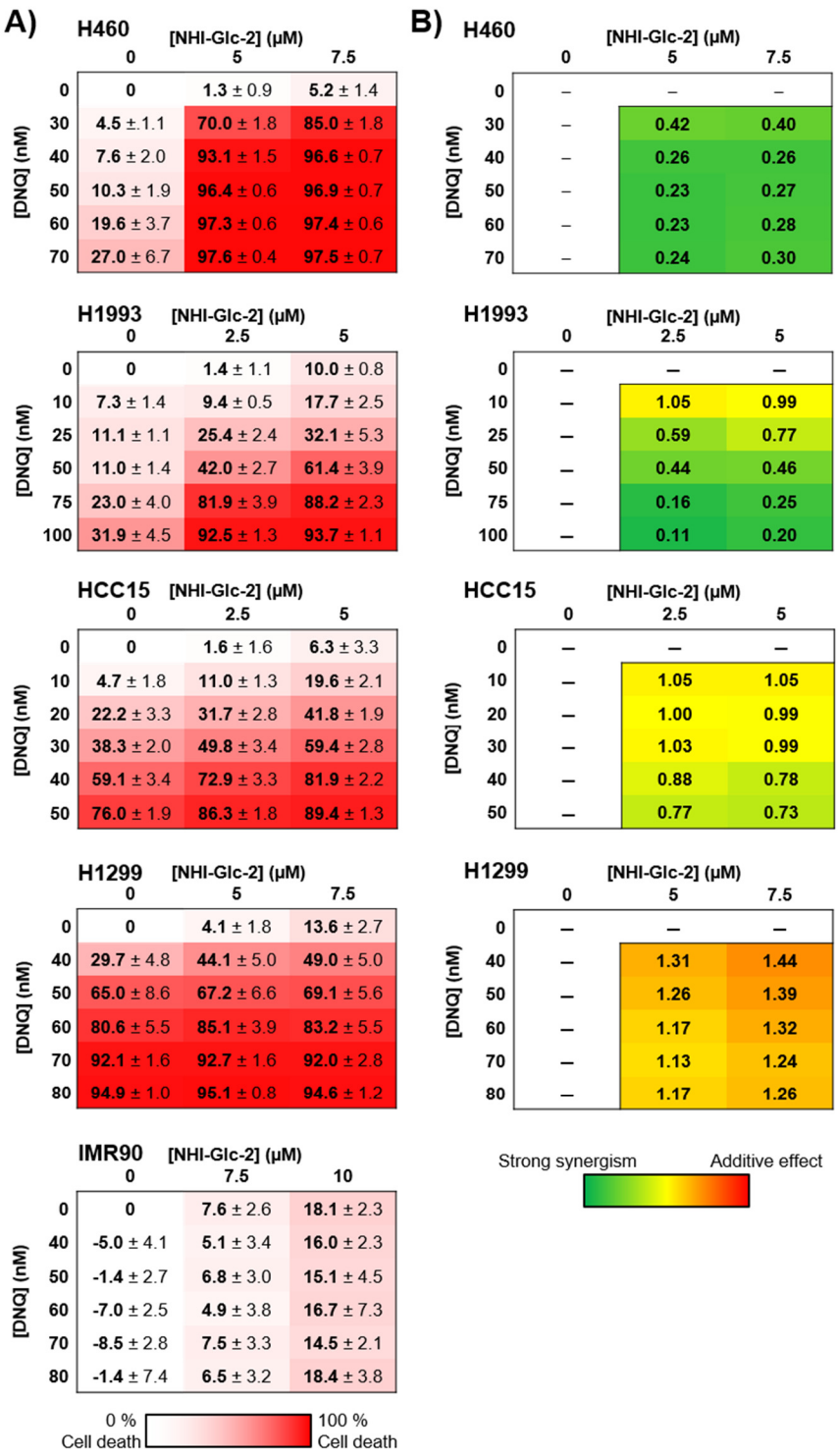


Supporting Figure S1. Expression of LDHA and NQO1 in various cancer cell lines and lung fibroblast as observed using western blot. A) Total cell lysates of lung cancer cells (A549, H460, H1993, HCC15, H1299) and lung fibroblast (IMR90) were immunoblotted for the confirmation of LDHA and NQO1 expression. B) Total cell lysates of MDA-MB-231 cells transfected with empty vector or vector with the gene encoding NQO1 were assessed by Western blot with antibodies for LDHA and NQO1.

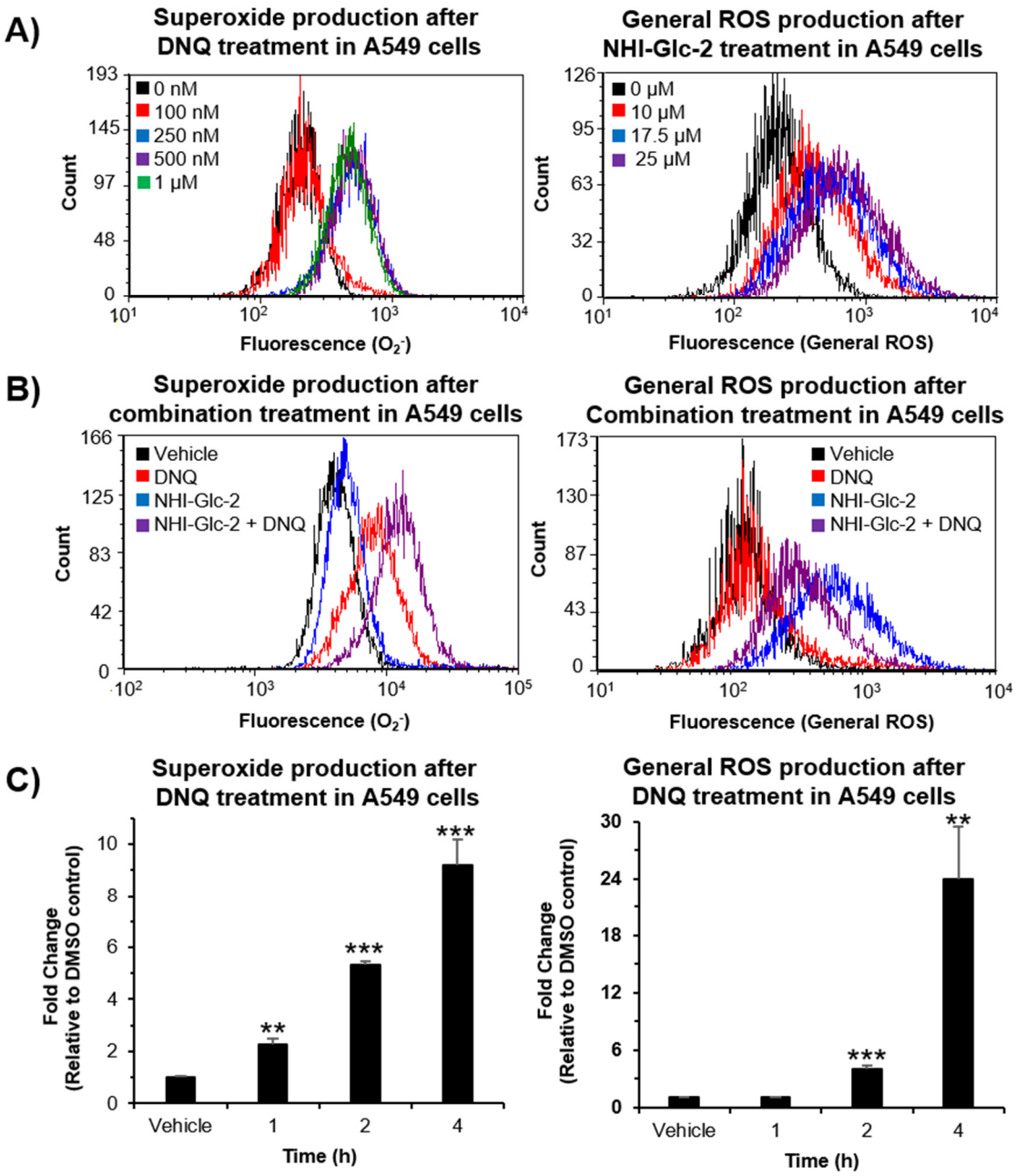


Concentration of NHI-Glc-2 (μM)	IC_{50} of DNQ (nM)
0	72.1 ± 6.9
1	38.0 ± 1.5
5	28.0 ± 0.7

Supporting Figure S2. The combination of DNQ and NHI-Glc-2 synergize to induce death in A549 NSCLC cells. Cells were treated with the indicated concentrations of DNQ and NHI-Glc-2 for 48 h. Percent cell death was assessed using the SRB assay and plotted against concentration. Error bars represent the s.e.m. of four replicates. Statistical analysis was performed on the IC_{50} values of DNQ using an unpaired, two-tailed student's t test. * $0.01 \leq p < 0.05$, ** $0.001 \leq p < 0.01$, *** $p < 0.001$ relative to DNQ only treatment.

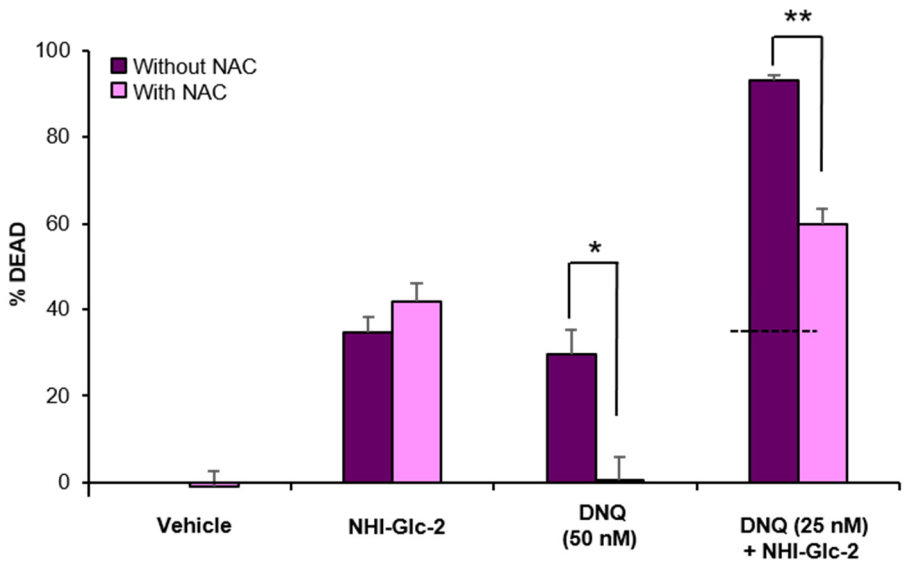


Supporting Figure S3. The combination of DNQ and NHI-Glc-2 induces synergistic cell death in lung cancer cell lines with elevated NQO1 levels. A) Human lung cancer cell lines (H460, H1993, HCC15, and H1299) and human lung fibroblast cells (IMR90) were treated with the indicated concentrations of DNQ and NHI-Glc-2 for 48 h. Percent cell death was assessed using SRB assay and represents in matrix format. Error represents the s.e.m of three or four replicates. B) Combination Index values calculated for each combination with Compusyn software (<1 indicates synergistic interaction).

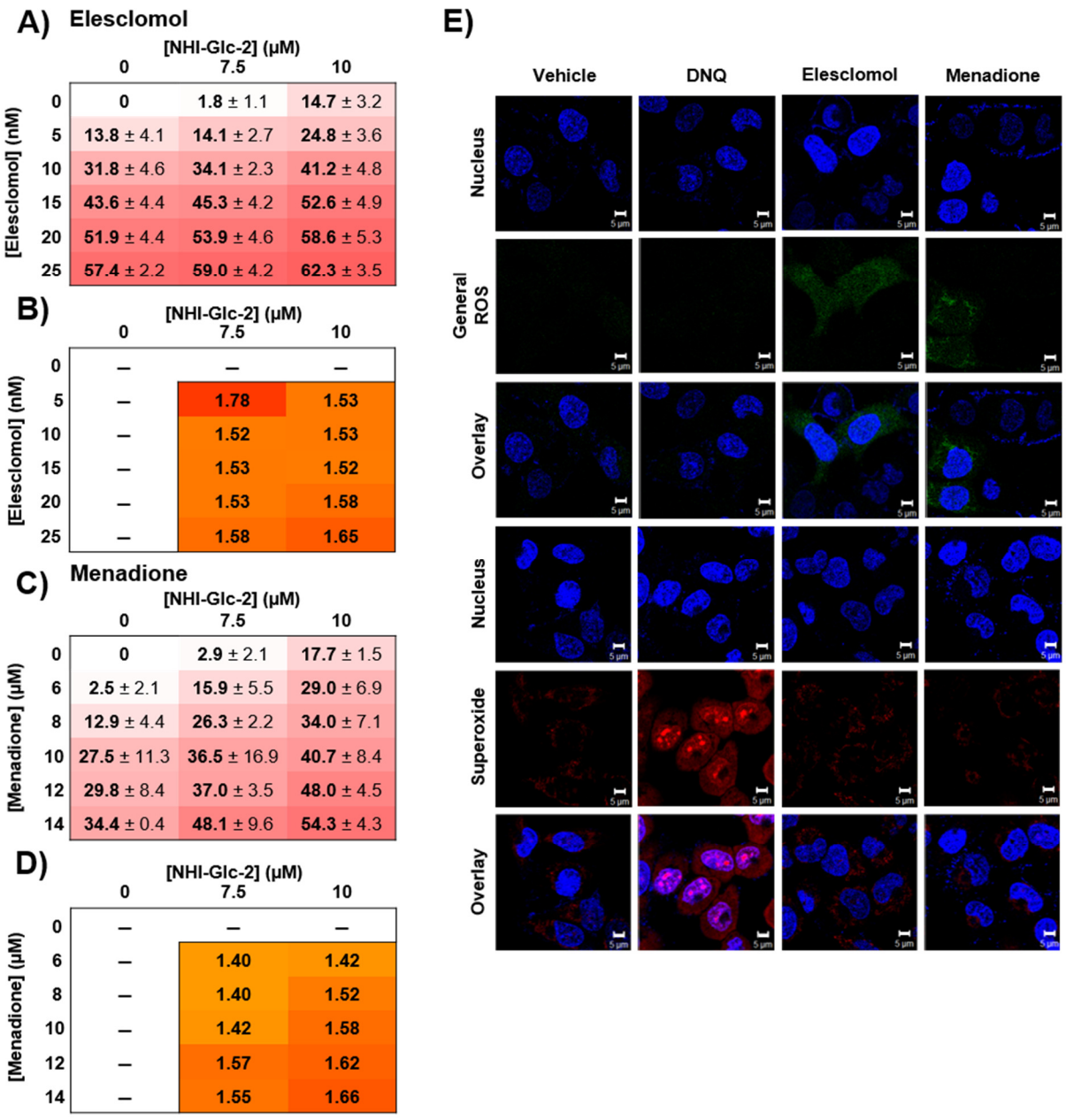


Supporting Figure S4. DNQ and NHI-Glc-2 produce mitochondrial superoxide and general ROS, respectively, and ROS generation is significantly increased by the combination. A) DNQ and NHI-Glc-2 produce mitochondrial superoxide and intracellular general ROS, respectively, in A549 cells in a dose dependent manner. A549 cells were treated with DNQ or NHI-Glc-2 for 1 h. Then the cells were treated with MitoSox Red (5 µM) for 10 min or carboxy-H_2DCFDA (10 µM) for 30 min. Fluorescence intensity was measured using flow cytometry. B) Combination of DNQ and NHI-Glc-2 induces significant ROS production in A549 cells. A549 cells were treated with the DNQ (500 nM) and/or NHI-Glc-2 (15 µM) for 1 h. Superoxide and general ROS production after compounds treatment were visualized using MitoSox Red and carboxy-H_2DCFDA respectively. Fluorescence intensities of each ROS indicator were measured using flow cytometry. C) DNQ produces ROS in time dependent manner. A549 cells were treated with DMSO or DNQ (250 nM) for 1, 2, or 4 h. Then the cells were treated with MitoSox Red (5 µM) for 10 min or carboxy-H_2DCFDA (10 µM) for 30 min. Fluorescence intensity was measured using flow cytometry. Error represents the s.e.m.

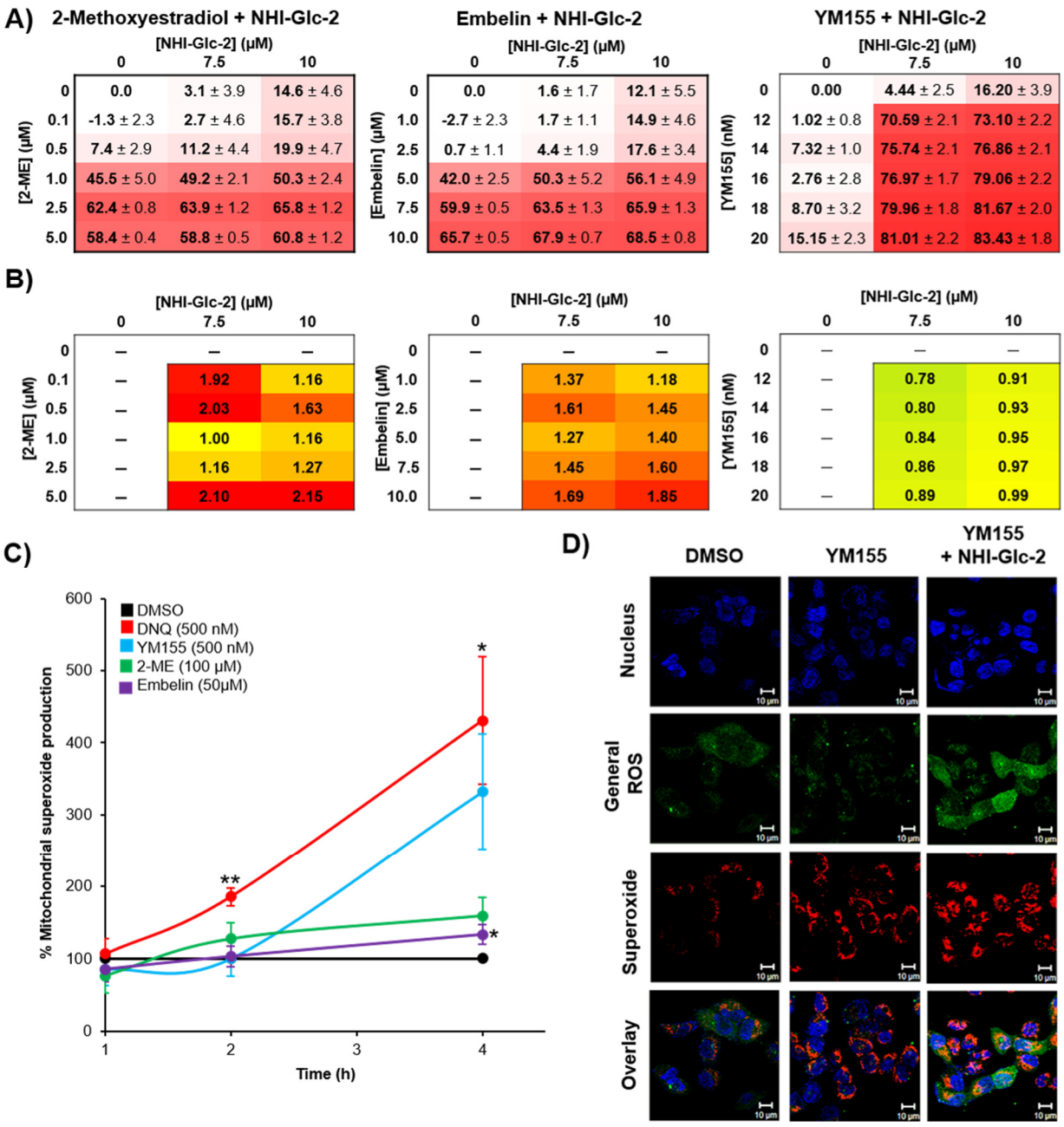
of four replicates (n = 4). Statistical analysis was performed using an unpaired, two-tailed student's t test. ** $0.001 \leq p < 0.01$, *** $p < 0.001$ relative to vehicle treatment.



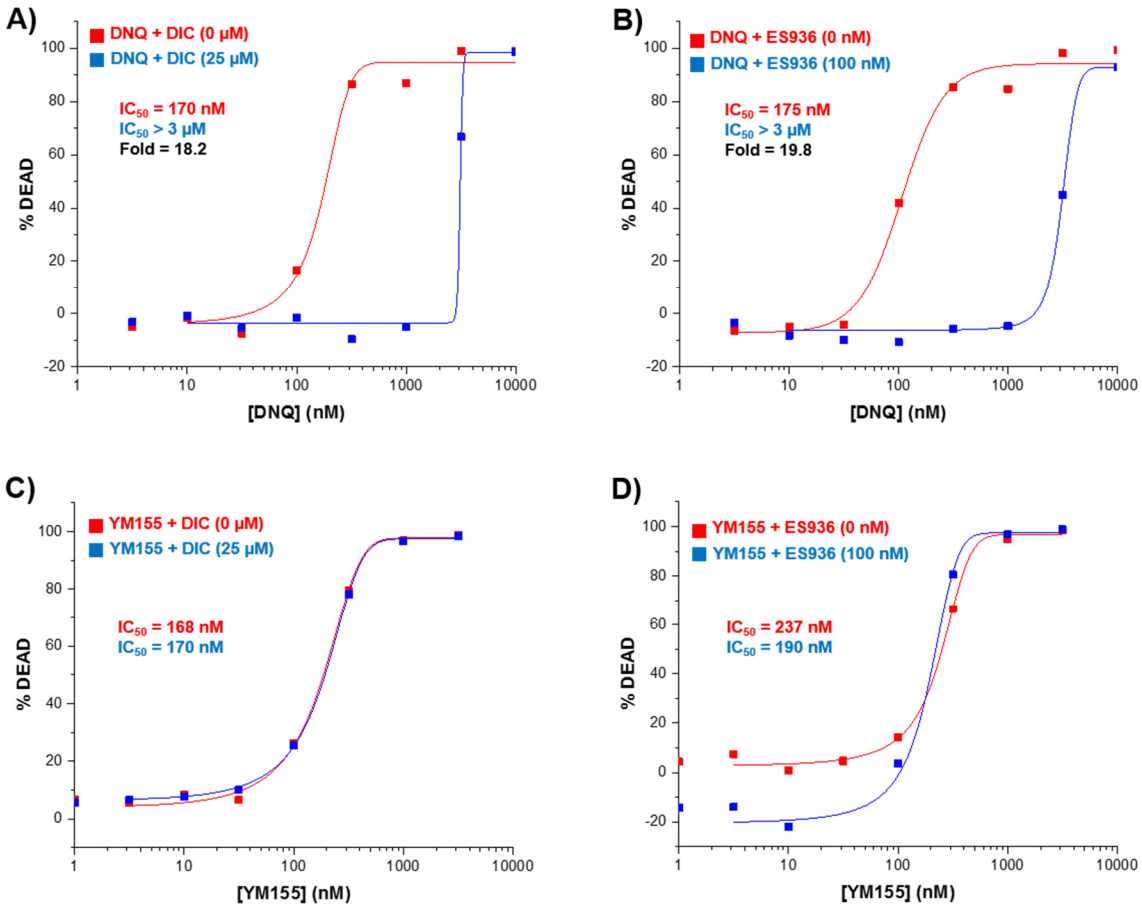
Supporting Figure S5. ROS generation by the combination of DNQ and NHI-Glc-2 synergizes to induce cancer cell death, and the antioxidant *N*-Acetyl Cysteine (NAC) protects against DNQ or DNQ/NHI-Glc-2 –mediated cell death in A549 cells. A549 cells were treated with NAC (5 mM) 1h prior to treatment with DNQ (25 or 50 nM) and/or NHI-Glc-2 (15 μM). Percent cell death observed after 48 h treatment of DNQ and/or NHI-Glc-2 in the absence and the presence of NAC using SRB assay, and the dashed horizontal lines represent the additive effect of DNQ and NHI-Glc-2. Error represents the s.e.m. of three replicates (n = 3). Statistical analysis was performed using an unpaired, two-tailed student's t test. * $0.01 \leq p < 0.05$, ** $p < 0.01$



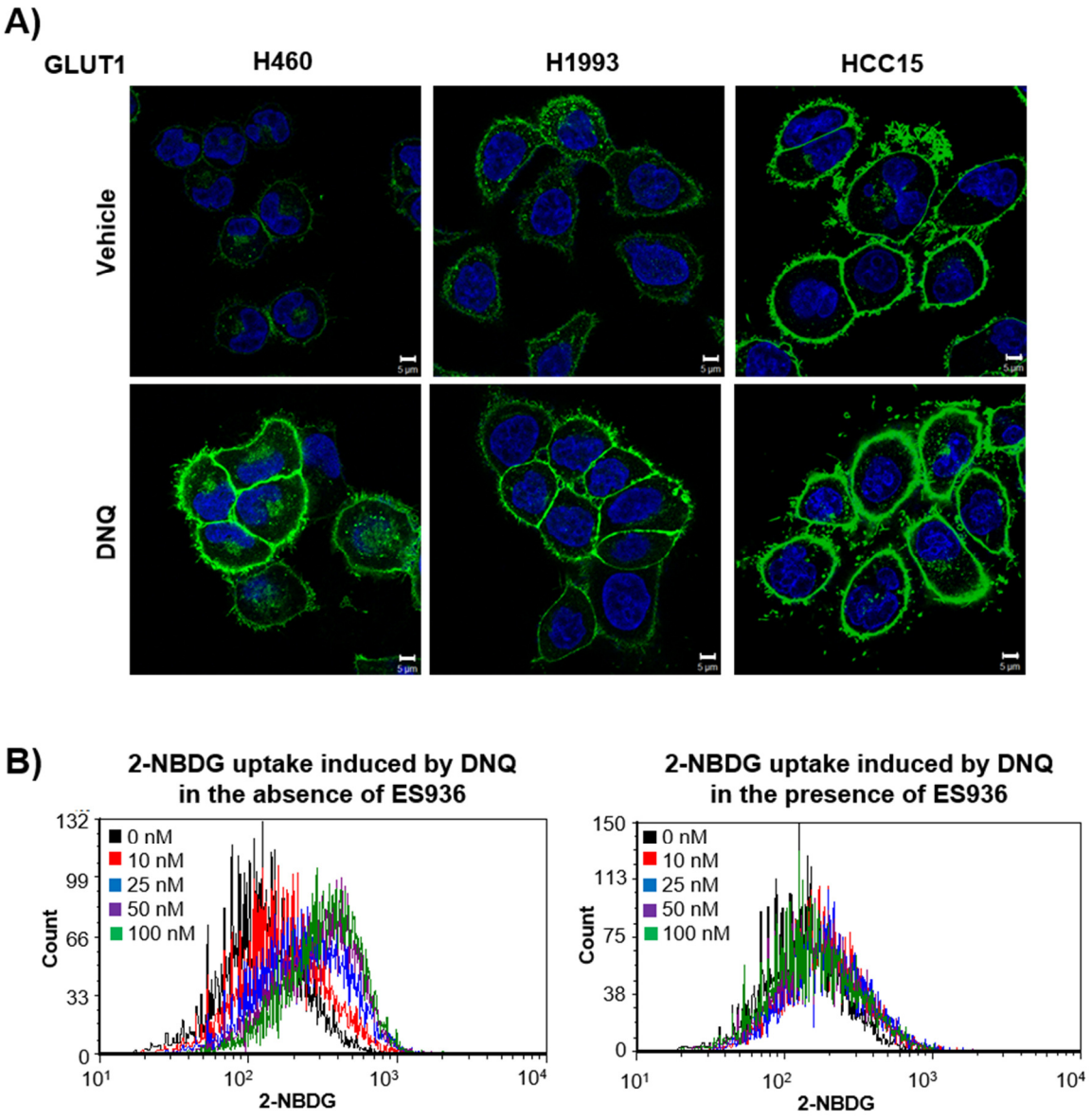
Supporting Figure S6. NHI-Glc-2 does not synergize with other ROS inducers (elesclomol and menadione) in A549 cells. A, C) A549 cells were treated with the indicated concentrations of NHI-Glc-2 and the ROS inducers, elesclomol (A) and menadione (C), for 48 h. Percent cell death was assessed using SRB assay and represented in matrix format. Error represents the s.e.m. of three replicates. B, D) Combination Index values calculated for each combination with Compusyn software (<1 indicates synergistic interaction) of combination of NHI-Glc-2 with elesclomol (B) and menadione (D). E) A549 cells were treated with DMSO, DNQ (500 nM), elesclomol (500 nM) or menadione (100 μM) for 1h. Superoxide and general ROS production after each compound treatment were visualized using DHE and carboxy-DCFDA respectively. Fluorescence intensities of each ROS indicator were observed using LSM 700. Scale bar indicates 5 μm.



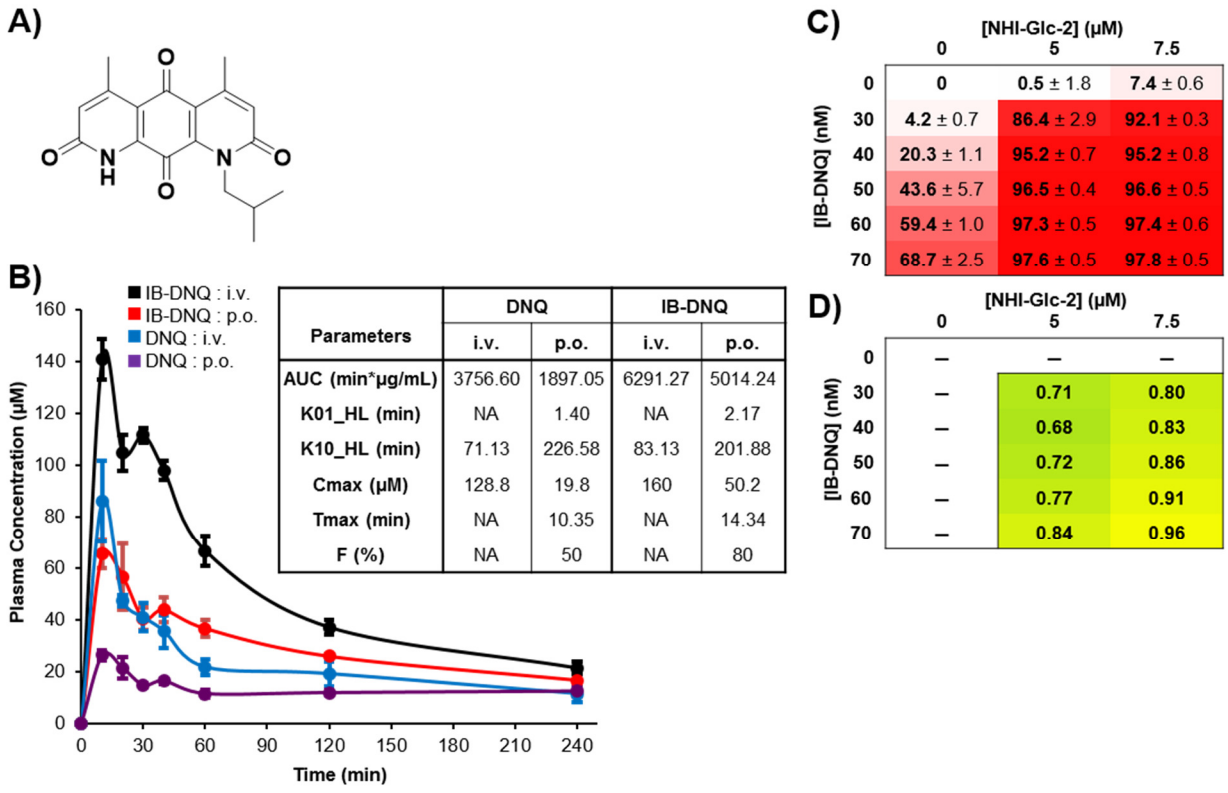
Supporting Figure S7. 2-ME or Embelin treatment does not potentiate NHI-Glc-2-mediated A549 cell death. In contrast, YM155 synergizes with NHI-Glc-2 to enhance cell death. A) A549 cells were treated with the indicated concentrations of NHI-Glc-2 and 2-ME, Embelin, or YM155 for 48 h. Percent cell death was assessed by the SRB assay. B) CI values were calculated using compusyn software. C) Mitochondrial superoxide production was measured using MitoSox Red after DNQ (500 nM), YM155 (500 nM), 2-ME (100 μM), or Embelin (50 μM) treatment for 1, 2, and 4 h in A549 cells. Fluorescence intensities were measured using microplate reader and normalized with cell density.[6] Statistical analysis was performed using an unpaired, two-tailed student's t test. * $0.01 \leq p < 0.05$, ** $p < 0.01$ relative to DMSO treatment. D) The combination of YM155 and NHI-Glc-2 induces ROS production in A549 cells. A549 cells were treated with YM155 (250 nM) and/or NHI-Glc-2 (15 μM) for 1h. Superoxide and H_2O_2 production after each compound treatment were visualized using MitoSox Red and Carboxy-H_2DCFDA respectively. Fluorescence intensities of each ROS indicator were measured using LSM 700. Scale bar indicates 10 μm.



Supporting Figure S8. DNQ induced cell death was protected by NQO1 inhibitor DIC (A) or ES936 (B). In contrast, cell death induced by YM155 was not altered in the presence of NQO1 inhibitor DIC (C) or ES936 (D). A, C) A549 cells were treated with DNQ or YM155 in the absence and the presence of DIC (25 µM) for 2 h. After washing, cells were incubated for 72 h in drug free fresh media. B, D) A549 cells were pre-treated with ES936 (100 nM) for 1h. Then DNQ or YM155 was added and the cells were co-treated for 2 h. After washing, cells were incubated for 72 h in drug free fresh media. Percent cell death was assessed using the SRB assay.



Supporting Figure S9. DNQ increases GLUT1 expression and glucose uptake in cancer cells in a dose dependent manner. A) GLUT1 expression in the membrane is enhanced by DNQ treatment in H460, H1993, and HCC15 cells. The cancer cells were treated with DNQ (250 nM) for 4h. GLUT1 expression was visualized by immunofluorescence using LSM700. Scale bar indicates 5 μm. B) The difference of cellular uptake of 2-NBDG, a fluorescence glucose probe, upon the DNQ treatment is measured in the absence (left) and the presence (right) of NQO1 inhibitor ES936. 2-NBDG uptake is increased by DNQ treatment in dose dependent manner only when NQO1 is active. A549 cells were treated with DNQ (0─100 nM) for 2 h in the absence (left) and the presence of 100 nM ES936 (right) and then incubated with 2-NBDG (50 μM) for 30 min. 2-NBDG cellular uptake was analyzed using flow cytometry.

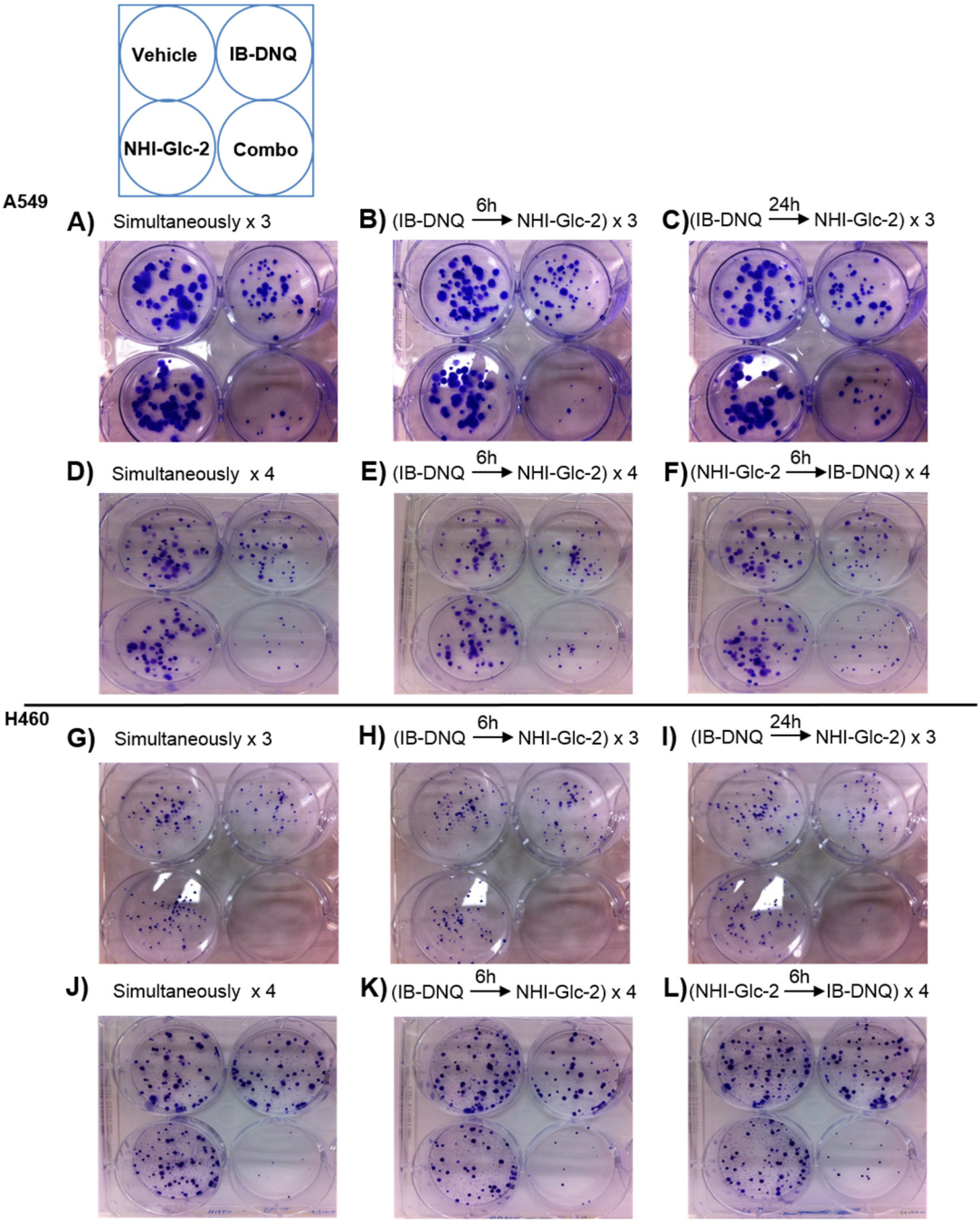


Parameters	DNQ		IB-DNQ	
	i.v.	p.o.	i.v.	p.o.
AUC (min*μg/mL)	3756.60	1897.05	6291.27	5014.24
K01_HL (min)	NA	1.40	NA	2.17
K10_HL (min)	71.13	226.58	83.13	201.88
Cmax (μM)	128.8	19.8	160	50.2
Tmax (min)	NA	10.35	NA	14.34
F (%)	NA	50	NA	80

[IB-DNQ] (nM) \ [NHI-Glc-2] (μM)	0	5	7.5
0	0	0.5 ± 1.8	7.4 ± 0.6
30	4.2 ± 0.7	86.4 ± 2.9	92.1 ± 0.3
40	20.3 ± 1.1	95.2 ± 0.7	95.2 ± 0.8
50	43.6 ± 5.7	96.5 ± 0.4	96.6 ± 0.5
60	59.4 ± 1.0	97.3 ± 0.5	97.4 ± 0.6
70	68.7 ± 2.5	97.6 ± 0.5	97.8 ± 0.5

[IB-DNQ] (nM) \ [NHI-Glc-2] (μM)	0	5	7.5
0	–	–	–
30	–	0.71	0.80
40	–	0.68	0.83
50	–	0.72	0.86
60	–	0.77	0.91
70	–	0.84	0.96

Supporting Figure S10. IB-DNQ synergizes with NHI-Glc-2 to enhance cell death in cell culture. A) Structure of IB-DNQ. B) Pharmacokinetics of DNQ and IB-DNQ was analyzed using female C57BL/6 mice. DNQ (9 mg kg^{-1}) and IB-DNQ (11 mg kg^{-1}) were administered via tail vein injection or oral gavage. The plasma concentration of DNQ or IB-DNQ was measured using HPLC analysis at 360 nm. PK parameters were determined using GraphPad Prism Version 5.00 for Windows. Oral bioavailability of IB-DNQ (80 %) is higher than DNQ (50 %). C) H460 cells were treated with the indicated concentrations of IB-DNQ and NHI-Glc-2 for 48 h. Percent cell death was assessed using SRB assay. Error represents the s.e.m. of three replicates. D) Combination index values calculated for each combination with CompuSyn software (<1 indicates synergistic interaction).



Supporting Figure S11. Colony forming assay conducted to compare the timing of drug addition in A549 (A-F) and H460 (G-L) cells. A, G) Cells were co-treated with NHI-Glc-2 (5 µM) and IB-DNQ (30 nM) every other day for total three times. B, H) Cells were treated with IB-DNQ (30 nM) and NHI-Glc-2 (5 µM) was added 6 h later. The treatment was repeated every other day for total three times. C, I) Cells were treated with IB-DNQ (30 nM) and NHI-Glc-2 (5 µM) was added 24 h later. The treatment was repeated every other day for a total of three times. D, J) Cells were treated with NHI-Glc-2 (5 µM) and IB-DNQ (30 nM) every other day for total four times. E, K) Cells were treated with IB-DNQ (30 nM) and NHI-Glc-2 (5 µM) was added 6 h later. The treatment was repeated every other day for total four times. F, L) Cells were treated with NHI-Glc-2 (5 µM) and IB-DNQ (30 nM) was added 6 h later. The treatment was repeated every other day for four times. Resulting colonies were stained with crystal violet.

4. Supporting Information References

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