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Complete List of Authors:	Lee, Hyang Yeon; University of Illinois , Parkinson, Elizabeth; University of Illinois, Granchi, Carlotta; Università di Pisa, Farmacia Paterni, Ilenia; Università di Pisa, Farmacia Panigrahy, Dipak; Harvard Medical School, Dana-Farber Cancer Institute Seth, Pankaj; Beth Israel Deaconess Medical Center Minutolo, Filippo; Università di Pisa, Dipartimento di Farmacia Hergenrother, Paul; University of Illinois at Urbana-Champaign, Department of Chemistry

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

Hyang Yeon Lee[†], Elizabeth I. Parkinson[†], Carlotta Granchi[‡], Ilaria Paterni[‡], Dipak Panigrahy[§], Pankaj Seth[⊥], Filippo Minutolo[‡], and Paul J. Hergenrother^{*,†}

[†]Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801, United States

[‡]Dipartimento di Farmacia, Università di Pisa, Via Bonanno 33, 56126 Pisa, Italy

[§]Center for Vascular Biology Research and [⊥]Division of Interdisciplinary Medicine and Biotechnology, Beth Israel Deaconess Medical Center, Boston, Massachusetts 02115, United States

ABSTRACT

A distinctive feature of cancer cells is their elevated levels of reactive oxygen species (ROS), a trait that can cause cancer cells to be more sensitive to ROS-inducing agents than normal cells. ROS takes several forms, each with different reactivity and downstream consequence. Here we show that simultaneous generation of superoxide and hydrogen peroxide within cancer cells results in significant synergy, causing potent and selective cancer cell death. In these experiments superoxide is generated using the NAD(P)H quinone oxidoreductase 1 (NQO1) substrate deoxyxyboquinone (DNQ), and hydrogen peroxide is generated using the lactate dehydrogenase A (LDH-A) inhibitor NHI-Glc-2. This combination reduces tumor burden and prolongs survival in a mouse model of lung cancer. These data suggest that simultaneous induction of superoxide and hydrogen peroxide can be a powerful and selective anticancer strategy.

INTRODUCTION

Cancer cells harbor increased levels of reactive oxygen species (ROS) relative to normal cells, and ROS plays an important role in signaling, tumorigenesis, proliferation, angiogenesis, and metastasis.¹ Due to the elevated ROS levels in cancer, treatment with drugs that generate ROS (either directly or indirectly) has been suggested as an effective strategy for selectively

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3 pushing cancer cells over the ROS threshold and into cell death.²⁻⁷ Unfortunately, cancer cells
4 also have adaptations that enable them to survive under increased oxidative stress, and these
5 mechanisms can lead to reduced efficacy of ROS-inducing drugs, including in clinical studies.^{8,9}
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9 Given the recognized potential for ROS induction as an anticancer strategy, various
10 approaches have been attempted to enhance the efficacy of ROS generators, for example, the
11 combination of a ROS inducer with an inhibitor of ROS scavenging.¹⁰⁻¹³ While such strategies
12 can increase the quantity of general ROS, dramatic synergy is rarely observed.
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16 An alternative approach is to combine two different ROS generators. Several forms of
17 ROS can exist, including hydrogen peroxide (H₂O₂), superoxide (O₂^{•-}), hydroxyl radical (•OH),
18 and peroxynitrite (ONOO⁻), with each having a different reactivity and function.¹⁴⁻¹⁶ Superoxide
19 is typically converted to H₂O₂ by superoxide dismutase (SOD). H₂O₂ has relatively low
20 reactivity, but it can generate highly reactive hydroxyl radical through the reaction with
21 transition metals such as Fe²⁺ (Fenton reaction).¹⁷⁻¹⁹ We hypothesized that superoxide and H₂O₂
22 generators would potently synergize due to their ability to induce the superoxide-driven Fenton
23 reaction. Specifically, the superoxide generator is predicted to drive the Haber-Weiss reaction
24 (Fe³⁺ + O₂^{•-} → Fe²⁺ + O₂), providing the ferrous iron necessary to react with H₂O₂ in the Fenton
25 reaction (Fe²⁺ + H₂O₂ → Fe³⁺ + OH⁻ + •OH). The production of the highly reactive hydroxyl
26 radical then is capable of damaging nearly all macromolecules found within the cell.^{8, 20} If
27 superoxide and H₂O₂ could be generated selectively in cancer cells, they might induce significant
28 synergy and selective cancer cell death.
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39 Described herein we test this hypothesis through use of two experimental therapeutics,
40 deoxyxyboquinone (DNQ²¹⁻²⁴) and NHI-Glc-2²⁵ (Figure 1). These well-characterized compounds
41 operate through two distinct mechanisms, superoxide formation by DNQ through activation by
42 NAD(P)H quinone oxidoreductase 1 (NQO1), and H₂O₂ formation by NHI-Glc-2 through lactate
43 dehydrogenase A (LDH-A) inhibition. NQO1 is an NAD(P)H dependent two-electron reductase
44 and typically acts as a detoxification enzyme. NQO1 is highly expressed in many solid tumors
45 with minimal expression in normal tissues.²⁶⁻²⁸ Additionally, elevated levels of NQO1 correlate
46 with poor prognosis in many cancers including lung cancer.²⁹ Reduction of DNQ by NQO1
47 generates an unstable hydroquinone, which is rapidly and spontaneously oxidized back to the
48 parent, forming superoxide in the process (Figure 1A).²⁴ Greater than 60 mols of superoxide are
49 generated by each mole of DNQ;^{22, 24} this burst of superoxide overwhelms the cellular capacity
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to convert it to hydrogen peroxide, thus DNQ is an outstanding compound for generation of rapid and persistent cellular superoxide. LDH-A catalyzes the conversion of pyruvate to lactate, and high LDH-A levels are frequently found in tumors and correlate with poor prognosis and low response to chemotherapy.^{30, 31} Inhibition of LDH-A results in cancer cell death in culture and *in vivo* by increased oxidative stress (Figure 1B).^{32, 33} Here, we show that the combination of DNQ and NHI-Glc-2 significantly increases cancer cell death in a mechanistically distinct fashion. This synergism was shown to be dependent upon NQO1 activity and is operational *in vivo*. These results suggest that the combination of therapeutics that generate different forms of ROS can potentially synergize to induce selective cancer cell death.

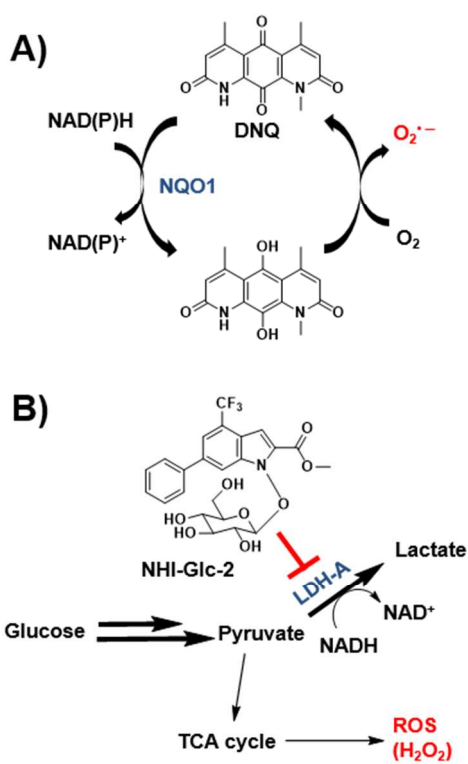


Figure 1. ROS generation by DNQ and NHI-Glc-2. A) DNQ is reduced by NQO1, and the resulting hydroquinone is unstable and spontaneously oxidizes to the parent in the presence of oxygen, producing superoxide. The regenerated DNQ can be used as a substrate for another round of NQO1-mediated redox cycling. B) Aerobic glycolysis is increased in cancer cells. Inhibition of LDH-A increases oxidative phosphorylation, ultimately generating H₂O₂.

RESULTS AND DISCUSSION

The combination of NHI-Glc-2 and DNQ synergistically induce death of cancer cells in culture. The ability of a combination treatment of NHI-Glc-2 and DNQ to induce cell death was examined in A549 non-small cell lung cancer (NSCLC) cells. These cells express high levels of both LDH-A and NQO1 (Supporting Figure S1A). A549 cell death induced by DNQ was dramatically increased by the addition of NHI-Glc-2 in a dose dependent manner (Figure 2A and Supporting Figure S2). For example, as shown in Figure 2A, single agent treatment with

DNQ (50 nM) or NHI-Glc-2 (5 μ M) has little effect, but the combination results in >90% cell death. To determine synergy, the combination index (CI) was calculated with the Chou and Tallay method using Compusyn software.³⁴ Combinations describe synergistic interactions when CI value <1, and the lower the CI value, the stronger the synergy. The combination index shows that the combination of DNQ and NHI-Glc-2 in A549 NSCLC cells is markedly synergistic (Figure 2B).

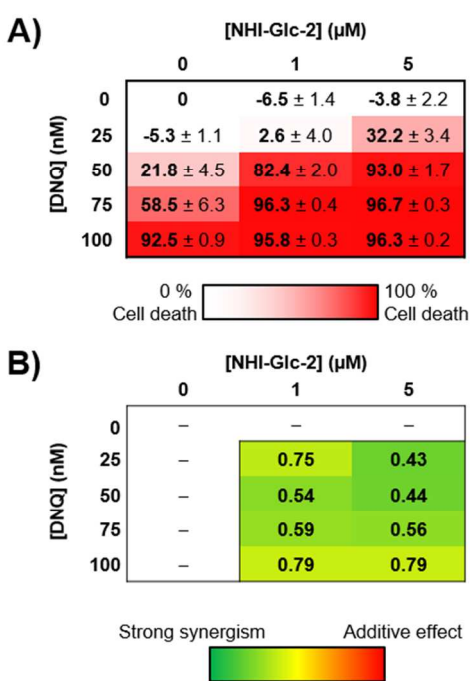


Figure 2. DNQ synergizes with NHI-Glc-2 to enhance cell death in culture. A) A549 NSCLC cells were treated with the indicated concentrations of DNQ and NHI-Glc-2 for 48 h. Percent cell death was assessed using the SRB assay.³⁵ Error represents the s.e.m. of four replicates. B) Combination index values calculated for each combination with CompuSyn software (<1 indicates synergistic interaction).

The synergistic cell death induced by NHI-Glc-2 and DNQ is dependent on NQO1. DNQ is an excellent substrate for NQO1, and its anticancer activity is dependent on NQO1.²⁴ The NQO1 dependency of the combination was investigated by comparing cell death induction in an isogenic cell line pair, MDA-MB-231 (human breast cancer cells) with or without NQO1 expression. In MDA-MB-231 cells that do not express NQO1 (Supporting Figure S1B), DNQ does not induce cell death (Figure 3A, left panel), and no synergy is observed. However, using MDA-MB-231 transfected to express NQO1,²² DNQ induces cell death in a dose dependent manner and synergy is observed upon co-treatment with NHI-Glc-2 (Figure 3A, right panel). Cellular NQO1 activity was also modulated by treating A549 cells with the NQO1 inhibitors ES936 or dicoumarol (DIC) prior to treatment with DNQ (50 nM) and/or NHI-Glc-2 (15 μ M). Consistent with previous reports,^{22, 23} cell death induced by DNQ is reduced by co-treatment with

ES936 or DIC, whereas the activity of NHI-Glc-2 is not changed by treatment with NQO1 inhibitors. In the absence of NQO1 inhibitors, dramatic increases of cell death are observed in the combination treatment with NHI-Glc-2 and DNQ. This dramatic increase is not observed when NQO1 is inhibited (Figure 3B). These experiments demonstrate that both the activity of DNQ and the synergy observed with DNQ in combination with NHI-Glc-2 are dependent on NQO1.

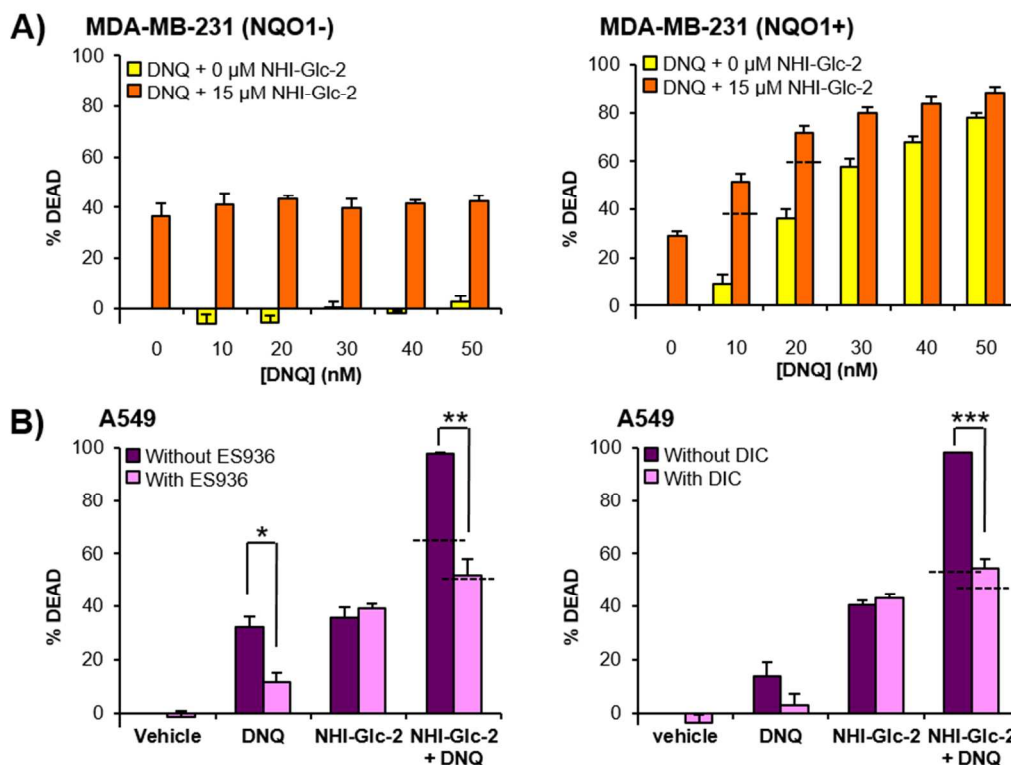


Figure 3. DNQ and NHI-Glc-2 synergize to induce cancer cell death in an NQO1 dependent manner. A) MDA-MB-231 cells transformed with empty plasmid (MDA-MB-231 NQO1-) and with a plasmid containing the gene for NQO1 (MDA-MB-231 NQO1+) were treated with the indicated concentrations of DNQ and NHI-Glc-2 for 48 h, and cell death was assessed using the SRB assay. B) The NQO1 inhibitor ES936 or DIC protects against and DNQ/NHI-Glc-2 mediated cell death in A549 cells. A549 cells were treated with ES936 (100 nM) or DIC (25 μ M) prior to treatment with DNQ (50 nM) and/or NHI-Glc-2 (15 μ M). Percent cell death observed after 48 h treatment of DNQ and NHI-Glc-2 in the presence and the absence of NQO1 inhibitor using the SRB assay. Error bars represent the s.e.m. of three replicates, and the dashed horizontal lines represent the additive effect of DNQ and NHI-Glc-2 for each drug combination. Statistical analysis was performed using an unpaired, two-tailed student's t test. * $0.01 \leq p < 0.05$, ** $0.001 \leq p < 0.01$, *** $p < 0.001$.

NHI-Glc-2 and DNQ synergistically induce death in NQO1-expressing lung cancer cells. The generality of the synergy of NHI-Glc-2 and DNQ was examined in various human

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3 lung cancer cell lines (H460, H1993, HCC15, H1299) and normal lung fibroblast cells (IMR90).
4 H460, H1993, and HCC15 cells show high expression of NQO1, while H1299 and IMR90 cells
5 show little-to-no expression of NQO1 (Supporting Figure S1A). Co-treatment of NHI-Glc-2 and
6 DNQ in the cells with high NQO1 expression such as H460, HCC15, and H1993 dramatically
7 induces increased cell death (Supporting Figure S3A). In particular, in H460 cells where single
8 agent treatment with NHI-Glc-2 (5 μ M) or DNQ (40 nM) induces less than 10% cell death,
9 greater than 90% of the cells are killed by the co-treatment; the combination indices in H460 and
10 H1993 cell show strong synergism (Supporting Figure S3B). In contrast, the combination in
11 H1299, which has low NQO1 expression, demonstrates minimal increases in cell death and the
12 combination indices indicate an additive effect or no synergism. IMR90 cells are normal lung
13 fibroblast and do not express NQO1.²³ Single agent treatment with DNQ in IMR90 cells is
14 ineffective, and co-treatment with NHI-Glc-2 only reflects the activity of NHI-Glc-2 (Supporting
15 Figure S3). These results are consistent with the experiments performed in the NQO1 +/-
16 isogenic cell lines and with NQO1 inhibitors shown in Figure 3. Taken together, these
17 experiments demonstrate the synergistic cell death induced by the combination of NHI-Glc-2 and
18 DNQ is dependent on NQO1.
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32 **ROS generation increases substantially in A549 cells treated with NHI-Glc-2 and**
33 **DNQ.** NQO1-mediated bio-reduction of DNQ to the hydroquinone, and its subsequent oxidation
34 to DNQ rapidly generates ROS in a catalytic fashion (Figure 1A). To observe ROS in cells, ROS
35 detection after 1 hr was accomplished using the fluorescent probes MitoSox Red for superoxide
36 detection, and carboxy-H₂DCFDA for detection of general ROS including cytosolic hydrogen
37 peroxide (H₂O₂); importantly, carboxy-H₂DCFDA does not detect superoxide.³⁶ In DNQ-treated
38 A549 cells mitochondrial superoxide generation was markedly increased, while high levels of
39 general ROS (presumably H₂O₂) were detected in NHI-Glc-2 treated cells; high levels of both
40 superoxide and general ROS were detected in the combination treated cells (Figure 4A). This
41 phenomenon was quantified by flow cytometric analysis, showing superoxide generation by
42 DNQ and H₂O₂ generation by NHI-Glc-2 are dose dependent (Supporting Figure S4A), with
43 high level of both forms of ROS observed in combination treated cells (Supporting Figure S4B).
44 Importantly, while NHI-Glc-2-treated cells produce H₂O₂ within the 1 h duration of this
45 experiment, DNQ-treated cells do not produce H₂O₂ in 1 h, but instead generate large quantities
46 of superoxide during this timeframe (Supporting Figure 4C).
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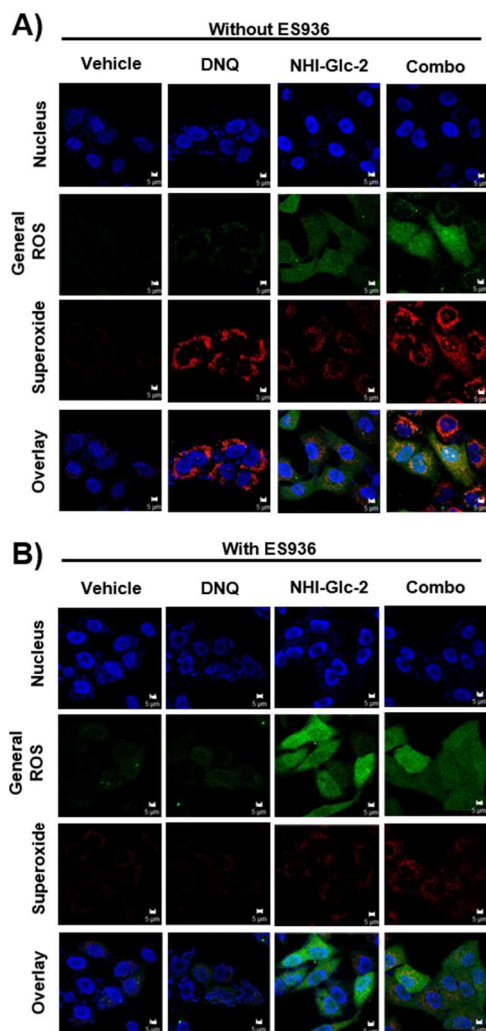


Figure 4. The combination of DNQ and NHI-Glc-2 induces ROS production in A549 cells. A) A549 cells were treated with DNQ (250 nM) and/or NHI-Glc-2 (15 μ M) for 1 h. Superoxide and H₂O₂ production after each compound treatment were visualized using MitoSox Red and carboxy-H₂DCFDA respectively. Fluorescence intensities of each ROS indicator were measured using LSM 700. B) The NQO1 inhibitor ES936 protects against DNQ or DNQ/NHI-Glc-2-mediated ROS production in A549 cells. A549 cells were treated with DNQ (250 nM) and/or NHI-Glc-2 (15 μ M) for 1 h after pretreatment of ES936 (100 nM) for 1h. Scale bar indicates 5 μ m.

To investigate the role of ROS in the synergistic cell death, cells were treated in the absence and the presence of the reducing agent *N*-Acetyl Cysteine (NAC). The results show that cell death induced by NHI-Glc-2 (15 μ M) was not affected by NAC, while cell death induced by DNQ (50 nM) was reduced by the pre-incubation with NAC (Supporting Figure S5). Additionally, cell death induced by the combination of DNQ (25 nM) and NHI-Glc-2 (15 μ M) was significantly reduced by addition of NAC. These results suggest ROS plays an important role in DNQ and combination-mediated cell

death.

Increased ROS generation is NQO1 dependent. Because the dramatic synergy observed with the combination of NHI-Glc-2 and DNQ is NQO1 dependent, an assessment was made of whether the ROS generation was also due to NQO1 activity. For this experiment, A549 cells were pretreated with the NQO1 inhibitor ES936 for 1 h. In the absence of ES936, DNQ treatment greatly increased production of superoxide in both the single agent and combination treatment as seen in Figure 4A. However, increased superoxide generation was not detected in DNQ and the combination treated cells after pre-treatment with ES936 (Figure 4B). ROS generation by NHI-Glc-2 was unchanged regardless of NQO1 inhibition. Combination treated cells only showed ROS produced by NHI-Glc-2 after the inhibition of NQO1.

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The combination of NHI-Glc-2 with most other ROS-inducing small molecules does not synergistically induce cancer cell death. To test whether NHI-Glc-2 synergizes with other ROS-inducing compounds, A549 were co-treated with NHI-Glc-2 and elesclomol³ or menadione³⁷ for 48h and biomass was assessed. Unlike DNQ, cell death induced by elesclomol or menadione did not synergize with co-treatment of NHI-Glc-2 (Supporting Figure S6A-D). Little to no increase in cell death was observed and combination indices showed additive effects or no synergism. As shown in Supporting Figure S6E, some cells treated with elesclomol or menadione show increased H₂O₂ generation but no superoxide formation; only DNQ-treated cells displayed a high level of superoxide. Thus, superoxide produced by DNQ is an important and distinctive factor for potentiation with NHI-Glc-2. 2-Methoxyestradiol (2-ME)³⁸ and Embelin³⁹ are reported to be superoxide generators by inhibition of SOD activity and expression respectively. However, cell death induced by 2-ME or Embelin was not increased by addition of NHI-Glc-2 and combination indices showed no synergism (Supporting Figure S7A-S7B). In contrast to the results with 2-ME and Embelin, the compound YM155^{40, 41} produces dramatic levels of superoxide similar to DNQ and causes synergistic cancer cell death when treated in combination with NHI-Glc-2 (Supporting Figure S7). As shown in Supporting Figure S8, YM155 is not a substrate for NQO1, and therefore YM155 produces superoxide via a different mechanism from DNQ. Regardless, generation of rapid and persistent cellular superoxide by DNQ or YM155 is needed to produce synergistic cytotoxicity with NHI-Glc-2 (Supporting Figure S7C and S7D), likely explaining the dramatic difference in the ability of these compounds to potentiate H₂O₂.

The combination of DNQ with hydrogen peroxide or *tert*-butyl hydroperoxide (TBHP) increases cell death. A549 cells were treated with DNQ and hydrogen peroxide (H₂O₂) or *tert*-butyl hydroperoxide (TBHP) for 48 h and cell death was assessed. Similar to NHI-Glc-2, H₂O₂ and TBHP potentiate DNQ-mediated cell death (Figure 5A and 5B), with treatment of DNQ (50 nM) and TBHP (10 μM) inducing <20% cell death, but with ~90% of cells killed by the combination (Figure 5B). To determine whether the superoxide-driven Fenton reaction is responsible for this synergy, cell death mediated by the combination of DNQ and NHI-Glc-2 or DNQ and TBHP were compared in the absence and the presence of the iron chelator deferoxamine (DFO).⁴² In A549 cells, cell death induced by TBHP (15 μM) was 35%, but it was reduced to 6% by the pretreatment of DFO (Figure 5C) suggesting that TBHP dependent cell

death is primarily caused by highly reactive *tert*-butoxy radical formation via the Fenton reaction.¹⁹ As a single agent, NHI-Glc-2 and DNQ induced cell death were not significantly altered by DFO. However, DNQ + NHI-Glc-2, or DNQ + TBHP mediated cell death was protected by DFO (Figure 5C). These results suggest that the combination of DNQ + NHI-Glc-2 or DNQ + THBP cause synergistic cell death by their ability to induce the Haber-Weiss reaction followed by the Fenton reaction resulting in production of highly reactive and toxic radicals such as hydroxyl or alkoxy radical.

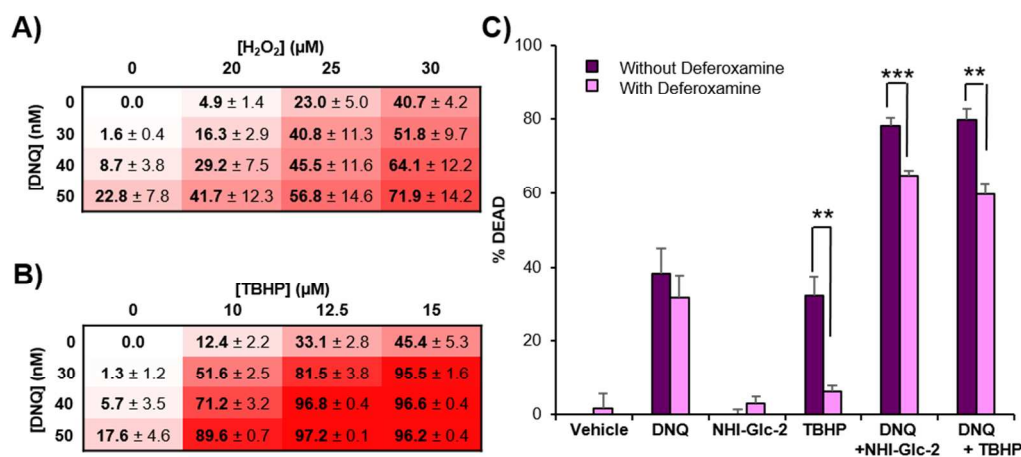


Figure 5. H₂O₂ or TBHP treatment potentiates DNQ-mediated cell death. A, B) A549 cells were treated with the indicated concentrations of DNQ and H₂O₂ (A) or TBHP (B) for 48 h. Percent cell death observed after 48 h using SRB assay. Error represents the s.e.m of three replicate. C) Treatment with the iron chelator deferoxamine (DFO) protects from TBHP, DNQ + NHI-Glc-2, or DNQ + TBHP-mediated cell death. A549 cells were pretreated with DFO (100 μM) for 1 h and then DNQ (100 nM), NHI-Glc-2 (10 μM), or TBHP (15 μM) were added and co-treated for 2 h. After washing, the cells were incubated for 48 h in drug free media. Percent cell death was assessed using SRB assay. Error bars represent the s.e.m of four replicates. Statistical analysis was performed using an unpaired, two-tailed student's t test. ** 0.001 ≤ p < 0.01, *** p < 0.001.

ROS generation by DNQ increases glucose uptake into cells by inducing GLUT1 over-expression. ROS is closely correlated with carbohydrate metabolism,^{43, 44} inducing hypoxia inducible factor 1 (HIF1) activation, which alters the expression level of glucose transporters (GLUT) and glycolytic proteins including LDH-A.⁴⁵ Previously, it was shown that GLUTs transcription level in U-937 cells is increased upon the treatment with DNQ.²¹ As NHI-Glc-2 is taken up by cells in a GLUT specific fashion,²⁵ the manner in which DNQ-derived ROS affects GLUT1 expression and glucose uptake in synergy with NHI-Glc-2 was assessed. GLUT1 expression dramatically increased following DNQ treatment in A549, H460, and H1993 cells (Figure 6A and Supporting Figure S9A). GLUT1 expression was most dramatically increased by

DNQ treatment in H460 cells and the synergism was greatest in this cell line, consistent with a strong relationship between ROS, glucose uptake, and synergistic cell death. To further explore this connection, uptake of a fluorescently-labeled glucose probe, 2-NBDG, was measured by flow cytometry. Cellular uptake of 2-NBDG was increased with increasing concentrations of DNQ, and inhibited by ES936 (Figure 6B and Supporting Figure S9B). In order to assess if ROS generation resulted in increased cellular NHI-Glc-2 uptake, cellular uptake of NHI-Glc-2 following DNQ treatment was measured (using HPLC) and found to be increased 1.3-fold (Figure 6C). Taken together, these results demonstrate that ROS generation by DNQ is NQO1 dependent and results in increased GLUT1 expression, leading to increased cellular uptake of glucose and NHI-Glc-2. In cells, NHI-Glc-2 reduces lactate production and aerobic glycolysis by inhibition of LDH-A.²⁵ Therefore, these cells produce more ATP through oxidative phosphorylation, resulting in increased ROS (H_2O_2) production. DNQ-generated superoxide synergizes with the resulting H_2O_2 to generate highly reactive hydroxyl radical through the superoxide-driven Fenton reaction. A schematic of how ROS produced by the combination of NHI-Glc-2 and DNQ induces dramatic cancer cell death is shown in Figure 6D.

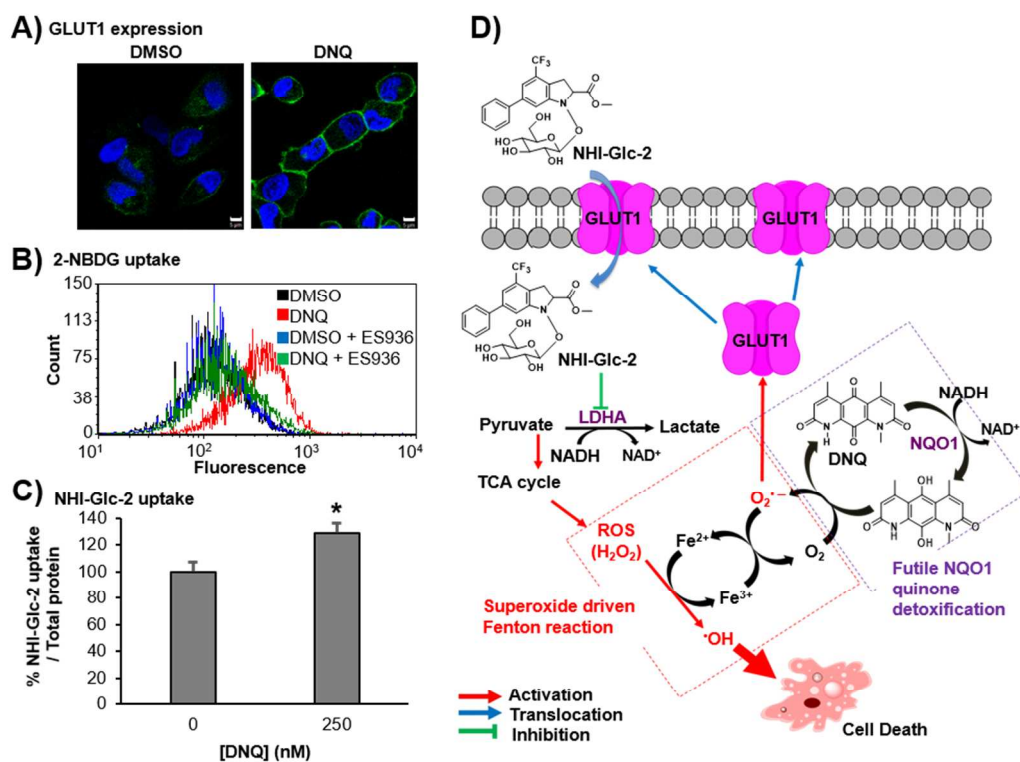


Figure 6. DNQ induces ROS production and glucose uptake in A549 cells. A) GLUT1 expression in A549 cells is increased by DNQ treatment. A549 cells were treated with DNQ (250 nM) for 4 h, and

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3 GLUT1 expression in plasma membrane was assessed using immunofluorescence. Scale bar indicates 5
4 μm . B) 2-NBDG cellular uptake is increased by DNQ treatment. A549 cells were treated with DNQ (100
5 nM) for 2 h in the absence and the presence of ES936 (100 nM) and then incubated with 2-NBDG (50
6 μM) for 30 min. 2-NBDG cellular uptake was analyzed using flow cytometry. C) Cellular uptake of NHI-
7 Glc-2 is increased by DNQ treatment in A549 cells. A549 cells were treated with DNQ (250 nM) for 4 h
8 and then incubated with NHI-Glc-2 (20 μM) for 30 min. NHI-Glc-2 cellular uptake was analyzed by
9 HPLC after cell lysis. Error bars represent the s.e.m. of three replicates. Statistical analysis was performed
10 using an unpaired, two-tailed student's t test. * $p < 0.05$ relative to DMSO treatment. D) Proposed
11 mechanism of action.
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15 **The combination of NHI-Glc-2 and IB-DNQ is active in a murine tumor model.** In
16 order to explore the antitumor activity of the combination *in vivo*, IB-DNQ, a derivative of DNQ
17 that possess superior pharmacokinetic properties (Supporting Figure S10A and S10B) was used.
18 Like DNQ, IB-DNQ-mediated cell death is potent and NQO1-dependent,^{23, 46} and IB-DNQ also
19 powerfully synergizes with NHI-Glc-2 to enhance cancer cell death in H460 cells (Supporting
20 Figure S10C and S10D). To determine the optimal timing and order of compound addition, a
21 colony forming assay was used to assess multiple dose of NHI-Glc-2 and IB-DNQ. A549 cells
22 were treated either simultaneously with IB-DNQ and NHI-Glc-2 (Supporting Figure S11A) or
23 with NHI-Glc-2 being added 6 (Supporting Figure S11B) to 24 h (Supporting Figure S11C) after
24 IB-DNQ treatment. At these concentrations, treatment of IB-DNQ or NHI-Glc-2 alone has little
25 to no effect on inhibition of colony formation. Simultaneous treatment of compounds and NHI-
26 Glc-2 addition 6 h post IB-DNQ treatment were equally effective. A span of 24 h between IB-
27 DNQ and NHI-Glc-2 was less effective at reducing colony formation. Next, the order of
28 compound addition was examined. A549 cells were treated with IB-DNQ and NHI-Glc-2
29 simultaneously (Supporting Figure S11D), NHI-Glc-2 was added 6 h after treatment with IB-
30 DNQ (Supporting Figure S11E), or IB-DNQ was added 6h after treatment with NHI-Glc-2
31 (Supporting Figure S11F). The compound treatments were repeated every other day for four total
32 treatments. More colonies were observed when cells were treated with NHI-Glc-2 prior to IB-
33 DNQ addition. In contrast, the number of colonies was similar when NHI-Glc-2 was added
34 simultaneously or 6 h after IB-DNQ. This trend was also observed with H460 cells (Supporting
35 Figure S11G-S11L).
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52 Based upon the results of the colony forming assays, dosing strategies were designed
53 using oral gavage of IB-DNQ followed by oral gavage of NHI-Glc-2 six hours later. To assess
54 efficacy *in vivo*, a H460 surgical intervention metastasis model was utilized, where tumor cells
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were implanted subcutaneously, allowed to reach 2 cm³, and then removed prior to drug treatment. The four groups of mice received either vehicle, IB-DNQ, NHI-Glc-2, or the combination of IB-DNQ and NHI-Glc-2. Kaplan-Meier survival curves showed an increased survival of mice treated with the combination of IB-DNQ and NHI-Glc-2 (Figure 7A). 60% of the combination treated mice were alive 60 days post resection, while all of vehicle treated mice were dead. In addition, tumor burden in the lungs (lung metastases) was significantly reduced in combination treated mice (Figure 7B-7D).

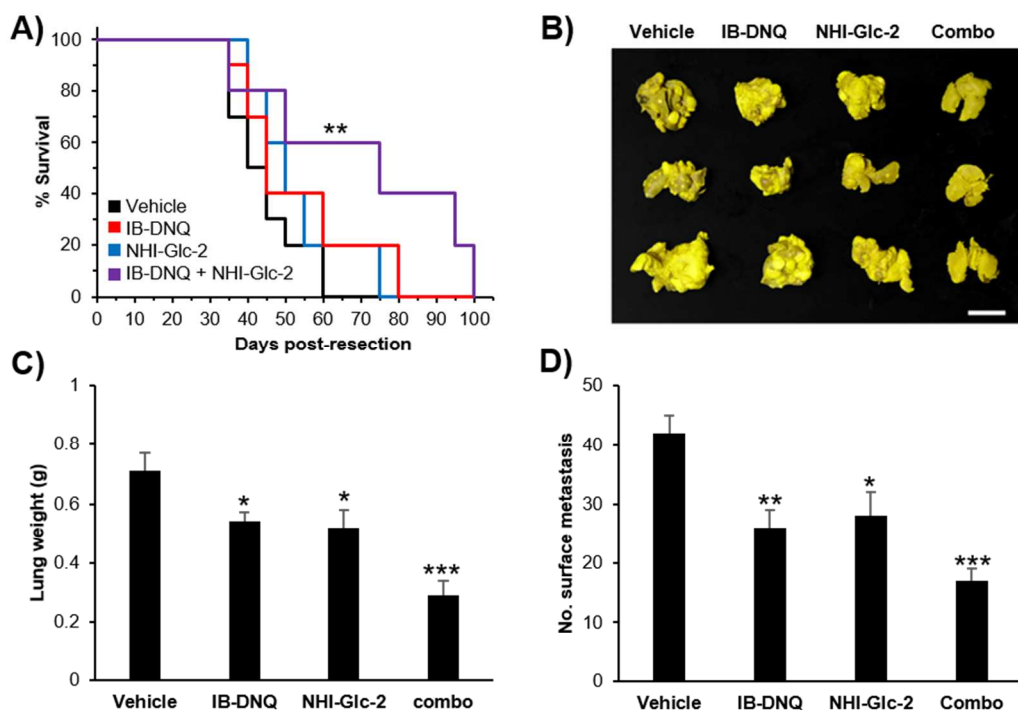


Figure 7. The combination of IB-DNQ and NHI-Glc-2 has an antitumor effect *in vivo*. H460 cells were inoculated subcutaneously into SCID mice. After 8 weeks the size of tumors were 2 cm³, and at this point the tumors were resected. The animals were split into four groups and treated with vehicle, IB-DNQ (15 mg/kg), NHI-Glc-2 (125 mg/kg), and IB-DNQ and NHI-Glc-2 (15 mg kg⁻¹ and 125 mg kg⁻¹, respectively). The drugs were given by oral gavage twice-a-week for 11 weeks. A) Kaplan-Meier survival curve shows prolonged survival of mice by treatment with IB-DNQ and/or NHI-Glc-2. Statistical analysis was performed using Log Rank Survival Test using Origin Pro 9. ** 0.001 ≤ p < 0.01 relative to vehicle treatment. B-D) Lung tumor burden is reduced by the combination treatment. Scale bar indicates 1 cm. Error bars show s.e.m. (n = 5–10 mice per group). Statistical analysis was performed using an unpaired, two-tailed student's t test. * 0.01 ≤ p < 0.05, ** 0.001 ≤ p < 0.01, *** p < 0.001 relative to vehicle treatment.

CONCLUSION

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Cancer cells often have elevated ROS compared to normal tissues thus making them, in principle, more sensitive to changes in ROS levels. In practice, the general toxicity of ROS-generating drugs has led to narrow therapeutic windows and diminished clinical impact.⁴⁷⁻⁵⁰ While the combination of ROS generators and cancer drugs with other mechanisms of action is an active area of investigation,^{1,8} the synergistic combination of different forms of ROS remains underexplored. As ROS signaling is essential for normal cell function and extreme levels of ROS are toxic to normal tissues, selective production of ROS in cancer cells is critical. Described herein is a strategy whereby selective generation of superoxide in cancer cells synergizes with drug-generated hydrogen peroxide, resulting in potent and selective cancer cell death. This strategy capitalizes on mechanism-based synergy, with many aspects that are cancer cell specific: 1) Cancer cells have an enhanced antioxidant defense due to high levels of ROS, and NRF2 acts as the master antioxidant regulator by controlling the expression of the enzymes with antioxidant functions, including NQO1.^{1, 29} As such the expression of NQO1 is dramatically increased in many cancer types compared to normal cells. 2) DNQ-derived superoxide is continuously generated by a futile NQO1-mediated process, driving the Fenton reaction. 3) Finally, the cellular uptake of glucose and aerobic glycolysis are also dramatically increased in many cancers via the Warburg effect. Thus, high LDH-A levels are frequently found in cancer cells.

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The work described herein utilized the combination of the NQO1 substrate DNQ and LDH-A inhibitor NHI-Glc-2 as the superoxide and hydrogen peroxide generators, respectively. However, alternative small molecule sources of superoxide^{51, 52} and hydrogen peroxide can be envisioned; for example as shown herein DNQ strongly synergizes TBHP (Figure 5). As DNQ operates at the diffusion-controlled limit for NQO1 processing,²³ this compound appears to be a particularly strong choice for rapid, potent, and selective superoxide generation in cancer cells.

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The selectivity of this strategy, as shown by the minimal effects in normal lung fibroblast IMR-90 cells (or in cancer cells with low levels of NQO1), is worthy of note and suggests *in vivo* promise. In addition, while the work herein has focused on NSCLC, this drug combination is very likely to be effective in the many other cancer types with overexpression of NQO1,^{27, 28} including pancreatic,⁵³ breast,⁵⁴ and head-and-neck cancers.⁵⁵ More generally, the potential of different ROS forms to synergize is intriguing and could be a general manner to exploit mechanism-based synergy to induce selective cancer cell death.

METHODS

See the Supporting Information for details.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available for free of charge on the [ACS Publications website](#) at

DOI:

Experimental procedures and supporting figures S1 – S11

AUTHOR INFORMATION

Corresponding Author

*E-mail: hergenro@illinois.edu

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Graphical Table of Contents

