

Chemistry and Biology of Deoxyxyboquinone, a Potent Inducer of Cancer Cell Death

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Abstract: Deoxyxyboquinone (**DNQ**) is a potent antineoplastic agent with an unknown mechanism of action. Here we describe a facile synthetic route to this anthraquinone, and we use this material to determine the mechanism by which **DNQ** induces death in cancer cells. **DNQ** was synthesized in seven linear steps through a route employing three palladium-mediated coupling reactions. Experiments performed on cancer cells grown in hypoxia and normoxia strongly suggest that **DNQ** undergoes bioreduction to its semiquinone, which then is re-oxidized by molecular oxygen, forming superoxide that induces cell death. Furthermore, global transcript profiling of cells treated with **DNQ** shows elevation of transcripts related to oxidative stress, a result confirmed at the protein level by Western blotting. In contrast to most other antineoplastic agents that generate reactive oxygen species (ROS), **DNQ** potently induces death of cancer cells in culture, with IC_{50} values between 16 and 210 nM. In addition, unlike the experimental therapeutic elesclomol, **DNQ** is still able to induce cancer cell death under hypoxic conditions. This mechanistic understanding of **DNQ** will allow for a more comprehensive evaluation of the potential of direct ROS generation as an anticancer strategy, and **DNQ** itself has potential as a novel anticancer agent.

Reactive oxygen species (ROS) are generated through mechanisms incidental to cellular respiration, or from environmental insults such as UV irradiation.¹ The predominant initial reactive species is the superoxide radical anion ($O_2^{\cdot-}$), produced by the one-electron reduction of molecular oxygen, generally by mitochondrial complex I or III of the electron transport chain. As part of the endogenous antioxidant system, superoxide dismutase converts superoxide radicals into less reactive hydrogen peroxide, which is further degraded to water and oxygen by catalase or reduced by glutathione as mediated by glutathione peroxidase.

Cancer cells are known to have elevated levels of ROS.² This increase is due in large part to increased metabolism and, hence, increased superoxide formation in the mitochondria. The role of hydrogen peroxide in activating pro-growth pathways^{3–6} is consistent with evidence that suggests increased ROS levels correlate with aggressive tumor growth.^{2–4} It has been postulated that disrupting the equilibrium concentration of ROS in cancer cells may be an effective anticancer strategy.^{2,5–8} The cancer-selective toxicity of enhanced ROS levels rests on the

notion that cancer cells already have elevated ROS levels and therefore have a reduced antioxidant capacity relative to normal cells.^{2,7} Thus, an increase in ROS sufficient to kill cancer cells would still be within the antioxidant capacity of healthy cells. There is reasonable data to support this hypothesis, and indeed a handful of compounds whose mode of action is strongly related to ROS induction are in advanced clinical trials (elesclomol, fenretinide, motexafin gadolinium, menadione, β -lapachone) or are FDA approved (As_2O_3) for the treatment of cancer (Figure 1A).^{8–10} Notably, fenretinide,^{11,12} motexafin gadolinium,¹³ menadione,^{14–19} β -lapachone,^{20,21} and As_2O_3 ^{22–25}

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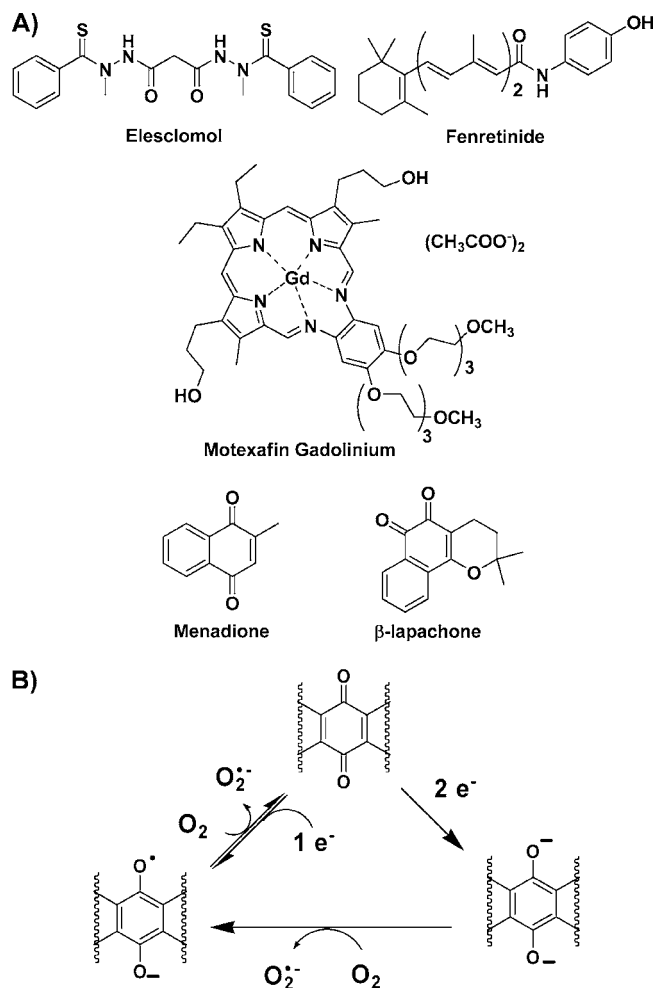


Figure 1. (A) Anticancer experimental therapeutics that have ROS generation as part of their mechanism of cell death induction. (B) Quinones can be bioreduced to semiquinones via a one-electron process. In the presence of oxygen the quinone can be regenerated, forming superoxide. Alternatively, a two-electron bioreduction of quinones to leads to the hydroquinone anion.³⁶

are relatively modest inducers of death in cancer cells, with IC_{50} values in the low micromolar range versus cancer cell lines in culture. Importantly, some of the most drug-refractory cancers are known to have the highest levels of ROS, including metastatic melanoma and pancreatic cancer.^{8,26–28}

A mechanism for intracellular ROS generation is the bioreduction and subsequent oxidation of a quinone, typically occurring through a one-electron mechanism (Figure 1B). The semiquinone can then be oxidized by molecular oxygen, forming superoxide and the parent quinone. A complicating factor in

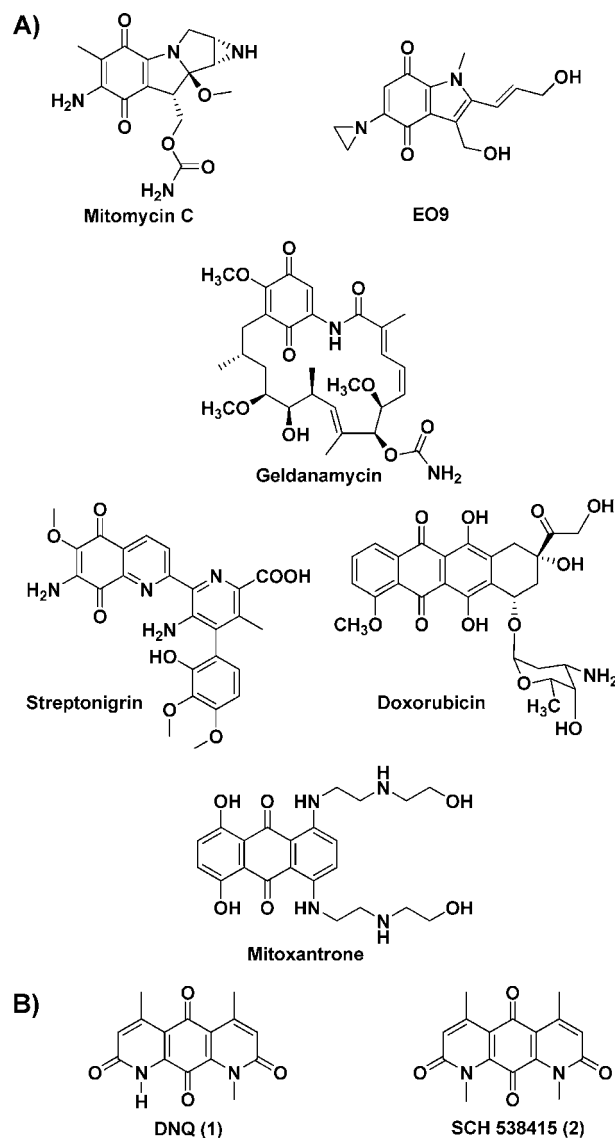


Figure 2. (A) Some anticancer quinones. (B) The structures of DNQ and SCH 538415.

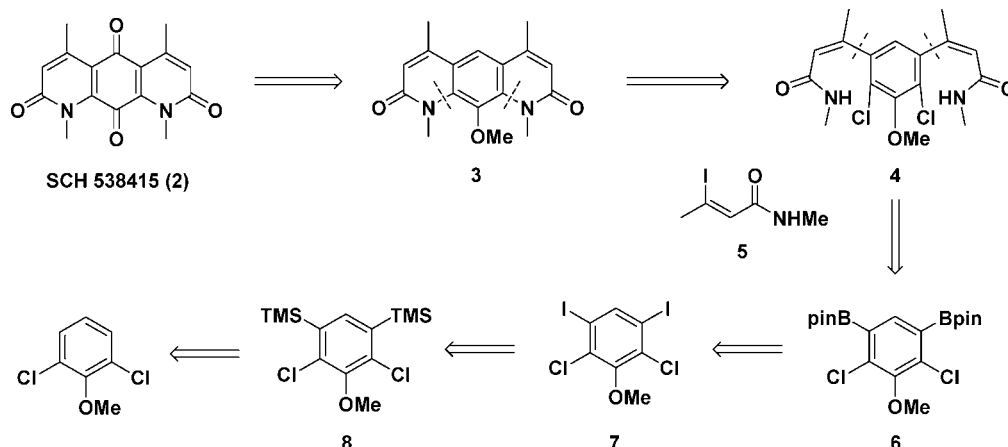
determining the value of quinone redox cycling in cancer therapy is that quinone-containing anticancer agents (Figure 2A) typically have other more important modes of action such as DNA alkylation (e.g., mitomycin C, EO9),^{29–32} Hsp90 inhibition (e.g., geldanamycin),^{7,33} and topoisomerase inhibition (e.g., streptonigrin, doxorubicin, mitoxantrone).^{34,35} These mixed modes of action make it difficult to assess the effectiveness of a single anticancer strategy.

Antraquinone **1** (Figure 2B) was originally synthesized during studies of the antibiotic nybomycin,³⁷ we have named

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Scheme 1. Retrosynthetic Analysis of SCH 538415



this compound deoxyxyboquinone (**DNQ**). The only published biological study on **DNQ** showed that it potently induces apoptosis in cancer cell lines through cytochrome *c* release.³⁸ Although cells treated with **DNQ** contained ROS, it was not known if ROS was a cause or effect of the antineoplastic activity of **DNQ**.³⁸ A nearly identical compound, **SCH 538415**, is a natural product that has recently been isolated and characterized (compound **2**, Figure 2B).^{39,40} Interestingly, assays reveal **DNQ** to be ~10-fold more potent in inducing death of cancer cells than **SCH 538415**,^{38,39} although they differ in structure by only a single methyl group.

Intrigued by the potency of **DNQ** and the potential of ROS-generating compounds as anticancer agents, we sought to develop a concise and modular synthesis of **DNQ** and to use the resulting material in mode-of-action studies. Herein is detailed a convenient synthetic route to **DNQ**, followed by the evaluation of this compound in a battery of biological experiments. These experiments reveal **DNQ** to be an extremely potent antineoplastic agent that induces death of cancer cells primarily through a ROS-based mechanism, likely due to a uniquely stable semiquinone species that enables facile ROS generation. As the only known quinone that potently induces cancer cell death predominately through a ROS-based mechanism, **DNQ** will be an excellent tool by which to further probe the value of direct ROS generation as an anticancer strategy and itself has potential as a therapeutic agent.

Results

Synthesis. The synthesis of **SCH 538415** was first developed and then applied to the nonsymmetric **DNQ**. Although synthetic routes analogous to those used to make structurally related compounds were considered,^{41–43} we ultimately elected to

devise a new route to the diazaanthraquinones that draws heavily on palladium-mediated cross-couplings, shown retrosynthetically for **SCH 538415** in Scheme 1. Tricycle **3** was envisioned through an intramolecular aryl amidation of intermediate **4** preceded by a double intermolecular Suzuki coupling between vinyl iodide **5** and aryl bis(pinacolboronate) **6**. Bisboronate **6** would be formed by a double Miyaura borylation of tetrahalide **7**, which was predicted to arise from a double directed *ortho*-metalation/silylation of 2,6-dichloroanisole to make **8** followed by iododesilylation by an electrophilic iodine reagent.

Isomerically pure β -iodoamide coupling partner **5** was easily accessed on a multigram scale starting from ethyl 2-butyrate (Scheme 2). Direct amidation with methylamine in methanol produced alkyne **9** in 87% yield. Treatment of **9** with sodium iodide in acetic acid produced **5** in 96% yield, with only the *Z*-isomer being detected by NMR.⁴⁴

The specific disposition of halides in compound **7** was envisioned to arise through directed *ortho*-lithiation of 2,6-dichloroanisole. As diiodination under such conditions is difficult, intermediate disilane **8** was targeted. Chloride is known to be a weak directing group for *ortho*-lithiation but has been successfully utilized in a number of settings.^{45,46} However, lithium–chloride exchange appeared to be the dominant reaction in a variety of *n*- and *sec*-butyllithium-mediated reactions, presumably due to the strong directing effects of the methoxy group. Fortunately, deprotonation with lithium diisopropyl amide (LDA) was both efficient and selective for the 3- and 5-positions (Scheme 2). In addition, the two-step sequence could be carried out in one pot. Trimethylsilyl chloride was an effective *in situ* quench reagent, with highest conversions when additions of reagent were sequential, beginning with LDA.⁴⁷ Iododesilylation of **8** by the action of iodine monochloride was rapid and quantitative, producing **7**.

Miyaura borylation conditions then provided cross-coupling partner **6** in good yield but contaminated with variable amounts of bis(pinacolborane).⁴⁸ The two-step yield after the subsequent cross-coupling was higher when **6** was used without purification; thus, after workup, **6** was typically taken directly into the cross-

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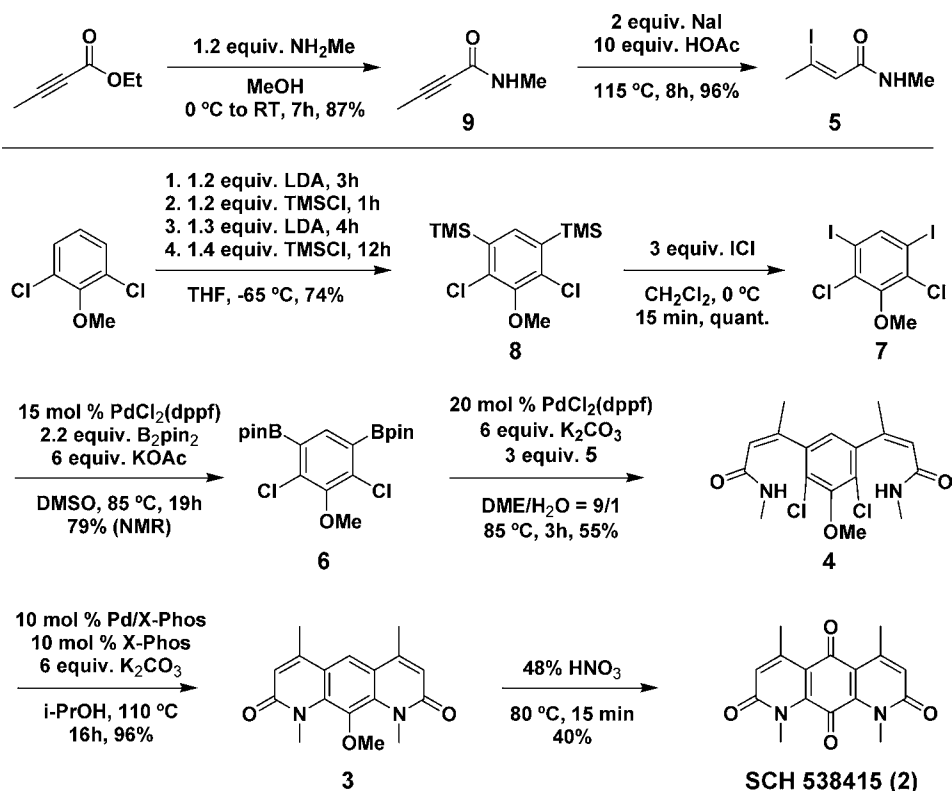
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Scheme 2. Total Synthesis of SCH 538415



coupling. The Suzuki coupling between **5** and **6** is a challenging process representing two individual cross-coupling events and linking a relatively unactivated aryl boronate with base-sensitive vinyl iodides. This reaction provided product **4** in a 55% yield.

A brief survey of known conditions for aryl chloride coupling with amides revealed a Pd/X-Phos precatalyst recently described by Buchwald,⁴⁹ and this reagent was used to convert **4** to diazaanthracene **3** in 96% yield. Oxidation of **3** by brief heating in concentrated nitric acid produced **SCH 538415** as a bright red-orange solid in 40% yield. By this route the first total synthesis of the natural product was completed in six steps and 9.7% overall yield from 2,6-dichloroanisole. Spectral data matched those of the natural product (see Supporting Information).

To apply this route to the synthesis of **DNQ**, primary amide **11** was synthesized but found to be an unreactive partner in the Suzuki coupling with **6** (Scheme 3). The *N-p*-methoxybenzyl amide **13** was then synthesized in 82% yield from 2-butynoic acid by hydroiodination^{44,50} and treatment of the corresponding acid chloride with *p*-methoxybenzyl amine (Scheme 3). This route was employed because the reaction of ethyl 2-butynoate with *p*-methoxybenzyl amine resulted primarily in 1,4-addition to the alkyne.

A mixed cross-coupling between bisboronate **6** and iodoamides **5** and **13** was found to be the simplest method to form the nonsymmetric diamide **14**. Separation of **14** from the accompanying symmetric products was easily effected by chromatography. Aryl amidation under the previously employed conditions efficiently formed tricycle **15** along with variable amounts of unprotected amide **16**. Isolation at this step was unnecessary, as subjection of the crude amidation products to

acidic hydrolysis produced **17** in 76% yield over two steps. Oxidation of **17** catalyzed by salcomine under O₂ produced **DNQ** in 77% yield. The synthesis of **DNQ** consisted of seven steps in the longest linear sequence, 12% overall yield. This compares to 11 steps and <0.84% yield for the previous synthesis of **DNQ**.^{37,51}

Activity versus Cancer Cells in Culture. The abilities of **DNQ** and **SCH 538415** to induce death of cancer cell lines in culture were determined using the sulforhodamine B assay.⁵² For these experiments, four cancer cell lines were used: SK-MEL-5 (human melanoma), MCF-7 (human breast cancer), HL-60 (human leukemia), and HL-60/ADR (doxorubicin-resistant HL-60). In addition to **DNQ** and **SCH 538415**, doxorubicin (DOX), elesclomol, fenretinide, β -lapachone, and arsenic trioxide were evaluated to obtain a side-by-side comparison with compounds that have ROS generation as part of their mechanism of anticancer activity. As shown in Table 1, **DNQ** potently induces death in these cancer cell lines, with nanomolar toxicity similar to that of the most potent compounds, DOX and elesclomol. **DNQ** is on average an order of magnitude more potent than **SCH 538415** against all cell lines. Importantly, while the HL-60/ADR cells are extremely resistant to DOX (~80-fold), they are only minimally resistant (~3-fold) to **DNQ**.

Hypoxia and Antioxidants. A 2003 study comparing the biochemical and antineoplastic properties of multiple quinones suggested that **DNQ** has an unusually stable semiquinone and that ROS are generated in cells treated with **DNQ**.³⁸ Thus, **DNQ** could generate ROS through redox cycling in the presence of O₂ (as shown in Figure 1B), ROS could be generated through another mechanism, or ROS could be a product of the cell death

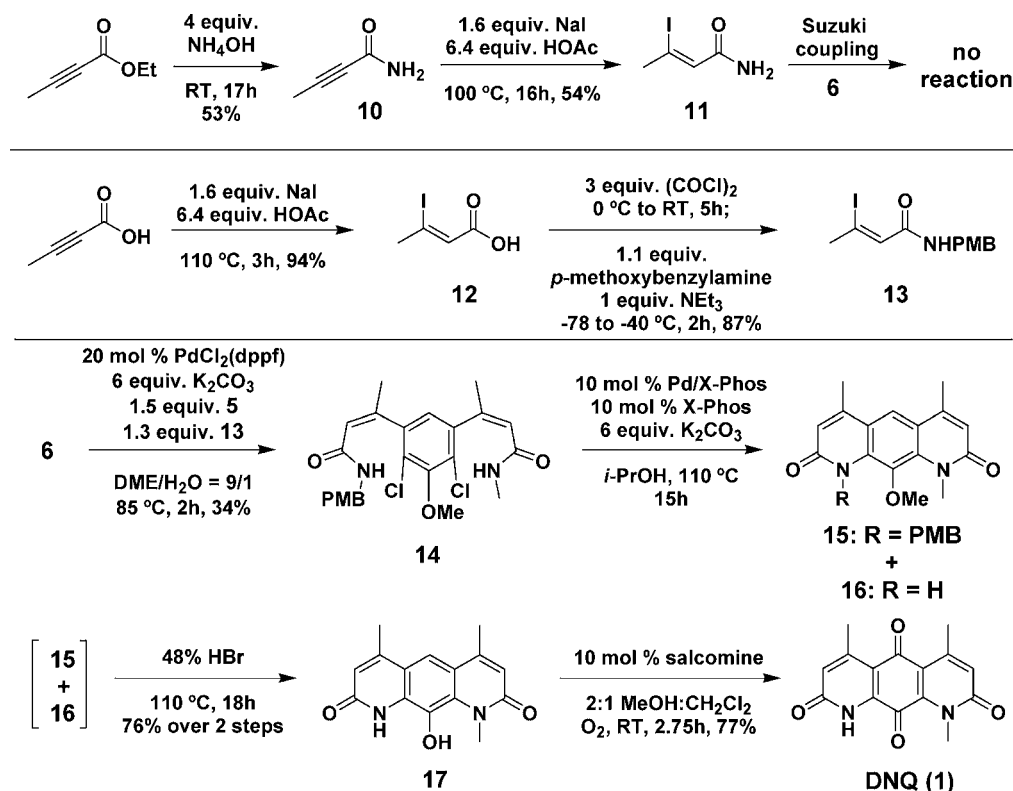
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Scheme 3. Synthesis of DNQ

Table 1. Toxicity of Compounds to Cancer Cell Lines in Culture^a

	SK-MEL-5	MCF-7	HL-60 ^b	HL-60/ADR
DNQ	0.024 ± 0.002	0.016 ± 0.006	0.21 ± 0.13	0.67 ± 0.26
SCH 538415	0.76 ± 0.25	0.87 ± 0.16	1.30 ± 0.04	2.5 ± 0.8
DOX	0.16 ± 0.02	0.33 ± 0.04	0.064 ± 0.004	5.1 ± 2.1
elesclomol	0.11 ± 0.04	0.024 ± 0.010	0.009 ± 0.004	
fenretinide	>10	12.5 ± 0.7	3.2 ± 1.9	
β-lapachone	1.0 ± 0.4	1.5 ± 0.1	0.72 ± 0.26	
As ₂ O ₃	2.8 ± 1.0	5.9 ± 1.6	3.7 ± 1.6	

^a Cell lines were incubated with compound for 72 h, biomass was quantified using the SRB assay, and IC₅₀ values were calculated from logistical dose–response curves. All IC₅₀ values are in μM. Error is standard deviation of the mean, *n* ≥ 3. ^b Cell viability assessed by the MTS assay.

process. To explore the connection between ROS and cell death, cytotoxicity evaluations were made in the presence of antioxidants and under different partial pressures of O₂. Antioxidants can quench ROS, and thus antioxidant treatment may decrease the cytotoxicity of an anticancer compound for which ROS is critical to cell death. In a similar fashion, if a compound generates cytotoxic ROS through redox cycling with O₂ (Figure 1B), cells grown in hypoxia should be less sensitive to the effect of the compound. Thus, the effects of DNQ and other cytotoxins were evaluated against HeLa cells in normoxia (~20% O₂), in the presence of the antioxidant *N*-acetylcysteine (NAC, 5 mM), under hypoxia (1% O₂), or combined with NAC under hypoxia. Tirapazamine, a compound whose cytotoxicity is more pronounced under hypoxia,^{30,53–55} was utilized as a control.

The most striking results were found for DNQ and elesclomol (Table 2 and Figure 3). NAC provided significant protection

Table 2. IC₅₀ Values for Compounds versus HeLa Cells under Normoxic (20% O₂) and Hypoxic (1% O₂) Conditions in the Presence and in the Absence of NAC^a

	normoxia	normoxia + 5 mM NAC	hypoxia	hypoxia + 5 mM NAC
DNQ	0.20 ± 0.02	0.75 ± 0.06 fc = 3.8	1.80 ± 0.44 fc = 8.4	5.1 ± 1.3 fc = 26
SCH 538415	1.40 ± 0.25	1.4 ± 0.2 fc = 1.0	5.2 ± 2.4 fc = 3.6	7.9 ± 3.9 fc = 5.4
DOX	0.10 ± 0.02	0.12 ± 0.02 fc = 1.2	0.05 ± 0.01 fc = -2.2	0.07 ± 0.03 fc = -1.4
etoposide	1.3 ± 0.7	1.2 ± 0.2 fc = 1.1	0.52 ± 0.17 fc = -2.1	0.64 ± 0.27 fc = -1.6
mitomycin C	0.10 ± 0.03	0.14 ± 0.04 fc = 1.40	0.06 ± 0.02 fc = -1.5	0.12 ± 0.04 fc = 1.3
menadione	5.9 ± 1.2	19.0 ± 2.4 fc = 3.5	7.0 ± 0.7 fc = 1.3	22.0 ± 5.4 fc = 4.0
As ₂ O ₃	1.30 ± 0.25	3.7 ± 1.2 fc = 2.9	2.3 ± 1.1 fc = 1.8	6.0 ± 1.8 fc = 4.5
β-lapachone	1.2 ± 0.2	1.6 ± 0.5 fc = 1.3	3.8 ± 1.0 fc = 3.2	6.1 ± 3.2 fc = 5.4
fenretinide	4.4 ± 1.2	4.0 ± 0.5 fc = -1.1	4.40 ± 0.95 fc = 1.0	3.90 ± 0.87 fc = -1.1
elesclomol	0.012 ± 0.004 fc = 20	0.26 ± 0.21 fc = 20	X ^b	X
tirapazamine	38 ± 6	42 ± 9 fc = 1.1	1.9 ± 0.3 fc = -20	2.10 ± 0.06 fc = -18

^a Fold change (fc) is with respect to normoxia. Cells were treated with compounds for 48 h, biomass was quantified using the SRB assay, and IC₅₀ values were calculated from logistical dose–response curves. All IC₅₀ values are in μM. Error is standard deviation of the mean, *n* ≥ 3. ^b X indicates that cells were >50% viable at every concentration.

from both DNQ- and elesclomol-induced toxicity (4- and 20-fold, respectively).⁵⁶ Incubation under hypoxia also strongly protected the cells from these two compounds, but whereas complete cell death was still observed at the highest concentration of DNQ (10 μM), elesclomol-treated hypoxic cells were

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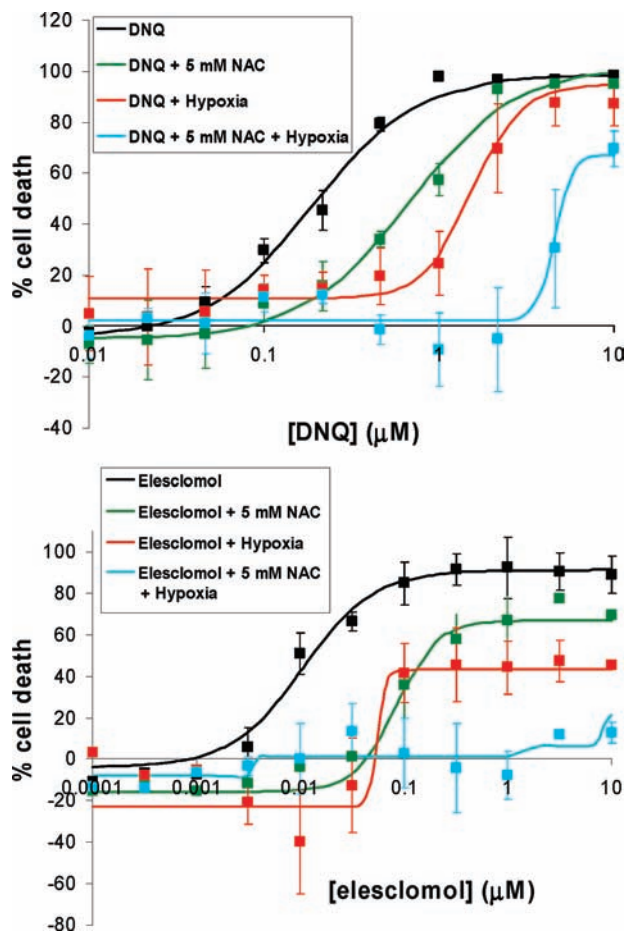


Figure 3. Effect of DNQ and elesclomol on HeLa cells in hypoxia and normoxia, and in the presence of NAC. Cells were treated with compound for 48 h, and death was determined via the SRB assay. Error is standard deviation of the mean, $n \geq 3$.

>50% alive even at the highest concentrations (Figure 3). When co-treated with NAC under hypoxic conditions, elesclomol was essentially nontoxic, whereas DNQ, although 25-fold less toxic than under normoxic conditions, still caused ~60% cell death at 10 μM (Figure 3). These results suggest that DNQ induces cell death through a ROS-based mechanism that relies on the production of superoxide radical anion from O_2 . SCH 538415 responded less strongly than DNQ in these assays, with no protection being observed by co-treatment with NAC (Table 2).

The behavior of the other compounds was generally consistent with literature reports (Table 2): tirapazamine became significantly more toxic under 1% O_2 , whereas the toxicity of mitomycin C (known to require more severe hypoxia to increase in potency)⁵⁷ remained unchanged. As expected, both menadione and arsenic trioxide were less toxic in the presence of NAC.^{22,23,58,59} The remaining compounds are known to display variable and cell line-dependent effects under these conditions.^{12,14–17,60–62}

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Transcript Profiling. To further investigate the mechanism by which DNQ induces death in cancer cells, cells treated with DNQ were analyzed by global transcript profiling, and the pattern of transcriptional response was compared to that of compounds with known modes of action. For this experiment, U-937 cells were treated with DNQ or vehicle control at 350 nM for 6 h, at which point the mRNA was harvested and whole genome transcript profiling was performed using the Illumina HumanHT-12 array. This DNQ concentration and time point were chosen such that the data could be readily compared with the transcript profile data from cells treated with other compounds, as outlined in the Connectivity Map.⁶³ The Connectivity Map consists of transcript profile data for >1300 compounds, many of which have known cellular targets. Previous studies have shown that, in many cases, compounds with similar modes of action will induce similar patterns of transcriptional response in cells.^{63,64} Comparing the transcript profile of DNQ with those compounds in the Connectivity Map database⁶³ showed that none of the >1300 compounds strongly correlate with DNQ (see Supporting Information Figure 1). Importantly, the Connectivity Map database contains hundreds of cytotoxins and multiple quinones including doxorubicin, daunorubicin, and mitoxantrone.

The top 20 up-regulated and repressed genes in response to DNQ treatment are listed in Table 3. The largest individual transcript elevated upon treatment of U-937 lymphoma cells with DNQ was from the *HMOX1* gene, encoding the antioxidant enzyme heme oxygenase 1 (HO-1) (8.8-fold change, Table 3). HO-1, a 32 kDa heat-shock protein, regulates cellular heme and iron concentrations.^{65,66} Elevated levels of this protein prevent cell death by converting heme to biliverdin, a potent antioxidant.⁶⁷ This conversion also results in the production of carbon monoxide (a potential neurotransmitter) and free iron, which serves as an oxidative stress signal.⁶⁵ Biliverdin produced by heme oxygenase is rapidly degraded into bilirubin, another potent small-molecule antioxidant.⁶⁷ Other transcripts elevated in DNQ-treated cells include those for various ferritins, which are under the transcriptional control of NRF2, a transcription factor that may be activated under oxidative stress.⁶⁸ Ferritins are responsible for the storage of free iron in a non-toxic and soluble form.⁶⁸ Other oxidative stress-related transcripts that are also elevated include oxidative stress-induced growth inhibitor 1 (OKL38) and sulfiredoxin 1 homologue (SRXN1).^{69,70} The NRF2-oxidative stress pathway was also the pathway most strongly implicated by Ingenuity Pathway Analysis software (see Supporting Information Figure 2). In summary, global tran-

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Table 3. Top 20 Elevated and Repressed Transcripts from Treatment of U-937 Cells with **DNQ** versus 0.2% DMSO for 6 h^a

symbol	protein	function	p value	fold change
HMOX1	heme oxygenase 1	antioxidant	0	8.8
ADM	adrenomedullin	hypotensive and vasodilator agent	0.0000002	4.0
IL8 ^b	interleukin 8	inflammation	0	3.7
IL8 ^b	interleukin 8	inflammation	0	3.5
DDIT4	DNA damage inducible transcript 4	cell growth inhibition	0.0000017	3.4
FTH1	ferritin, heavy polypeptide-like 1	intracellular iron storage	0.0000002	2.3
CDKN1A	cyclin-dependent kinase inhibitor 1A	p53-dependent G1 phase arrest	0	2.3
FTHL12	ferritin, heavy polypeptide-like 12	intracellular iron storage	0.0000012	2.3
FTHL8	ferritin, heavy polypeptide-like 8	intracellular iron storage	0.0000187	2.2
SLC2A3	facilitative glucose transporter	glucose transport	0.0000018	2.2
TNF	tumor necrosis factor	death ligand	0	2.2
OKL38	oxidative stress induced growth inhibitor 1	oxidative stress	0.0000005	2.2
FTHL11	ferritin, heavy polypeptide-like 11	intracellular iron storage	0.0000399	2.1
FTHL2	ferritin, heavy polypeptide-like 2	intracellular iron storage	0.0002126	2.1
CCL3L3	G0/G1 switch regulatory protein 19-2	cytokine	0	2.1
SRXN1	sulfiredoxin 1 homologue	oxidative stress	0	2.1
MAFB	V-maf musculoaponeurotic fibrosarcoma oncogene homologue B	represses ETS1-mediated transcription	0.0000005	2.1
CCL3	G0/G1 switch regulatory protein 19-1	HIV-suppressive factor	0.0000003	2.1
SLC2A14	glucose transporter type 14	glucose transport	0.0000044	2.0
NDRG1	N-myc downstream-regulated 1	p53-mediated caspase activation/apoptosis	0.0000098	2.0
VCX	variably charged protein X-B1		0.0000004	-2.0
VCX-C	variably charged protein X-C		0.0000005	-1.9
VCX3A	variably charged protein X-A		0.0000001	-1.9
GJB2	gap junction protein β 2	cell-to-cell channel for ion/small molecules transfer	0.0000094	-1.8
MS4A7	CD20 antigen	monocyte maturation	0.0000163	-1.7
RBBP9	retinoblastoma-binding protein 9	resistance to TGF- β 1 growth inhibition	0.0002341	-1.7
ZFYVE26	zinc finger, FYVE domain containing 26		0.0000159	-1.7
DHRS9	3- α -hydroxysteroid dehydrogenase	retinoic acid biosynthesis	0.0000457	-1.7
RBM17	RNA binding motif protein 17	sickle cell anemia	0.0000303	-1.6
FARP1	chondrocyte-derived ezrin-like protein		0.0000568	-1.6
CCDC26	coiled-coil domain containing 2		0.0003167	-1.6
CD47	CD47 molecule	membrane transport/signal transduction	0.0016747	-1.6
MEF2D	myocyte enhancer factor 2D	transcription factor	0.0001738	-1.5
CCDC136	coiled-coil domain containing 136		0.0005653	-1.5
CXCL10	interferon-inducible cytokine IP-10	immune cell migration	0.0045669	-1.5
CENPE	centromere protein E	G2-phase motor protein	0.001486	-1.5
MS4A7	CD20 antigen	monocyte maturation	0.0002341	-1.5
KIF20A	mitotic kinesin-like protein 2		0.0005729	-1.5
TXNIP	thioredoxin-binding protein 2	oxidative stress mediator	0.0252104	-1.5
C5orf29	GRB2-binding adaptor protein	transmembrane protein	0.0003523	-1.5

^a U-937 cells were treated with **DNQ** (350 nM) or 0.2% DMSO for 6 h, at which time the mRNA was isolated and the global transcriptional profile was determined. ^b These are different probes for the same transcript.

scriptional profiling provides strong evidence that **DNQ** induces the oxidative stress response, consistent with a ROS-based mechanism of cell death.

To confirm that the treatment of cells with **DNQ** elevates heme oxygenase 1 at the protein level, a Western blot for HO-1 was performed from cells treated with 0.5 or 1.0 μ M **DNQ** for 6 h. As shown in Figure 4A, **DNQ** treatment elevated HO-1 protein levels in U-937 cells, an effect that could be substantially prevented by co-treatment with the antioxidant NAC. The induction of antioxidant genes encoding HO-1 and the ferritins is likely in response to cellular ROS generated upon treatment of cells with **DNQ**.

It has been noted that oxidative stress can activate the heat shock response (HSR) expression pathway and upregulate HSP-70.^{56,59,71} Thus, HSP-70 protein levels in cells treated with **DNQ** were analyzed by Western blot. Both **DNQ** and elesclomol caused an increase in HSP-70 levels in U-937 cells (Figure 4B), consistent with oxidative stress-mediated upregulation of HSP-70.

Common biological properties of anthracycline-type compounds similar in structure to **DNQ** include cell cycle arrest in

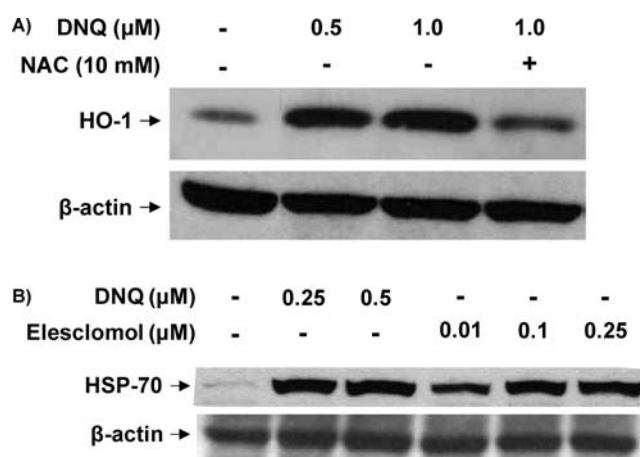


Figure 4. **DNQ** treatment enhances the levels of proteins involved in the oxidative stress response. U-937 cells were treated with **DNQ** and control compounds, and the lysates were then probed by Western blot. (A) Western blot for HO-1. (B) Western blot for HSP-70.

the S or G2/M phases, DNA binding, and topoisomerase inhibition.^{34,72-74} Thus, various *in vitro* and cell-based assays were performed to determine the importance of these mechanisms in cell death mediated by **DNQ**.

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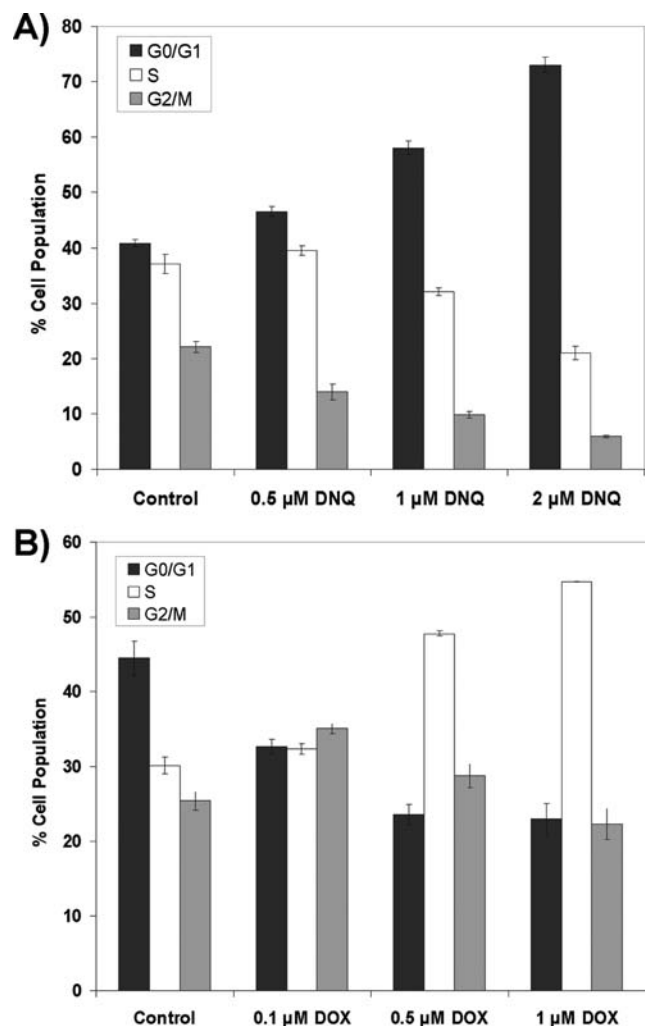


Figure 5. (A) **DNQ** treatment leads to arrest of cells in the G1-phase of the cell cycle, while (B) doxorubicin treatment leads to S-phase arrest. HL-60 cells were treated with the indicated concentrations of compound (or DMSO control) for 6 h, at which point DNA levels were analyzed by propidium iodide staining. Error bars represent standard deviation of the mean, $n = 3$.

Cell Cycle Arrest. Modes of cell death may be broadly categorized by their effects on the progression of actively dividing cells through the cell cycle. For example, DNA damaging agents and microtubule disruptors or stabilizers frequently arrest cells in the mitosis (M) phase, whereas compounds that inhibit DNA synthesis or replication arrest in the synthesis (S) phase. Gene expression analysis of **DNQ** revealed the elevation of several transcripts related to p53-dependent pathways and G0/G1 cell cycle regulation (CDKN1A, CCL3L3, CCL3, NDRG1, OKL38). The down-regulation of G2/M-associated motor kinesin transcripts was also observed (CENPE and KIF20A, Table 3). The effect of **DNQ** on cell cycle progression was measured by treating HL-60 cells with **DNQ** for 6 h (Figure 5A). Consistent with the transcript profiling results, **DNQ** caused significant cell cycle arrest in the G1-phase. In contrast, **DOX**-treated cells exhibited weak G2/M-phase arrest at low concentrations and a pronounced S-phase arrest at higher concentrations (Figure 5B).

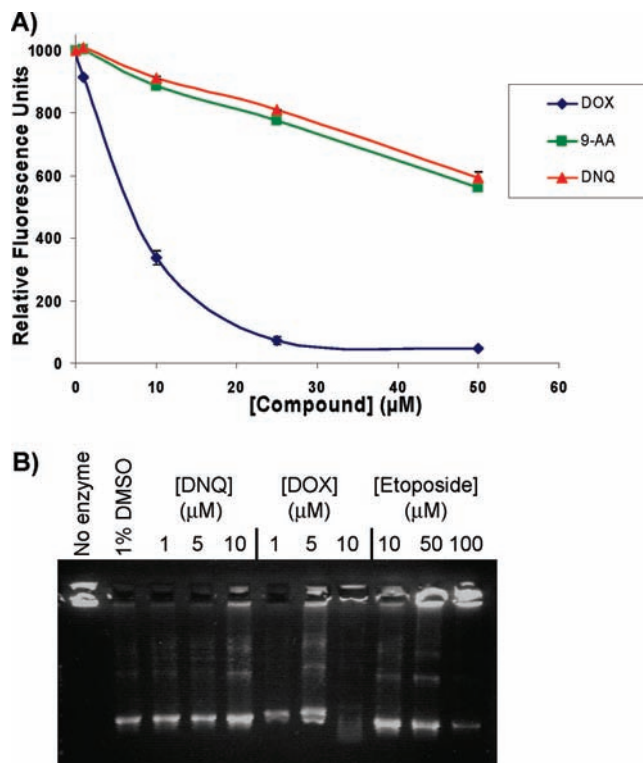


Figure 6. (A) Ethidium bromide displacement assay reveals large differences between **DNQ** and doxorubicin. Ethidium bromide was preincubated with herring sperm DNA prior to compound addition. Fluorescence was measured 30 min after compound addition. Error bars represent the standard deviation of the mean, $n = 3$. (B) **DNQ** does not inhibit topoisomerase II *in vitro*. Topoisomerase II was added to a solution of catenated DNA, ATP, and compound in assay buffer, and the reactions were allowed to proceed for 30 min at 37 °C. Products were analyzed by agarose gel electrophoresis.

DNA Interaction. The capacity of **DNQ** to interact with DNA was assessed using an ethidium bromide displacement assay,^{75,76} **DOX** and 9-aminoacridine (9-AA) were also evaluated for comparison. Double-stranded DNA was preincubated with ethidium bromide in buffer before compound addition (final DMSO concentration, 5%). After 30 min the competition reached equilibrium and the fluorescence was measured. As shown in Figure 6A, **DOX** has a very strong effect in this assay, while **DNQ** has a much weaker effect. Considering the nearly equipotent cellular toxicity of **DOX** and **DNQ**, these data are consistent with the notion that DNA binding is not of primary importance in the antineoplastic action of **DNQ**.

Topoisomerase II Inhibition. The ability of **DNQ** to inhibit topoisomerase II *in vitro* was evaluated using a decatenation assay^{77,78} and compared to the known topoisomerase II inhibitors **DOX** and etoposide. For this assay, catenated DNA was incubated at 37 °C for 30 min in a buffer containing purified topoisomerase II, ATP, and various concentrations of compound in DMSO. As shown in Figure 6B, **DOX** completely inhibited the topoisomerase II reaction at 10 μM , while **DNQ** showed

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virtually no inhibition at this concentration; the aqueous solubility of **DNQ** precluded its evaluations at higher concentrations. These data, together with the transcriptional profiling, cell cycle arrest, and DNA interaction assay results, support the notion that **DNQ**-mediated death does not involve mechanisms common to anthracycline-type compounds.

Discussion

The facile synthesis of **DNQ** described herein has allowed for the comprehensive biological evaluation of this interesting antineoplastic agent. As shown by cytotoxicity assays (Tables 1 and 2, Figure 3), **DNQ** induces death of cancer cells in culture with potencies on par with the front-line anticancer drug doxorubicin and the experimental therapeutic elesclomol. Among compounds evaluated that are believed to induce death predominantly through a ROS-based mechanism of action, **DNQ** and elesclomol are by far the most potent and respond the most strongly to NAC and hypoxia. These latter results suggest that **DNQ** and elesclomol most directly cause death by ROS production and not by other mechanisms. However, despite these commonalities, the mechanisms by which these compounds produce superoxide appear to be very different. Elesclomol, a clinically promising anticancer agent,⁷⁹ is believed to produce ROS through the chelation of copper and facilitation of copper redox, resulting in superoxide formation.^{80,81} In contrast, **DNQ** appears to induce death in cancer cells through rapid redox cycling of the quinone, a process that directly generates superoxide. ESR measurements of live cells minutes after treatment with **DNQ** indicate the presence of a semiquinone.³⁸ Semiquinones are, in general, not detected until all the oxygen in the cell has been consumed through redox cycling,³⁶ thus, the semiquinone of **DNQ** must be remarkably stable. To the best of our knowledge, **DNQ** is the most potent antineoplastic agent that operates predominantly through this direct ROS generation, bioreduction mechanism.

Additional evidence for the role of ROS in **DNQ**-mediated cell death was uncovered by global transcript profiling. As shown by the transcript profiles of a number of agents, oxidative stress results in the upregulation of genes related to three pathways: the oxidative stress response (NRF2),⁸² the heat shock response,⁷¹ and metallothioneins.^{83,84} Small molecules that induce oxidative stress (arsenic trioxide,^{59,85,86} menadione,⁸⁷

hydrogen peroxide,^{87,88} *tert*-butyl peroxide,⁸⁷ elesclomol,⁵⁶ motexafin gadolinium^{89,90}) generally activate one or more of these pathways. The transcript profiling data presented suggest that **DNQ** strongly activates the NRF2 pathway, resulting in upregulation of transcription from genes such as *HMOX1* and many ferritins which are downstream of antioxidant response elements. Western blot analysis demonstrates that **DNQ** also activates the HSR pathway, resulting in upregulation of HSP70. Finally, experimental evidence indicates that common targets of planar polycyclic compounds (such as the anthracyclines, acridines, and ethidium bromide) are of minimal importance in **DNQ**-mediated toxicity, as **DNQ**-treated cells show a lack of S- or G2/M-phase arrest, no topoisomerase II inhibition, and minimal DNA intercalation.

At least three important findings are derived from the experiments described herein: (1) A facile synthetic route to **DNQ** has been developed that can be used to produce large quantities of the compound for animal testing and is flexible enough to allow for derivative synthesis. (2) The data strongly indicate that ROS is a direct cause, rather than a downstream effect, of **DNQ**-induced cell death. (3) **DNQ** is considerably more potent than other compounds that generate ROS through a bioreduction process. Direct ROS formation is an anticancer strategy that has generated much interest and success. However, most compounds that act through a ROS-generating mechanism (with the exception of elesclomol) are only moderately potent, with IC₅₀ values in the low micromolar range. As shown by the data in Table 1 and Figure 3, elesclomol and **DNQ** are both quite potent to multiple cancer cell lines under normoxic conditions. However, under conditions of hypoxia (1% O₂), **DNQ** is still able to kill cancer cells in culture, whereas elesclomol is almost completely ineffective. These data suggest that even under low oxygen conditions, the **DNQ** semiquinone is able to convert molecular oxygen to superoxide. This trait has potential translational value, as hypoxic environments are found in the interior of many solid tumors.⁵³

In conclusion, a modern and modular synthetic route to **DNQ** has allowed for the full investigation of the activity of this compound versus cancer cells in culture. Notably, we find that **DNQ** has a potency that rivals doxorubicin in cell culture, with the added benefit of being effective against a doxorubicin-resistant cell line. Transcript profiling and other data strongly indicate that **DNQ** induces death through ROS generation and oxidative stress, and it is considerably more potent than other compounds that act through this mechanism. Importantly, **DNQ** provides a complement to elesclomol, as both compounds induce ROS and are potent antineoplastic agents, but through very different mechanisms. Thus, with these two compounds there is now an opportunity to probe the relative importance of one-electron bioreduction versus copper chelation for the formation of cytotoxic ROS. Given the documented elevation in ROS levels in many tumor types, especially those cancers with few treatment options such as melanoma and pancreas carcinoma, the continued exploration of the therapeutic utility of ROS generators is critical. **DNQ** is an important addition to the repertoire of compounds used to study ROS generation as a

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strategy for treatment of cancer and has potential as a novel anticancer agent.

Acknowledgment. We thank Dr. Karson S. Putt (UIUC) for initial work on **DNQ**. This research was supported by the University of Illinois. We acknowledge Dr. Justin Lamb (Broad Institute) for advice regarding experimental design of transcript profiling experiments for comparison to the Connectivity Map Database. We thank the W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana–Champaign for performing

the gene transcript profiling and preliminary data processing. We thank the W. M. Keck Cell Flow Cytometry facility at the University of Illinois at Urbana–Champaign.

Supporting Information Available: Supporting figures, spectral data for all new compounds, full experimental protocols, and complete ref 63. This material is available free of charge via the Internet at <http://pubs.acs.org>.

JA100610M