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Deoxynyboquinones as NQO1-Activated Cancer Therapeutics

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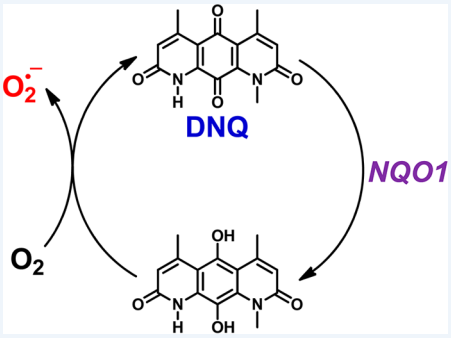
CONSPECTUS: One of the major goals of cancer therapy is the selective targeting of cancer cells over normal cells. Unfortunately, even with recent advances, the majority of chemotherapeutics still indiscriminately kill all rapidly dividing cells. Although these drugs are effective in certain settings, their inability to specifically target cancer results in significant dose-limiting toxicities. One way to avoid such toxicities is to target an aspect of the cancer cell that is not shared by normal cells.



A potential cancer-specific target is the enzyme NAD(P)H quinone oxidoreductase 1 (NQO1). NQO1 is a 2-electron reductase responsible for the detoxification of quinones. Its expression is typically quite low in normal tissue, but it has been found to be greatly overexpressed in many types of solid tumors, including lung, breast, pancreatic, and colon cancers. This overexpression is thought to be in response to the higher oxidative stress of the cancer cell, and it is possible that NQO1 contributes to tumor progression. The overexpression of NQO1 and its correlation with poor patient outcome make it an intriguing target.

Although some have explored inhibiting NQO1 as an anticancer strategy, this has generally been unsuccessful. A more promising strategy is to utilize NQO1 substrates that are activated upon reduction by NQO1. For example, in principle, reduction of a quinone can result in a hydroquinone that is a DNA alkylator, protein inhibitor, or reduction−oxidation cycler. Although there are many proposed NQO1 substrates, head-to-head assays reveal only two classes of compounds that convincingly induce cancer cell death through NQO1-mediated activation. In this Account, we describe the discovery and development of one of these compounds, the natural product deoxynyboquinone (DNQ), an excellent NQO1 substrate and anticancer agent.

A modular synthesis of DNQ was developed that enabled access to the large compound quantities needed to conduct extensive mechanistic evaluations and animal experiments. During these evaluations, we found that DNQ is an outstanding NQO1 substrate that is processed much more efficiently than other putative NQO1 substrates. Importantly, its anticancer activity is strictly dependent on the overexpression of active NQO1. Using previous crystal structures of NQO1, novel DNQ derivatives were designed that are also excellent NQO1 substrates and possess properties that make them more attractive than the parent natural product for translational development. Given their selectivity, potency, outstanding pharmacokinetic properties, and the ready availability of diagnostics to assess NQO1 in patients, DNQ and its derivatives have considerable potential as personalized medicines for the treatment of cancer.

■ INTRODUCTION

Traditional chemotherapeutics, such as DNA alkylators, antimetabolites, and antimitotics are not typically targeted to cancer cells and thus have dose-limiting toxicities in rapidly dividing normal tissues.[1] A complementary approach to classic chemotherapy is utilization of drugs that target a specific molecular defect of cancer cells (e.g., translocation, mutation, or overexpression) to selectively kill cancer cells while leaving normal cells intact. The natural product deoxynyboquinone (DNQ) and its derivatives are substrates for NAD(P)H quinone oxidoreductase 1 (NQO1), an enzyme that is overexpressed in many solid tumors; NQO1-catalyzed reduction of DNQ leads to DNQ reoxidation and concurrent reactive oxygen species (ROS) generation, inducing cancer cell death. In this manner, DNQ is able to act in a catalytic fashion with >60 mol of ROS generated per mole of DNQ. The discovery and development of DNQ is detailed herein, along with its advantages as a targeted agent that catalytically induces selective cancer cell death.

■ NQO1 AND CANCER

NQO1 is a 2-electron reductase responsible for the detoxification of xenobiotics such as quinones.[2] It is known to be overexpressed in many solid tumors, including nonsmall cell lung carcinoma,[3−10] breast cancer,[3,6,7,11] colon cancer,[3,7,12,13] pancreatic cancer,[14−17] and ovarian cancer,[7,18] among others.[3,7,19−21] High NQO1 expression correlates with later clinical stage, metastasis, lower disease-free survival, and lower overall survival.[10,11,13,18−20] Although the reason for NQO1 overexpression in cancer is not fully understood, it possibly enables cancer cells to cope with increased oxidative stress.[22] NQO1 expression is under the control of the transcription factor Nrf2, which with its repressor KEAP1 makes up one of the major signaling cascades for stress response.[23] Normally, Nrf2 is bound to KEAP1 and cannot induce expression of its target proteins. Upon exposure to

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electrophiles or ROS, KEAP1 is inactivated, thus allowing activation of Nrf2.[22,23] In cancer, including lung,[24,25] breast,[26] and ovarian,[27] Nrf2 is often constitutively activated by mutations to itself or KEAP1 or by changes in copy number.[22] These mutations and their downstream effects (e.g., overexpression of NQO1) are hypothesized to be drivers of oncogenesis.[25,28,29] On the basis of this hypothesis, it was speculated that inhibiting NQO1 would be an effective anticancer strategy as this inhibition could lead to toxic oxidative stress levels.[30] However, studies with NQO1 inhibitors indicate that there is no correlation between NQO1 inhibition and cancer cell cytotoxicity.[31,32]

NQO1 has two common polymorphisms, NQO1*2 and NQO1*3, either of which results in inactivation of the enzyme.[2] NQO1*2 is the more common polymorphism with ~4% of Caucasians and ~20% of Asians homozygous for it.[33] Although some studies suggest that individuals with the NQO1*2 polymorphism have a higher risk of developing cancer, others suggest that the effect is small if it even does exist.[34,35] Regardless, individuals with these polymorphisms that have cancer would obviously not benefit from a therapy that targets overexpression of NQO1; facile methods exist for the detection of this polymorphism, allowing for convenient patient screening for NQO1 status.[36]

NQO1 SUBSTRATES AS REDUCTION−OXIDATION CYCLERS AND ANTICANCER AGENTS

Given its dramatic overexpression in most solid tumors, exploitation of the ability of NQO1 to *activate* certain compounds to toxic species has been explored. NQO1 catalyzes a 2-electron reduction of a variety of substrates, including quinones (to give hydroquinones, Figure 1), quinoneimines,

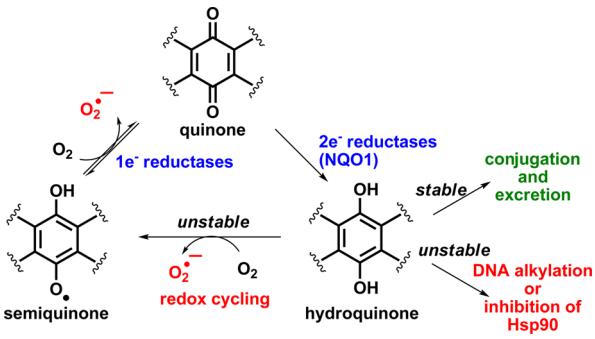

Figure 1. Reduction pathways and subsequent reactions of quinones.

nitroaromatics, and azo dyes.[37] The 2-electron reduction of quinones, including endogenous quinones (e.g., estradiol-3,4-quinone,[37] Figure 2A) and exogenous quinones (menadione[38] and benzo[α]pyrene 3,6-quinone,[38] Figure 2A), is generally believed to be a detoxification process as most hydroquinones are stable (or at least more stable than their semiquinone counterparts) and are easily conjugated to glutathione or glucuronic acid and excreted.[38,39] However, certain hydroquinones are not stable and have the potential to induce cell death (Figure 1),[2] and three main mechanisms of NQO1-dependent death as induced by quinone reduction have been proposed. The first is a direct alkylation of DNA by the hydroquinones.[33,40−42] Although there is evidence that some quinones (e.g., mitomycin C and RH1) are activated by NQO1 to DNA alkylators,[33,40−42] there is controversy over the importance and magnitude of this effect.[2,33,43−45] Recent

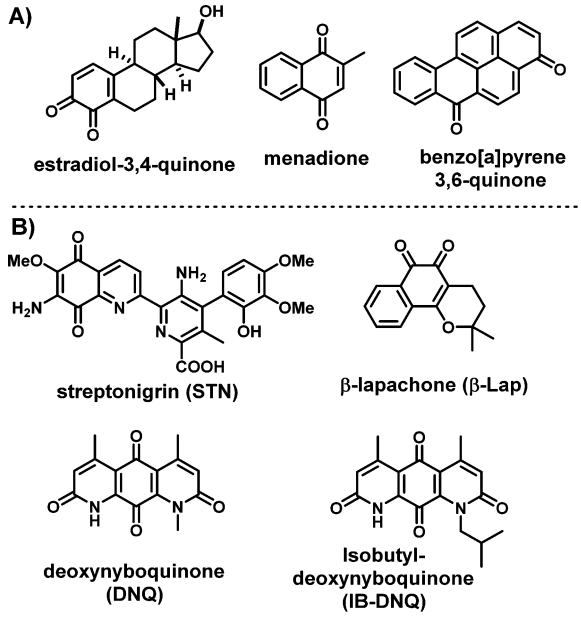

Figure 2. (A) Quinones detoxified by NQO1. (B) Redox cycling quinones bioactivated by NQO1.

head-to-head comparisons (as discussed herein) indicate that most of these compounds are not bioreductively activated by NQO1 in cancer cells in culture.[46] The second mechanism is inhibition of Hsp90 by hydroquinones.[47,48] It appears that certain quinones (e.g., geldanamycin and its derivative 17-AAG) upon reduction to their corresponding hydroquinones by NQO1 become potent inhibitors of Hsp90.[2,47,48] However, the NQO1 dependence of this conversion has been questioned in other studies.[49]

The final and most substantiated NQO1-dependent mode-of-action for anticancer agents is through redox cycling.[9,46,50] Upon reduction of an appropriate quinone by NQO1, the respective hydroquinones rapidly react with molecular oxygen in the cell to give two moles of superoxide and regenerate the quinone (Figure 1). Because these compounds rapidly and catalytically generate large quantities of toxic ROS only in NQO1-expressing cells, they have considerable potential as anticancer agents. Three main NQO1 substrates that are redox cyclers have been described (Figure 2B): streptonigrin (STN), β-lapachone (β-Lap), and deoxynyboquinones (DNQ and IB-DNQ, the focus of this Account). STN is a moderate NQO1 substrate[46,51] but is also activated by other enzymes[52] and likely because of this has severe in vivo toxicity.[53] β-Lap is a good substrate for NQO1[46,54] that has been convincingly shown to induce NQO1-dependent death of cancer cells[46,54−60] and has shown promise in initial clinical trials.[2,61] As described in detail herein, DNQ has also been found to be activated by NQO1 and undergo redox cycling.[46,50]

DISCOVERY AND SYNTHESIS OF DNQ

DNQ was initially synthesized in 1961 by Rinehart and Renfroe as part of studies aimed at elucidating the structure of the natural product nybomycin[62] and in 2011 was found to be a natural product.[63,64] The potent anticancer activity of DNQ was discovered during a high throughput screen of the UIUC Heritage library[65] as well as in a study by the National Cancer Institute.[66]

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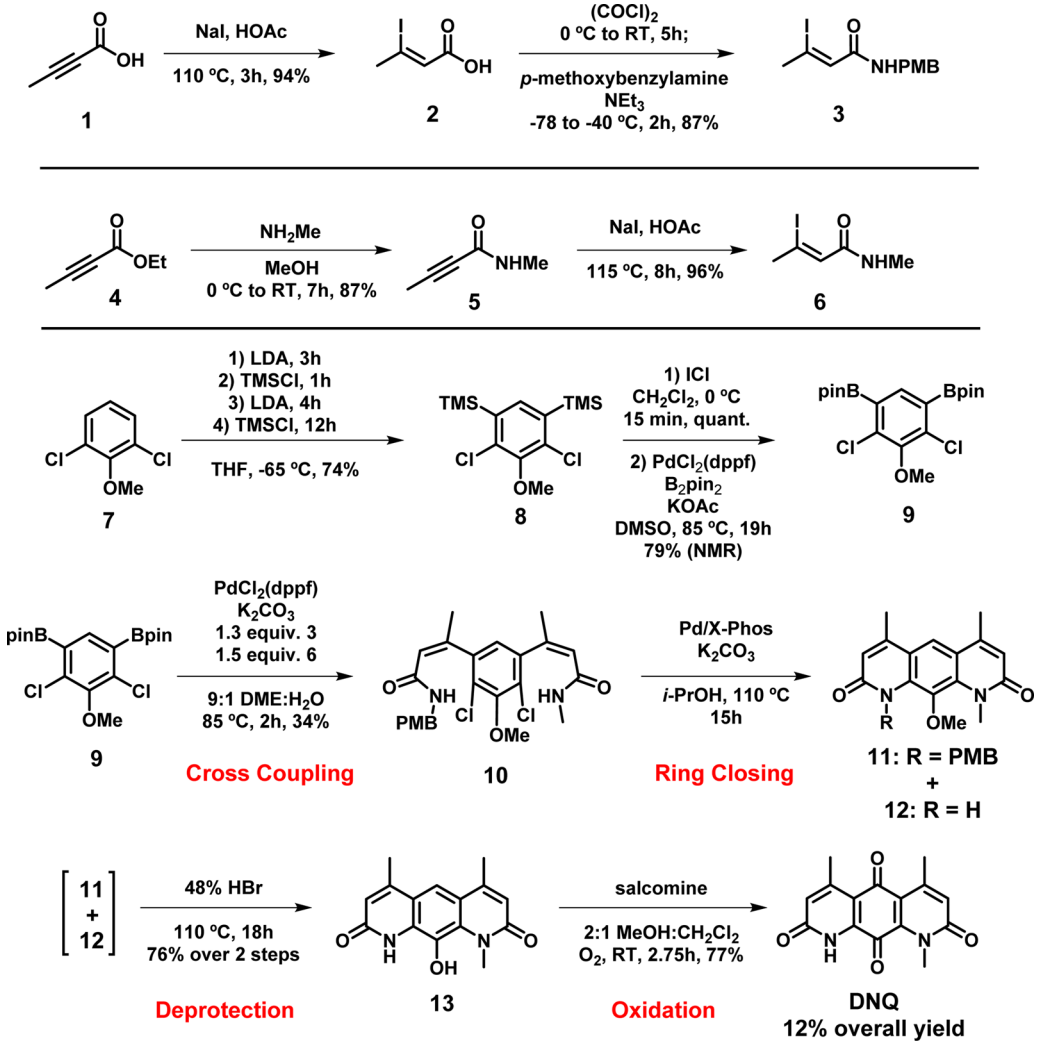
Scheme 1. Synthesis of DNQ[69]



To further assess its activity and generate derivatives, a scalable synthetic route to DNQ was required. Although Rinehart and co-workers developed a synthesis of the structurally related compound deoxynybomycin (DNM),[67,68] a compound that could then be converted to DNQ using concentrated nitric acid,[62] this route was low yielding (<0.8% overall yield) and not amenable to derivative synthesis. For this reason, we developed a novel, modular route to DNQ that could provide the large quantities of compound required for animal studies and allow facile construction of derivatives. This route involves a mixed Suzuki cross-coupling followed by ring closing to afford the core, deprotection, and salcomine-catalyzed oxidation to the quinone (Scheme 1).[69] Using this route, we have generated gram quantities of compound for biological evaluations and have synthesized 25 novel derivatives that will be discussed further below.[46] We have also shown that a variation of this route can be used to efficiently construct DNM, and through derivative synthesis and screening, we identified a non-natural version of DNM that has impressive antibacterial activity in mice infected with MRSA.[70]

MODE OF ACTION OF DNQ

When NQO1-overexpressing cancer cells are treated with DNQ, large amounts of ROS are generated,[50,69] and the potency of DNQ is reduced either in cotreatments with antioxidants (e.g., *N*-acetylcysteine) or treatment under reduced oxygen atmosphere (hypoxia), suggesting that its mechanism is dependent upon ROS generation (Figure 3A).[69] This mechanism was further supported by transcript profiling data of cells treated with DNQ where the top upregulated genes were *HMOX1*, the gene that encodes the antioxidant enzyme heme oxygenase-1, and other oxidative stress-related genes.[69]

For quinones, two major pathways for ROS generation exist: (1) 2-electron reduction by NQO1 followed by redox cycling of the unstable hydroquinone (as described earlier), or (2) 1-electron reduction by one of the various 1-electron reductases to an unstable semiquinone that then redox cycles (Figure 1).[69,71] To distinguish between these possibilities, we explored whether DNQ is a substrate for NQO1. Remarkably, we found that DNQ is processed by NQO1 in vitro near the diffusion controlled limit ($k_{cat}/K_m = 6.2 \times 10^7\ M^{-1}\ s^{-1}$, Figure 3B); other known redox cyclers β-Lap and STN are less efficient NQO1 substrates (Figure 3B).[46] The correlation between NQO1 activity in vitro and anticancer potency in cell culture was also investigated. As shown in Table 1, 2 h exposure of cells to DNQ leads to potent cell death (IC_{50} values of 0.05 to 0.12 μM) in cell lines expressing a threshold level (~200 U) of NQO1, but little-to-no activity against cell lines with low levels

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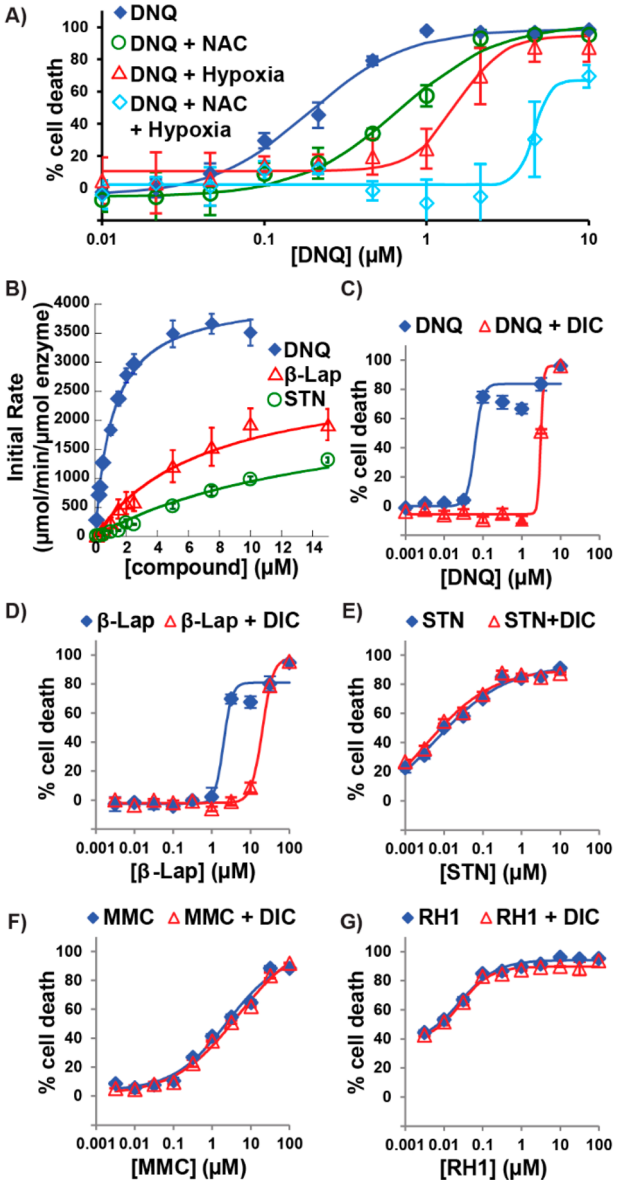

Figure 3. (A) Effect of DNQ on HeLa cells in hypoxia and normoxia and in the presence of *N*-acetylcysteine (NAC).[69] (B) Michaelis–Menten curves for DNQ, β-Lap, and STN with NQO1. Virtually no activity is observed with RH1 and MMC at these concentrations.[46] (C–G) Cell death curves of A549 cells treated for 2 h with DNQ, β-Lap, STN, MMC, and RH1 in the presence and absence of the NQO1 inhibitor dicoumarol (DIC, 25 μM).[46]

of NQO1, including normal lung fibroblast cells.[46,50] A similar pattern of activity, at IC_{50} values ~20–100 fold higher, is observed for β-Lap (Table 1).

Several other experiments led to additional data that confirmed the connection between NQO1 activity and DNQ sensitivity:

(1) The potent activity of DNQ observed in cell lines overexpressing NQO1 can be prevented by coincubation with NQO1 inhibitors (dicoumarol and ES936), as shown in Figure 3C.[46,50] For A549 cells (a lung cancer cell line that overexpresses NQO1) treated with DNQ, there is a 53-fold protection with dicoumarol and >170-fold protection with ES936.[46] Similar results were observed for other NQO1-overexpressing cell lines, such as MCF-7, MIA PaCa-2, and PC-3.[46,50]

Table 1. NQO1 Activity of Cancer Cell Lines and Sensitivity to DNQ[50]

cell line	NQO1 activity (μmol Cyt C reduced min^{-1} mg^{-1} protein)	DNQ IC_{50} (μM)	β-Lap IC_{50} (μM)
A549	3000 ± 300	0.12	2.5
MIA PaCa-2	410 ± 17	0.05	4.5
MCF-7	2641 ± 555	0.12	2.2
H596 NQ+	260 ± 5	0.1	3.8
H596 NQ–	<0.01	>1	>20
MDA-MB-231 NQ+	1800 ± 50	0.06	6.6
MDA-MB-231 NQ–	<0.01	>1	14.2
PC-3	200 ± 5	0.07	3.2
PC-3 shNQ	26 ± 10	>1	>20

(2) shRNA knockdown of NQO1 in cell lines expressing high levels of NQO1 causes the cell lines to be less susceptible to DNQ.[46] MIA PaCa-2 cells with shRNA knockdown of NQO1 (that have at least 8-fold less NQO1 activity than the WT control[58]) were ~5-fold less sensitive to DNQ compared to the cell line with the scrambled shRNA control.[46]

(3) Cell lines that express an inactive NQO1 variant (NQO1*2 polymorphism) are not responsive to DNQ, but upon stable transfection with the gene encoding WT NQO1, they become sensitized.[46,50] Both MDA-MB-231 and H596 cell lines have the NQO1*2 polymorphism and are insensitive to DNQ (IC_{50} > 1 μM). Stable transfection of these cell lines with the WT NQO1 gene results in sensitization to DNQ (≥10 fold increase in potency) (Table 1).[46,50]

COMPARISON OF DNQ TO OTHER PUTATIVE NQO1-ACTIVATED ANTICANCER AGENTS

The experiments described above allowed us to conclude that NQO1 is responsible for activating DNQ. We next decided to directly compare DNQ to the other putative NQO1 substrates reported in the literature. As shown in Figure 3B, DNQ is the best in vitro substrate for NQO1, processed approximately 9 times faster than the next best substrate (β-Lap), and 24 times faster than STN (Figure 3B).[46] RH1 and mitomycin C (MMC) were poor substrates for NQO1 in this assay with observed activity of <100 μmol/min/μmol enzyme.[46]

The protection offered by NQO1 inhibitors in cell culture was also explored. These experiments clearly show that DNQ induces cell death in an NQO1-dependent fashion, as cells treated with NQO1 inhibitors are protected (Figure 3C).[46,50] These experiments also indicate that β-Lap exerts its activity in an NQO1-dependent fashion (Figure 3D) consistent with multiple literature reports from the Boothman laboratory.[9,58] However, the NQO1 inhibitor dicoumarol offered no significant protection from cell death induced by STN, MMC, or RH1, strongly suggesting that these compounds are not highly dependent on NQO1 activation for their activity (Figures 3E–G).[46] The assays described here are an outstanding series of tests that can be used to determine if future compounds induce cell death in an NQO1-dependent fashion.

On the basis of this data set, DNQ and β-Lap appear to be the most promising NQO1-activated anticancer drugs. β-Lap is currently in clinical trials, and there are reasons to be optimistic about DNQ, given its superiority to β-Lap in several areas:

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potency (10−100-fold more potent, Table 1), larger therapeutic window,[50] extended hepatic stability,[50] and dramatically longer in vivo half-life (576 vs 18 min).[50]

DNQ INDUCES PARTHANATOS

As described earlier, DNQ induces the formation of ROS in NQO1-expressing cancer cells. For each mole of DNQ, it is estimated that >60 mol of superoxide are generated each minute, highlighting the catalytic nature of this compound.[50] Superoxide is known to cause DNA damage, specifically DNA base and single strand break lesions.[50] Poly(ADP-ribose) polymerase 1 (PARP-1) is typically recruited to these breaks, where it synthesizes poly(ADP-ribose) polymers (PAR) that recruit DNA repair proteins.[72] In cases of extensive DNA damage, PARP-1 can be overactivated, resulting in PARP-1-dependent cell death called parthanatos.[72,73] Within 5 min of treating an NQO1-overexpressing cancer cell line with DNQ, significant PAR formation is observed (Figure 4A).[50] Additionally, there is a significant reduction in both NAD+ and ATP levels, consistent with overactivation of PARP-1 and parthanatos (Figure 4B,C).[50] Transient knockdown of PARP-1 decreased NAD+ reduction and overall cell death following exposure to DNQ,[50] further implicating parthanatos as the major form of DNQ-induced cell death. Similar results were observed in vivo: treatment of mice inoculated with orthotopic Lewis Lung Carcinoma (LLC) tumors with DNQ resulted in significantly decreased tumor burden (Figure 4D,E).[50] Additionally, tumor cells showed PARP-1 overactivation, suggesting that the mechanism of action in vivo is the same as that seen in cell culture (Figure 4F). The studies above utilized the A549 or LLC cell lines, and PARP-1-dependent death induced by DNQ has also been observed with another lung cancer cell line.[50]

DESIGN, SYNTHESIS, AND ACTIVITY OF DNQ DERIVATIVES

As DNQ is processed by NQO1 near the diffusion controlled limit, it would be unlikely to find a derivative that is a better NQO1 substrate. However, derivatives were sought to determine what makes an excellent NQO1 substrate and to find compounds with improved solubility and tolerability profiles. DNQ and derivatives were modeled in silico into the active site of NQO1. Crystal structures of NQO1 with substrates such as duroquinone[74] show that substrates typically π-stack with the bound FAD cofactor and that the quinone moiety often hydrogen bonds to nearby tyrosine residues. When DNQ was docked into the crystal structure, similar interactions were observed (Figure 5A,B).[46] Derivatives with further substitution on the nitrogen (e.g., IB-DNQ) show computational binding modes similar to DNQ, suggesting that they would also be excellent NQO1 substrates, whereas derivatives with multiple substitutions (e.g., DNQ-3) do not, suggesting that they would be less efficiently processed by the enzyme (Figure 5C−E).

Using a modified version of our synthetic route, 25 DNQ-derivatives were constructed.[46] In agreement with the modeling data, most derivatives with further substitution at the nitrogen are good substrates for NQO1 and have potent anticancer activity against NQO1-expressing cell lines (Figure 6A,B). Several compounds also have increased solubility in either PBS or hydroxypropyl-β-cyclodextrin (HPβCD, an adjuvant used to increase solubility of lipophilic drugs) compared to DNQ (Figure 6C).[75] The low solubility of DNQ is likely due to its

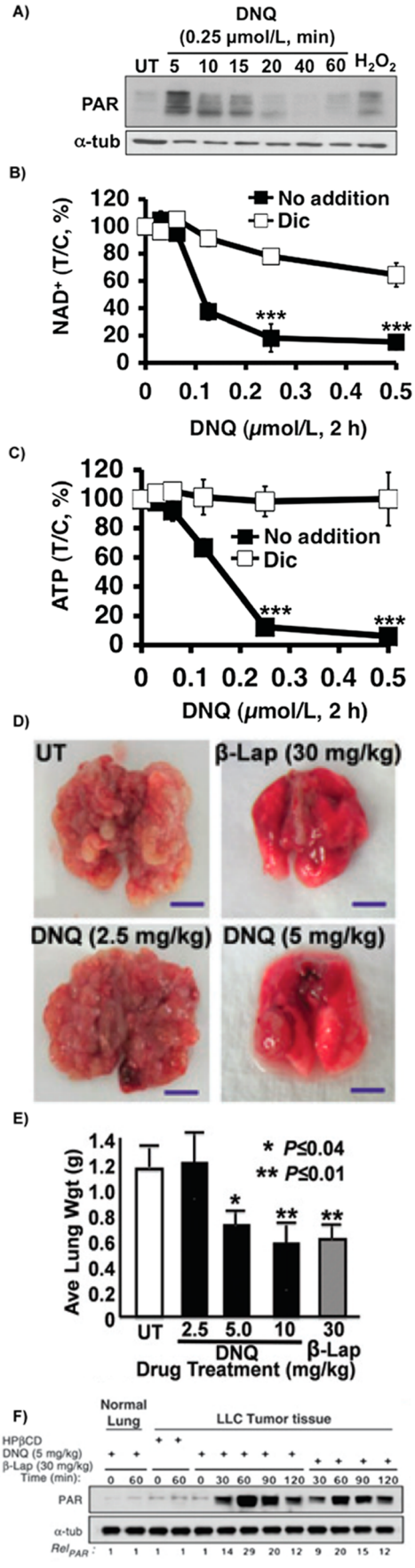

Figure 4. (A) Immunoblot demonstrating PAR formation in A549 cells treated with or without 0.25 μM DNQ. UT is vehicle-treated

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Figure 4. continued

control. H_2O_2 is the positive control.[50] (B,C) Dose-dependent NAD+ and ATP loss in A549 cells after treatment with DNQ ± dicoumarol (Dic, 40 μM). T/C% = % treated/control ± SEM ***P < 0.001.[50] (D) Lungs of mice inoculated with LLC tumors and treated with vehicle (UT), β-Lap (30 mg/kg), or DNQ (2.5 or 5 mg/kg).[50] (E) Average weight of lungs of mice from (D).[50] (F) Western blot similar to (A) confirming PAR formation in tumor but not normal tissues after treatment of mice bearing LLC tumors with either DNQ or β-Lap.

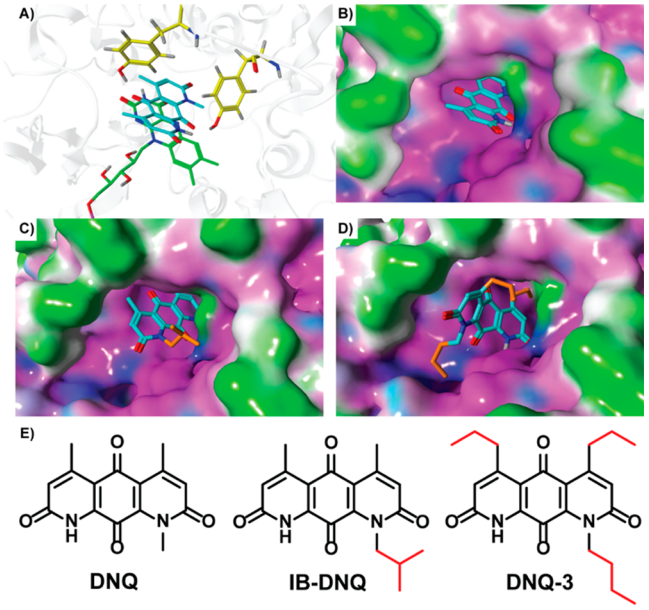

Figure 5. Modeling of DNQ and derivatives in the NQO1 active site.[46] (A) The π-stacking and hydrogen bonding interactions among DNQ (blue), the cofactor FAD (green), and Tyr-126 and 128 (yellow) of NQO1. DNQ (B) and IB-DNQ (C) fit deeply into the active site. (D) DNQ-3 does not fit as deeply into the active site. (E) Structures of the compounds in (B–D).

ability to π-stack with itself, as was observed in its crystal structure.[63] The addition of alkyl chains likely interferes with the π-stacking, allowing for better aqueous and organic solubility; we recently reported a similar phenomenon for DNM and its derivatives.[70] IB-DNQ and some of the other derivatives appear to be very promising for in vivo applications.

CONCLUSIONS AND OUTLOOKS

NQO1-mediated activation of a nontoxic compound to a toxic compound is an excellent targeted strategy as NQO1 is often dramatically overexpressed in cancer cells compared to normal cells. Utilizing a small molecule substrate like DNQ that is capable of cycling back to the parent molecule is particularly promising both because of the targeted nature of the activation and the low amount of compound that is required. Further studies exploring why DNQ is such an efficient substrate and redox cycler would be informative for designing additional derivatives. The cancer cell selectivity, potency, and promising in vivo properties of DNQ all suggest its outstanding potential as a personalized anticancer therapy.

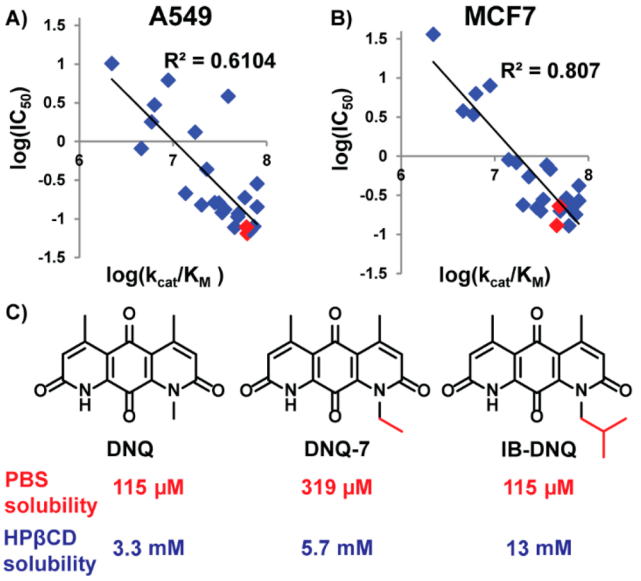

Figure 6. Correlation between in vitro processing of DNQ analogues by NQO1 (k_{cat}/K_M in M^{-1} s^{-1}) and their ability to induce death (IC_{50} in μM) in (A) A549 and (B) MCF-7 cells.[46] Red points are DNQ and IB-DNQ. (C) The structure and solubility of DNQ and select derivatives.[46]

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Notes

The authors declare the following competing financial interest(s): The University of Illinois has filed patents on DNQ and its derivatives with E.I.P. and P.J.H. listed as inventors.

Biographies

Paul J. Hergenrother was born in Akron, OH in 1972, received a B.S. in chemistry from the University of Notre Dame in 1994, and a Ph.D. in chemistry from the University of Texas, Austin in 1999. After a stint as an American Cancer Society postdoctoral fellow at Harvard University, he joined the faculty at the University of Illinois, Urbana–Champaign in 2001 where he is currently the Kenneth L. Rinehart Jr. Endowed Chair in Natural Products Chemistry. His laboratory seeks to use small molecules to identify novel biological targets for the treatment of cancer and drug-resistant bacteria.

Elizabeth I. Parkinson was born in Greenville, MS in 1988, obtained a B.S. in chemistry from Rhodes College in 2010, and her Ph.D. in chemistry at the University of Illinois at Urbana–Champaign in 2015 in the laboratory of Prof. Paul J. Hergenrother. Her research interests include the development of targeted anticancer agents and novel antibiotics for the treatment of antibiotic-resistant bacteria.

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