**SUPPLEMENTAL MATERIAL: MATERIALS AND METHODS**

**Materials and Methods for Supplemental Tables 1 and 2.**

Reduction of DNQ and β-lap by NQO1 In vitro.

**Supplemental Table 1.** To further understand the enhanced potency of DNQ relative to β-lap, the ability of these compounds to act as substrates in vitro for NQO1 was assessed (via loss of NADH signal at 340 nm) according to the method of Pink et al. JBC, 2000 (see ref #10). The results show that at concentrations of 1 µmol/L NQO1 processes DNQ approximately 13-fold more efficiently than β-lap (1400 vs. 110 µmol/min/µmol enzyme) (Table 1). Other quinones, such as streptonigrin, menadione, mitomycin C, and RH1 were also assessed for their ability to be reduced by NQO1. NQO1 reduced streptonigrin at a similar rate as β-lap, but the other quinones were much less efficiently reduced by the enzyme.

**Supplemental Table 2.** DNQ, β-lap, and menadione were also tested at their respective equitoxic doses, using log-phase A549 NSCLC cancer cells as an example (See Figures 2 and 3). The rates of reduction of equitoxic doses of DNQ and β-lap were roughly the same, while menadione was a much less efficient substrate at these equitoxic doses. The equal rates of reduction of DNQ and β-lap agrees with oxygen (O2) consumption and NAD+/ATP depletion data (Figures 3 and 4), further supporting the similar mode of action of these two compounds within the cell, and suggesting that DNQ is a far better substrate for the enzyme than β-lap. Menadione’s lower rate of reduction was not surprising and suggests a different mode of action, since this quinone is known to be detoxified by NQO1, as noted in Figure 2. These data agree with past literature suggesting that menadione is primarily activated by one-electron reductases, b5R and p450R oxidoreductases (as noted in Pink et al., JBC, 2000; ref. #10).

**Supplemental Figure 8A. Plasma levels of β-lap and DNQ after IV dosing.** Mice were dosed IV with either 30 mg/kg of β-lap or 10 mg/kg DNQ formulated with hydroxypropyl-β-cyclodextrin. At varying times after dosing, the mice were sacrificed and plasma samples taken out to 120 mins for β-lap and 1440 mins for DNQ. Plasma levels were monitored by LC/MS/MS after extraction of the compounds from the plasma matrix by solid phase extraction (β–lap) or simple removal of plasma proteins by precipitation with acetonitrile (DNQ). Recoveries of both compounds were near 100% for both methods.

**Conclusions:** Plasma levels of DNQ were significantly higher and more sustained than those of β-lap, despite the use of a lower dose. Calculated non-compartmental pharmacokinetic parameters are shown in Table S4.

**Methods:** Mice were dosed IV with either 30 mg/kg of β-lap or 10 mg/kg DNQ formulated with hydroxypropyl-β-cyclodextrin as described (Blanco and Bey et al., Cancer Research, 2010). At varying times after dosing, the mice were sacrificed and whole blood isolated with an ACD (acidified sodium citrate-dextrose) coated syringe and needle. Samples were taken out to 120 mins for β-lap and 1440 mins for DNQ. Plasma was isolated from whole blood by centrifugation at 4°C for 10 mins at 9,300xg. For β-lap: 100 µL of plasma was mixed with 200 µL of acetonitrile. Samples were vortexed 15 s and set at room temperature for 10 mins. 700 µL of ddH2O was added and the samples were vortexed. Samples were spun for 5 mins at 16,100xg. The supernatant was collected and 1 mL of ddH2O was
added. The supernatant solution was then loaded onto an Oasis HLB 3cc extraction column (Waters) that had been previously washed with 2 mL MeOH and equilibrated with 2 mL dH2O. The sample column was then washed twice with 2 mL of 5% MeOH/95% dH2O. The sample was eluted with 2 mL of 100% MeOH. 2 mL of ddH2O and 0.2% formic acid were added to the sample and then analyzed by HPLC/MS/MS using an Applied Biosystems/MDS Sciex 3200-QTRAP coupled to a Shimadzu Prominence LC. Chromatography conditions were as follows. Buffer A consisted of 25% Isopropanol/75% HPLC grade H2O + 0.1% formic acid. Buffer B consisted of 25% Isopropanol/75% Methanol + 0.1% formic acid. The column flow rate was 0.5 ml/min using a Phenomenex Synergi Fusion RP 75 X 2 mM, 4 micron packing column. The gradient conditions were: 0-2 min 100% A, 2-3 min gradient to 100% B, 3-7 min 100% B, 7-7.5 min gradient to 100% A, 7.5-8.5 min 100%A. β-lap was detected in MRM mode following the 243.1 to 187.2 transition. Back-calculation of standard curve and quality control samples were accurate to within 25% for 74% of these samples at concentrations ranging from 5 to 10000 ng/ml. For DNQ, 100 µl of plasma was mixed with 400 µl of acetonitrile containing 0.5% formic acid and 10 ng/ml tolbutamide (internal standard, Sigma-Aldrich). Samples were vortexed 15 s and set at room temperature for 10 mins. Samples were spun for 5 mins at 16,100xg. The supernatant (480 µl) was removed and spun a second time for 5 mins at 16,100xg. The supernatant was then analyzed by HPLC/MS/MS again using an Applied Biosystems/MDS Sciex 3200-QTRAP coupled to a Shimadzu Prominence LC. Chromatography conditions were as follows. Buffer A consisted of HPLC grade dH2O + 0.1% formic acid and Buffer B consisted of MeOH + 0.1% formic acid. The column flow rate was 0.5 ml/min using a Phenomenex Synergi Fusion RP 75 X 2 mM, 4 micron packing column. The gradient conditions were as for β-lap. Both DNQ and tolbutamide were monitored in MRM mode following the 283.0 to 255.0 transition for DNQ and the 269.1 to 169.9 transition for tolbutamide. Back-calculation of standard curve and quality control samples were accurate to within 20% for 75% of these samples at concentrations ranging from 1 ng/ml to 10,000 ng/ml.

**Supplemental Figure 8B.** Stability of β-lap and DNQ after incubation with cryopreserved murine hepatocytes. β-lap and DNQ were incubated at 37°C at 2 µM for up to 240 mins with cryopreserved murine hepatocytes purchased from Celsis/In Vitro Technologies. At varying points after the start of the incubation, compounds were extracted by precipitation of protein with MeOH and levels monitored by LC/MS/MS. The natural logarithm (Ln) of the % of intact compound remaining relative to time 0 is plotted.

**Conclusions:** DNQ shows better stability than β-lap upon *in vitro* incubation with cryopreserved murine hepatocytes, suggesting that better metabolic stability may contribute in part to the lower clearance, higher plasma levels, and more sustained compound levels of DNQ *in vivo* relative to β-lap. Such observations are in agreement with the hypothesis that DNQ is a less efficient substrate than β-lap for one-electron oxidoreductases (Figure 3) such as p450R and b5R, which unlike NQO1 are well expressed in normal hepatocytes.

**Methods:** Male ICR/CD-1 mouse hepatocytes, InVitroGRO HI and HT Medium, and Celsis Torpedo Antibiotic Mix were purchased from Celsis/In Vitro Technologies (Baltimore, MD). Cryopreserved hepatocytes were thawed in HT Media containing antibiotics, resuspended in HI media at 2 X 10⁶/ml and plated in 96 well plates at 0.05 ml (10⁵ cells)/well. Compounds to be tested were dissolved in DMSO at 2mM, further diluted to 4 µM in HI media, and added to the cells in 50 µl so that the final
compound concentration was 2 μM. Two additional wells containing compound and no cells were plated to serve as time 0 (C₀) and endpoint solvent control (Cₑp). The cells were then placed in a 37°C, 5% CO₂ incubator. Reactions were quenched with 200 μL of MeOH containing 300 ng N-benzylbenzamide (internal standard, Sigma-Aldrich) and 0.15% formic acid, vortexed for 15 seconds, incubated at room temperature for 10 minutes and spun at 4°C for 5 mins at 16,100xg. Supernatant (200 μL) was transferred to an HPLC vial and analyzed by HPLC/MS/MS using the conditions described above. The 212.1 to 91.1 transition was monitored for n-benzylbenzamide. Metabolism of 7-ethoxycoumarin was used to monitor hepatocyte performance.

**Supplemental Table 4. Comparison of in vitro metabolic stability and pharmacokinetic properties for β-lap and DNQ.**

**Conclusions:** A comparison of calculated metabolic stability and PK properties for β-lap and DNQ indicates that in vitro intrinsic hepatic clearance is 6-fold higher for β-lap than DNQ. In vivo a larger difference is observed suggesting other factors may contribute to the observed clearance rates, including differences in protein binding, distribution, metabolism at sites other than the liver, and differences in final sampling times for the two compounds. β-lap data were acquired only out to 120 mins versus 1440 mins for DNQ. A comparison of the present data to previously published β-lap PK data (1) where data were acquired out to longer times indicates the present data capture exposure (AUC) and Cl values appropriately but may under-represent terminal T ½ and Vz values, although the comparison is complicated by different route of administration and dose.

**Methods:** An in vitro metabolic T ½ and corresponding intrinsic clearance were calculated for β-lap and DNQ as follows. The method described in McNaney, et al (2) was used with modification for determination of metabolic stability half-life by substrate depletion. Briefly, a “% recovered” number was calculated for the C₀ and Cₑp samples plated in media only to control for compound-related issues such as solubility and stability in the assay media. This value was obtained by dividing Cₑp LC/MS/MS peak area by the C₀ peak area and multiplying by 100. Typically, acceptable values are between 70-140% (3). A “% remaining” value was used to assess metabolic stability of a compound over time. The LC/MS/MS peak area of the incubated sample at each time point was divided by the LC/MS/MS peak area of the time 0 (T₀) sample and multiplied by 100. The natural Log (LN) of the % remaining of compound was then plotted versus time (in min) and a linear regression curve plotted going through y-intercept at LN(100). The metabolism of some compounds failed to show linear kinetics at later time point, so those time points were excluded. The half-life (T ½) was calculated as T ½ = -0.693/slope. An intrinsic clearance rate is then calculated from the half-life as

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V (\mu l/10^6 \text{ cells}) = \frac{\text{Incubation volume (}\mu l\text{)}}{\text{Number of cells in incubation (x}10^6\text{)}}
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Intrinsic Clearance (Clₐ₉) (μl/min/million cells) = \(\frac{V \times 0.693}{T \ ½}\)

The non-compartmental analysis tool in WinNonlin (Version 5.3, Pharsight) was used in sparse sampling mode to calculate pharmacokinetic parameters for plasma data from mice administered either β-lap (30 mg/kg IV) or DNQ (10 mg/kg IV).
