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# Metabolic and electrochemical mechanisms of dimeric naphthoquinones cytotoxicity in breast cancer cells

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ABSTRACT

Cancer cells reprogram their metabolism due to genetic alteration to compensate for increased energy demand and enhanced anabolism, cell proliferation, and protection from oxidative damage. Here, we assessed the cytotoxicity of three dimeric naphthoquinones against the glycolytic MCF-7 versus the oxidative MDA-453 breast carcinoma cell lines. Dimeric naphthoquinones **1** and **2** impaired MDA-453, but not MCF-7, cell growth at $IC_{50}$ = 15 μM. Significant increase in reactive oxygen species, decrease in oxygen consumption and ATP production were observed in MDA-453 cells but not in MCF-7 cell. These findings suggest that oxidative stress and mitochondrial dysfunction are mechanisms by which these agents exert their cytotoxic effects. Cyclic voltammetry and semi-empirical molecular orbital calculations further characterized the electrochemical behavior of these compounds. These results also suggest that dimeric naphthoquinones may be used to selectively target cancer cells that depend on oxidative phosphorylation for energy production and macromolecular synthesis.



## 1. Introduction

Breast cancer is the most prevalent cancer in women in industrialized countries. In the United States, approximately 207,000 women were diagnosed with breast cancer and 40,000 died of it in 2010.[1] Although advanced therapies have contributed to decreased mortality from breast cancer in recent years, the global burden of this heterogeneous disease highlights the need for new therapies against more aggressive and advanced forms of the disease.

In 2006, we prepared a series of novel dimeric naphthoquinones for the purpose of developing structurally less complex HIV-integrase inhibitors that mimicked the action of conocurvone, a naturally occurring trimeric naphthoquinone with potent anti-HIV activity.[2] While the less complex dimeric naphthoquinones inhibited HIV-1 mediated cytopathogenicity, they also showed cytotoxicity in CEM-$T_4$ lymphoblastic cells at varying concentrations, suggesting their possible use as anti-neoplastic agents.[2]

Recently, we reported that dimeric naphthoquinones possessed cytotoxic activity and synergistic effects with radiation against prostate cancer cells.[3] Substitution patterns on the quinone double bond and aromatic ring of the quinone units were shown to influence cytotoxicity. Mechanistic studies using a chemical genetic screen in yeast revealed that their cytotoxicity was probably related to their ability to undergo redox cycling, produce free radicals and cause mitochondrial dysfunction.[4]

Targeting aerobic glycolysis (Warburg effect) has been the focus of numerous studies in cancer, but more recent data have identified a number of cancer cells that use mitochondria and oxidative phosphorylation as a main energy source.[5,6] Our previous studies of the cytotoxicity of dimeric naphthoquinones in prostate cancer cells and yeast suggested that one mechanism by which these compounds affect cell survival may be through induction of mitochondrial dysfunction.[3,4] We hypothesized that dimeric naphthoquinones would be selectively cytotoxic for cancer cells which utilize oxidative phosphorylation while cancers relying on glycolysis would not be affected by these agents.[3,4]

In the present study, we investigated how exposure to dimeric naphthoquinones affected MDA-453 and MCF-7 differently as two metabolically distinct breast cancer cell lines.[7] We report that

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dimeric naphthoquinones **1–3** are selectively cytotoxic to a MDA-453, which depend on oxidative phosphorylation for energy production, with $IC_{50}$'s in the micromolar range. We further show that exposure of MDA-453 breast cancer cells to dimeric naphthoquinones results in the accumulation of reactive oxygen species, depletion of intracellular oxygen and inhibition of ATP generation, while have no or minimal effect on MCF-7 breast cancer cells. The ability of the quinone dimers to undergo redox cycling was studied using cyclic voltammetry. Molecular orbital calculations were performed to predict lowest unoccupied molecular orbital (LUMO) energies of the dimers in an attempt to see if there is a correlation between their LUMO's and cytotoxicity (Fig. 1).

## 2. Results and discussion

A high yielding one step synthesis of dimeric naphthoquinones **1–3** was described by us previously[8] and has been subsequently utilized by other research groups[9,10] to study the synthesis and biological properties of this unique class of compounds.

To determine whether dimeric naphthoquinones affected the viability of breast cancer cells, we performed a preliminary screen using three structurally related chloro-hydroxy dimeric naphthoquinones **1–3** in MDA-453 and MCF-7 breast cancer cell lines using an MTT assay. As mentioned previously, MDA-453 is a basal-type breast cancer cell line, which depends more on oxidative phosphorylation for survival, proliferation, invasion and energy production.[7,11,12] MCF-7 is a luminal-type breast cancer cell line that depends more on glycolysis for survival.[7,11,12] At concentrations of 40 μM or less dimeric naphthoquinones **1–3** significantly impaired conversion of MTT to formazan in MDA-453 breast cancer cells ($p$ <0.05, compared to DMSO) but not in MCF-7 cells, suggesting selective, dose-dependent cytotoxicity by possibly affecting oxidative phosphorylation (Fig. 2). Trypan blue exclusion assays for cell viability confirmed cell deaths. Dimeric quinones **1** and **2** showed cytotoxic effect against MDA-453 with $IC_{50}$s of 15 and 17 μM, respectively, compared to the pyranylated dimeric quinone **3** with an $IC_{50}$ of 34 μM. In contrast, approximately 80% of MCF-7 cells survived at 50 μM of dimeric naphthoquinones concentration (**1**, 80 ± 4%; **2**, 83 ± 5%; **3**, 85 ± 7%).

To test if dimeric naphthoquinones selectively affect energetic metabolism in cancer cells by disrupting mitochondrial function, we performed a series of metabolic experiments in MDA-453 and MCF-7 breast cancer cells. Indeed, after treatment of MDA-453 breast cancer cells with dimeric naphthoquinones at a concentration of 10 μM, quinones **1** and **2** induced a 53% and a 37% decrease in ATP in the remaining viable cells compared to DMSO, respectively (Fig. 3). At 10 μM, quinone **3** did not decrease ATP production significantly. When the quinone concentration was increased to 20 μM, quinones **1** and **2** decreased ATP production by approximately 60%, while quinone **3** induced a 27% decrease in ATP compared to DMSO (Fig. 3). As expected, treatment of glycolysis-dependent MCF-7 cells with dimeric naphthoquinones affected ATP production to a much lesser extent (Supplementary Fig. 1). At concentrations greater than 20 μM, ATP production was not further decreased, suggesting the presence of other mechanisms involved in cytotoxic effects of these compounds.

To test if reactive oxygen species (ROS) increased in breast cancer cells treated with dimeric naphthoquinones, we preloaded MDA-453 breast cancer cells with 5-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) followed by exposure to dimeric

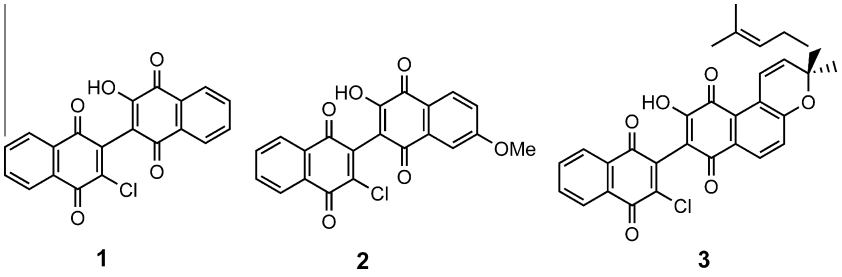



**Figure 1.** Dimeric naphthoquinones.

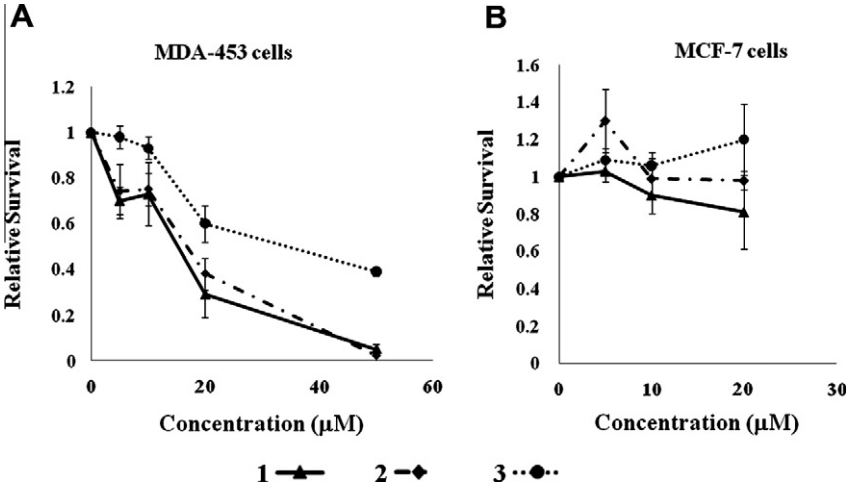



**Figure 2.** Effect of dimeric naphthoquinones **1**–**3** on MDA-453 (A) and MCF-7 (B) breast cancer cell proliferation (compared to DMSO). All cells were grown at $1 \times 10^5$ cells/mL. Cell counts were performed in triplicate and shown as mean ± SD, and the entire experiment was replicated, with similar results.

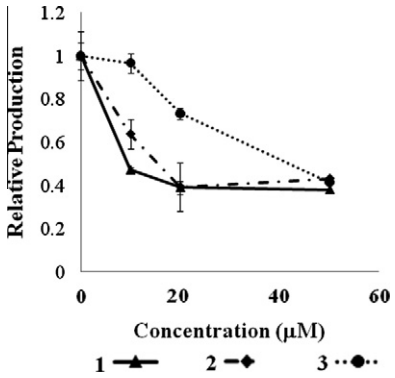



**Figure 3.** Relative (compared to DMSO) decrease in ATP production in MDA-453 breast cancer cells by dimeric naphthoquinones **1**–**3**.

naphthoquinones. Intracellular oxidation of DCFDA by ROS yields a fluorescent compound which can be measured using flow cytometry. All three dimeric naphthoquinones increased production of ROS in MDA-453 cells as shown by a 50–63% ($p$ <0.05) absolute increase in the percentage of fluorescent cells compared to DMSO (20%), suggesting that oxidative stress may contribute to cytotoxicity through free radical production in these cell lines (Fig. 4). Interestingly, when we performed similar experiments in MCF-7 cells, dimeric naphthoquinones did not increase ROS production significantly (Supplementary Fig. 2). Furthermore, when we measured $O_2$ consumption by MDA-453 cells in the presence and absence of dimeric naphthoquinones, we found that dimeric quinones **1**–**3** decreased $O_2$ consumption to different degrees, suggesting interference with mitochondrial oxidative phosphorylation. As expected, dimeric naphthoquinones did not alter $O_2$ consumption in MCF-7 cells which rely on non-oxygen non-mitochondrial glycolysis for survival (Supplementary Fig. 3).[11] These results suggest that dimeric naphthoquinones may be used to selectively target cancer cells that depend on oxidative phosphorylation for energy production and macromolecular synthesis.

In view of the ability of dimeric naphthoquinones to selectively generate ROS in MDA-453 breast cancer cell lines, it was of interest to examine the electrochemical behavior of these compounds in order to determine whether they had the inherent ability to undergo reversible oxidation–reduction reactions. The correlation of electrochemical potentials of monomeric quinones and their inhibitory effects on Epstein–Barr virus activation,[13–15] and cytotoxic activities[16,17] have been reported. However, to the best of our knowledge, such correlations have not been investigated in dimeric naphthoquinones. Thus, 2 mM stock solutions of the dimeric quinones were prepared in acetonitrile and analyzed by cyclic voltammetry using a standard Ag/AgCl reference electrode and tetrapropyl ammonium bromide as the supporting electrolyte.[18] The cyclic voltammograms are shown in Figure 5.

The electrochemical behavior of 2-hydroxynaphthoquinones are complex.[19] Quinones are known to undergo disproportionation reactions, which could account for the non-uniform peak shapes in the cyclic voltammograms of the hydroxyquinone dimers. In Figure 5, the first and second waves correspond to formation of the radical anion and dianion (presumably by reduction of the more electron deficient chloronaphthoquinone unit first), while the third wave corresponds to radial anion formation of the more electron rich hydroxyquinone unit at higher potential. The fourth wave was not definable. Table 1 shows cathodic ($E^c$) and anodic ($E^a$) peak potentials for each of the four redox steps ($E_1$–$E_4$) as well as reduction potentials ($E^{1/2} = E^a + E^c/2$).[20,21] Similar values for $E_1^{1/2}$ reduction potentials for each of the dimeric quinones supports the idea that reduction of the chloroquinone occurs first because all three quinones dimers contain a structurally similar chloroquinone unit (i.e., with no substituents on the aromatic ring). The peaks selected for Table 1 are depicted in the Supplementary Figure 4.

To study whether regioselective reduction of the chloroquinone unit could be predicted, we used semi-empirical molecular orbital

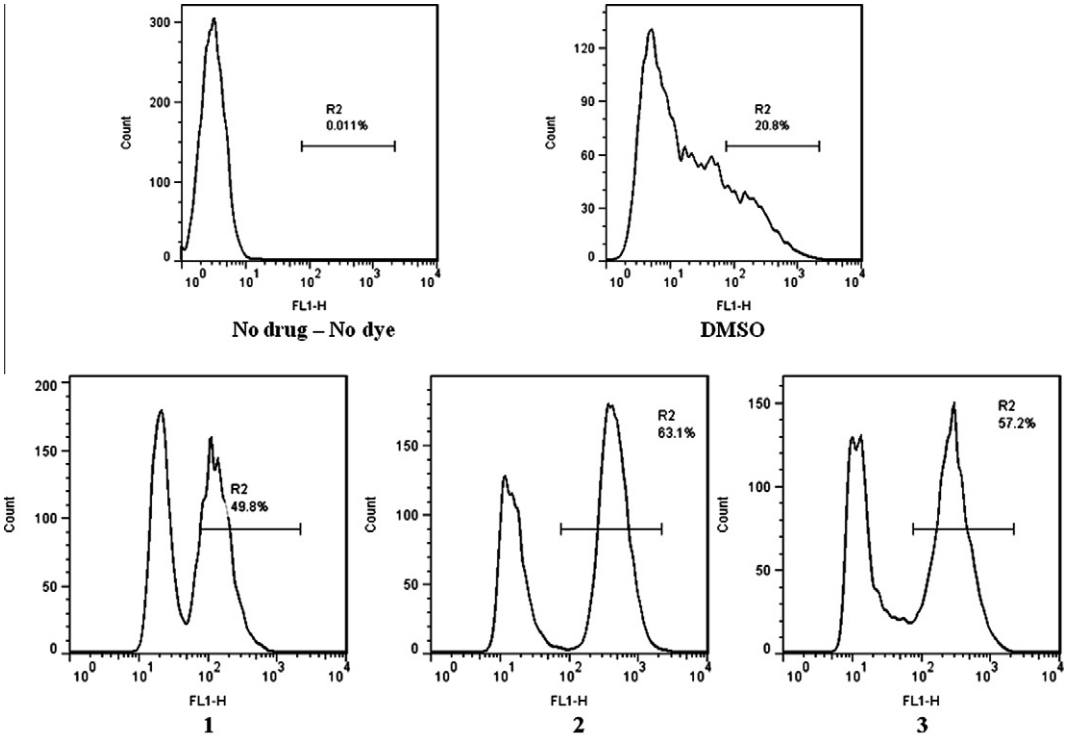



**Figure 4.** Generation of free oxygen radicals by dimeric naphthoquinones **1**–**3** compared to DMSO in MDA-453 cells. ROS levels were determined by DCFDA fluorescence in the cells. Data are representative of triplicate samples of two separate experiments. For comparison with MCF-7 cells, see Supplementary Figure 2.

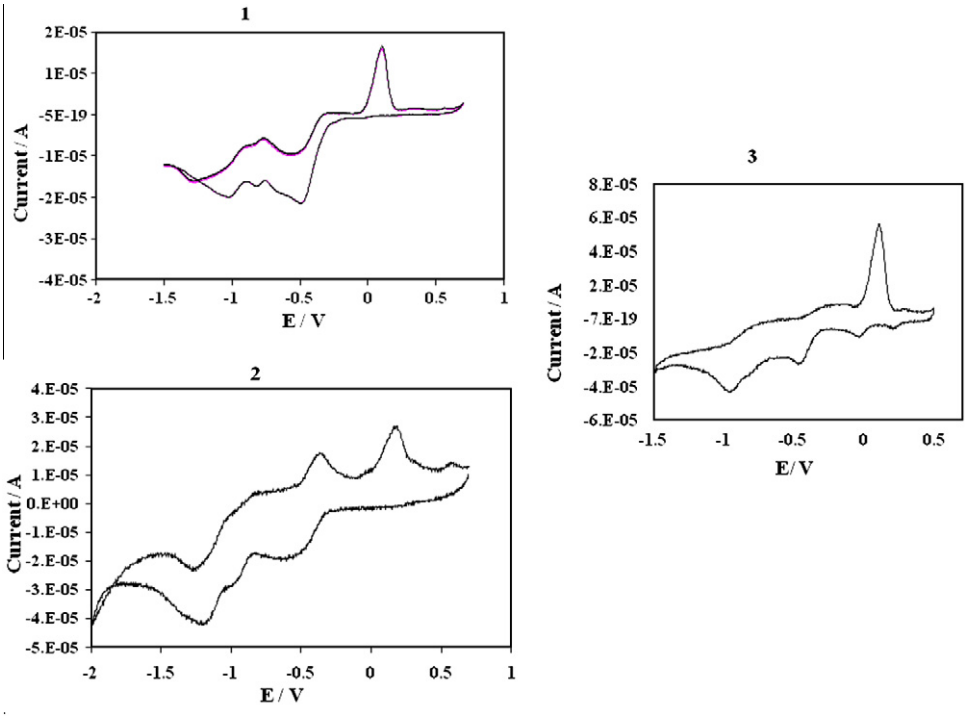



**Figure 5.** Cyclic voltammetry of 2 mM dimeric naphthoquinones in acetonitrile plus 0.1 M tetrapropyl ammonium bromide (TBAP), with a Ag/AgCl reference electrode (RE), and the scan rates between 0.1 and 2 V/s, E = electrochemical potential, V = volt, A = ampere. Cyclic voltammetry of background electrolyte did not show any waves.

**Table 1**
Cathodic ($E^c$) and anodic ($E^a$) potentials

| | $E_1^c$ | $E_1^a$ | $E_1^{1/2}$ | $E_2^c$ | $E_2^a$ | $E_2^{1/2}$ | $E_3^c$ | $E_3^a$ | $E_3^{1/2}$ | $E_4^c$ | $E_4^a$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | −0.99 | −0.94 | −0.97 | −0.79 | −0.79 | −0.79 | −0.48 | −0.34 | −0.41 | nd | 0.08 |
| 2 | −1.21 | −1.04 | −1.12 | −0.96 | −0.90 | −0.93 | −0.56 | −0.39 | −0.48 | nd | 0.15 |
| 3 | −0.92 | −0.84 | −0.88 | −0.43 | −0.34 | −0.39 | −0.02 | 0.12 | 0.05 | 0.22 | 0.29 |

calculations to determine the partial charges and molecular orbital energies of the quinone dimers (Fig. 6).[22–24] The nearly identical partial charges of the carbon atoms of the four carbonyl groups at C-1, C-4, C-6 and C-8 (ranges between 0.34 and 0.39) revealed that oxygen n-donation to the carbonyl $\pi$-system was similar in all three dimeric naphthoquinones. Furthermore, the charges of the chlorinated carbons at C-2 (i.e., 0.17–0.18) were nearly identical in all three of the quinone dimers making it difficult to predict selective reduction or correlate cytotoxicity merely from partial charge distributions. Interestingly, calculation of the lowest unoccupied molecular orbital (LUMO) and the second LUMO (SLUMO) energies of the quinones (Fig. 6) revealed a slightly narrower LUMO–SLUMO orbital energy gap [ΔE (LUMO–SLUMO)] in quinones **1** and **2** (5 kcal/mol) compared to quinone **3** (7.6 kcal/mol). This slightly narrow energy gap may make the SLUMO more accessible to the reacting cellular nucleophiles to form Michael adducts as additional mechanism for cytotoxicity.[4] Images of the calculated LUMO and SLUMO for dimeric naphthoquinones are presented in Supplementary Figure 5.

In conclusion, the potential mechanisms of cytotoxicity of three structurally related 2-chloro-2′-hydroxyl dimeric naphthoquinones in MDA-453 breast carcinoma cells were investigated. Dimeric naphthoquinone cytotoxicity is likely mediated by oxidative stress and mitochondrial dysfunction as evidenced by increased levels of ROS, and decreased ATP production and oxygen consumption. Cyclic voltammetry and semi-empirical molecular orbital calculations assisted to further characterize the electrochemical behavior of the quinone dimers. Our data suggests that participation in redox cycling by dimeric naphthoquinones generate significant levels of reactive oxygen radicals which may contribute to their cytotoxicities. Further in vitro and in vivo studies are warranted to continue to explore anti-neoplastic properties of this class of compounds in different cancer cell lines as single agents and in combination with other chemotherapeutics.

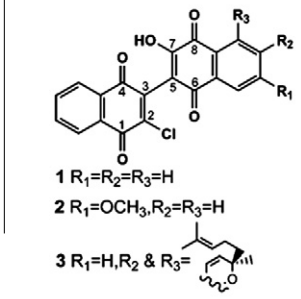
## 3. Material and methods

### 3.1. Chemistry

Binaphthoquinones were synthesized and characterized using previously described methods, and dissolved in DMSO.[8] We already performed and reported a comprehensive study of biological activities and physical properties of halohydroxy monomeric naphthoquinones. None of the monomeric quinones showed anti-viral or anti-neoplastic activities.[2,3,8,25]

### 3.2. Cell lines

Breast cancer cell lines, MCF-7 and MDA-453 were grown in a humidified incubator at 37 °C and 5% $CO_2$ in DMEM media (Life Technology, Rockville, Maryland) containing 10% fetal bovine serum and 1% antibiotics.




ΔE (LUMO-SLUMO) of
Quinone 1: 0.00897 au = 5.63 kcal/mol
Quinone 2: 0.00772 au = 4.84 kcal/mol
Quinone 3: 0.0121 au = 7.59 kcal/mol

au= atomic unit

| | C # | Mulliken Charge | LUMO Coefficient | SLUMO Coefficient |
|---|---|---|---|---|
| Quinone 1 | 1 | 0.3575 | 0.0228 | -0.4051 |
| | 2 | -0.1755 | 0.0375 | **-0.5241** |
| | 3 | -0.0568 | -0.0326 | **0.5345** |
| | 4 | 0.3702 | 0.0012 | **0.5019** |
| | 5 | -0.1845 | 0.0362 | 0.0323 |
| | 6 | 0.3805 | -0.0097 | -0.1191 |
| | 7 | 0.0728 | -0.022 | -0.0024 |
| | 8 | 0.3453 | 0.0832 | 0.0391 |
| Quinone 2 | 1 | 0.3589 | -0.0188 | -0.0958 |
| | 2 | -0.1843 | 0.0126 | **-0.2605** |
| | 3 | -0.0485 | 0.0002 | **0.2815** |
| | 4 | 0.3719 | -0.0911 | **0.1812** |
| | 5 | -0.1919 | -0.3516 | 0.0151 |
| | 6 | 0.3779 | -0.254 | 0.0625 |
| | 7 | 0.0789 | 0.4057 | -0.001 |
| | 8 | 0.3512 | 0.5273 | -0.0004 |
| Quinone 3 | 1 | 0.3546 | 0.3563 | -0.0506 |
| | 2 | -0.1871 | **0.4722** | -0.0209 |
| | 3 | -0.0795 | **-0.4599** | 0.0481 |
| | 4 | 0.369 | **-0.4294** | 0.0556 |
| | 5 | -0.2649 | -0.0083 | 0.0087 |
| | 6 | 0.3931 | -0.1253 | -0.0685 |
| | 7 | 0.1069 | 0.0281 | 0.0007 |
| | 8 | 0.3592 | -0.0098 | 0.1772 |

**Figure 6.** Semi-empirical partial charges and molecular orbital energy calculations for dimeric naphthoquinones using the PM3 Hamiltonian carried out with the Gaussian03 program package. All geometries were initially sketched and cleaned and molecular orbitals were generated and viewed using GaussView 3.09. Geometry optimizations were performed in Gaussian03 using the very tight optimization convergence criteria with no symmetry constraints. The contribution of each of the carbonyl and vinylogous carbons' (C-1–C-8) atomic orbitals into the first and the second lowest unoccupied molecular orbitals are represented as LUMO and SLUMO coefficients.

### 3.3. Cytotoxic activity assays

Cell survival was assessed by MTT assays and trypan blue exclusion.[26] For MTT assays, an equal numbers of cells were plated in three or more replicates and allowed to attach overnight. Cells were then treated with the vehicle (DMSO) or individual solutions of dimeric naphthoquinones at 5, 10, 20 and 50 μM in complete media for 4 h. Following treatment, cells were incubated with 0.5 mg/ml MTT in media for 1 h and then the media was removed and PBS was added. Absorbance was assayed at 570 nm (signal) and 690 nm (background).

### 3.4. ATP measurement

Cells were plated at 10,000 cells per well in a 96-well plate and treated in sextuplet with either DMSO or 5, 10, 20 or 50 μM of dimeric naphthoquinones for 4 h. ATP production was measured using the Cell Titer-Glo Luminescent assay (Promega) and adjusted for viable cell number. The resultant luminescence was normalized to the average number of viable, trypan blue excluding cells per treatment condition.

### 3.5. Reactive Oxygen Species (ROS) measurement

Flow cytometric analysis of ROS production was performed using a FACScan instrument and CellQuest Pro software (Becton Dickinson) as previously described.[11] To measure reactive oxygen species generation, breast cancer cells were preloaded with 5 μM of 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) for 30 min. Cells were then treated with DMSO or 20 μM of naphthoquinones for 2 h, collected and analyzed using an excitation wavelength of 488 nm produced by an argon laser and measuring emission at 530 nm.

### 3.6. Oxygen consumption

Oxygen consumption was measured using a Clark-type oxygen electrode (Oxytherm System; Hansatech Instruments Ltd).[11] Cells were treated with 20 μM dimeric naphthoquinones or DMSO for 3 h. Then the media were removed, the cells were detached using trypsin, washed with PBS and spun down. Then, $5 \times 10^6$ cells in 0.8 mL of PBS containing 10% FCS were placed in a chamber above a membrane that is permeable to oxygen. Oxygen diffuses through the membrane and is reduced at the cathode surface so that a current flows through the circuit, which is completed by a thin layer of KCl solution. The current that is generated bears a direct stoichiometric relation to the oxygen reduced and is converted to a digital signal. Determinations were done in duplicate.

### 3.7. Cyclic voltammetry (CV)

Cyclic voltammetry was performed on a CV 130 system from Gamry Instruments (Warminster, PA, USA).[27] The electrochemical cells were installed in a Faraday cage to reduce externally generated noise in the sensor circuits. The initial time delay before starting CV was 30 s. A three electrode cell (gold disk working electrode (WE), platinum wire counter electrode (CE), and Ag/AgCl reference electrode (RE)[13,28]) was used for all electrochemical experiments. The WE was scanned between −0.5 and +0.5 V or less versus RE. Resolution was set to collect a current data point every 2 mV. Under this condition, the scan rates ranged from 0.1 to 2 V/s as indicated in the data. All solutions were deoxygenated for 10 min by bubbling of nitrogen gas. A blanket of nitrogen was maintained over the solution during all experiments. Test solutions contained 2 mM dimeric naphthoquinones and 0.1 M tetrapropyl ammonium bromide (TPAB) in anhydrous acetonitrile. The potentials of oxidation (anodic $E$, $E_{pa}$) and reduction (cathodic $E$, $E_{pc}$) values were measured. $E_{redox}$ is calculated as $E_{pa} + E_{pc}/2$.[20,21]

### 3.8. Semi-empirical molecular orbital calculations

Semi-empirical molecular orbital calculations using the PM3 Hamiltonian[29] were carried out with the Gaussian03 program package.[30] All geometries were initially sketched and cleaned and molecular orbitals were generated and viewed using GaussView 3.09. Geometry optimizations were performed in Gaussian03 using the very tight optimization convergence criteria with no symmetry constraints.

### 3.9. Statistical analysis

Statistical analyses were performed using Stata® Software version 10.1 (StataCorp, College Station, Tx). All *p*-values are two-sided and those <0.05 were considered as statistically significant.

### Acknowledgments

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### Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.10.005.

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