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Synthesis and evaluation of (±)-dunnione and its *ortho*-quinone analogues as substrates for NAD(P)H:quinone oxidoreductase 1 (NQO1)

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ABSTRACT

Natural product (±)-dunnione (**2**) and its *ortho*-quinone analogues (**3**–**8**) were synthesized and found to be substrates for NQO1. The structure–activity relationship study revealed that the biological activity was favored by the presence of methyl group at the C ring and methoxy group at the A ring. The docking studies supported the rationalization of the metabolic studies. Deeper location in the active site of NQO1, interactions with hydrophobic pocket and C–H...π interactions with the adjacent Phe178 residue contributed to the better catalytic efficiency and specificity to NQO1. Cytotoxicity studies and determination of superoxide ($O_2^{\cdot-}$) production in the presence and absence of the NOQ1 inhibitor dicoumarol confirmed that the *ortho*-quinones exerted their antitumor activity through NQO1-mediated ROS production by redox cycling.

NAD(P)H:quinone oxidoreductase 1 (NQO1) is a ubiquitous flavoenzyme that catalyzes the direct two-electron reduction of quinones to hydroquinones, and it is highly expressed in many solid tumors.[1] This forms the development of efficient quinone substrates as potential NQO1-directed antitumor agents.[2] A number of quinone-based chemotherapeutic agents such as *ortho*-naphthoquinone, *para*-naphthoquinone, *para*-indolequinone, *para*-benzimidazolequinone, and *para*-quinolinequinone derivatives can be bioactivated by NQO1.[3] Compared to the structurally diverse *para*-quinone substrates, the reported *ortho*-quinone substrates for NQO1 are limited. Among them β-lapachone (β-lap, **1**, Fig. 1), a natural tetrahydropyran-fused *ortho*-naphanoquinone isolated from the Bignoniaceae family, is the most prominent and representative example.[3] **1** is currently in multiple phase II clinical trials for the treatment of pancreatic adenocarcinoma.[4,3a] It has been reported to kill many human cancers selectively through rapid reactive oxygen species (ROS) generation mediated by NQO1 bioreduction.[4]

(±)-Dunnione (**2**) is a natural dihydrofuran-fused *ortho*-naphthoquinone with similar *ortho*-quinone moiety as compared to **1** (Fig. 1). Recently, **2** has been reported to exhibit potent anti-*Trypanosoma cruzi* activity[5,6] as well as antitumor activity.[7] However, the molecular target of **2** to elicit its biological effect remains unrevealed. The structural similarity of the dihydrofuran-fused *ortho*-naphthoquinone **2** to **1** prompted us to determine whether NQO1 plays a pivotal role on initiating its antitumor effect. In addition, few studies have investigated the structure–activity relationship (SAR) of the *ortho*-quinones such as **1** and **2** for their NQO1 mediated bioactivity. Thus we planned to explore the preliminary SAR for this series of *ortho*-quinone compounds. Because the quinone pharmacophore (B ring) is considered to be essential for NQO1 reduction, we mainly focus our efforts on investigating compounds with substitution at A ring and C ring in this study.

Compound **2** and its analogues **3**–**8** were synthesized in only three steps (O-allylation, Claisen rearrangement and cyclization) from lawsone[8] (**9a**) or methoxy-substituted lawsone (**9b**–**9d**). O-allylation of **9a** (3,3-dimethyl allyl bromide, K_2CO_3, DMF) gave 3,3-dimethyl-allyl ether (**10a**) in 71% yield. Claisen rearrangement of **10a** (120 °C, toluene) gave 3,3-dimethyl allyl lawsone (**11a**) (82%). **11a** was cyclized to dunnione (**2**) mediated by Lewis acid $NbCl_5$ at room temperature in 85% yield (Scheme 1). Similarly,

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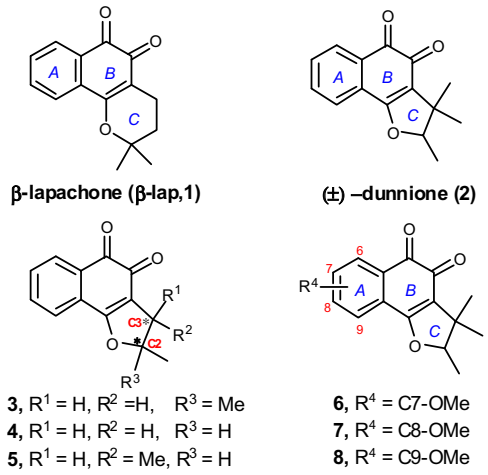


Figure 1. Structures of β-lap, dunnione and analogues of dunnione.

compounds **3**–**8** were prepared by analogous routes in similar yields. Most of these compounds were obtained with a mixture of stereoisomers, and compound **4** was mainly in its *trans*-isomer The steric configuration of structural formula was shown in the Supporting information (Fig. S2).

After achieving the synthesis, the ability of NQO1 to process these *ortho*-quinones (**2**–**8**) in vitro was assessed as previously reported.[3a] From the results, Michaelis–Menten curves were generated (Fig. 2), and the NQO1 kinetic parameter (k_{cat}/K_m) was calculated (Table 1). The highest value of the NQO1 kinetic parameter (k_{cat}/K_m) reflects the highest catalytic efficiency and specificity. As shown in Table 1, it was observed that **4** ($k_{cat}/K_m = 0.8 \pm 0.2 \times 10^6\ M^{-1}\ s^{-1}$) showed worse catalytic efficiency and specificity than the control β-lap ($k_{cat}/K_m = 2.6 \pm 0.5 \times 10^6\ M^{-1}\ s^{-1}$), most likely because of the impact of C ring narrowing on binding of *ortho*-quinone in the active site of NQO1. To further probe the effect, we examined the *ortho*-quinone with two methyl groups (**3** and **5**) and three methyl groups (**2**) at the C ring. It was found that compounds with more substituted methyl groups were more specific toward enzyme. Especially for **2**, which had three methyl groups at C ring, showed efficient catalyzed reduction rate ($k_{cat}/K_m = 1.9 \pm 0.4 \times 10^6\ M^{-1}\ s^{-1}$). Compounds (**6**–**8**) with methoxy group at the A ring showed better enzyme catalytic efficiency and specificity than **2**, indicating that methoxy-substitution was tolerable for NQO1 substrates. Among them, compound **7** ($k_{cat}/K_m = 3.1 \pm 0.5 \times 10^6\ M^{-1}\ s^{-1}$) was the most efficient substrate, which possessed slightly better kinetic parameters than the control β-lap. It indicated that the introduction of methoxy group at the A ring improved the catalytic efficiency and specificity of these *ortho*-quinones.

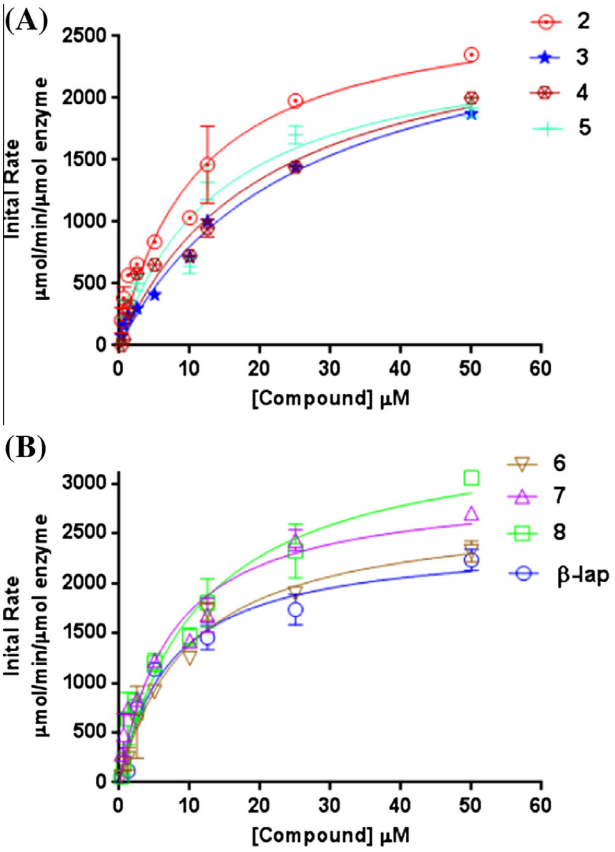
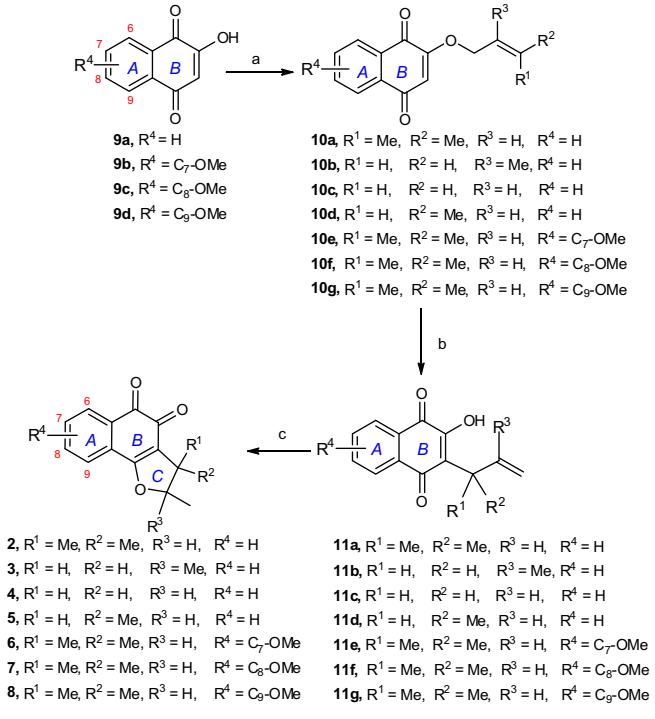


Figure 2. Michaelis–Menten curves for the *ortho*-quinone substrates for NQO1. (A) Michaelis–Menten curves for compounds **2**–**5**. (B) Michaelis–Menten curves for compounds **6**–**8** and the control β-lap.

Table 1
Kinetic and computation parameters for the dihydrofuran-fused *ortho* naphthoquinones and β-lap

Compd	k_{cat}/K_m ($10^6\ M^{-1}\ s^{-1}$)	ChemScore[a]	C=O5...NH5[a] (Å)
2	1.9 ± 0.4	70.52 (69.82)	3.52 (3.64)
3	1.0 ± 0.2	66.98	3.59
4	0.8 ± 0.2	66.02 (65.50)	3.60 (3.91)
5	1.4 ± 0.5	70.92 (68.98)	3.57 (3.72)
6	2.0 ± 0.4	71.88 (70.23)	3.41 (3.97)
7	3.1 ± 0.5	72.57 (70.29)	3.33 (3.88)
8	2.4 ± 0.6	69.37 (67.01)	3.45 (3.66)
β-Lap (**1**)	2.6 ± 0.5	69.43	3.31

[a] The computation parameters of the C2-*R*-isomer; the C2-*S*-isomer was shown in parentheses.



Scheme 1. Reagents and conditions: (a) substituted allyl bromide, K_2CO_3, DMF, 45 °C, 57–72%; (b) toluene, 120 °C, 63–90%; (c) $NbCl_5$, DCM, rt, 70–85%.

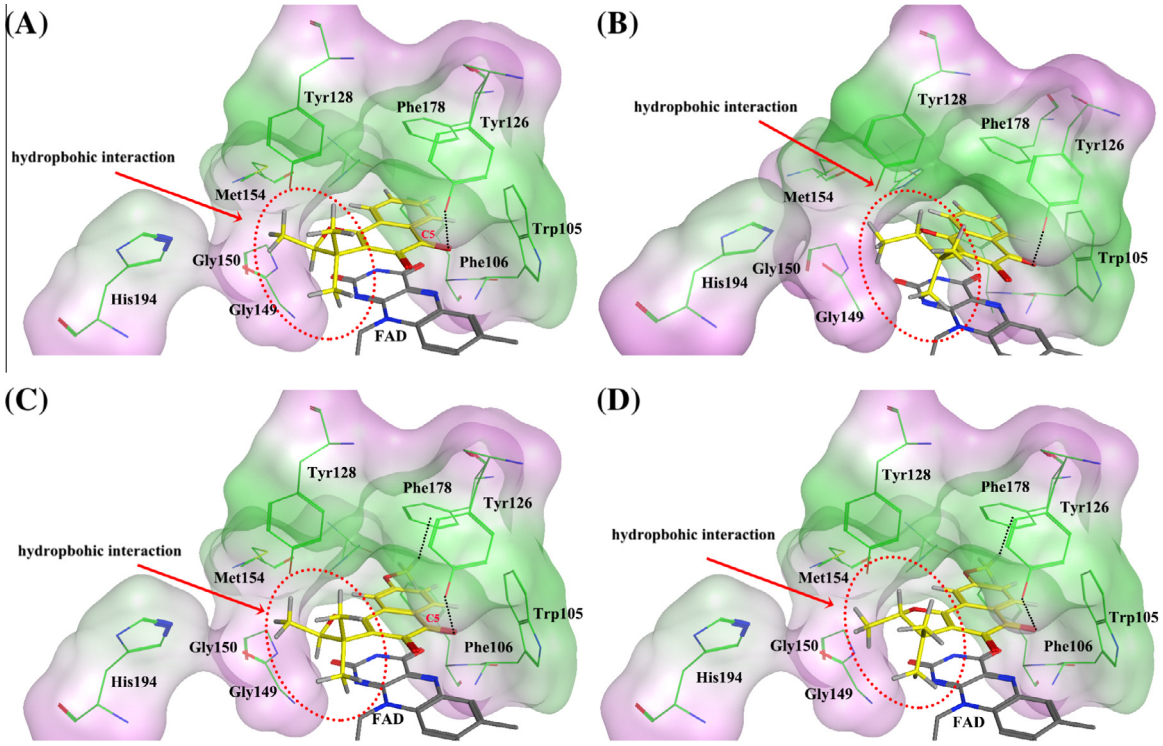


Figure 3. Docked conformation of compounds **2** C2-*R*-isomer (A), **2** C2-*S*-isomer (B), **7** C2-*R*-isomer (C) and **7** C2-*S*-isomer (D) into the active binding site of NQO1. The interaction mode was obtained through molecular docking (PDB ID: 1DXO) and depicted using MOE 2013.08. The carbon atoms of the compounds and the key residues in the active site of NQO1 were colored in yellow and green, respectively. The H-bonds and H–π-bonds were shown as black dot lines.

To elucidate the possible binding mode of NQO1 with the *ortho*-quinones, a molecular docking study was performed based on the crystal structure of NQO1 (PDB code 1DXO). All of the compounds were found to form π-stacking interactions with the isoalloxazine ring of FAD which was very similar to the parent duroquinone.[9] A correlation was previously observed between the substrate (quinone carbonyl) and the FAD cofactor (atom N5 which transfer the hydride), with compounds that have a shorter predicted hydride donor–acceptor distance being better NQO1 substrates.[10] Therefore, it was necessary to calculate and analyse the corresponding distances between the *ortho*-quinones and FAD. As shown in Table 1, all were within a reasonable distance (about 4 Å) for hydride transfer, suggesting that they were potential substrates for NQO1. Notably, compounds with methoxy substituent at the C ring such as **7** (distance 3.33 Å, Table 1) fitted deeper into the active site compared to **2** (distance 3.52 Å, Table 1), providing a rationale for the better catalytic efficiency and specificity of the compounds with methoxy substituent at the A ring on one hand. The binding modes of these *ortho*-quinones with NQO1 were carefully analysed and the results for representative compounds **2** and **7** were shown in Figure 3 (Other compounds see Supplementary data, the representative conformations were selected). As shown in Figure 3, the methyl groups at the C ring can fit into the hydrophobic pocket formed by Tyr128, Gly149 and Gly150. It explained the fact that **2** which contained three methyl substituents exhibited higher NQO1 catalytic efficiency and specificity than other compounds (**3**–**5**) with fewer methyl substituents at the C ring. Particularly, as for compounds **7** and **8** with methoxy group at the C-8 or C-9 position of the A ring, C–H…π interaction with the adjacent Phe178 residue was observed for enhancing π-stacking interaction with surrounding residues, supporting that they would be excellent substrates for NQO1 from the other hand (Fig. 3B). It should be noted that most of the synthesized compounds (**2**, **4**–**8**) are mixtures of stereoisomers. The docked structures were further analysed relative to the stereoisomers of these compounds (take compound **1** as examples), observed as the likely products of chemical synthesis (Fig. 3). For both C2-*R*-isomer and C2-*S*-isomer, the substituted methyl (C ring) can be placed into the pocket formed by Tyr128, Gly149 and Gly150. The Gold5.1 ChemScore scores for the *R* and *S* isomers are 70.57 and 69.82, respectively, favouring the *R*-isomer slightly. As for the corresponding distances between the *ortho*-quinones and FAD, C2-*R*-isomer (distance 3.52 Å, Table 1) was also fitted slightly deeper into the active site than the C2-*S*-isomer (distance 3.64 Å, Table 1), but the difference is not significant. The docking results indicated that both isomers can be catalyzed by NQO1 which is consistent with the result of the indistinguishable kinetic data of these isomers from enzymatic assays. The docking poses of the *R*, *S*-isomers of the representative compounds (**1** and **7**) were shown in Figure 3.

Cytotoxicity studies and determination of ROS production were also performed on all of the naphthoquinones. Cell survival was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT assay. We utilized the human non-small

Table 2
Cytotoxicity of all of the naphthoquinones toward A549 cells in the presence and absence of the NQO1 inhibitor (DIC, 25 μmol L^{-1})

Compd	Cytotoxicity IC_{50} (μmol L^{-1})		Selectivity ratio IC_{50} (A549 + DIC)/IC_{50} (A549)
	A549	A549+DIC	
2	6.1 ± 1.2	24 ± 5.9	3.9
3	10 ± 6.5	20 ± 6.0	2.0
4	7.7 ± 0.8	30 ± 3.5	3.8
5	7.0 ± 1.3	17 ± 2.1	2.4
6	9.2 ± 2.2	34 ± 5.9	3.7
7	6.8 ± 1.1	41 ± 4.9	6.0
8	16 ± 5.0	79 ± 4.3	4.9
β-lap (**1**)	7.0 ± 0.5	25 ± 2.3	3.6

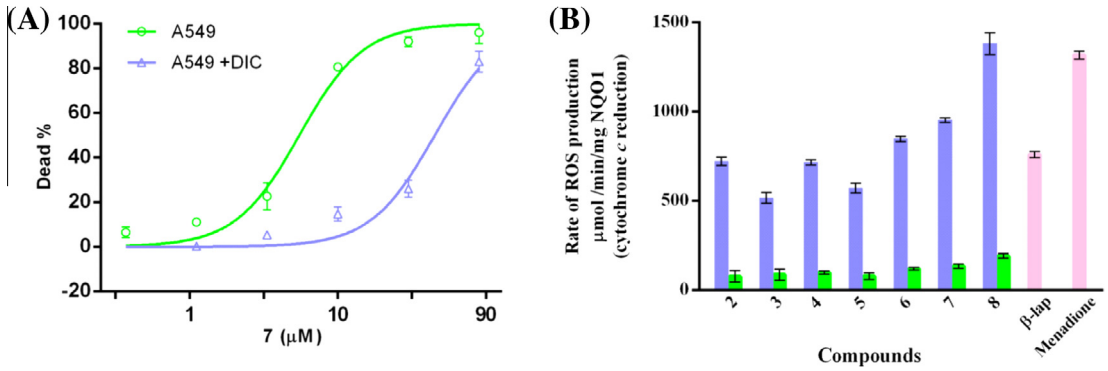


Figure 4. (A) Cell death of A549 Cells treated for 2 h with compound **7** in the presence and absence of the NQO1 inhibitor (DIC, 25 μM). (B) Rate of ROS production of all of the naphthoquinones (**2**–**8**) in the absence (the blue bars) and presence (the green bars) of the NQO1 inhibitor (DIC, 25 μM) with NQO1. Rate of ROS production of β-lap and menadione (the pink bars) in the absence of DIC were showed as control. The rates expressed mean ± SD, P <0.001 versus control (n = 3).

cell lung cancinoma A549 cell lines in the absence and presence of the NQO1 inhibitor dicoumarol (DIC, 25 μmol L^{-1}) to compare the cytotoxicity of the *ortho*-quinones (Table 2). DIC has been widely used in studies of NQO1-mediated cell death, as incubation of cells with the inhibitor was effective in blocking the enzymatic activity of NQO1.[11] As illustrated in Table 2, coincubation with DIC protects A549 cells from the cell death mediated by these *ortho*-quinones, shifting the IC_{50} from 2-fold to 6-fold. (The fold is the ratio of the IC_{50} of cotreatment with *ortho*-quinones and DIC to the IC_{50} of treatment with only *ortho*-quinones, and a higher ratio indicates greater protection and greater NQO1 specificity). Compounds (**6**–**8**) with methoxy group at the A ring of **2** even showed better toxic selectivity to NQO1-rich A549 cell lines (selectivity ratio ⩾3.7) compared to the control β-lap (select ratio = 3.6). The results suggested that the *ortho*-quinones exerted their antitumor activity by an NQO1-dependent mechanism. As more clearly shown in Figure 4A, coincubation with DIC greatly protected A549 cells from the cell death mediated by the best compound **7**, shifting the IC_{50} = 6-fold.

In addition, NQO1 substrates have been suggested to exert their antitumor activity through rapid ROS generation.[4] We thus further investigated the ability of these compounds to produce ROS. As we previously reported, menadione was used as positive control.[3a] The production of the superoxide ($O_2^{\cdot-}$), the main constitute of ROS, was measured by a spectrophotometric assay with cytochrome *c* as the terminal electron acceptor.[3a] The initial rates (μmol cytochrome *c* reduced/min/mg NQO1) were calculated from the liner portion (0–30 s) of the reduction graphs. The highest value represented it can produce maximum amount of ROS. As shown in Figure 4B, compounds **2**, **3**, **4** and **5** showed similar rates of ROS generation to β-lap. However, compounds **6**, **7** and **8** showed high rates of ROS generation, being much more efficient than β-lap. In fact, the rate of ROS production for compound **8** (1380 ± 120 μmol cyt *c*/min/mg NQO1) was even shown better than the positive control menadione (1315 ± 25 μmol cyt *c*/min/mg NQO1). The results indicated that these *ortho*-quinone substrates could generate a high amount of ROS via NQO1-directed redox cycling. These compounds were further tested in the presence of DIC (25 μmol L^{-1}) for its rate of ROS production. As illustrated in Figure 4B, coincubation with DIC dramatically reduced the rate of ROS production by these compounds.

In summary, natural product **2** and its several novel *ortho*-quinone analogues were synthesized and evaluated as efficient substrates for NQO1. The preliminary structure–activity relationship (SAR) study revealed that the metabolic rates for NQO1 were favored by the presence of methyl group at the C ring and methoxy group at the A ring. Molecular modelling was used to analyse the interactions between the *ortho*-quinones and NQO1. Our docking studies supported the rationalization of the metabolic studies. Deeper location in the active site of NQO1, interactions with hydrophobic pocket and C–H…π interaction with the adjacent Phe178 residue provided the rationale for the better catalytic efficiency and specificity of compound **7** (named as **DDO-7102**). The evaluation of the antitumor activity in human non-small cell lung carcinoma A549 cell lines in the absence and presence of the NQO1 inhibitor DIC provided **7** as lead, with a better toxic selectivity than the parent drug **2**. Determination of superoxide ($O_2^{\cdot-}$) production in the presence and absence of the NQO1 inhibitor dicoumarol confirmed that these *ortho*-quinones exerted their antitumor activity through NQO1-mediated ROS production by redox cycling. These findings encourage further investigations of the novel dihydrofuran-fused *ortho*-naphthoquinones as NQO1-directed antitumor agents.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.01.057.

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