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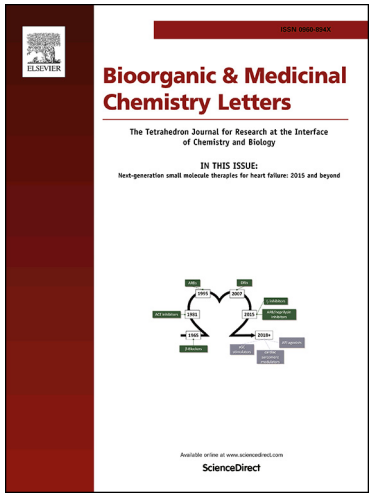

Investigation of Chemical Reactivity of 2-Alkoxy-1,4-naphthoquinones and their Anticancer Activity

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PII: S0960-894X(18)30372-X
DOI: https://doi.org/10.1016/j.bmcl.2018.04.060
Reference: BMCL 25807

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 22 February 2018
Revised Date: 20 April 2018
Accepted Date: 25 April 2018

Please cite this article as: Manickam, M., Reddy Boggu, P., Cho, J., Nam, Y.J., Lee, S.J., Jung, S-H., Investigation of Chemical Reactivity of 2-Alkoxy-1,4-naphthoquinones and their Anticancer Activity, *Bioorganic & Medicinal Chemistry Letters* (2018), doi: https://doi.org/10.1016/j.bmcl.2018.04.060

Investigation of Chemical Reactivity of 2-Alkoxy-1,4-naphthoquinones and their Anticancer Activity

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Abstract

To establish the structure-activity relationship of 5-hydroxy-1,4-naphthoquinones toward anticancer activity, a series of its derivatives were prepared and tested for the activity (IC_{50} in μM) against three cell lines; colo205 (colon adenocarcinoma), T47D (breast ductal carcinoma) and K562 (chronic myelogenous leukemia). Among them **2** (IC_{50}: 2.3; 2.0; 1.4 μM), **6** (IC_{50}: 1.9; 2.2; 1.3 μM), **9** (IC_{50}: 0.7; 1.7; 0.9 μM) and **10** (IC_{50}:1.7; 1.0; 1.2 μM) showed moderate to excellent activity. Our perception toward the DNA substitution of alkoxy groups at the C2 position of these naphthoquinones for the anticancer activity led us to investigate their reactivity of substitution toward dimethylamine as a nucleophile. The ease of the substitution of alkoxy groups at the C2 position with dimethylamine is strongly accelerated by hydroxyl group at C5 position and is well correlated with the found anticancer activity results.

Keywords: 2-Alkoxy-5-hydroxy-1,4-naphthoquinone, anticancer activity, chemical reactivity.

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Cancer is a major health problem worldwide and the second most common cause of death.[1] The number of people living beyond a cancer diagnosis reached nearly 14.5 million in 2014 and is expected to rise to almost 19 million by 2024.[1] Screening for new anticancer drugs is still important and is one of the major goals in medicinal chemistry.[2] Mass screening programs of natural products by the National Cancer Institute have identified the quinone moiety as an important pharmacophoric element for cytotoxic activity.[3] Quinones are widely distributed in nature,[4] and are represented in many clinically used drugs (e.g. doxorubicin, daunorubicin, mitroxantrone, mitomycin-C (Fig.1)).[5,6] Two major mechanisms have been proposed for the anticancer action of quinones in a variety of cell systems. First, quinones undergo one electron reduction by enzymes such as microsomal NADPH-cytochrome P-450 reductase or mitochondrial NADH-ubiquinone oxidoreductase, yielding the corresponding semiquinone radicals.[7,8] Under aerobic conditions, the semiquinone radical then participates in redox cycling to generate superoxide anion, all of which are believed to be responsible for most of the drug activity.[9,10] Second, quinones are potent electrophiles, capable of reacting with the thiol groups in proteins as well as GSH.[11,12] Depletion of GSH has been associated with menadione-induced cytotoxicity.[11,13] In addition, cytotoxic quinone derivatives were proven to be topoisomerase I and II inhibitors.[14] Among the quinones, 1,4-naphthoquinones have been found to possess a diverse range of biological activities, in particular, they exhibit potent anticancer activity.[15-19] For example, starting from simple menadione to calothrixins A and B (Fig. 1), they exhibit potent anticancer activities.[20,21] The attachment of variety of functional groups such as amines, esters, sulfonamides, to the 1,4-naphthoquinone system are proven to improve their anticancer activities.[22-25] In a similar fashion, the fusion of many heterocycles to 1,4-naphthoquinone system such as furan, pyran etc., have also been synthesized and checked for anticancer

activity.[26] An interesting subgroup of 1,4-naphthoquinones is 5-hydroxy-1,4-naphthoquinones. For e.g. juglone and plumbagin possess anticancer property (Fig. 1).[27-30] Some of the esters attached to 5-hydroxy-1,4-naphthoquinone have also been prepared and showed anticancer activity.[31]

Since 5-hydroxy-1,4-naphthoquinones are not well explored for its anticancer activity, we were interested to design and synthesis a series of 5-hydroxy-1,4-naphthoquinone analogs and establish its structure-activity relationship.

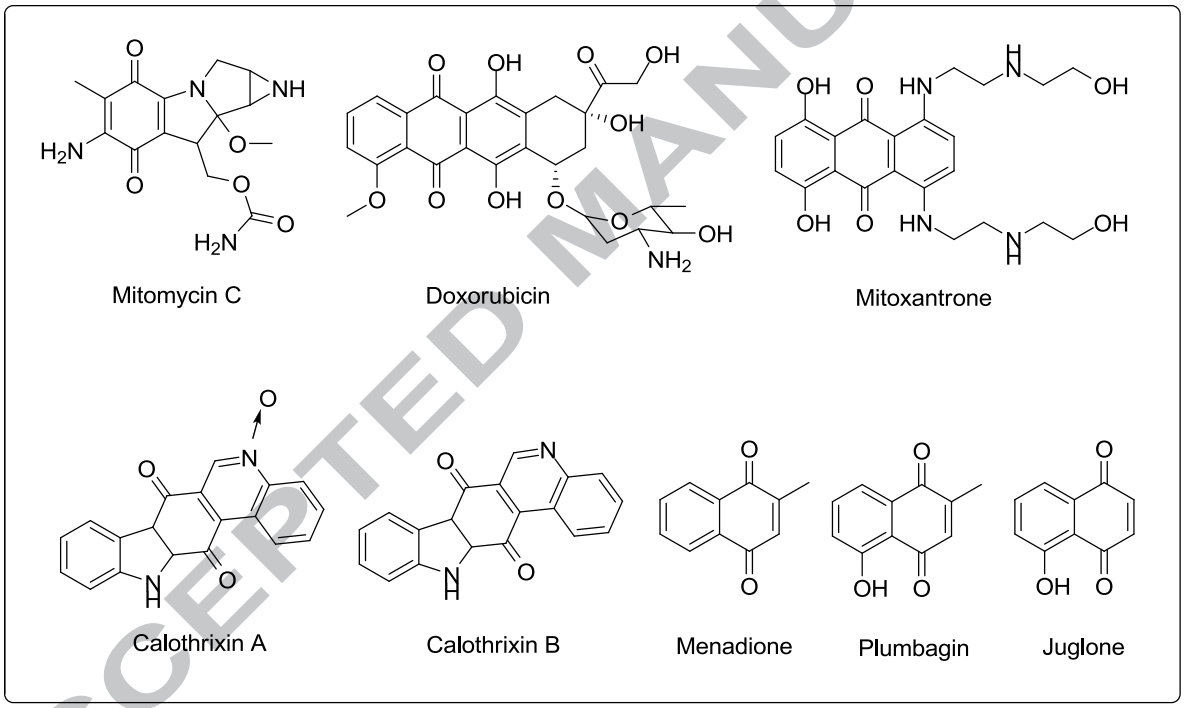
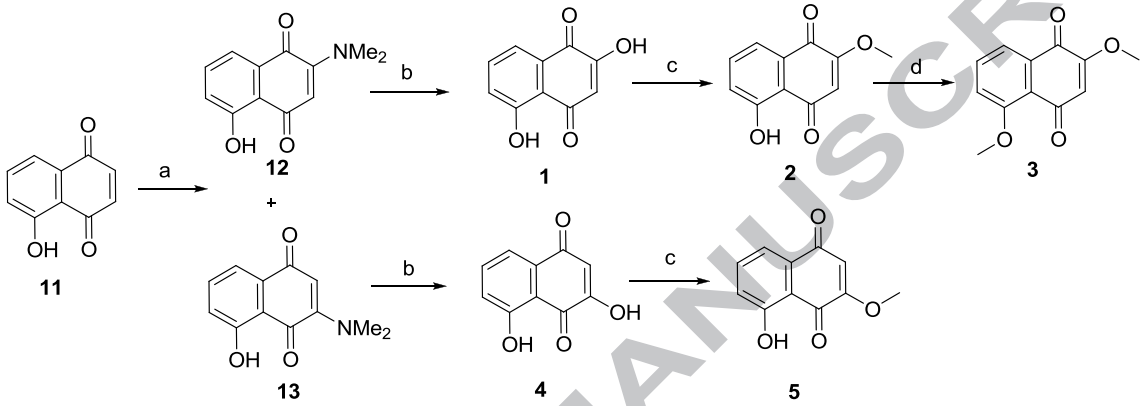


Figure 1. Anticancer 1,4-naphthoquinones and 5-hydroxy-1,4-naphthoquinones

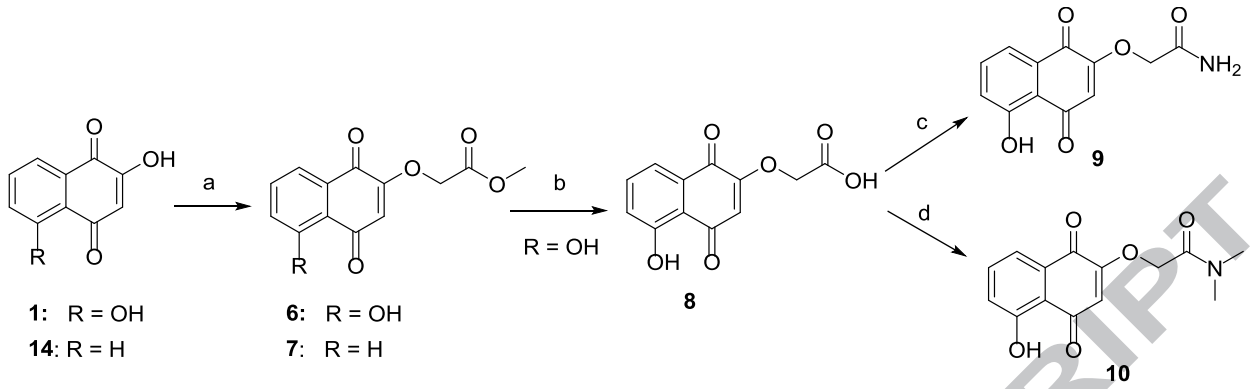
Scheme 1 represents the preparation of compounds **1-5**. Compounds **1** and **4** were prepared according to the literature procedure with slight modifications.[32] The commercially available juglone (**11**) was reacted with dimethylamine at -20 °C to obtain **12** as major product and **13** as

minor product. Both the products upon refluxing with 10% HCl yielded the corresponding **1** and **4**. Compounds **1** and **4** on methylation with methyl iodide in presence of sodium carbonate yielded **2** and **5**, respectively. The dimethoxy derivative **3** was prepared by the methylation of **2** with methyl iodide using potassium carbonate as base.



Scheme 1. Preparation of compounds **1-5.** *Reagents and condition:* a) Dimethylamine (2M in THF), toluene, -20 °C, 24 h b) 10% HCl, dioxane, reflux, 5 h c) CH_3I, Na_2CO_3, DMF, RT, 10 h d) CH_3I, K_2CO_3, DMF, RT, 15 h.

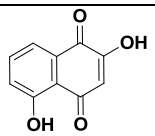
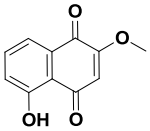
The synthesis of compounds **6-10** is denoted in Scheme 2. The prepared compound **1** (R = OH) and the commercially available compound **14** (R = H) on reaction with methyl bromoacetate using sodium carbonate as base afforded the corresponding C2-O-alkylated analogs **6** and **7**. The occurrence of O-alkylation at C2-position of **1** to form **6** was supported by the literature.[31] Further it was substantiated by the assignment through NOESY spectrum. The one proton singlet at 3rd position (H-3) of the naphthoquinone appears at δ 5.99 ppm. The OCH_2 proton at 2nd position appears δ 4.75 ppm and has a correlation with H-3 at δ 5.99 ppm as shown in the NOESY spectrum (see supporting information).

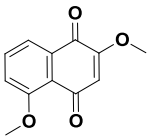
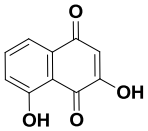
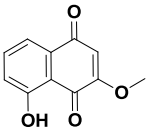
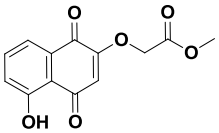
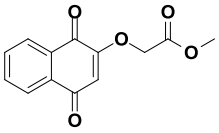
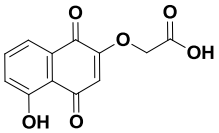
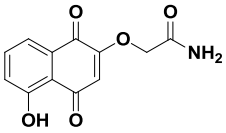
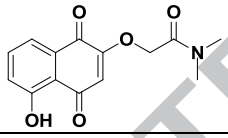


Scheme 2. Preparation of compounds **6-10.** *Reagents and condition:* a) $BrCH_2COOCH_3$, Na_2CO_3, DMF, 0 °C to RT, 5 h b) 3M H_2SO_4, 10 h, RT c) (i) $SOCl_2$, DCM, reflux, 2 h (ii) NH_4OH, 0.5 h, RT d) dimethylamine (2M in THF), DCC, HOBT, THF -5 °C to RT, 8 h

The hydrolysis of the methyl ester of **6** to its carboxylic acid **8** was carried out using 3*M* sulfuric acid at ambient temperature. The carboxylic acid **8** was converted to its amide **9** by refluxing with thionyl chloride to get the acid chloride followed by the reaction of ammonium hydroxide solution. In another reaction, **8** was reacted with *N,N*-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) followed by the addition of dimethylamine to get the dimethylamine analog **10**.

Table 1. 5-Hydroxy-1,4-naphthoquinones (1-10) and their anticancer activity

No.	**Compound**	**colo205** (colon adenocarcinoma) **IC_{50} (µM)**	**T47D** (ductal carcinoma) **IC_{50} (µM)**	**K562** (chronic myelogenous leukemia) **IC_{50} (µM)**
1[32]		>10	>10	>10
2		2.3	2.0	1.4

3		>10	>10	>10
4[32]		>10	>10	>10
5		>10	>10	>10
6		1.9	2.2	1.3
7		>10	>10	>10
8		>10	>10	>10
9		0.7	1.7	0.9
10		1.7	1.0	1.2

The naphthoquinones **1-10** were evaluated for their *in vitro* inhibitory activities against three human cancer cell lines (colo205 (colon adenocarcinoma) T47D (ductal carcinoma) and K562 (chronic myelogenous leukemia). Colo205, T47D and K562 cell lines were purchased from the American Type Culture Collection and regularly tested to confirm the absence of mycoplasma contamination using an e-Myco™ Mycoplasma PCR detection Kit (iNtRON Biotechnology). Cells were maintained under RPMI media with 10% fetal bovine serum, 1% penicillin/streptomycin, and 2 mM L-glutamine. Cells were seeded in 96-well plates, incubated

for 24 h, and then exposed to compounds at the dose of 0.3, 1, 3, and 10 μM for 72 h. Cell viability was measured with CellTiter Glo® assay following to the manufacturer's instructions (Promega) and calculated relative to vehicle (0.1% DMSO)-treated controls. IC_{50} was determined with standard curve analysis from SigmaPlot ver.12.0 as described previously.[33] Doxorubicin (Sigma) was used as positive control. Independent three experiments were performed, each in triplicate. The results are listed in Table 1 as concentrations required for 50% growth inhibition ($IC_{50,}$ μM).

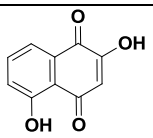
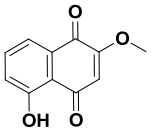
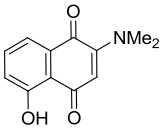
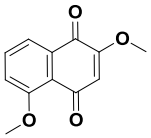
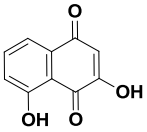
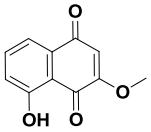
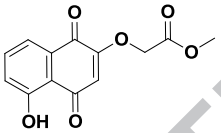
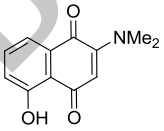
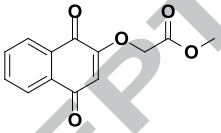
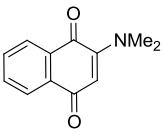
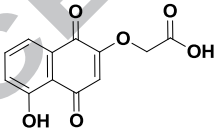
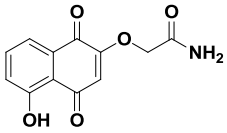
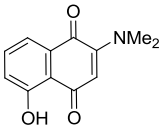
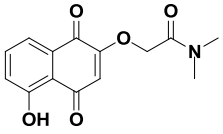
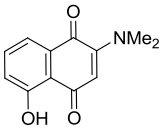
IC_{50} values are listed in parentheses below along with this sequence of these cell lines written here (colo205 (colon adenocarcinoma), T47D (breast ductal carcinoma) and K562 (chronic myelogenous leukemia).

2,5-Dihydroxynaphthoquinone (**1**) did not show any activity (>10; >10; >10) whereas the change of 2-hydroxy group to 2-methoxy group as shown in compound **2** (2.3; 2.0; 1.4) fetched good activity. The reason could be visualized such that the existence of the tautomerism of the 2-hydroxy group to its keto group in **1** disfavors the nucleophilic substitution of DNA, whereas in case of **2**, DNA can readily substitute the 2-methoxy group, thus showing anticancer activity. The change of 5-hydroxy group to 5-methoxy group in compound **2** as shown in compound **3** (>10; >10; >10) abolished the activity which shows the importance of 5-hydroxy group for the anticancer activity in this naphthoquinone series. The change of 2-hydroxy group to the next position as shown in compound **4** (>10; >10; >10) or its methylation to methoxy group as shown in compound **5** (>10; >10; >10) did not show any activity. From these results, it is evident that the methoxy group and the hydroxy group in the naphthoquinone should be placed in 2 and 5 positions respectively.

In the next series, the 2-methoxy group was changed to 2-methoxycarbonylmethyoxy group as represented in compound **6** (1.9; 2.2; 1.3) for increasing the electrophilicity of naphthoquinone moiety toward DNA substitution. The activity was similar to that of compound **2** in all the three cell lines. The removal of 5-hydroxy group from compound **6** as shown in compound **7** (>10; >10; >10) abolished the anticancer activity which once again proved the importance of 5-hydroxy group. The change of the ester group of **6** to carboxylic acid as shown in compound **8** (>10; >10; >10) did not give the activity, probably due to its poor cell permeability. Next the replacement of carboxylic acid **8** to its amide functionality as demonstrated in compound **9** (0.7; 1.7; 0.9) gained potent activity. Methylation of the amide of **9** to its *N*,*N*-dimethyl analog as shown in compound **10** (1.7; 1.0; 1.2) also retained the activity.

Naphthoquinones were also reported as potential bioreductive alkylating agents and inhibits the synthesis of DNA in neoplastic cells.[34] The nucleophilic substitution of DNA to the C2 position of the naphthoquinone favored by the hydroxy group at C5 position was envisioned as one of the reason for the anticancer activity in this naphthoquinone series. To support this view, dimethyl amine was reacted with the prepared compounds **1-10** and the results are tabulated in Table 2.

Table 2. Reactivities of the naphthoquinones with dimethylamine

No.	Reactant	Product	* Reaction conditions	Time period	Yield %
1		no reaction	THF or EtOH, -10 °C to 60 °C	>48 h	---
2			THF or EtOH, -10 °C	10 h	>95
3		no reaction	THF or EtOH, -10 °C to 60 °C	>48 h	---
4		no reaction	THF or EtOH, -10 °C to 60 °C	>48 h	---
5		no reaction	THF or EtOH, -10 °C to 60 °C	>48 h	---
6			THF or EtOH, -10 °C	2-3 h	>95
7		 **15**	THF or EtOH, -10 °C	24 h	>95
8		precipitation due to salt formation	THF or EtOH, -10 °C	---	---
9			THF or EtOH, -10 °C	30-40 min	>95
10			THF or EtOH, -10 °C	30-40 min	>95

* *Procedure:* Naphthoquinones **1-10** was dissolved in THF or EtOH. Dimethylamine (2M inTHF, 1.1equivalent) was added to the reaction mixture at -10 °C. If the reaction did not proceed, the temperature was gradually increased upto 60 °C. The resulting mixture was evaporated under vacuum and analyzed.

Compound **1** did not react with dimethyl amine even at 60 °C, whereas compound **2** reacted at -10 °C and the reaction was completed after 10 h. This result indicates that the presence of 2-methoxy group in **2** favors substitution with dimethyl amine whereas the 2-hydroxy group in **1** tautomerizes to keto form and disfavors the substitution reaction. Similarly compounds **3**, **4**, **5** did not react with dimethyl amine which clearly denote the importance of location of 5-hydroxy group from the leaving group to facilitate the substitution reaction at 2nd position holding alkoxy group such as methoxy group (Fig 2).

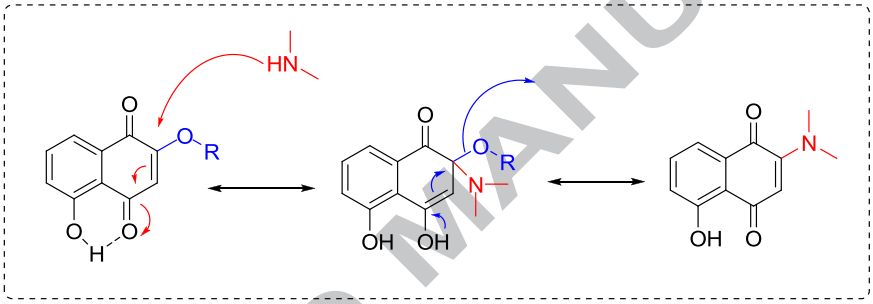


Figure 2. Substitution reaction of dimethylamine with naphthoquinone

This statement is further supported by the reaction of compound **6** with dimethylamine which only took 2-3 h for completion. The importance of 5-hydroxy group for the substitution reaction at 2nd position was once again proved from the reaction of compound **7** with dimethylamine as it took 24 h for completion. The reaction of dimethylamine with compound **8** holding a carboxylic acid group resulted in precipitation, undoubtedly due to salt formation. Finally, the reactions of compounds **9** and **10** with dimethyl amine resulted in products within 30-40 minutes. This result proves the presence of amide functionality over ester group favors the substitution reaction at C2 position. The reactivity sequence of the naphthoquinones **1-10** with dimethylamine is well correlated with its anticancer activity along all the three cell lines.

In conclusion, a series of 5-hydroxy-1,4-naphthoquinone analogs have been prepared to establish the structure-activity relationship for the anticancer activity (IC_{50} in µM) against three cell lines; colo205 (colon adenocarcinoma), T47D (breast ductal carcinoma) and K562 (chronic myelogenous leukemia). Among them 5-hydroxy-2-methoxynaphthalene-1,4-dione (**2,** 2.3; 2.0; 1.4 µM), methyl 2-(5-hydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yloxy)acetate (**6,** 1.9; 2.2; 1.3 µM), 2-(5-hydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yloxy)acetamide (**9,** 0.7; 1.7; 0.9 µM) and 2-(5-hydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yloxy)-*N*,*N*-dimethylacetamide (**10,** 1.7; 1.0; 1.2 µM) showed moderate to excellent activity. Hydroxyl group at 5th position is crucial for the activity. Hydrophobic alkoxyl group at C2 position such as methoxy, methoxycarbonylmethoxyl or amidomethoxyl is important. Our speculation towards the DNA substitution at the C2 position of this naphthoquinone for the anticancer activity directed us to react dimethylamine as a nucleophile with the naphthoquinones **1-10**. The ease of the substitution of the alkoxy groups present in the C2 position of naphthoquinones with dimethylamine is strongly supported by hydroxyl group at C5 position and is well correlated with the anticancer activity results (Fig.3). The observed results were considered as preliminary and the active compounds **9** and **10** can be taken as lead to find more potent 5-hydroxy-2-substituted naphthoquinones as novel anticancer agents.

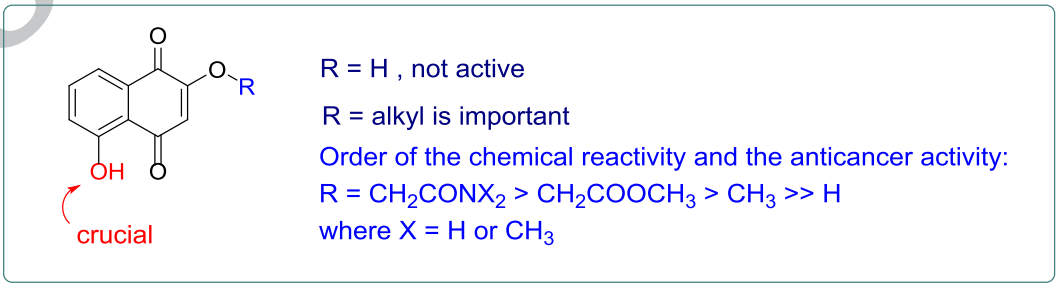


Figure 3. Structure activity relationship of 5-hydroxy-1,4-naphthoquinone analogs

Acknowledgment

This work was supported by Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0093815).

Supplementary data

The detailed experimental part of this article can be found in the Supplementary data.

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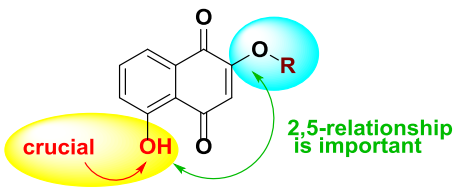
Highlights

- SAR of 1,4-naphthoquinones toward anticancer activity was established.
- C2-Alkoxy and C5-hydroxyl is crucial for activity.
- The substitution of alkoxyl at the C2 with amine is accelerated by hydroxyl at C5.
- The chemical reactivity of these analogs is correlated with their anticancer activity.

Graphical abstract:

Investigation of Chemical Reactivity of 2-Alkoxy-1,4-naphthoquinones and their Anticancer Activity



R	IC_{50} (μM) colo205	T47D	K562
H	> 10	> 10	> 10
CH_3	2.3	2.0	1.4
CH_2COOCH_3	1.9	2.2	1.3
CH_2COONH_2	0.7	1.7	0.9
$CH_2COON(CH_3)_2$	1.7	1.0	1.2

The substitution reactivity of alkoxy group at position 2 is parallel to anticancer activity.