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Naphthazarin Derivatives: Synthesis, Cytotoxic Mechanism and Evaluation of Antitumor Activity

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The rate of the GSH conjugate formation, the inhibition of DNA topoisomerase-I and the cytotoxic activity against L1210 cells of the naphthoquinones showed the same order; 5,8-dimethoxy-1,4-naphthoquinone (DMNQ) > 6-(1-hydroxyethyl)-DMNQ > 2-(1-hydroxyethyl)-DMNQ; the steric hindrance of the substituents, particularly 2-substutuent, in reacting with cellular nucleophiles must be the main cause for lowering the bioactivities. Acetylation of 2-(1-hydroxyethyl)-DMNQ producing 2-(acetyloxyethyl)-DMNQ potentiated the bioactivities; 2-(1-hydroxyethyl)-DMNQ did not react with GSH and the enzyme, and showed ED_{50} of 0.680 μg/ml, whereas the values of 2-(1-acetyloxyethyl)-DMNQ were the conjugate formation of 0.14 μM, IC_{50} value of 81 μM for the enzyme inhibition and ED_{50} of 0.146 μg/ml for the cytotoxcity. Furthermore, the acetylation 2-(1-hydroxyethyl)-DMNQ (T/C, 119%) enhaced the T/C values for the mice bearing S-180 tumor [T/C of 2-(1-acetyloxyethyl)-DMNQ, 276%]. It was assumed that the difference in bioactivities ensued by acetylation was based on the mechanism of the so-called bioreductive alkylation.

Key words : 1,4-Naphthoquinone derivatives, Formation of glutathione conjugate, Antitumor activity, Inhibition of DNA topoisomerase-I, Structure-activity relationship, Steric hindrance, Bioreductive activation

INTRODUCTION

Shikonin derivatives isolated from the root of *Lithospermum erythrorhizon* showed a strong cytotoxic activity against L1210 and a good antitumor activity in mice bearing S-180 cells (Sankawa *et al.*, 1977 & 1981; Kim *et al*, 1990). The pharmacophoric moiety of the shikonin structure was found to be 5,8-dihydroxy-1,4-naphthoquinone (naphthazarin), which is positioned in the structure of tricyclic anthracycline antitumor agents such as mitoxanthrone and doxorubicin. Naphthazarin itself showed more potent cytotoxicity against L1210 cells and more toxicity in mouse than shikonin (Kim *et al.*, 1990).

Quinone's cytotoxic activity has been explained by two major mechanisms (O'BRIAN, 1991). First it has a capability to form covalent bonds with cellular nucleophiles such as protein thiols and basic parts of DNA (DiMonte *et al.*, 1984). Secondly, quinones are readily reduced by flavoenzymes to semiquinone radical anions, which is further converted to hydroquinones by quinone oxidoreductase (Pisani *et al.*, 1986; Ross *et al.*, 1986). Once the semiquinone radical anion reacts with oxygen, it can generate superoxide anions, which are known to cause the oxidative stress.

Especially naphthazarin is very liable for the formation of the semiquinone radical anion because the semiquinone radical is stabilized by tautomeric equilibrium (Moore *et al.*, 1966). In contrast, the formation of semiquinone radical anions in 5,8-di-methoxy-1,4-naphthoquinone (DMNQ), a permethylated product of naphthazarin, must be limited to the quinoid moiety. Instead, on arylating capacity is expected to be enhanced, since the electron density should be localized in the quinoid moiety of DMNQ.

Additionally, a 2-substituted naphthazarin derivatives could be reduced by quinone oxidoreductase to generate 2-substituted 1,4,5,8-tetraoxynaphthalene derivatives which could modify cellular nucleophiles through the so-called bioreductive alkylation mechanism (Lin *et al.*, 1975).

In this study, we synthesized 5,8-dimethoxy-1,4-naphthoquinone and its derivatives, and evaluated their cytotoxicity and antitumor activity. The rate of formation of glutathione conjugates and the inhibitory effect on DNA topoisomerase-I were also determined to rationalize the electrophilic arylation as a cytotoxic

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mechanism.

MATERIALS AND METHODS

Chemical reagents were obtained from Aldrich Chemical Company. Solvents were of reagent grade and used without further purification. L1210 cells were obtained from Korea Institute for Chemical Technology. RPMI 1640, Fetal bovine serum and other reagents used for cell culture were purchased from Gibco Co. Proton NMR spectra were recorded on a JEOL 90 MHz spectrometer using tetramethylsilane as an internal standard. Analytical thin layer chromatography was performed on plastic sheet (0.2 mm) coated with silica gel 60 F254 (E. Merk). Silica gel 60 (70~230 mesh, E. Merk) was used for column chromatography.

Synthesis of compounds

The compounds were synthesized using Baik's method (Baik *et al.*, 1997).

Measurement of cytotoxicity

Cytotoxicity was measured against L1210 cells using the reported method (Thayer *et al.*, 1971). Fisher's medium supplemented with horse serum in 10% was used for the proliferation of L1210 cells. Cell numbers were counted using a haemacytometer, and ED50 value was defined as the concentration of drug to produce a 50% reduction in viability relative to the control in three independent experiments.

Antitumor activity in mice

The test sample dissolved in a predetermined amount of 50% PEG200 were stored 4°C Sarcoma 180 cells (0.1 ml per mouse) suspended in saline (1×10^7 cells/ml) were inoculated intraperitoneally to male ICR mice (National Cancer Institute USA, 1972). 24 Hrs after the transplantation, mice were divided so that each group contains 8 mice. The sample was administered into the intraperitoneal cavity of the mouse daily for 7 days. The survival rate (T/C, %) was calculated by following equation;

$$\frac{\text{Average survival period in the test group}}{\text{Average suvival period in the control group}}\times 100$$

RESULTS AND DISCUSSION

The rate of formation of GSH conjugates, inhibition of DNA topoisomerase-I, cytotoxicity and antitumor action of naphthoquinone derivatives were summarized in Table I. 5,8-Dimethoxy-1,4-naphthoquinone (DMNQ) was about 4,6-fold more reactive with GSH than 5,8-dihydroxy-1,4-naphthoquinone (DHNQ). The lower reacting of DHNQ, compared to DMNQ, might be due to the dispersion of electron in DHNQ molecule caused by the tautomerism as evidenced from one aromatic peak at 7.14 ppm in ^{1}H-NMR spectrum (Moore *et al.*, 1966). On the other hand, 1,4-naphthoquinone (1,4-NQ) lacking electron-releasing group such as hydroxy or methoxy group formed the GSH conjugate more rapidly than DMNQ, similar to the earlier observation by Öllinger (Öllinger *et al.*, 1991) who found that the electrophilic reactivity was in the order of 1,4-NQ>5-hydroxy-1,4-NQ>DHNQ. Thus, it is evidenced that the presence of hydroxyl group in the naphthoquinone ring decreased the electrophilicity of the quinoid moiety.

1,4-NQ, the most effective in forming the GSH conjugate, was the most potent in the inhibition of DNA topoisomerase-I (IC_{50}, 39 μM). Although the electrophilic aryltion appears to govern the cytotoxicity, DHNQ, which is less electrophilic, expressed a higher inhibition of Topo-I activity than DMNQ, suggesting that other

Table I. Relationship between formation of GSH conjugate, DNA topo-I inhibition and antitumor activities of 1,4-dioxynaphthoquinone derivatives

Compound	GSH-conjugate (mM)[a]	Topo-I[b] IC_{50} (μM)	ED_{50} (μg/ml) In L1210	T/C (%) (μmole/kg/day)
1,4-NQ	0.368±0.082	39.43±1.87	0.135±0.005	248 (1.6)[c]
DHNQ	0.072±0.025	46.42±2.21	0.030±0.006	260 (5.3)
DMNQ	0.331±0.054	96.00±7.11	0.097±0.019	294 (14)
6he-DMNQ	0.302±0.061	110.63±0.80	0.222±0.013	279 (25)
6ae-DMNQ	0.351±0.104	84.07±2.14	0.125±0.013	250 (15)
2he-DMNQ	N.D.	>1500	0.680±0.016	119 (25)
2ae-DMNQ	0.143±0.015	81.63±7.43	0.146±0.006	276 (14)

1,4-NQ; 1,4-naphthoquinone, DHNQ; 5,8-dihydroxy-1,4-naphthoquinone, DMNQ; 5,8-dimethoxy-1,4-naphthoquinone, 6he-DMNQ; 6-(1-hydroxyethyl)-DMNQ, 6ae-DMNQ; 6-(1-acetyloxyethyl)-DMNQ, 2he-DMNQ; 2-(1-hydroxylethyl)-DMNQ, 2ae-DMNQ; 2-(1-acetyloxyethyl)-DMNQ.

[a]mM of conjugate formed in 5min by reacting 1mM of naphthoquinone with GSH 0.5 mM.

[b]Topo-I; DNA topoisomerase-I.

[c]Parenthesis shows the daily dose to the animal.

*All the values are the mean of three measurements.

factors such as hydrogen bonding might also be involved in the Topo-I inhibition. Among the derivatives tested, moreover, the highest cytotoxicity against L1210 cells was demonstrated by DHNQ, which is the least efficient in conjugation with GSH. This is an analogous to the observation by Ollinger that the cytotoxicity of hydroxylated 1,4-naphthoquinones against hepatocytes was the order of naphthazarin > 5-hydroxy-1,4-NQ > 1, 4-NQ, in support of the oxidative stress by naphthoquinones.

Next, the antitumor action of naphthoquinones was examined (Table I). On the whole, the antitumor action (T/C value) of all naphthoquinones was within the similar range. However, the optimal doses were different; DMNQ showed the highest antitumor action with a T/C value of 294% against intraperitoneal S-180 tumor in mice at a dose of 3 mg/kg/day, while T/C value of DHNQ was 260% at 1 mg/kg/day. Actually the optimal dose of DMNQ corresponded to a toxic dose of DHNQ. The permethylation of DHNQ decreased the cytotoxicity but appeared to increase the T/C values. This might be explained by the assumption that the metabolic rate of DMNQ was slower than that of DHNQ in the body of the animal, so that its action persisted for a longer period.

Based on these data, it is suggested that both DHNQ and DMNQ molecules are useful lead compounds for the synthesis of antitumor naphthoquinone derivatives. However, in order to enhance the electrophilic arylation selectively, DMNQ was selected as lead compound for further study (Baik *et al.*, 1997; Terada *et al.*, 1987).

As for the synthesis of DMNQ derivatives, DMNQ was formylated to provide 2-formyl-5,8-dimethoxy-1, 4-naphthoquinone(2-formyl DMNQ), which was further treated with ethylmagnesium bromide.2-(1-Hydroxyethyl)-DMNQ was oxidized and demethylated to an isomer pair, 2-(1-hydroxyethyl)-DMNQ and 6-(1-hydroxyethyl)-DMNQ. The respective isomer, separated chromatographically, was acetylated to provide 2-(1-acetoxyethyl)- and 6-(1-acetoxyethyl)-DMNQ. Table I demonstrates the formation of GSH-conjugate and bioactivites of different DMNQ derivatives.

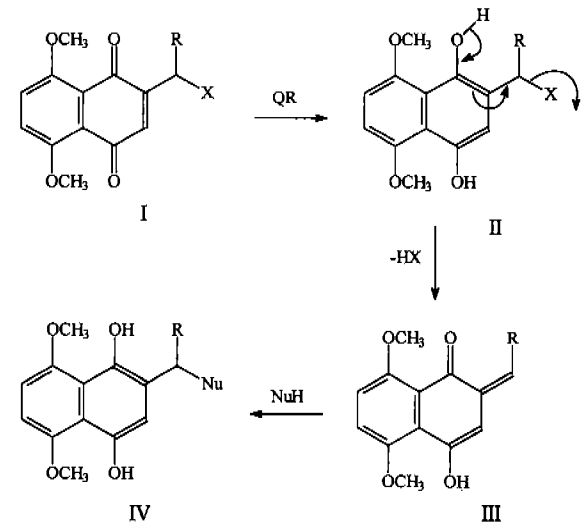
6-(1-Hydroxyethyl)-5,8-dimethoxy-1,4-naphthoquinone (6he-DMNQ) was almost equal to DMNQ in the formation of GSH conjugate, the inhibiton of Topo-I and the cytotoxicity. In contrast, 2-(1-hydroxyethyl)-5, 8-dimethoxy-1,4-naphthoquinone (2he-DMNQ) which was not so interactive with GSH and DNA topoisomerase-I showed a lower cytotoxicity, implying that the steric hindrance of hydroxyethyl group at C-2 of DMNQ molecule is the main reason for the reduced activity of 2-(1-hydroxyethyl)-DMNQ. It seemed that the higher activities of 6he-DMNQ, compared to 2he-DMNQ, was due to the higher arylation capability of its exposed quinone moiety.

Next, 6-heDMNQ and 2heDMNQ were acetylated, and the bioactivities of acetylated derivatives were assessed as in Table I. The acetylation of 6he-DMNQ enhanced slightly the rate of conjugate formation, the Topo-I inhibition and the cytotoxicity, rather tended to decrease the T/C value. Meanwhile, acetylation of 2he-DMNQ increased all of three biological activities to a larger extent; from >1500 to 81.63 μM; formation of GSH conjugate, from 0.00 to 0.14 mM; cytotoxicity, from 0.680 to 0.146 μg/ml.

Still, it seems that a steric hinderance is responsible for the difference of electrophilicity and cytotoxicity of 2ae- and 6ae-DMNQ derivatives. However, the modest difference of cytotoxicity between 6ae-DMNQ and 2ae-DMNQ suggests that the role of arylation may not be decisive for the cytotoxicity of acetylated DMNQ derivatives. Instead, the oxidative stress could be considered as an additional mechanism as well evidenced generally for the quinone cytotoxicity.

An alternative mechanism of 2-(1-oxyalkyl)-DMNQ derivatives could be bioreductive alkylation (scheme 1); Earlier, Lin and coworkers found that some of 2-chloromethylnaphthoquinone derivatives showed a higher T/C value (around 200%) than chloro or bromomethylnaphthazarin derivatives against S-180 ascites tumor. It was suggested that 2-chloromethylnaphthoquinone is more easily subjected to reductive alkylation than 2-chloro or bromo derivatives. In the same way the naphthoquinone I is reduced to a 1,4-dihydroxynaphthoquine II by quinone oxidoreductase in L1210 cells (Maliepaard *et al.*, 1996). Then elimination of HX produces a semiquinone methide, which reacts as a Michael acceptor with cellular nucleophiles (NuH) to form the adduct IV.

The higher cytotoxicity and antitumor action of 2-



Scheme 1. Bioreductive activation of 2-(1-oxyalkyl)-5,8-dimethoxy-1,4-naphthoquinone derivatives. QR: quinone reductase, NuH: cellular nucleophiles.

(1-acetyloxyethyl)-DMNQ (2ae-DMNQ), compared to 2-(1-hydroxyethyl)-DMNQ (2he-DMNQ), might be explained by the mention that compared to 2he-DMNQ, 2ae-DMNQ bearing acetyl moiety, a better leaving group than hydroxyl group, may be more liable for the formation of the semiquinone methide.

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