

Naphthazarin Derivatives (V): Formation of Glutathione Conjugate and Cytotoxic Activity of 2-or 6-Substituted 5,8-Dimethoxy-1,4-naphthoquinones in the Presence of Glutathione-S-transferase, in Rat Liver S-9 Fraction and Mouse Liver Perfusate

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Formation of glutathione (GSH) conjugates with 2- or 6-(1-hydroxymethyl)- and 2-(1-hydroxyethyl)-DMNQ derivatives (DMNQ, 5,8-dimethoxy-1,4-naphthoquinone) was carried out in phosphate buffer (pH 7.4), in the presence of glutathione-S-transferase (GST), in rat liver S-9 fraction and by perfusion, and the rates of conjugates formation were compared and correlated to cytotoxicity. The GSH conjugates of 6-(1-hydroxyalkyl)-DMNQ derivatives were formed faster than 2-(1-hydroxyalkyl)-DMNQ derivatives under all of the media, implying that steric hindrance was the cause of lowering the rate of conjugate formation of 2-substituted derivatives. For both isomers, addition of GST did not improve the reaction rate, compared with that in buffer, while the reaction in the S-9 fraction and the perfusate was accelerated to a great extent. The catalytic effect of the S-9 fraction and the perfusion on 2-isomers was greater than on 6-substituted ones, suggesting that S-9 fraction and the perfusate contain an effective system relaxing the steric hindrance of 2-(1-hydroxyalkyl)-DMNQ derivatives. Furthermore, a good correlation between the formation of the GSH conjugates and the cytotoxic activity of both naphthazarin isomers suggests that the steric hindrance is a cause of lowering the cytotoxicity of 2-isomers.

Key words: Naphthazarin derivatives, Formation of glutathione conjugates, Glutathione-S-transferase, Rat liver S-9 fraction, Mouse liver perfusion

INTRODUCTION

Glutathione (GSH) is an essential substance participating in phase II of drug metabolism. It is well known that GSH attacks a nucleophilic site of drug structure or its metabolic intermediate. The naphthoquinones are good electrophiles which should be attacked by GSH in the liver, resulting in the formation of various reaction products (Miller *et al.*, 1986; Gant *et al.*, 1987 and 1988; Monks *et al.*, 1990; Thor *et al.*, 1982; Finley *et al.*, 1974). The reaction produces GSH-quinone conjugates as main products and others such as the GSH-hydroquinone adducts, hydroquinones and polygluta-

thionylated quinones. The GSH-hydroquinones and hydroquinones so produced are readily oxidized to release toxic oxygen radicals, which are mostly harmful to the cell. Both the electrophilicity and the formation of the harmful oxygen radicals have been interpreted as cause of cytotoxicity of quinones (Belisario *et al.*, 1994; Gant *et al.*, 1988).

Recently, we have discovered that enhancement of the electrophilicity of 5,8-dimethoxy-1,4-naphthoquinone derivatives led to the increase of antitumor activity, while steric hindrance by 2-substitution lowered the activity. (You and Zheng *et al.*, 1998; Song *et al.*, 1999). It was also confirmed that the rate of GSH conjugates formation was good correlated with the cytotoxic activity of the naphthoquinone derivatives. Furthermore, the details on the synthetic pathways and the structure of the conjugates were reported (Zheng *et al.*, 1999).

Present study is targeting to observe effect of glutathione-S-transferase and rat liver S-9 fraction on the

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formation of the reaction products between GSH and 5,8-dimethoxy-1,4-naphthoquinones, as their metabolic products, and to correlate the result with antitumor activity.

MATERIALS AND METHODS

Chemicals and instruments

Glutathione-S-transferase (GST), NADPH and EDTA (Sigma Chemical Co., USA), LS-3E Fluorescence spectrophotometer (Perkin-Elmer, England), HPLC (Shimadzu, Japan) were used. HPLC column; Polyosil C18 (100 × 250 mm, 10 μm, Metachem Tec, USA) for preparative purpose and Inertsil (ODS-3 (4.6 × 150 mm, GL Science Inc., USA) for analytics. Male ICR mice (18-20g) were purchased from Samyouk Co. (Korea).

All other chemicals used were of reagent grade and commercially available. The GSH conjugates reported in previous study (Zheng *et al.*, 1999) were used for comparison. 5,8-Dimethoxy-1,4-naphthoquinone derivatives used were synthesized using Baik's method (Baik *et al.*, 1997).

Formation of glutathione conjugates in phosphate buffer

To 0.1 ml of 1 mM naphthoquinone derivative in methanol was added 0.1 ml of 1 mM GSH in 0.1 M potassium phosphate buffer (pH=7.4) and the mixture was stirred at room temperature for 5 min. 10 μl of the reaction mixture underwent an HPLC analysis. For HPLC analysis Inertsil ODS-3 column and 28% methanol in 0.1 M sodium acetate buffer (pH 4.4) were used. The flow rate was 2 ml/min, and the components were detected with diode array detector (SPD-M10AVP, Shimadzu, Japan) at 254 and 450 nm.

Formation of GSH conjugates in the presence of glutathione-S-transferase

To mixture of 1mM GSH (0.1 ml) in 0.1 M potassium phosphate buffer (pH 7.4) and 0.1 mg/ml glutathione-S-transferase (0.1 ml) suspended in the same buffer was added 0.1 ml of 1 mM naphthoquinone derivative in methanol, and the resulting solution was diluted to 2 ml with the same buffer solution. After the mixture solution was stirred at room temperature for 5min, 10 μl of reaction solution were analyzed by HPLC.

Formation of GSH conjugates in rat liver S-9 fraction

Mouse liver was homogenized with 3 volumes of 0.1 M potassium phosphate buffer (50 mM KCl, 5 mM EDTA, pH=7.4) and the homogenate was centrifuged at 9,000 × g for 30 min at 4°C. The supernatants were stored at -70°C as stock solution. 0.5 ml of the supernatant was added to a mixture of 0.1 M MgCl₂ (0.1 ml)

and 10 mM NADPH (0.1 ml) and 1 mM GSH (0.1 ml) in 0.1 M potassium phosphate buffer. To this mixture, 0.1 ml of 1 mM naphthoquinone derivative in methanol was added and incubated for 5 min at 37°C. Then, 1.0 ml of acetonitrile was added and centrifuged at 13,000 rpm for 5min, and 20 μl of the clear supernatant were analyzed by HPLC.

Formation of GSH conjugates in mouse liver perfusate

Naphthoquinone derivative (3 mg) was dissolved in 10 μl of DMSO and diluted to 1 ml with the 0.1 M potassium phosphate buffer solution. 0.1 ml of the solution was injected into the portal vein of the rat and perfused with a syringe pump at a flow rate of 1.0 ml/min. The perfusate was collected from inferior vena cava at a interval of 1.0 min and the 20 μl of collected perfusate was analyzed by HPLC.

Determination of cytotoxic activity against L1210 cells

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

RESULTS AND DISCUSSION

In a previous study (Song *et al.*, 1999), it was found that the rate of formation of GSH conjugates in phosphate buffer was good correlated to cytotoxicity of 2- or 6-(1-hydroxyalkyl)-DMNQ derivatives (DMNQ, 5, 8-dimethoxy-1,4-naphthoquinone). Based on this result, it was suggested that the electrophilicity of the quinone moiety was an important factor for the cytotoxicity of the naphthoquinone derivatives.

Present study intended to observe the relationship between the rates of GSH conjugates formation under presence of GST (glutathione-S-transferase) and rat liver S-9 fraction, and antitumor activity of the naphthoquinone derivatives. 2-(1-Hydroxymethyl)-and 2-(1-hydroxyethyl)-DMNQ as the steric hindered species, and 6-(1-hydroxymethyl)-and 6-(1-hydroxyethyl)-DMNQ as less hindered ones, were selected as studying materials.

Formation of GSH conjugates

As notified in Table I, the GSH conjugates of 6-(1-hydroxymethyl)-and 6-(1-hydroxyethyl)-DMNQ were formed about eighteen times more rapidly than 2-(1-hydroxymethyl)- and 2-(1-hydroxyethyl)-DMNQ in phosphate

Table I. GSH conjugate formation of 2-(1-hydroxymethyl)-, 2-(1-hydroxyethyl)-, 6-(1-hydroxymethyl)-and 6-(1-hydroxyethyl)-5,8-dimethoxy-1,4-naphthoquinones in buffer and in the presence of glutathione-S-transferase, rat liver S-9 fraction, and in mouse liver perfusate.

	Rate of GSH conjugation($\mu\text{m}/\text{min}$)			
	Buffer	GST	S-9 fraction	
2-(1-hydroxymethyl)-DMNQ	9.0	9.52	72	
2-(1-hydroxyethyl)-DMNQ	6.8	8.75	60	120
6-(1-hydroxymethyl)-DMNQ	165	168	229	
6-(1-hydroxyethyl)-DMNQ	121	130	225	242

DMNQ; 5,8-dimethoxy-1,4-naphthoquinone

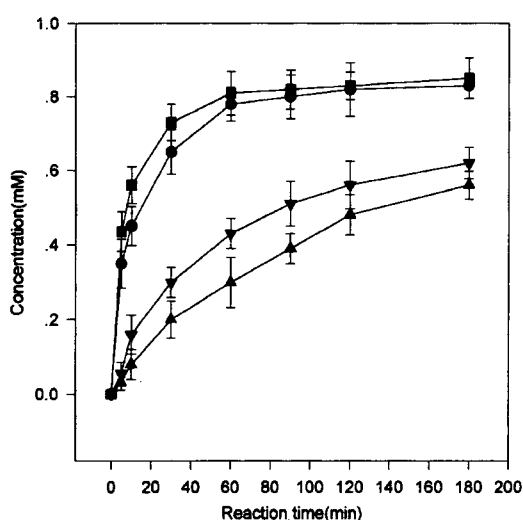


Fig. 1. Time dependent formation of DMNQ-GSH conjugates in the absence or presence of GST. (▲)-2he-DMNQ, ▼-2he-DMNQ-GST, ●-6he-DMNQ, ■-6he-DMNQ-GST

buffer (pH 7.4). Meanwhile, it was found that there was only a little difference between the products formed at 5 min in buffer alone and under GST. The addition of GST showed no significant change in the reaction rate in 5 min. However, a time course follow-up of the GSH conjugate formation revealed that addition of GST to the buffer accelerated the formation of GSH conjugate for both of the DMNQ isomers by a longer time exposure (Fig. 1). The conjugation of 6-isomer in the presence of the enzyme was completed at about 60 min after start of the reaction, while that of 2-isomer was done at around 180 min, disclosing that the reaction of 2-isomers occurred more delayed than 6-isomers did. The time difference between the reactions suggests that the GST requires some induction period for reaction with the sterically hindered 2-isomers, while the reaction occurs more directly with 6-isomers. The time difference between the reactions suggests did that the GST requires some induction period for reaction with the sterically

hindered 2-isomers, while the reaction occurs more directly with 6-isomers.

Contrary to the enzymatic effect, the formation of the GSH conjugates was markedly accelerated in rat liver S-9 fraction for both of 2- and 6-isomers, and as expected, 6-isomers were formed faster. The GSH conjugates of 2-(1-hydroxymethyl)- and 2-(1-hydroxyethyl)-DMNQ were formed in the S-9 fraction 7.6 and 6.9 times faster than the reaction in presence of GST, respectively, while for 6-(1-hydroxymethyl)-and 6-(1-hydroxyethyl)-DMNQ it was 1.4 and 1.7 times faster. The effect of the S-9 addition on 2-isomers is greater than that on 6-isomers.

In order to observe the metabolic behavior of the naphthoquinones in an ex vivo condition, 2-(1-hydroxyethyl)- and 6-(1-hydroxyethyl)-DMNQ derivatives were perfused through the mouse liver, respectively and the perfusates were HPLC-analyzed. As shown in Table I, 6-(1-hydroxyethyl)-DMNQ was more rapidly metabolized in the mouse liver than 2-(1-hydroxyethyl)-DMNQ, implying that the steric hindrance is effective in the ex vivo condition as well. However, compared to the metabolic rate in microsome, 2-isomer transformed to 2-(1-hydroxyethyl)-3- glutathionyl-DMNQ in 2 times faster than 6-isomer did to 6-(1-hydroxyethyl)-2(3)-glutathionyl-DMNQ in the perfusion.

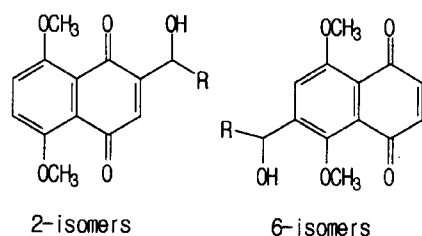
This finding implies that the steric hindrance of 2-isomers, which was appeared strongly in the reaction under phosphate buffer, could be overcome by addition of the S-9 fraction or through the perfusion leading to a considerable acceleration of GSH conjugate formation.

The difference in the rate of the conjugate formation between the three conditions (GST, S-9 fraction, and perfusion) suggests that GST alone is not enough for catalyzing the formation of the GSH conjugates. Thus, beside GST, a cascade of other catalytic reactions being present in the intact cells or tissue is required for a rapid metabolism of DMNQ derivatives in the liver cells.

Relationship between the rate of formation of GSH conjugates and cytotoxicity

As demonstrated in Table II, the rate of the conjugate formation of 2-isomers in buffer was 10- 16 times slower than that of 6-isomers. The cytotoxic activity of 2-isomers was weaker than 6-isomers. From this parallel relationship it is suggested that the steric hindrance is a cause for lowering the cytotoxic activity of 2-isomers. The cytotoxic activity of 2-isomers seemed not to be dependent on the size of the alkyl side chain, while there was an optimal range of the size for that of 6-substituted DMNQ derivatives. The presence of R (ethyl, propyl, butyl and pentyl) enhanced the cytotoxicity of 6-isomers (ED₅₀ of 0.08 0.06 $\mu\text{g}/\text{ml}$), despite decreasing tendency of the rate of the conjugate

Table II. GSH conjugate formation in phosphate buffer (pH 7.4) and cytotoxic activity of 2-(1-hydroxyalkyl)-5,8-dimethoxy-1,4-naphthoquinone (2-isomer) and 6-(1-hydroxyalkyl)-5,8-dimethoxy-1,4-naphthoquinone (6-isomer) derivatives.



R	2-isomer		6-isomer	
	Rate($\mu\text{M}/\text{min}$)	ED ₅₀ ($\mu\text{g}/\text{ml}$)	Rate($\mu\text{M}/\text{min}$)	ED ₅₀ ($\mu\text{g}/\text{ml}$)
H	9.0	0.524	165	0.062
Methyl	6.8	0.487	121	0.132
Ethyl	8.4	0.516	125	0.085
Propyl	7.6	0.409	121	0.043
Butyl	9.2	0.412	124	0.057
Pentyl	6.0	0.378	120	0.066
Hexyl	5.2	0.732	117	0.220
Heptyl		1.043	109	0.274

The rate of formation of GSH conjugates was measured in phosphate buffer (pH 7.4).

formation in the buffer with the increasing chain size. This implies that smaller alkyl chains and a higher rate of GSH conjugate formation were not the conditions for a higher cytotoxic activity. It is possibly due to a rapid metabolism of the smaller molecules in the cells and to a disturbed uptake of a larger molecule into the cells eventually because of poor water solubility, while the naphthoquinone with a medium size of side chain exerts a sustained action inside the cells.

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REFERENCES

Ahn, B. Z. and Sok, D. E. Michael Acceptors as a Tool for Anticancer Drug Design, *Current Pharmaceutical*

Design, **2**, 247~262 (1996).

Baik K. U., Song G. Y., Kim Y., Sok D. E., Ahn B. Z.: 2-Substituted Naphthazarins: Synthesis and Antitumor Activity. *Arch. Pharm. Med. Chem.* **330**, 377-382 (1997).

Belisario, M. A., Pecce, R., Maturo, M., De Rosa, S. Arylation of sulfhydryl groups in vitro by the naturally occurring sesquiterpenoid benzoquinone avarone. *Toxicology*, **86**, 89-108 (1994).

Finley, K. T. The additional and substitution chemistry of quinones. In *The Chemistry of the Quinonoid Compounds* (S.Patai, Ed.), Part 2, pp. 878-1144. Wiley, London (1974).

Gant, T. W., and Cohen, G. M. Reaction of glutathione or amino acids with quinones forming semiquinone radicals, In *Free Radicals, Oxidants Stress and Drug Action* (C. Rice-Evans, Ed.), pp. 377-399 (1987).

Gant, T. W., Rao, D. N. R., Mason, R. P., and Cohen, G. M. Redox cycling and sulphhydryl arylation; Their relative importance in the mechanism of quinone cytotoxicity to isolated hepatocytes, *Chem. Biol. Interactions*, **65**, 157-173, (1988).

Miller, M. G., Rodgers, A., and Cohen, G. M. Mechanisms of toxicity of naphthoquinones to isolated hepatocytes, *Biochem. Pharmacol.*, **35**, 1177-1184 (1986).

Monks, T. J., Anders, M. W., Dekant, W., Stevens, J. L., Lau, S. S., and van Bladeren, P. J. Glutathione Conjugate Mediated Toxicology. *Toxicol. Appl. Pharmacol.* **106**, 1-19 (1990).

Song, G. Y., Zheng, X. G., Kim, Y., You, Y. J., Sok D.E., and Ahn, B. Z. Naphthazarin Derivatives (II): Formation of Glutathione Conjugate, Inhibition of DNA topoisomerase-I and Cytotoxicity. *Bioorg. Med. Chem. Lett.*, **9**, 2407-2412 (1999).

Thor, H., Smith, M. T., Bellomo, G., Jewell, S. A., and Orrenius, S. The metabolism of menadione (2-methyl-1,4-naphthoquinone) by isolated hepatocytes, *J. Biol. Chem.*, **257**, 12419-12425 (1982).

You, Y. J., Zheng, X. G., Kim Y. and Ahn B. Z. Naphthazarin derivatives; Naphthazarin Derivatives: Cytotoxic Mechanism and Evaluation of Antitumor Activity. *Arch. Pharm. Res.*, **21**(5), 595-598 (1988).

Zheng, X. G., Ahn, B. Z. (1999). Glutathione conjugates of some 2- or 6-substituted 5,8-dimethoxy-1,4-naphthoquinone derivatives: synthesis and structure. *Arch. Pharm. Res.* **22**, 384-390 (1999).