J. Microbiol. Biotechnol. (2000), **10**(4), 514–517

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Cytotoxicity of Shikonin Metabolites with Biotransformation of Human Intestinal Bacteria

MIN, BYUNG SUN[1], MESELHY R. MESELHY[1], MASAO HATTORI[1], HWAN MOOK KIM[2], AND YOUNG HO KIM[3]*

[1]*Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan*
[2]*Advanced Biomaterial Research Center, Korea Research Institute of Bioscience and Biotechnology, Taejon 305-600, Korea*
[3]*College of Pharmacy, Chungnam National University, Taejon 305-764, Korea*

Received: April 20, 2000
Accepted: June 30, 2000

Abstracts Six shikonin metabolites were obtained from human intestinal bacteria, *Bacteriodes fragilis* subsp. *thetaotus*, following biotransformation. The transformation of shikonin (**1**) was performed anaerobically for 3 day at 37°C in the bacterial suspension of *B. fragilis* which was cultured overnight in GAM broth. The incubation mixture was extracted with EtOAc to give a dark-brown residue. The residue was applied to a silica gel column, which was eluted successively with hexane (Fr. A), $CHCl_3$ (Fr. B), and $CHCl_3$:MeOH (9:1) (Fr. C). Six metabolites, Fr.A (**2** and **3**), Fr. B (**6** and **7**), and Fr. C (**4** and **5**) were isolated by repeated silica gel column chromatography, preparative TLC, followed by Sephadex LH-20. *In vitro* cytotoxicities were tested against human tumor cell lines; PC-3 (prostate), ACHN (renal), A549 (lung), SW620 (colon), K562 (leukemia), and Du145 (prostate). The shikonin metabolites **2**, **4**, **5**, and **6** showed weaker cytotoxicity than the parent shikonin (**1**), whereas shikonin monomeric metabolite **3** (ED_{50} 0.44–1.22 μg/ml) and dimeric metabolite **7** (ED_{50} 0.48–2.35 μg/ml) exhibited stronger activities compared with adriamycin, which was used as the positive control.

Key words: Shikonin, biotransformation, *Bacteriodes fragilis* subsp. *thetaotus*, cytotoxicity

The radix of *Lithospermum erythrorhizon* Sieb. et Zucc. (Boraginaceae) has been used as an anti-inflammatory and antipyretic agent in treatment of measles and eczema in Chinese folk medicine [19]. Shikonin (**1**) is a naphtoquinone pigment found in the root of *L. erythrorhizon*. It is produced commercially from *Lithospermum* cell cultures [16] and used as a natural coloring agent for foods, drugs, and cosmetics. Shikonin and its derivatives from this plant have been considered as important medicinal compounds, potentially for antibacterial [17], anti-inflammatory [18], and antiamebic activities after studies in rats with induced intestinal amebiasis [13]. Shikonin derivatives, such as alkannane, dihydroshikonin, cycloshikonin, and acetylshikonin, have all shown to have potent antitumor activities against L1210 (mouse leukemia) and Sarcoma 180 cells [5, 7, 14]. Synthetic 1,4-naphthoquinone analogues have also been evaluated for their antitumor activity [2, 3, 4, 8, 9, 12, 15] and inhibitory activity of DNA topoisomerase type I [1]. Accordingly, this potent antitumor activity of shikonins encouraged the current investigation of the cytotoxicity of shikonin metabolites obtained by the biotransformation of human intestinal bacteria. The present study reports on the cytotoxic activity of shikonin metabolites (**2**-**7**) obtained after the anaerobic incubation of shikonin with *Bacteriodes fragilis* subsp. *thetaotus*.

Materials and Methods

General Experimental Procedures

The melting points were determined by a Yanagimoto micro melting point apparatus and were presented uncorrected. The CD spectra were recorded with a JASCO J-720 automatic recording spectropolarimeter (JASCO Co., Tokyo, Japan) in MeOH and $CHCl_3$. The IR spectra were measured using a Hitachi 260-10 infrared spectrometer. The UV spectra were recorded on a Shimadzu UV-2200 UV/VIS spectrophotometer. The ^1H- and ^{13}C-NMR spectra were measured with a JEOL GX-400 spectrometer with TMS as the internal standard and the chemical shifts were recorded in δ values. The EI-MS and FAB-MS spectra were measured with a JEOL JMS DX-300 mass spectrometer. Kieselgel 400 (Wako Pure Chemical Co.), Merck Kieselgel 60 (Merck), and Sephadex LH-20 (Pharmacia LKB) were

*Corresponding author
Phone: 82-42-821-5933; Fax. 82-42-823-6566.
E-mail: yhk@cnu.ac.kr

used for the column chromatographies. Merck Kieselgel 60 F_{254} with thicknesses of 0.25 mm and 0.5 mm was used for TLC and preparative TLC, respectively. Spots were detected under a UV lamp in day light after spraying with $Ce(SO_4)_2$-H_2SO_4.

Chemicals

The shikonin (**1**) was purchased from Mitsui Petrochemical Industries, Ltd. (Japan) and its purity was confirmed before use by TLC, HPLC, CD, and ^{1}H- and ^{13}C-NMR spectroscopies. A general anaerobic medium (GAM) was purchased from Nissui Co. (Japan). Fetal calf serum, media, and other supplemental materials for the cell culture were all purchased from GIBCO (U.S.A.). Adriamycin was obtained from Sigma Chemical Co. (U.S.A.).

Microorganism

The *Bacteroides fragilis* subsp. *thetaotus*, a human intestinal bacterial strain, was provided by Prof. T. Mitsuoka, Tokyo University, Japan.

Cytotoxic Sulforhodamine B (SRB) Assay

Human cancer cell lines were used and cultured with RPMI 1640 containing 5% fetal calf serum (FCS). For SRB assay, the cells were cultured in RPMI 1640 containing 5% FCS. The SRB assay was performed according to the previously described method [6]. A cell suspension (30,000–40,000 cells/ml) was prepared in the culture medium which was then inoculated into each well of a 96-well microtiter plate. One day after the plating, a time-zero control plate was made. The cells were directly treated with the compounds and incubated for 48 h in a CO_2 incubator. The cells were fixed with 50 µl of 50% trichloroacetic acid (TCA) solution for 1 h at 4°C, and the plates were then washed 5 times with tap water and air-dried. One hundred microliters of SRB solution (0.4% in 1% acetic acid) was added, and the cells were stained at room temperature for 30 min. The residual dye was washed with 1% acetic acid and the cells were air-dried. Tris solution (10 mM, pH 10.5) was added to each well, and the optical density (OD) was measured at 540 nm with a microtiter plate reader. The growth inhibition was calculated according to a previous method. Briefly, the OD of the treated well was subtracted from the OD of the time-zero (Tz) plate and divided by the calculated value of the untreated control. A growth inhibition of 50% (ED_{50}) was calculated using the Probit method [20].

Transformation of Shikonin (1) with *Bacteroides fragilis* subsp. *thetaotus*

The transformation of shikonin (**1**) with *B. fragilis* and the isolation of the metabolites were performed as follows: Stock cultures of *B. fragilis* (2 l) were added to a GAM broth (18 l) and cultured overnight at 37°C under anaerobic conditions. The culture was then centrifuged at 7,800 ×g for 10 min and the resulting pellets washed twice with saline and suspended in 0.1 M phosphate buffer (20 l, pH 7.3). A 200 ml of shikonin (**1**, 0.1 g/ml DMSO) was added to the bacterial suspension and the mixture was anaerobically incubated for 3 day at 37°C. The incubation mixture was adjusted to pH ca. 3.0 with 5% HCl, and extracted with EtOAc (20 l×5). The EtOAc layer was washed with H_2O and evaporated *in vacuo* to give a dark-brown residue (34 g). The residue was applied to a silica gel column (50×10 cm) and successively eluted with hexane (Fr. A), $CHCl_3$ (Fr. B), and $CHCl_3$:MeOH (9:1) (Fr. C). The fractions (1,500 ml each) were collected and subjected to TLC. Fraction A was concentrated and applied to a silica gel column. The elution with hexane:Me_2CO (9:1) gave two compounds, **2** and **3**. Fraction B was first subjected to silica gel column chromatography (hexane:$CHCl_3$, 7:3 and 1:9) and then to preparative-TLC (hexane:Me_2CO, 8:3) to collect compounds **6** and **7**. Repeated column chromatography of fraction C on a silica gel (hexane:$CHCl_3$, 1:9 and $CHCl_3$:MeOH, 9:1), preparative TLC, followed by Sephadex LH-20 ($CHCl_3$:MeOH, 7:3) yielded two dark blue compounds **4** and **5**. The physico-chemical properties and spectral data of each compound were reported previously (Meselhy *et al.*, 1994).

Results and Discussion

Six metabolites of shikonin (**1**) were obtained from *B. fragili* after a 3-day anaerobic incubation, and their structures were identified as anhydroalkannin (**2**), deoxyshikonin (**3**), and shikometabolins A (**4**), B (**5**), C (**6**), and D (**7**) as described in a previous study [11]. The naphthoquinone type monomeric metabolites (**2** and **3**) were yielded through the dehydration and reduction of **1**. The major dimeric metabolites (**4** and **5**) were obtained by the tautomerization and condensation of **1** and **2**. In addition, the minor dimeric metabolites (**6** and **7**) were produced by the condensation of **2** and **3**. The coupling of C3-C3' and C2-C12' resulted in the formation of **6**, while that of C3-C12' and C11-C1' yielded **7**.

Shikonin (**1**) was reported to exhibit potent anti-inflammatory, antitumor, and other biological activities. In a previous study, the current authors tested the metabolites of shikonin for their anti-inflammatory and antiviral activities on the reverse transcriptase of human immunodeficiency virus (HIV). However, these metabolites exhibited no inhibitory activity on carragenin-induced hind-paw edema in mice. Only the violet dimers (**4** and **5**) showed a weaker inhibitory effect on reverse transcriptase activity on HIV than shikonin [11]. This is supported by the fact that the anti-inflammatory and antiviral effects of **1** were reduced after anaerobic incubation with human intestinal bacteria.

In continuous study of the biological activity of these metabolites using tumor cell lines, the cytotoxicities of

Table 1. Cytotoxicity of shikonin metabolites (**1**-**7**) against human tumor cell lines.

Compound	ED_{50} values (μg/ml)					
	PC-3	ACHN	A549	SW620	K562	Du145
Shikonin (**1**)	1.85	0.42	2.19	1.00	3.56	1.39
Anhydroalkannin (**2**)	3.25	2.67	4.13	2.80	5.89	2.85
Deoxyshikonin (**3**)	0.85	0.64	1.03	0.44	1.22	0.80
Shikometabolin A (**4**)	5.21	5.33	5.46	5.26	6.48	>10
Shikometabolin B (**5**)	5.80	6.74	>10	8.09	8.24	>10
Shikometabolin C (**6**)	>10	>10	>10	>10	>10	>10
Shikometabolin D (**7**)	1.45	1.14	0.94	0.48	1.50	2.35
Adriamycin[a]	0.94	0.60	0.95	0.58	0.91	0.81

[a]Positive control.

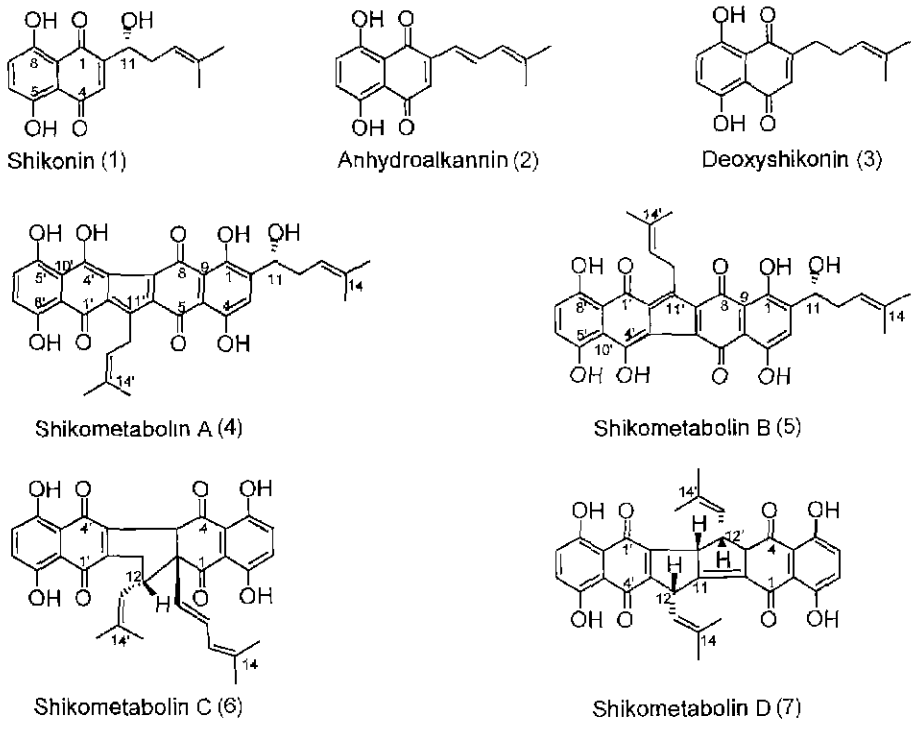

Fig. 1. Structures of shikonin (**1**) and its metabolites (**2**-**7**).

shikonin (**1**) and its metabolites (**2**-**7**) were measured against six human tumor cell lines: PC-3 (prostate), ACHN (renal), A549 (lung), SW620 (colon), K562 (leukemia), and Du145 (prostate). Shikonin (**1**) showed a relatively potent cytotoxicity (ED_{50} values, <4.0 μg/ml) against all the tumor cell lines. The metabolites **2**, **4**, **5**, and **6** showed a weaker cytotoxicity than the parent shikonin (**1**), whereas compounds **3** and **7** exhibited a stronger activity.

Between the two monomeric metabolites (**2** and **3**), compound **3**, which had a double bond in the naphthoquinone side chain, demonstrated an increased cytotoxicity of about two-fold against all the tested tumor cells, with the exception of ACHN, when compared to the parent compound **1**. In addition, compound **3** showed similar activities to adriamycin against PC-5, ACHN, A549, SW620, K562, and Du145 tumor cell lines with ED_{50} values of 0.85, 0.64, 1.03, 0.44, 1.22, and 0.80 μg/ml, respectively. In contrast, compound **2**, which had a diene structure in its side chain, exhibited a reduced activity of about two-fold when compared to that of **1**. A comparison of the shikonin derivatives (**1**-**3**) revealed that the number of double bonds and functional groups in the naphthoquinone side chain were important factors for enhancing the cytotoxicity in tumor cell lines.

Among the dimeric metabolites (**4**-**7**), compound **7** showed the most potent cytotoxicity against PC-3, ACHN, A549, SW620, K562, and Du145 cell lines with ED_{50} values of 1.45, 1.14, 0.94, 0.48, 1.50, and 2.35 μg/ml, respectively. Compounds **4** and **5**, both condensed dimeric metabolites with shikonin tautomers, showed a weaker cytotoxicity than the parent shikonin (**1**). These two compounds were

stereoisomers with a mirror image-like structure of each other and had almost the same activity against all the tested tumor cell lines. Compound **6** was completely inactive with all the cells tested. Compound **7** presented a bicyclopentane spacer between two naphthoquinones, showed an increased cytotoxicity compared to the parent shikonin (**1**), and exhibited the most potent activity among the dimeric metabolites (**4-7**), thus indicating that the bicyclopentane spacer of the dimer has an important role in enhancing cytotoxicity. Among the compounds tested, compounds **3** and **7** exhibited significant cytotoxicities against six tumor cell lines, especially SE620 colon tumor cells. Based on the cytotoxic activity of compounds **3** and **7** against several tumor cell lines, further *in vivo* studies are needed to evaluate the respective antitumor activities of shikonin metabolites.

REFERENCES

1. Ahn, B. Z., K. U. Baik, G. R. Kweon, K. Lim, and B. D. Hwang. 1995. Acetylshikonin analogues: Synthesis and inhibition of DNA topoisomerase-I. *J. Med. Chem.* **38:** 1044–1047.
2. Antonini, I., T. S. Lin, L. A. Cosby, Y. R. Dai, and A. C. Sartorelli. 1982. 2- and 6-Methyl-1,4-naphthoquinone derivatives as potential bioreductive alkylating agents. *J. Med. Chem.* **25:** 730–735.
3. Hodnett, E. M., C. Wongwiechintana, W. J. Dunn, and P. Marrs. 1983. Substituted 1,4-naphthoquinones vs. the ascitic Sarcoma 180 of mice. *J. Med. Chem.* **26:** 570–574.
4. Holshouser, M. H., L. J. Loeffler, and J. H. Hall. 1981. Synthesis and antitumor activity of a series of sulfone analogues of 1,4-naphthoquinone. *J. Med. Chem.* **24:** 853–858.
5. Kim, H. and B. Z. Ahn. 1990. Antitumor effects of acetylshikonin and some synthesized naphthazarin on L1210 and S-180 systems *Yakhak Hoeji* **34:** 262–266.
6. Kim, H. M., S. B. Han, M. S. Kim, J. S. Kang, G. T. Oh, and D. H. Hong. 1996. Efficient fixation procedure of human leukemia cells in sulforhodamine B cytotoxicity assay. *J. Pharm. Tox. Method* **36:** 163–169.
7. Lee, J. H. and B. Z. Ahn. 1986. Antineoplastic natural products and the analogues (XI); cytotoxic activity against L1210 cells of some raw drugs from the oriental medicine and folklore. *Kor J. Pharmacogn.* **17:** 286–291.
8. Lin, A. J., R. S. Pardini, L. A. Cosby, B. J. Lillis, C. W. Shansky, and A. C. Sartorelli. 1973. Potential bioreductive alkylating agents. 2. Antitumor effect and biochemical studies of naphthoquinone derivatives. *J. Med. Chem.* **16:** 1268–1271.
9. Lin, A. J., B. J. Lillis, and A. C. Sartorelli. 1975. Potential bioreductive alkylating agents. 5. Antineoplastic activity of quinoline-5,8-diones, naphthazarines, and naphthoquinones. *J. Med. Chem.* **18:** 917–921.
10. Linardi, M. C., M. M. Oliveria, and M. R. P. Sampaio. 1975. A lapachol derivative active against mouse lymphocytic leukemia P-388. *J. Med. Chem.* **18:** 1159–1161.
11. Meselhy, M. R., S. Kadota, K. Tsubono, M. Hattori, and T. Namba. 1994. Biotransformation of shikonin by human intestinal bacteria. *Tetrahedron* **50:** 3081–3098.
12. Prakash, G. and E. M. Hodnett. 1978. Discriminant analysis and structure-activity relationship. 1. Naphthoquinones. *J. Med. Chem.* **21:** 369–374.
13. Rubinchik, M. A. 1972. Antiamoebic properties of shikonin. *Bittsa USSR*, 236–237.
14. Sankawa, U., Y. Ebizuka, T. Miyazaki, Y. Isomura, H. Otsuka, S. Shibata, M. Inomata, and F. Fukuoka. 1977. Antitumor activity of shikonin and its derivatives. *Chem. Pharm. Bull.* **25:** 2392–2395.
15. Sankawa, U., H. Otsuka, Y. Kataka, Y. Iitaka, A. Hoshi, and K. Kuretani. 1981. Antitumor activity of shikonin, alkannin and their derivatives. II. X-ray analysis of cyclo-alkannin leucoacetate, tautomerism of alkannin and cyclo-alkannin and antitumor activity of alkannin derivatives. *Chem. Pharm. Bull.* **29:** 116–122.
16. Seo, W. T., Y. H. Park, and T. W. Choe. 1992. Production of shikonin by a hairy root culture of *Lithospermum erythrorhizon*. *J. Microbiol. Biotechnol.* **2:** 41–45.
17. Tabata, M., H. Mizukami, S. Naoe, and M. Konoshima. 1975 Antimicrobial activity of *Lithospermum erythrorhizon* callus culture. *Yakugaku Zasshi* **95:** 1376–1379.
18. Tanaka, S., M. Tajima, M. Tsukada, and M. Tabata. 1986. A comparative study on anti-inflammatory activities of the enantiomer, shikonin and alkannin. *J. Nat. Prod.* **49:** 466–469.
19. Tang, W. and G. Eisenbrand. 1992. pp. 204–205. *Chinese Drugs of Plant Origin*. Springer-Verlag, Berlin, Germany.
20. Wu, L., A. M. Smythe, S. F. Stinson, L. A. Mullendore, A. Monks, D. A. Scudiero, K. D. Paull, A. D. Koutsoukos, L. V. Rubinstein, M. R. Boyd, and R. H. Shoemaker. 1992. Multidrug-resistant phenotype of disease-oriented panels of human tumor cell lines used for anticancer drug screening. *Cancer Res.* **52:** 3029–3034.