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Farnesyl Protein Transferase Inhibitory Components of *Lithospermum erythrorhizon*

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Abstract – The methanolic extract of the roots of *Lithospermum erythrorhizon* (Boraginaceae) was found to show inhibitory activity towards farnesyl protein transferase (FPTase). Bioassay-guided fractionation of the methanolic extract resulted in the isolation of three naphthoquinone compounds, as inhibitors of FPTase. These compounds inhibited the FPTase activity in a dose-dependent manner.
Keywords – *Lithospermum erythrorhizon*, Boraginaceae, naphthoquinone, FPTase

Introduction

Farnesyl protein transferase (FPTase), a member of the prenyltransferase enzyme family, is a key post-translational modification step for Ras protein and this is a mandatory process for retention of transforming ability (Oliff, 1999; Reiss, *et al*., 1990). The protein encoded by *ras* is an important part of the signal transduction pathway in the life cycle of the cell. Mutations in the *ras* gene can lead to uncontrolled cell growth and have been reported in human tumors (Suzanne, *et al*., 2001). A crucial post-translational modification required for activation of the Ras protein is farnesylation of the C-terminus. The *ras* gene, discovered in 1978, has attracted a great deal of attention because it was among the first oncogenes found to be associated with human cancer, and studies on the function of Ras have helped in the elucidation of many mitogenic cell signaling pathways (Lowy and Willumsen, 1993; Gibbs, *et al*., 1993; Gibbs, 2000). For Ras oncoproteins to transform mammalian cells, they must be post-translationally modified with a farnesyl group, in a reaction catalyzed by the enzyme, FPTase (Omer, *et al*., 2000). When a farnesylation of these proteins is blocked, their oncogenic activity is abolished (Downward, *et al*., 2003; Oliff, 1999). Therefore, the identification and synthesis of FPTase inhibitors has become an active area for the development of anti-tumor agents (Gibbs, *et al*., 1994; Oh, *et al*., 2005).

Recent work has demonstrated that specific inhibitors of the FPTase might be interesting chemical leads in the development of effective therapeutic agents for the treatment of cancer (Doll, *et al*., 2004; Sepp-Lorenzino, *et al*., 2001; Kohl, *et al*., 1994). Therefore, the discovery of FPTase inhibitors is becoming an active area for the development of anti-tumor agents.

In the course of our screening for potent inhibitors of FPTase from herbal medicines, the total extract of the roots of *Lithospermum erythrorhizon* (Boraginaceae) was found to show inhibitory activity towards FPTase. Subsequent activity-guided fractionation of the methanolic extract led to the isolation of three naphthoquinone compounds, as active principles.

The roots of *L. erythrorhizon* have been used in traditional medicine for skin diseases, dermatitis, burns, hemorrhoid, eczema, and measles in Oriental countries (Kimura, *et al*., 1996). Earlier investigations on the chemical constituents of *L. erythrorhizon* dealt with several naphthoquinone derivatives (Cho, *et al*., 1999; Chung and Lee, 1994; Morimoto, *et al*., 1966, Morimoto

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and Hirata, 1966). Previous pharmacological studies showed that anti-tumor, DNA topoisomerase-I inhibitory, and anti-inflammatory activities (Tanaka, *et al*., 1986; Kim and Ahn, 1990; Lee and Ahn, 1986). This paper describes the isolation of three naphthoquinone compounds from *L. erythrorhizon,* and the inhibitory effects of these compounds towards FPTase.

Experimental

General procedure – ^{1}H- and ^{13}C-NMR spectra were obtained on a JEOL JMN-EX 400 spectrometer. TLC was carried out on Merck precoated silica gel F_{254} plates, with Kiesel gel 60 (230 - 400 mesh, Merck) used as the silica gel. All other chemicals and solvents were of analytical grade and used without further purification. Farnesyl transferase was purified from rat brain homogenates by sequential ammonium sulfate fractionation and Q-sepharose column chromatography (Reiss, *et al*., 1990). Human FPTase was expressed in baculovirus, and purified by affinity column chromatography.

Plant materials – The roots of *L. erythrorhizon* were purchased from the Oriental drug store, Bowha Dang (Jeonju, Korea). A voucher specimen was deposited in the herbarium of the College of Pharmacy, Woosuk University.

Extraction and isolation – The plant material (1 kg) was extracted three times with MeOH at room temperature, and then filtered. The filtrate was evaporated *in vacuo* to give a dark red residue. The resultant methanolic extract (240 g) was subjected to successive solvent partitioning to give *n*-hexane (8 g), $CHCl_3$ (4 g), EtOAc (3 g), *n*-BuOH (20 g) and H_2O soluble fractions. Each fraction was tested for its inhibitory effects on FPTase. Among these fractions, the *n*-hexane-soluble fraction showed the most significant FPTase inhibitory activity. Silica gel column chromatography of the *n*-hexane-soluble fraction, with *n*-hexane-$CHCl_3$-MeOH (20 : 6 : 1), gave five fractions (H1-H5). Fraction H1 was chromatographed by silica gel with *n*-hexane-$CHCl_3$-EtOAc (8 : 2 : 1), and purified by Sephadex LH-20 (MeOH) to give compound **1** (18 mg). Fraction H2 and H3 were chromatographed by Lobar A column with *n*-hexane-$CHCl_3$-MeOH (20 : 6 : 1), and purified by Sephadex LH-20 (MeOH) to give compound **2** (52 mg) and **3** (15 mg), respectively.

Deoxyshikonin (1) – ^{1}H-NMR (400 MHz, $CDCl_3$, δ ppm) : 12.62, 12.46 (each 1H, s, OH), 7.20 (2H, s, H-6,7), 6.84 (1H, s, H-3), 5.15 (1H, m, H-13), 2.64 (2H, m, H-11), 2.29 (2H, m, H-12), 1.70 (3H, s, H-16), 1.60 (3H, s, H-15), ^{13}C-NMR (100 MHz, $CDCl_3$, δ ppm) : 183.0 (C-1, 4), 163.0 (C-5), 162.3 (C-8), 151.5 (C-2), 134.5 (C-3), 133.6 (C-14), 131.2 (C-6), 130.9 (C-7), 122.4 (C-13), 111.7 (C-9, 10), 29.7 (C-12), 26.6 (C-11), 25.7 (C-16), 17.8 (C-15)

Acetylshikonin (2) – ^{1}H-NMR (400 MHz, $CDCl_3$, δ ppm) : 12.54, 12.38 (each 1H, s, OH), 7.15 (2H, s, H-6,7), 6.99 (1H, s, H-3), 6.02 (1H, dd, J = 7.6, 4.4, H-11), 5.13 (1H, m, H-13), 2.62, 2.47 (each 1H, m, H-12), 2.16 (3H, s, $CO\underline{C}H_3$), 1.70 (3H, s, H-16), 1.59 (3H, s, H-15), ^{13}C-NMR (100 MHz, $CDCl_3$, δ ppm) : 178.2 (C-4) , 176.8 (C-1), 169.7 ($\underline{C}OCH_3$), 167.3 (C-5), 166.8 (C-8), 148.2 (C-2), 136.0 (C-14), 132.8 (C-6), 132.6 (C-7), 131.5 (C-3), 117.8 (C-13), 111.8 (C-9), 111.5 (C-10), 69.5 (C-11), 32.9 (C-12), 25.8 (C-16), 20.9 ($CO\underline{C}H_3$), 17.9 (C-15)

Shikonine (3) – ^{1}H-NMR (400 MHz, $CDCl_3$, δ ppm) : 12.57, 12.48 (each 1H, s, OH), 7.18 (2H, s, H-6,7), 7.14 (1H, s, H-3), 4.90 (1H, dd, J = 7.7, 4.3, H-11), 5.20 (1H, m, H-13), 2.64, 2.36 (each 1H, m, H-12), 1.75 (3H, s, H-16), 1.62 (3H, s, H-15), ^{13}C-NMR (100 MHz, $CDCl_3$, δ ppm) : 180.4 (C-4) , 179.6 (C-1), 165.6 (C-5), 165.0 (C-8), 151.5 (C-2), 137.2 (C-14), 132.4 (C-6, 7), 131.9 (C-3), 118.5 (C-13), 112.1 (C-9), 111.5 (C-10), 68.5 (C-11), 35.6 (C-12), 25.8 (C-16), 18.0 (C-15)

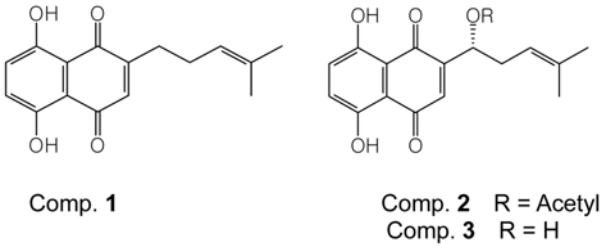
***In vitro* enzyme assay of FPTase** (Reiss, *et al*., 1990) – FPTase assays were performed using a Scintillation Proximity Assay (SPA) kit, following the protocol described by the manufacturer, with the exception of the use of a biotinylated substrate peptide containing the Ki-Ras carboxyl-terminal sequence. The C-terminal peptide of Ki-Ras (Biotin-KKKSKTKCVIM) was synthesized by solid-phase peptide synthesis. The FPTase activity was determined by measuring the transfer of ^{3}H-farnesyl pyrophosphate to Biotin-KKKSKTKCVIM. The inhibitory activity was expressed as follows; % inhibition of FPTase = [1 – (Sample – B2) / (C – B1)] × 100, Blank 1 (B1): without sample and enzyme, Blank 2 (B2): with sample, but without enzyme, Control (C): without sample, but with enzyme (Lee, *et al*., 2002).

Results and Discussion

To isolate the FPTase inhibitory constituents from *L. erythrorhizon*, the total methanolic extract was suspended in water and partitioned successively with *n*-hexane, $CHCl_3$, EtOAc and *n*-BuOH. Each fractions were monitored for their FPTase inhibitory activity (Table 1). The most active *n*-hexane-soluble fraction was subjected to silica gel column chromatography, and purified by Sephadex LH-20 and MPLC, to yield compounds **1 - 3**, as active constituents.

Table 1. FPTase inhibitory activities of the solvent fractions on the leaves of *Lithospermum erythrorhizon* using a Scintillation Proximity Assay

Fraction	Inhibition ratio of FPTase (%, 100 μg/ml)
n-hexane	67.7
$CHCl_3$	46.5
EtOAc	37.9
n-BuOH	30.6



Comp. **1** Comp. **2** R = Acetyl
Comp. **3** R = H

Fig. 1. Structures of compounds **1 - 3**.

Compounds **1 - 3** were dark red amorphous solid pigments obtained from chloroform. NMR spectral data of compounds **1 - 3** were similar each other. The ^{1}H-NMR spectrum of **2** revealed three methyl signals at δ 1.70 (3H, s, H-16), 1.59 (3H, s, H-15) and 2.16 (3H, s, $CO\underline{CH_3}$), four olefinic and/or oxygen bearing signals at δ 7.15 (2H, s, H-6,7), 6.99 (1H, s, H-3), 6.02 (1H, dd, J = 7.6, 4.4, H-11) and 5.13 (1H, m, H-13), and two hydroxyl proton at δ 12.54, 12.38 (each 1H, s, OH). The ^{13}C-NMR spectrum indicated the presence of eighteen carbon signals. From this evidence, the structure of **2** was deduced as one of the naphthoquinone compounds, the isolation from *L. erythrorhizon* of which have previously been reported in the literature. By direct comparison of its spectral and published data, the structure of **2** was established to be acetylshikonin, which has previously been isolated from this plant (Inoue, *et al*., 1985; Hwang, *et al*., 2000). The NMR spectra of **1** and **3** were similar to that of **2**, suggesting it had the same carbon skeleton. The main differences were the NMR chemical shift data for acetyl group. The acetyl groups were disappeared from the NMR spectrum of **1** and **3**. On the basis of the above evidence, together with a direct comparison of the above data with those published in the literature, the structures of **1** and **3** were determined to be deoxyshikonin and shikonin, respectively (Inoue, *et al*., 1985).

Compounds **1 - 3** inhibited the FPTase activity in a dose-dependent manner (Fig. 2), with IC_{50} values of 47.9, 47.6 and 32.8 μg/mL, respectively. Isorhamnetin, an FPTase inhibitor isolated from *Persicaria thunbergii*, showed an IC_{50} value of 12 μg/mL (positive control, Oh, *et al*., 2005).

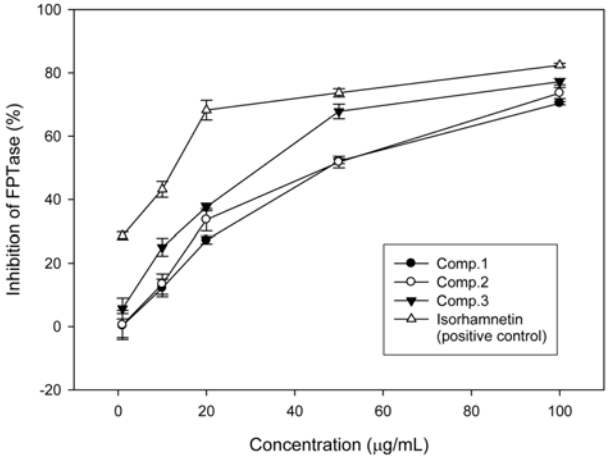


Fig. 2. The inhibitory activity of compounds **1 - 3** on FPTase.

It is well known fact that shikonin derivatives show good anti-inflammatory activities (Kundakovic, *et al*., 2006). In this study, FPTase inhibition assay was performed to prove the anti-tumor effect of isolated compounds **1 - 3** from the roots of *L. erythrorhizon*, and those compounds showed FPTase inhibitory activities in a dose-dependent manner. With anti-inflammatory activity, shikonin derivatives may be useful to treat cancer.

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