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RESEARCH ARTICLE

Inhibition of protein tyrosine phosphatase 1B by lupeol and lupenone isolated from *Sorbus commixta*

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Abstract
Protein tyrosine phosphatase 1B (PTP1B) appears to be an attractive target for the development of new drugs for type 2 diabetes and obesity. In our preliminary test, a MeOH extract of the stem barks of *Sorbus commixta* Hedl. (Rosaceae) showed strong PTP1B inhibitory activity. Bioassay–guided fractionation of the MeOH extract resulted in the isolation of two lupane–type triterpenes, lupenone (1) and lupeol (2). Compounds 1 and 2 inhibited PTP1B with IC_{50} values of 13.7 ± 2.1 and 5.6 ± 0.9 μM, respectively. Kinetic studies revealed that both the compounds 1 and 2 are non–competitive inhibitors of PTP1B that decrease V_{max} values with no effect on K_m values.

Keywords: *Protein tyrosine phosphatase 1B; Sorbus commixta Hedl.; Rosaceae; lupenone; lupeol; non–competitive inhibitors*

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Introduction

Metabolic diseases such as type 2 diabetes and obesity are associated with insulin resistance [1–3]. Recent studies suggest that protein tyrosine phosphatase 1B (PTP1B) plays a major role in the inhibition of insulin action [1–3]. At the cellular level, the insulin signaling is initiated by means of phosphorylation of insulin receptor (IR) and insulin receptor substrates (IRS), which then activates several signaling cascades leading to biological responses such as glucose transport into the cells and glycogen synthesis [1–3]. In this process, PTP1B blocks the signaling pathway by dephosphorylating the activated IR as well as IRS proteins. Accordingly, the enzyme is emerging as a potential therapeutic target for the treatment of type 2 diabetes and obesity [1–4]. Although several types of synthetic PTP1B inhibitors have been developed and applied for clinical trials, due to the toxicity, side effects, and low bioavailability, new types of PTP1B inhibitors still need to be discovered [1–4]. Because natural products are recognized as an attractive source for the development of new PTP1B inhibitors, we have screened for hundreds of plant extracts using in vitro enzyme assay [5]. In the continuing study, we found that a MeOH extract of the stem barks of *Sorbus commixta* Hedl. (common names: mountain ash, scarlet rowan) in the family Rosaceae inhibited PTP1B activity (65% inhibition at 30 μg/mL). The stem bark of *S. commixta* has been used in traditional medicine as a tonic and to treat various respiratory diseases [6]. The fruits have also been used as a laxative, gargle for sore throats, inflamed tonsils, and hoarseness [6]. The biphenyls acuparin and its 2′- and 4′-oxygenated derivatives have been reported as phytoalexins of the genus *Sorbus* [7]. Previous phytochemical investigations on this plant have resulted in the isolation of triterpenes, lignans and flavonoids [8]. In our previous work, the flavanol glycosides, catechin-7-*O*-β-D-xylopyranoside and catechin-7-*O*-β-D-apiofuranoside, isolated from this species, were found to have antioxidant activity [9]. Recently, its extract was demonstrated to have beneficial effects on atherosclerosis and protective effect on hepatic lipid peroxidation in acute-alcohol treated model [10,11]. However, there has been no study with regard to its inhibitory effect on PTP1B. Using a bioassay-guided fractionation, we finally purified two lupane type triterpenoids identified as lupenone (1) and lupeol (2) as active principles. In this paper, we describe the isolation of two active constituents, and the kinetic analyses of the compounds on the enzyme.

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Materials and Methods

Plant material

The stem barks of *S. commixta* were collected in Mt. Sulak, Korea in June 1998, and identified by Professor. KiHwan Bae, College of Pharmacy, Chungnam National University. A voucher specimen (CNU 1081) has been deposited in the herbarium of the College of Pharmacy, Chungnam National University (Korea).

Extraction and isolation

The dried stem barks of *S. commixta* (500 g) were extracted with MeOH (3 L) by reflux. The MeOH extract (48 g) was suspended in H_2O (1 L) and partitioned with EtOAc (900 mL × 3) and BuOH (900 mL × 3), sequentially. The EtOAc–soluble fraction showed PTP1B inhibitory activity (80% inhibition at 30 μg/mL). The EtOAc–soluble fraction (20 g) was chromatographed over silica gel (7 × 40 cm; 70 – 230 mesh) using a gradient of hexane–acetone (from 10:1 to 0:1) to yield seven fractions (Fr. 1 – Fr. 7). Of these, Fr. 2 and Fr. 3 showed the most potent PTP1B inhibitory activity (79 and 87% inhibition at 10 μg/mL). Fr. 2 (2.1 g) was chromatographed on silica gel (4.5 × 40 cm; 15 – 40 μm) with mixtures of hexane–acetone (15:1), to afford lupenone (1, 105 mg). The active fraction, Fr. 3 (3.2 g), was further separated by a silica gel column (4.5 × 40 cm; 15 – 40 μm), eluted with hexane–acetone (6:1), to give lupeol (2, 1100 mg).

Lupenone (1)

colorless needle (from $CHCl_3$-MeOH); mp 169-170°C; $[\alpha]_D$ +62.8° (*c* 1.0, $CHCl_3$); IR ν_{max} cm^{-1}: 3080, 1700, 1648, 890; EIMS *m/z*: 424 $[M]^+$, 409, 313, 218, 205, 189, 161; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 4.69 (1H, m, H-29β), 4.57 (1H, m, H-29α), 2.41 (1H, m, H-19), 1.68, 1.07, 1.07, 1.02, 0.96, 0.93, 0.80 (each 3H, s, 7×CH_3); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 39.6 (C-1), 34.1 (C-2), 217.9 (C-3), 47.3 (C-4), 55.0 (C-5), 19.6 (C-6), 33.6 (C-7), 40.9 (C-8), 49.8 (C-9), 36.9 (C-10), 21.5 (C-11), 25.2 (C-12), 38.2 (C-13), 42.9 (C-14), 27.4 (C-15), 35.6 (C-16), 42.9 (C-17), 48.3 (C-18), 47.9 (C-19), 150.7 (C-20), 29.9 (C-21), 40.0 (C-22), 26.6 (C-23), 21.0 (C-24), 15.8 (C-25), 15.9 (C-26), 14.4 (C-27), 18.0 (C-28), 109.2 (C-29), 19.3 (C-30).

Lupeol (2)

white amorphous powder; mp 210°C; $[\alpha]_D$ +26.0° (*c* 0.8, $CHCl_3$); IR ν_{max} cm^{-1}: 3235, 1640, 1490, 1382, 1185, 1105, 1040, 984, 943; EIMS *m/z*: 426 $[M]^+$, 218, 207, 189; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 4.69 (1H, m, H-29β), 4.57 (1H, m, H-29α), 3.18 (1H, dd, H-3), 2.29 (1H, m, H-19), 1.91 (1H, m, H-21), 1.68, 1.03, 0.97, 0.94, 0.83, 0.79, 0.76 (each 3H, s, 7×CH_3); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 38.6 (C-1), 27.3 (C-2), 78.9 (C-3), 38.8 (C-4), 55.2 (C-5), 18.2 (C-6), 34.2 (C-7), 40.7 (C-8), 50.3 (C-9), 37.1 (C-10), 20.9 (C-11), 25.0 (C-12), 38.0 (C-13), 42.7 (C-14), 27.4 (C-15), 35.5 (C-16), 42.9 (C-17), 48.2 (C-18), 47.9 (C-19), 150.8 (C-20), 29.8 (C-21), 39.9 (C-22), 27.9 (C-23), 15.3 (C-24), 16.1 (C-25), 15.9 (C-26), 14.5 (C-27), 17.9 (C-28), 109.3 (C-29), 19.2 (C-30).

Assays for protein tyrosine phosphatases

PTP1B assay: PTP1B (human, recombinant) was purchased from BIOMOL® International LP (USA). The enzyme activity was measured using *p*–nitrophenyl phosphate (*p*NPP) as described previously [12]. To each 96 well (final volume: 200 μL) was added 2 mM *p*NPP and PTP1B (0.05 – 0.1 μg) in a buffer containing 50 mM citrate (pH 6.0), 0.1 M NaCl, 1 mM EDTA and 1 mM dithiothreitol (DTT) with or without test compounds. Following incubation at 37°C for 30 min, the reaction was terminated with 1 M NaOH. The amount of produced *p*–nitrophenol was estimated by measuring the absorbance at 405 nm. The nonenzymatic hydrolysis of 2 mM *p*NPP was corrected by measuring the increase in absorbance at 405 nm obtained in the absence of PTP1B enzyme.

Dual-specificity protein tyrosine phosphatase (DS–PTP) assay

DS–PTP was assayed with the His tagged–VH1–related human protein (VHR) fusion enzyme. The full-length human VHR(residues 1-185) was expressed in *Escherichia coli* and the intact protein was purified [12]. The reaction mixture containing VHR enzyme, 2 mM *p*NPP and assay buffer (50 mM succinate, 1 mM EDTA, 140 mM NaCl, 0.05% Tween 20, pH 6.0) was incubated at 37°C for 30 min. The reaction was terminated by the addition of 1 M NaOH, and the dephosphorylation activity measured at 405 nm [13].

Protein serine/threonine phosphatase 1 (PP1) assay

The PP1 (Sigma Chemical Co., St. Louis, MO, USA) was measured at 37°C using *p*NPP as a substrate. Reactions were performed for 30 min in the assay buffer (50 mM Tris–HCl, 0.1% β-mercaptoethanol, 1 mM EDTA, 1 mM $MnCl_2$, 20 mM $MgCl_2$, pH 7.6). The reaction was stopped by the addition of 1 M NaOH, and the amount of *p*–nitrophenol was measured by absorbance at 405 nm [12].

Inhibition kinetics

In the kinetic analysis, the reaction mixture consisting of six different concentrations of *p*NPP (0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 mM) used as a PTP1B substrate in the absence or presence of compounds 1 and 2 [12]. The Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) of PTP1B were determined by Lineweaver–Burk plots using a GraphPad Prism® 4 program (GraphPad Software Inc., USA).

Results

Bioassay–guided fractionation on the EtOAc–soluble fraction led to the isolation of active compounds 1 and 2 (Figure 1). The structures of compounds were identified as lupenone (1) and lupeol (2) by physicochemical (mp, $[\alpha]_D$) and spectroscopic data measurement (MS, ^{1}H–NMR, ^{13}C–NMR) and by comparison with published values [8].

As shown in Table 1, lupenone (1) and lupeol (2) inhibited PTP1B activity in a dose–dependent manner with IC_{50} values of 13.7 ± 2.1 and 5.6 ± 0.9 μM, respectively. The known inhibitors RK-682 (IC_{50} = 4.5 ± 0.5 μM) and ursolic

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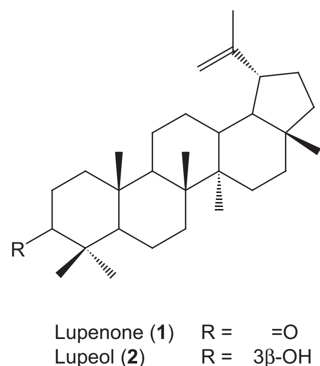

Figure 1. Structures of lupenone (1) and lupeol (2).

Table 1. Comparison of the inhibitory activity of the compounds 1 and 2 isolated from *S. commixta* against PTP1B, VHR and PP1.

	IC_{50} (μM)[a]		
Compounds	PTP1B	VHR DS–PTP	PP1
1	13.7 ± 2.1	> 100	> 100
2	5.6 ± 0.9	> 100	> 100
RK-682[b]	4.5 ± 0.5	NT[c]	NT[c]

[a] IC_{50} values were determined by regression analyses and expressed as mean ± SD of three replicates. [b] Positive control. [c] Not tested.

acid (IC_{50} = 3.5 ± 0.2 μM) were used as positive controls in this assay [13,14]. In addition, both the compounds were tested for the inhibitory effects on other types of protein phosphatases, and it was revealed that the compounds 1 and 2 had no inhibitory effects toward dual–specificity protein tyrosine phosphatase (VHR) and protein serine/threonine phosphatase (PP1) at levels up to 100 μM. This suggests that 1 and 2 have specific inhibitory activity against PTP1B. To elucidate the inhibition mode of 1 and 2 on the activity of PTP1B, kinetic analyses were performed with different concentrations of substrate. As shown in Figure 2, the mechanisms of inhibition by the two compounds were determined using a Lineweaver–Burk plot. When *p*NPP was used as substrate, both the 1 and 2 decreased the V_{max} values, but did not alter the K_m values of PTP1B (Figure 2). Accordingly, both the 1 and 2 were determined as non–competitive inhibitors with K_i values of 11.8 and 3.4 μM, respectively.

Discussion

PTP1B appears to be a promising therapeutic target because the level of PTP1B expression in muscles and adipose tissues is associated with the degree of insulin resistance in subjects with diabetes and obesity [1–4].

During the screening effort we found that a MeOH extract of the stem barks of *S. commixta* inhibited around 65% PTP1B activity at a level of 30 μg/ml. After solvent fractionation, the activity was concentrated in EtOAc–soluble fraction (80% inhibition at 30 μg/ml). Further bioassay–guided fractionation of the EtOAc–soluble fraction resulted in the isolation of two lupane–type triterpenes, lupenone (1) and lupeol (2) as the active principles. Both the compounds

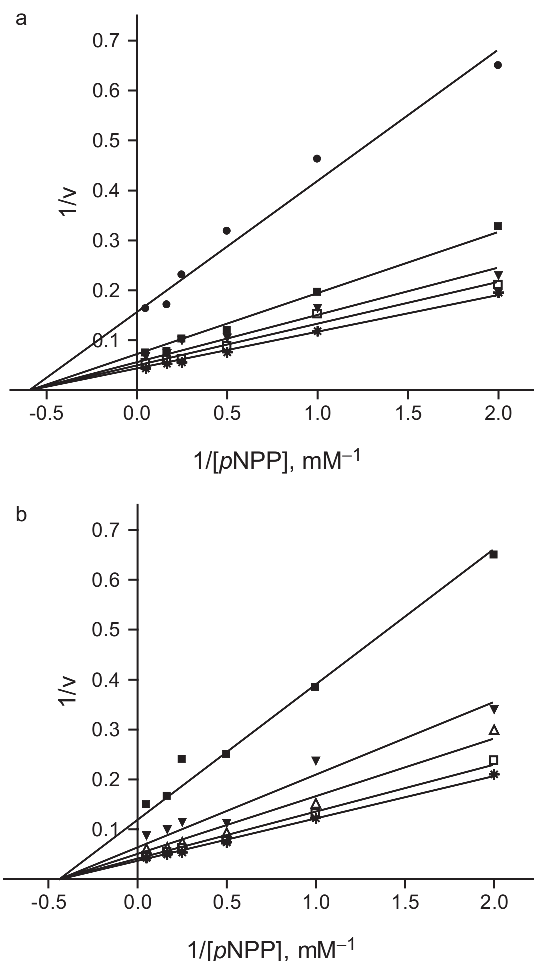

Figure 2. Inhibition kinetics of lupenone (1) and lupeol (2). Panel **a** shows a Lineweaver–Burk plot of the inhibitory effect of compound **1** on PTP1B–catalyzed hydrolysis of *p*NPP. Data are expressed as mean initial velocity for n = 3 replicates at each substrate concentration. Symbols: (*) 0 μM, (□) 1 μM, (▼) 5 μM, (■) 10 μM, (●) 20 μM lupenone (1). Panel **b** shows a Lineweaver–Burk plot of the inhibitory effect of compound 2 on PTP1B. Data are expressed as mean initial velocity for n = 3 replicates at each substrate concentration. Symbols: (*) 0 μM, (□) 1 μM, (Δ) 3 μM, (▼) 5 μM, (■) 10 μM lupeol (**2**).

isolated showed selective inhibitory activity against PTP1B. To examine whether 1 and 2 inhibit PTP1B by interacting with the enzyme's active site, we tested the inhibition kinetics of the compounds with *p*NPP as the substrate. From the kinetic studies, we found that both 1 and 2 inhibited PTP1B in a non–competitive manner, indicating that they may bind to the enzyme–substrate complex or interact with a specific binding site distinct from the active site of the enzyme [15]. Lupane–type triterpenes including lupenone and lupeol have been reported to possess a wide range of bioactivities that include anti-inflammatory, antiviral, antimicrobial, antioxidant, antitumor, antiangiogenic and antimalarial effects [16–21]. Recent studies suggest that various bioactivities of lupeol and lupenone are associated with the inhibition of key enzymes and/or transcription factors such as NF-κB and PI3K/Akt [19,22,23]. Those findings indicate that lupeol and lupenone are capable of modulating signaling cascades in cells, which may be useful for developing a new

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PTP1B inhibitor because PTP1B is an intracellular enzyme. Interestingly, some plants containing lupeol and lupeol itself were demonstrated to have hypoglycemic activity as well as α-amylase inhibitory activity useful for treating diabetes [24,25]. These evidences strongly support that lupane–type triterpenoids can be a lead moiety for the development of new PTP1B inhibitors. Among hundreds of PTP1B inhibitors developed so far, only a few types of molecules such as ursane and oleane type triterpenoids, and kaurane diterpenoids are known as non azole–type inhibitors of PTP1B with micromolar level IC_{50} values [12,26,27]. A lupane structure, as a non azole–type inhibitor, may provide us with better understanding of inhibition mechanism, and be useful in drug design.

In conclusion, bioassay–guided investigation of the MeOH extract of the stem barks of *S. commixta* afforded two lupane–type triterpenes, lupenone (1) and lupeol (2) as active constituents. The present study indicates that these compounds are selective and non–competitive inhibitors of PTP1B. To our knowledge, this is the first time that lupane–type triterpenes have been described as PTP1B inhibitors. Because PTP1B is an intracellular enzyme, further studies to confirm their cellular effects is in progress.

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