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β-Secretase (BACE1) Inhibitors from Pomegranate (*Punica granatum*) Husk

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In the course of screening for anti-dementia agents from natural products, two β-secretase (BACE1) inhibitors were isolated from the husk of pomegranate (*Punica granatum*) by activity-guided purification. They were identified as ellagic acid and punicalagin with IC_{50} values of 3.9 $\times 10^{-6}$ and 4.1$\times 10^{-7}$ M and *Ki* values of 2.4$\times 10^{-5}$ and 5.9$\times 10^{-7}$ M, respectively. The compounds were non-competitive inhibitors with a substrate in the Dixon plot. Ellagic acid and punicalagin were less inhibitory to α-secretase (TACE) and other serine proteases such as chymotrypsin, trypsin, and elastase, thus indicating that they were relatively specific inhibitors of BACE1.

Key words: Pomegranate (*Punica granatum*), Ellagic acid, Punicalagin, β-Secretase inhibitor, Alzheimer's disease

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder clinically characterized by progressive dementia that inevitably leads to incapacitation and death. A major histopathological characteristic of AD is the deposition of amyloid protein (amyloid plaques) in the parenchyma of the amygdala, hippocampus, and neocortex (Sisodia and Price, 1995). The major component of the amyloid plaques is the β-amyloid peptide (Aβ), which is a 39-43 amino acid peptide composed of a portion of the transmembrane domain and the extracellular domain of the amyloid precursor protein (APP) (Glenner and Wong, 1984). The Aβ peptide is generated by endoproteolysis of the large type I membrane protein APP (Selkoe, 2001). A protease called β-secretase initially cleaves the APP to form the *N*-terminus of Aβ at the Asp+1 residue of the Aβ sequence. Following β-secretase cleavage, C99 is the substrate of a second protease, γ-secretase, which cleaves the APP to generate the *C*-terminus of Aβ, and the mature peptide is secreted from the cell. A third protease, α-secretase, non-pathologically cleaves the APP in the middle of the Aβ domain; thus, precluding the formation of Aβ (Vassar, 2002).

Among the secretases, a novel transmembrane aspartic protease BACE1 (for β-site APP Cleaving Enzyme 1) (Anderson *et al.*, 1999; Vassar *et al.*, 1999), also known as Asp2 (for novel aspartic protease 2) and memapsin2 (for membrane aspartic protease/pepsin 2), is at present the most attractive target for the inhibition of amyloid production. There is strong evidence that it is the major β-secretase in neurons and that the absence of BACE1 inhibits production of amyloid and the C99 stubs without any major side-effect (Dewachter and van Leuven, 2002). The lack of Aβ production in BACE1 deficient mice clearly indicates that BACE1 is the β-secretase and that BACE1 inhibitors should reduce Aβ levels. Accordingly, as the key enzyme that initiates Aβ formation *in vivo*, BACE1 is a prime drug target for the inhibition of Aβ production.

Several transition-state analog peptide inhibitors of BACE1 modeled on the β-secretase cleavage site of the Swedish mutation have been reported with relatively low *Ki* values (Schmidt, 2003; Ghosh *et al.*, 2000). Natural product inhibitors, however, have rarely been investigated. All the drugs considered for treatment of Alzheimer's

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disease must cross the blood-brain barrier and the plasma membrane. For BACE1 inhibitors, this requirement might be difficult to meet because currently available BACE1 inhibitors are peptidomimetics of the β-cleavage site of APP. In this sense, secondary metabolites of plants and microbes with relatively low molecular weight and high lipophilicity might be BACE1 inhibitors for the drug candidate. In the course of screening natural products for small molecule BACE1 inhibitors, we tested 256 plant and fungal extracts. An ethanolic extract of pomegranate (*Punica granatum*) showed high inhibitory activity. Here, we report on the isolation and structure elucidation of active compound and on their inhibitory activity for additional proteolytic enzymes.

MATERIALS AND METHODS

General

Fluorescence was measured with a Bio-TEK ELISA microplate fluorescence reader FLx 800 (VT, U.S.A.). Optical density was measured by a Bio-TEK ELx 808 (VT, U.S.A.). The ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker Avance Digital 400 spectrometer (Karlsruhe, Germany) at 400 and 100 MHz, respectively. Chemical shifts were given in δ (ppm) from TMS. TLC was performed on a precoated silica gel plate (Kieselgel 60F254, Merck, NJ, U.S.A.). LC-MS was measured on an API 2000 (U.S.A.) under negative mode. Silica gel column chromatography was carried out using a Kiegelgel 60 (Merck, NJ, U.S.A.). Authentic ellagic acid, Sephadex LH-20, chymotrypsin, trypsin, elastase, and their substrates were purchased from Sigma (MO, U.S.A.).

Enzyme assays

A BACE1 (recombinant human BACE1) assay kit was purchased from PanVera, WI, U.S.A.. The assay was carried out according to the manufacturer's instructions and a reported method (Jeon *et al.*, 2003) with modifications. Briefly, a mixture of 10 μL of assay buffer (50 mM sodium acetate, pH 4.5), 10 μL of BACE1 (1.0 U/mL), 10 μL of the substrate (75 μM Rh-EVNLDAEFK-Quencher in 50 mM ammonium bicarbonate), and 10 μL of a sample dissolved in an assay buffer, was incubated for sixty min at 25°C in the dark. The mixture was excited at 530 nm and the light emitted at 620 nm was collected. The inhibition ratio was obtained by the following equation: Inhibition (%) = $[1 - \{(S - S_0)/(C - C_0)\}] \times 100$, where C was the fluorescence of a control (enzyme, assay buffer, and substrate) after 60 min of incubation, C_0 was the fluorescence of a control at zero time, S was the fluorescence of the tested samples (enzyme, sample solution, and substrate) after 60 min of incubation, and S_0 was the fluorescence of the tested samples at zero time. In order to check the quenching effect of samples, the sample solution was added to reaction mixture C, and any reduction in fluorescence by the sample was investigated. The compounds isolated had only a negligible quenching effect. α-Secretase activity was measured by an α-secretase assay kit with TACE according to the manual from R&D Systems, MN, U.S.A. Chymotrypsin, trypsin, and elastase were assayed according to the protocol described in the references (Chung *et al.*, 1983; Hubert *et al.*, 1992; Bieth *et al.*, 1974) using *N*-benzoyl-L-Arg-*p*NA, *N*-benzoyl-L-Tyr-*p*NA, and *N*-succinyl-Ala-Ala-Ala-*p*NA as substrates, respectively. All data presented are the mean values of triplicate experiments.

Material, extraction, and isolation

The pomegranates were purchased from the Kyungdong market, Seoul, Korea in October, 2002. The voucher specimen had been stored at the Innovative Research Laboratory of Natural Products Medicine, Kyungpook National University, Daegu, Korea. The 760.0 g of powdered pomegranate husk were extracted with 2 L of 95% ethanol in a water bath for two times. The extract was filtered through filter paper and the filtrate was conducted to be evaporated. The extract (190.2 g) was suspended in 1 L of water to be consecutively partitioned with the same volume of dichloromethane, ethyl acetate (EtOAc), and *n*-butanol (BuOH). The EtOAc-soluble fraction (2.20 g), which showed high inhibitory activity against BACE1, was chromatographed on a Sephadex LH-20 column (4.0 × 60.5 cm, 20 to 100% MeOH) to yield Fr. E-1 to E-10. Further purification of the Fr. E-6 (530.0 mg) by HPLC (μ-Bondapak C18, Waters, MA, U.S.A., 7.8×300 mm, 5 to 50% MeCN in 1% HOAc, UV254 nm, 1.8 mL min^{-1}) afforded compound **1** (23.0 mg). The *n*-BuOH fraction (30.81 g) was suspended in water and the suspension was chromatographed on a Diaion HP-20 (Mitsubishi, Tokyo, Japan, 6.0×20.0 cm, 100% water to 100% MeOH). Then, the active fraction (B-2, 15.3 g) was purified again by a Sephadex LH-20 (4.0×65.5 cm, 20 to 40% MeOH) to give compound **2** (800.0 mg).

Ellagic acid (1)

^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.49 (2H, *s*, H-5/5').

Punicallagin (2) (α- and β-anomer mixture)

^{1}H-NMR (400 MHz, Acetone-d_6) δ 2.6 (*m*, H-5), 3.16-3.36 (*m*, H-6), 4.06-4.30 (*m*, H-6), 6.53, 6.61, 6.68, 7.02 (each *s*, aromatic H). ^{13}C-NMR (100 MHz, Acetone-d_6) δ 64.3, 66.8, 71.1, 71.6, 72.6, 74.5, 76.5, 78.9, 90.2 (α-C_1), 94.4 (β-C_1), 157.9, 158.4 (δ-lactone), 168.1, 168.4, 169.3, 169.5 (carboxyl carbon).

RESULTS AND DISCUSSION

The activity-guided purification of the EtOAc soluble fraction and BuOH soluble fraction afforded **1** and **2**, respectively. Compound **1** was obtained as a light brown powder that was positive to the $FeCl_3$ reagent. The only one resonance was observed at δ 7.49. A molecular ion peak ([M^+-1]) was detected at *m/z* 301 in the LC-MS analysis. The ^{1}H-NMR data was identical with that of ellagic acid, which had been previously isolated from *Melaleuca quinquenervia* (Moharram *et al.*, 2003). Compound **2** was obtained as a brown powder that was positive to the $FeCl_3$ reagent. The purified **2** was rapidly converted into two peaks during HPLC analysis, suggesting that it consisted of two inter-convertible isomers. The two peaks appeared as one by increasing the column temperature (Fig. 2), indicating that it had a free anomeric hydroxyl group in sugar skeleton. The LC-MS (*m/z* 1083 [M^+-1], ^{1}H-, and ^{13}C-NMR data were identical with those of the α- and β-punicalagin mixtures, which had been previously isolated from a pomegranate (Tanaka *et al.*, 1986). No recognizable impure peaks were found in the HPLC analysis (more than 99% of purity under conditions described in Materials and Methods).

Compounds **1** and **2** inhibited BACE1 activity in a dose-dependant manner with IC_{50} of 3.9×10^{-5} M and 4.1×10^{-7} M, respectively, in the current study (Fig. 3). The inhibition of **1** and **2** were non-competitive with a substrate in the Dixon plot (Fig. 4). The *Ki* values of **1** and **2** were 2.4×10^{-5} M and 5.9×10^{-7} M, respectively.

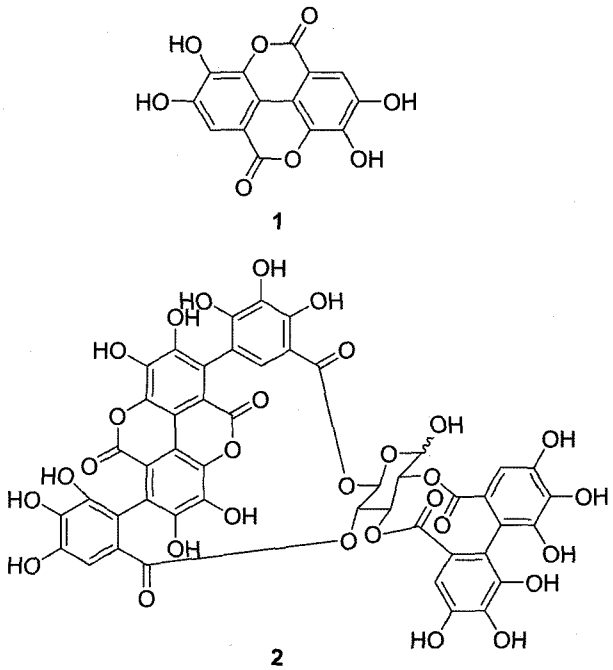

Fig. 1. Structures of ellagic acid and punicalagin

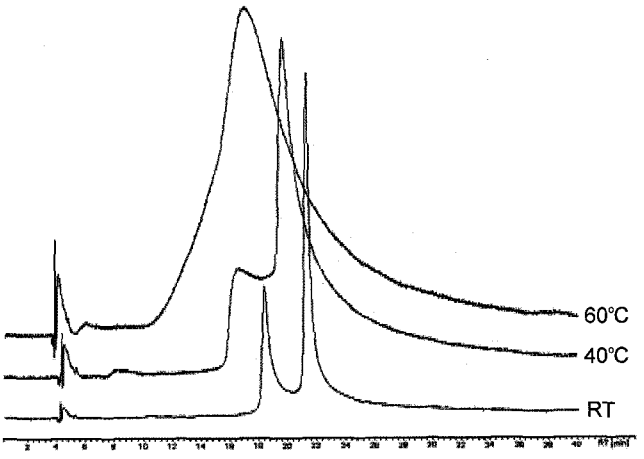

Fig. 2. Temperture-dependant change in the HPLC chromatogram of punicalagin isomers.

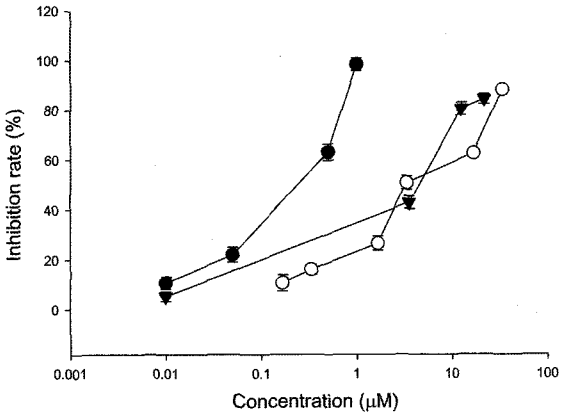

Fig. 3. Dose-dependant inhibition of BACE1 by ellagic acid and punicalagin. The values are the means of triplicate experiments. Punicalagin; -●-, ellagic acid; -○-, (-)-epi-gallocatechin gallate (positive control); -▼-.

In order to check the enzyme specificity, the inhibitory activities on TACE (tumor necrosis factor alpha converting enzyme), which is a candidate for a-secretase (Buxbaum *et al.*, 1998), and other serine proteases such as chymotrypsin, trypsin, and elastase were compared with those of BACE1. Up to 40 μM, **1** and **2** did not show a significant inhibition against the above enzymes, but they inhibited more than 80% of BACE1 at the same concentration level, indicating that they appeared to be relatively specific inhibitors of BACE1.

Peptidomimetic inhibitors such as OM99-1 and OM99-2 (Ghosh *et al.*, 2000) have been synthesized with the *Ki* values of 6.84×10^{-8} to 2.86×10^{-10} M and the IC_{50} values of 1.6×10^{-9} to 3.0×10^{-8} M. These large peptide-based inhibitors, however, have not been developed as drug candidates because the obstacles for Alzheimer therapy are even higher in comparison to those for the inhibition of rennin and HIV protease, which also belong to the

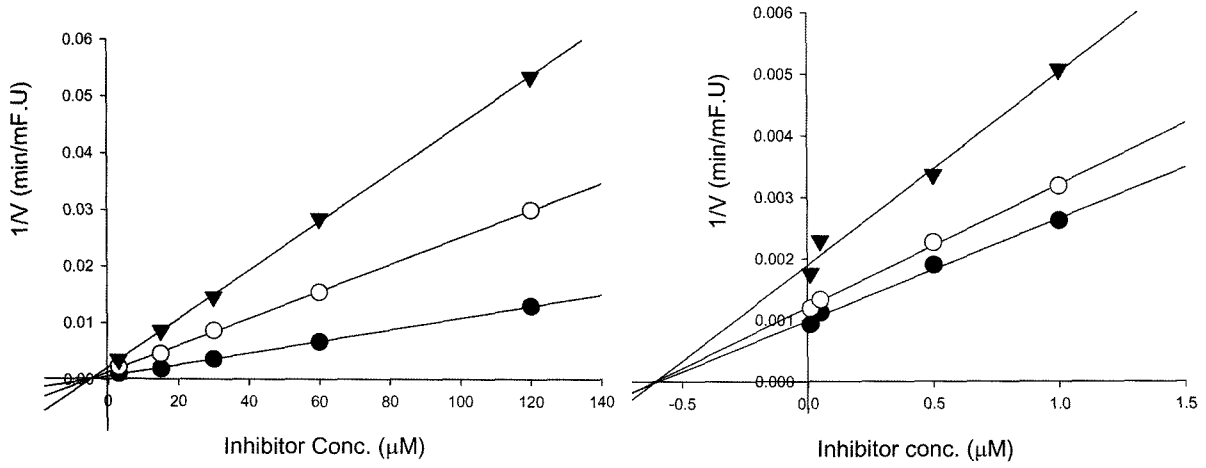

Fig. 4. Dixon plots of inhibition. Inhibition of BACE1 by ellagic acid (left) and punicalagin (right). Substrate concentration: Left; -▼- 375 nM, -○- 563 nM, -●- 750 nM.

Table I. Inhibitory activities[a] of ellagic acid and punicalagin against TACE and other serine proteases

Conc. (μM)	Chymotrypsin		Trypsin		Elastase		TACE	
	1	2	1	2	1	2	1	2
4.0	3.6±0.2	2.3±0.3	3.3±0.3	1.5±0.2	7.2±1.2	3.6±1.2	2.1±0.0	4.2±1.2
20.0	5.4±0.3	5.3±1.0	1.0±0.2	8.8±1.0	8.1±1.3	8.5±3.3	3.1±0.1	8.2±3.3
40.0	7.6±0.2	9.3±2.3	3.0±0.0	10.0±1.1	8.2±2.0	15.3±2.8	5.1±1.0	11.3±2.1
Control[b]	4.4±1.3	2.8±0.5	1.6±0.2	1.8±0.8	8.1±0.2	3.3±1.1	2.1±0.8	3.3±2.8

1, ellagic acid. **2**, punicalagin.
[a]The activity (%) is mean ± SE of the three independent experiments. The inhibition ratio was calculated as described in the experimental section.
[b]Ten μL of 5% MeOH was added to the reaction mixture instead of a sample solution. (-)-Epi-gallogcatechin gallate was used as a positive control for BACE1 assay (IC_{50} = 5.7×10^{-6} M).

aspartic protease group (Ghosh *et al.*, 2001). Takeda reported that the tetraline, which is not an obvious scaffold for protease inhibition, is likely to originate from high-throughput screening efforts. The inhibitory activity is poor and the mode of action is insecure (Miyamoto *et al.*, 2001). Latifolin, isolated from the heartwood of *Dalbergia sisso*, has been found to inhibit Aβ synthesis with an IC_{50} value of 180 μM, again a rather weak and insecure activity (Ramakrishna *et al.*, 2001). The *Ki* and IC_{50} values of ellagic acid and punicalagin were much higher than those of synthetic peptidomimetics. Their *Ki* and IC_{50} values were relatively similar (ellagic acid) to or higher (punicalagin) than those of a natural inhibitor EGCG (IC_{50} = 5.7×10^{-6} M, *Ki* = 2.1×10^{-7} M), which was introduced as a positive control for BACE1 inhibition in this study. Enzyme inhibitors with therapeutic potential are preferably smaller than 700 Da with an IC_{50} value of nM level, so ellagic acid and punicalagin might not be directly considered as a drug candidate. They, however, might be a starting point for rational non-peptidyl drug design and be a useful reagent for studying the enzyme properties of BACE1.

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