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Green Tea Catechins as a BACE1 (β-Secretase) Inhibitor

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Abstract—In the course of searching for BACE1 (β-secretase) inhibitors from natural products, the ethyl acetate soluble fraction of green tea, which was suspected to be rich in catechin content, showed potent inhibitory activity. (−)-Epigallocatechin gallate, (−)-epicatechin gallate, and (−)-gallocatechin gallate were isolated with IC_{50} values of 1.6×10^{-6}, 4.5×10^{-6}, and 1.8×10^{-6} M, respectively. Seven additional authentic catechins were tested for a fundamental structure–activity relationship. (−)-Catechin gallate, (−)-gallocatechin, and (−)-epigallocatechin significantly inhibited BACE1 activity with IC_{50} values of 6.0×10^{-6}, 2.5×10^{-6}, and 2.4×10^{-6} M, respectively. However, (+)-catechin, (−)-catechin, (+)-epicatechin, and (−)-epicatechin exhibited about ten times less inhibitory activity. The stronger activity seemed to be related to the pyrogallol moiety on C-2 and/or C-3 of catechin skeleton, while the stereochemistry of C-2 and C-3 did not have an effect on the inhibitory activity. The active catechins inhibited BACE1 activity in a non-competitive manner with a substrate in Dixon plots.

A major histopathological characteristic of Alzheimer's disease (AD) is the deposition of amyloid protein (amyloid plaque) in the parenchyma of the amygdala, hippocampus, and neocortex.[1] The major component of the amyloid plaque is the β-amyloid protein (Aβ), a 39–43 amino acid peptide composed of a portion of the transmembrane domain and the extracellular domain of the amyloid precursor protein (APP).[2] The Aβ peptide is generated by endoproteolysis of the large type I membrane protein APP.[3] 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. Until very recently, the secretases were known only as the APP-cleaving activities found in cells and tissues, but now molecular identities have been proposed for all. Some groups reported the identification of the β-secretase as the novel transmembrane aspartic protease BACE1 (for β-site APP Cleaving Enzyme 1),[4] also known as Asp2 (for novel aspartic protease 2) and memapsin2 (for membrane aspartic protease/pepsin 2). Enzyme inhibitors with therapeutic potential are preferably smaller than 700 Da, so large peptide-based inhibitors are not viable drug candidates. Thus, the secondary metabolites of plants and microbes which have relatively low molecular weight and high lipophilicity might be a good BACE1 inhibitor for the drug candidate.

By these backgrounds, more than 260 species of herbal drugs were tested for their inhibitory activity on the BACE1. The methanolic extract of commercial green tea showed high inhibitory activity. The activity-guided purification of the fraction yielded three active compounds **1**–**3**.[5] Structures were determined by EIMS, NMR, and a direct comparison with the spectral data of authentic samples. Compound **1** was positive to $FeCl_3$. The UV spectrum showed the typical catechin absorption at 220 and 280 nm.[6] The presence of a galloyl and a pyrogallol group were suggested from the proton resonances at δ 6.94 (2H, s) and δ 6.38 (2H, s). The aromatic signals at δ 5.95 and 5.88 (each 1H, d, $J=2.0$ Hz) and sp^3 proton signals at δ 5.02, 5.29, and 2.75 indicated that **1** had a catechin skeleton. The coupling constant (5.5 Hz) between H-2 and-3 implied that the relative stereochemistry was a *trans*.[7] In the ^{13}C NMR, an ester carbonyl carbon (δ 166.5), three sp^3 carbons (δ 79.0, 70.8, and 24.9), and 18 aromatic carbons (δ 95.8 to 146.9) were shown. From these data, **1** was postulated as (−)-gallocatechin gallate, and was finally confirmed by the comparison of its NMR data with those in the references.[7] **2** was obtained as a pale pinkish white

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powder, and positive to $FeCl_3$. In the EIMS spectrum, molecular ion peak was observed at m/z 458 along with the fragment ion at m/z 289 [M^+ −galloyl], indicating that **2** had a galloyl moiety. In the ^1H NMR spectrum, two aromatic singlets that detected at δ 7.01 (2H) and 6.61 (2H) could be assigned as the symmetric protons in the catechin B-ring and galloyl moiety, respectively. Two *meta*-coupled protons, originated from the catechin A-ring, were observed at δ 6.04 (1H, d, $J=2.0$ Hz) and 6.02 (1H, d, $J=2.0$ Hz). In addition, the signals at δ 5.05, 5.54, and 3.05 showed the typical resonance of H-2, -3, and -4 of the catechin skeleton. The major difference between **1** and **2** was the coupling constant of H-2. Considering the coupling constant of H-2 (broad singlet), the relative stereochemistry of H-2 and -3 should be a *cis*-form.[8–10] A carbonyl carbon (δ 166.5) and three sp^3 carbons (δ 78.5, 69.7, and 25.2) were detected in the ^{13}C NMR spectrum. Accordingly, **2** was assumed to be an epigallocatechin gallate, a 3-epimer of **1**. This was finally confirmed by comparing its NMR data with those in the reported data.[7] Compound **3** was positive to $FeCl_3$ and showed [M^+] at m/z 442 in the EIMS spectrum. The ^1H NMR data were very similar to those of **2** except for the resonances at δ 6.78 (1H, d, $J=8.5$ Hz), 6.91 (1H, dd, $J=8.5$, 2.0 Hz), and δ 7.08 (1H, d, $J=2.0$ Hz), suggesting that the B-ring of **3** was substituted with a catechol moiety instead of a pyrogallol. In the ^{13}C NMR spectrum, a carbonyl (δ 169.6), three sp^3 (δ 76.7, 68.0, and 25.9), and 18 aromatic carbon signals (δ 94.4–155.7) were detected. The coupling constant between H-2 and -3 was almost zero as in the case of **2**. The final structure was identified as (−)-epicatechin gallate by referring to the reported data[7] and a direct comparison with an authentic sample. The structures are presented in Figure 1 and the NMR data is listed in Table 1.

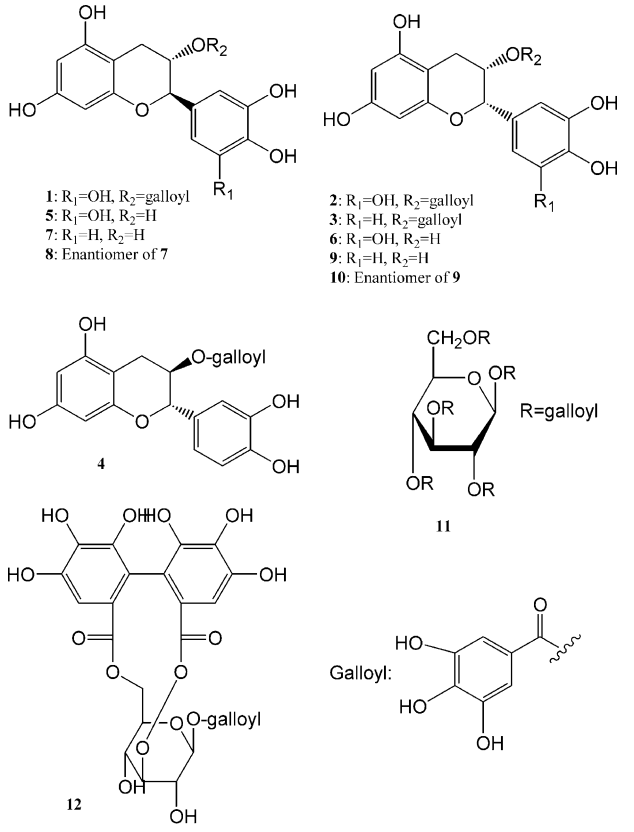

Figure 1. Structures of catechins and related compounds.

All three compounds inhibited BACE1 in a dose-dependent manner[8] and were non-competitive with a substrate in the Dixon plots (Figs. 2 and 3). The inhibition constant (K_i) of **1**, **2**, and **3** were 1.7×10^{-7}, 2.1×10^{-7}, and 5.3×10^{-6} M, respectively. The IC_{50} values are presented in Table 2. To check the fundamental structure–activity relationships, the inhibitory activities of authentic catechins such as (−)-catechin gallate (**4**), (−)-gallocatechin (**5**), (−)-epigallocatechin (**6**), (+)-catechin (**7**), (−)-catechin (**8**), (+)-epicatechin

Table 1. NMR data of compounds **1**, **2**, and **3**

No.	1		2		3	
	δC	δH (multi, *J*, Hz)	δC	δH (multi, *J*, Hz)	δC	δH (multi, *J*, Hz)
2	79.0 (d)	5.02 (d, 5.5)	78.5 (d)	5.05 (brs)	76.7 (d)	5.55 (brs)
3	70.8 (d)	5.29 (m)	69.7 (d)	5.53 (m)	68.0 (d)	5.14 (m)
4a	24.9 (t)	2.72 (dd, 17.0, 5.0)	25.2 (t)	3.02 (dd, 17.4, 4.6)	25.9 (t)	3.06 (dd, 17.5, 5.0)
4b		2.64 (dd, 17.0, 5.5)		2.89 (dd, 17.4, 2.2)		2.93 (dd, 17.5, 2.5)
5	157.6 (s)		157.8 (s)		156.1 (s)	
6	96.7 (d)	5.88 (d, 2.0)	96.8 (d)	6.02 (d, 2.0)	95.1 (d)	6.04 (d, 2.0)
7	158.4 (s)		158.2 (s)		156.4 (s)	
8	95.8 (d)	5.95 (d, 2.0)	96.2 (d)	6.04 (d, 2.0)	94.4 (d)	6.07 (d, 2.0)
9	156.6 (s)		157.5 (s)		155.7 (s)	
10	99.5 (s)		99.4 (s)		97.6 (s)	
1′	131.3 (s)		131.1 (s)		130.0 (s)	
2′	106.6 (d)	6.38 (s)	107.1 (d)	6.61 (s)	113.5 (d)	7.08 (d, 2.0)
3′	146.9 (s)		146.6 (s)		144.1 (s)	
4′	133.7 (s)		133.5 (s)		144.2 (s)	
5′	146.9 (s)		146.6 (s)		114.2 (d)	6.78 (d, 8.5)
6′	106.6 (d)	6.38 (s)	107.1 (d)	6.61 (s)	117.8 (d)	6.91 (dd, 8.5, 2.0)
1″	122.0 (s)		122.2 (s)		120.4 (s)	
2″,6″	110.3 (d)	6.94 (s)	110.3 (d)	7.01 (s)	108.5 (d)	6.94 (s)
3″,5″	146.5 (s)		146.3 (s)		144.6 (s)	
4″	139.4 (s)		139.2 (s)		137.5 (s)	
C=O	166.5 (s)		166.5 (s)		169.6 (s)	

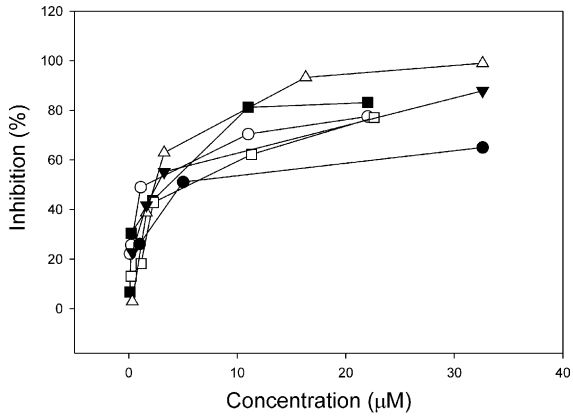

Figure 2. Concentration-dependant inhibition of BACE1 by catechins. (−)-Gallocatechin gallate (**1**) -■-; (−)-epigallocatechin gallate (**2**) -○-; (−)-epicatechin gallate (**3**) -□-; (−)-catechin gallate (**4**) -●-; (−)-gallocatechin (**5**) -△-; (−)-epigallocatechin (**6**); -▼-.

(**9**), and (−)-epicatechin (**10**) were tested. As a result, **7**, **8**, **9**, and **10** showed relatively low activity regardless of their stereochemistry (Table 2). Therefore, at least, a pyrogallol moiety in the catechin skeleton appeared to be essential for the stronger inhibitory activity. Catechins having a pyrogallol group on C-2 (compounds **1**, **2**, **5**, and **6**) were more than 2 times as strong as **3** and **4**, indicating that the pyrogallol ring on C-2 was more responsible for the stronger activity. The galloyl group alone did not seem to be important for the activity since pentagalloyl-β-D-glucopyranoside (**11**)[9] and corilagin (**12**)[10] did not show any significant activity ($IC_{50} > 1.6\times0^{-4}$ M) although they had several galloyl groups. There was no significant difference between catechin stereoisomers, suggesting that stereochemistry was not a great matter.

All the drugs considered as AD must cross the blood–brain barrier and the plasma membrane. For BACE1

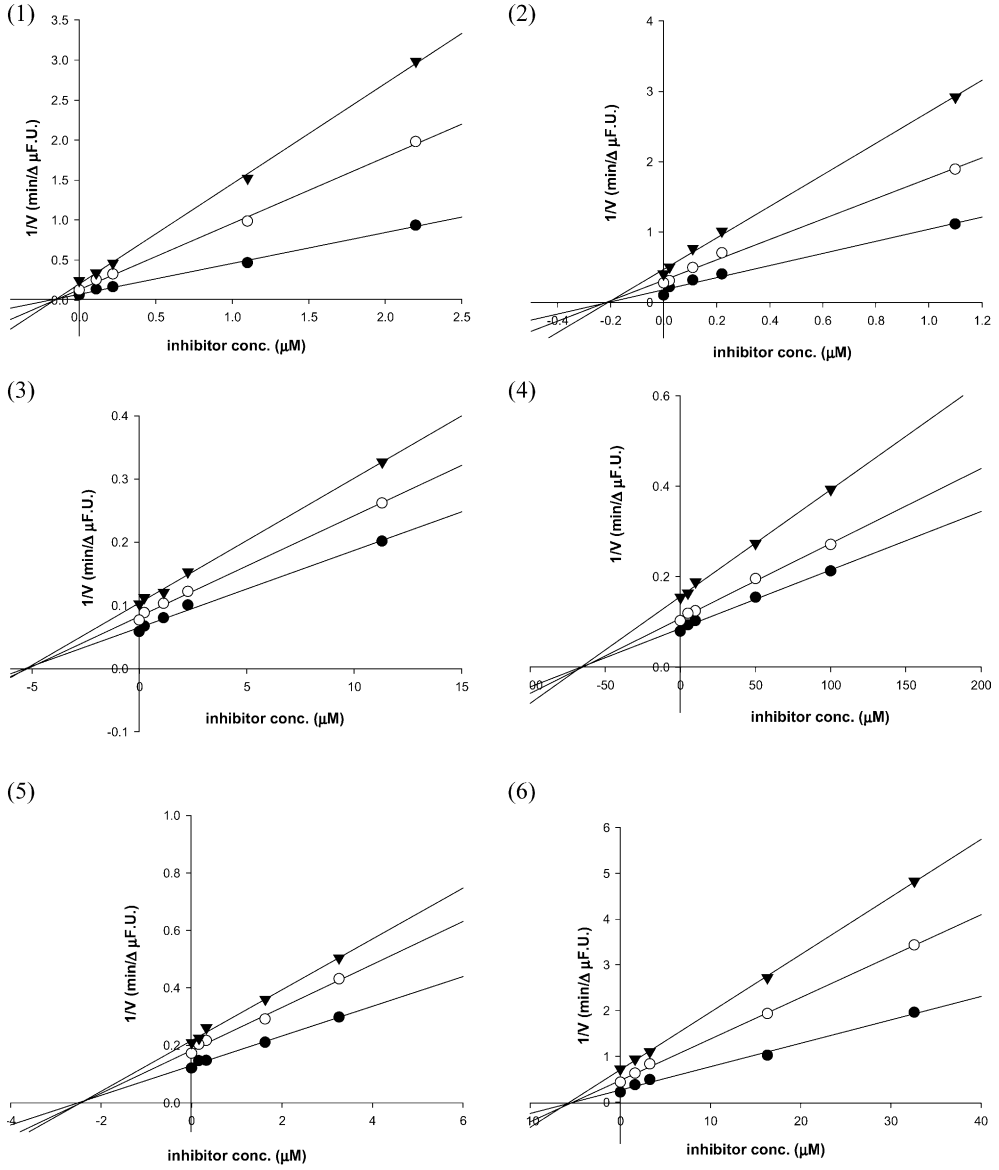

Figure 3. Dixon plots of compound **1**–**6**. Substrate concentration: -▼- 375 nM; -○- 563 nM; -●- 750 nM; **1**, (−)-gallocatechin gallate; **2**, (−)-epigallocatechin gallate; **3**, (−)-epicatechin gallate; **4**, (−)-catechin gallate; **5**, (−)-gallocatechin; **6**, (−)-epigallocatechin.

Table 2. IC_{50} values of catechins as BACE1 inhibitors

	1	2	3	4	5	6	7	8	9	10
IC_{50} (M)	1.8×10^{-6}	1.6×10^{-6}	4.5×10^{-6}	6.0×10^{-6}	2.5×10^{-6}	2.4×10^{-6}	3.5×10^{-5}	3.0×10^{-5}	2.8×10^{-5}	2.3×10^{-5}

inhibitors so far, this requirement might be difficult to meet because currently reported BACE inhibitors are synthetic peptidomimetics of β-cleavage site in APP. Green tea catechins are about 10 times less inhibitory than a statin-based synthetic peptidomimetic inhibitor ($IC_{50}=0.12$ μM),[11] however, it is meaningful in that this is the first report on natural and non-peptidyl BACE1 inhibitors.

The immune response is very active in Alzheimer's disease and may contribute to the disease rather than help. The brain's immune cells respond to the plaques and tangles and attempt to clean up this debris. This is a natural response. However, plaques and tangles are very difficult to dissolve. In the process of trying to digest the material within plaques and tangles, microglia also release pro-inflammatory proteins and free radicals, which cause secondary damage.[12] The isolated compounds, which inhibited not only BACE1 but active oxygen species[13] involved in the brain immune system, are expected to be used in the prevention and treatment of Alzheimer's disease.

Acknowledgements

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5. Dried commercial green tea leaves (100 g) were refluxed in MeOH and the extract was evaporated to dryness. The MeOH extract (14.5 g) was suspended in water and the suspension was partitioned with CH_2Cl_2 and ethyl acetate, consecutively. The ethyl acetate extract (7 g) was chromatographed on a silica gel column (4.5×85 cm, CH_2Cl_2–MeOH–HCOOH = 10:1:0.5→0:100:5) and the resultant active fraction was applied on a Sephadex LH-20 column (2.5×39 cm, 30→70% MeOH) to give Fr I–V. Fr III was suspended in 40 mL of acetone–chloroform mixture (1:1). Concentration of the soluble fraction gave compound **2** (220 mg). HPLC (μBondapak C18, 7.8×300 mm, Waters, 1% HOAc in 30% MeOH) of Fr IV and V afforded 5.2 mg of **3** and 4.8 mg of **1**, respectively. Authentic catechins were the product of Sigma, USA. ^1H and ^{13}C NMR (Bruker Avance Digital 400 spectrometer, Germany) was measure in acetone-d_6 at 400 and 100 MHz, respectively. Chemical shifts were given in δ ppm from TMS (tetramethylsilane). EIMS was recorded on VG QUATTRO II (UK).
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8. BACE1 (recombinant human BACE1) assay kit was purchased from the PanVera Co., USA. The assay was carried out according to the supplied manual with modifications. Briefly, the 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 (750 nM Rh-EVNLDAEFK-Quencher in 50 mM ammonium bicarbonate), and 10 μL of sample dissolved in the assay buffer was incubated for 60 min at 25 °C under dark condition. The mixture was allowed for excitation at 530 nm and the emitted light at 590 nm was collected. The inhibition ratio was obtained by the following equation: inhibition (%) = $[1-\{(S-S_0)/(C-C_0)\}]\times100$, where C was the fluorescence of a control (enzyme, assay buffer, and substrate) after 60 min of incubation, C_0 was the fluorescence of control at zero time, S was the fluorescence of 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. All data are the mean of duplicated experiments. To check the quenching effect of the samples, the sample solution was added to the reaction mixture C, and any reduction in fluorescence by the sample was then investigated. Catechins had only a negligible quenching effect.
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