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Traversal of the Blood Brain Barrier by Cleavable L-Lysine Conjugates of Apigenin

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Abstract

Apigenin, a flavone abundant in parsley and celery, is known to act on several CNS receptors, but its very poor water solubility (< 0.001 mg/mL) impedes its absorption *in vivo* and prevents clinical use. Herein, apigenin was directly conjugated with glycine, L-phenylalanine, and L-lysine to give the corresponding carbamate derivatives, all of which were much more soluble than apigenin itself (0.017, 0.018, and 0.13 mg/mL, respectively). The Lys-apigenin carbamate **10** had a temporary sedative effect on the mice within five minutes of intraperitoneal administration (single dose of 0.4 mg/g), and could be detected in the mice brain tissues at a concentration of 0.82 μg/g of intact Lys-apigenin carbamate **10** and 0.42 ug/g of apigenin at 1.5 h. This study accomplished the delivery of apigenin across the BBB in a manner that might be applicable to other congeners, which should inform the future development of BBB-crossing flavonoids.

Key words: flavonoid; water solubility; blood brain barrier; amino acids; prodrug; apigenin

INTRODUCTION

Apigenin (Figure 1), 4',5,7-trihydroxyflavone, is a flavonoid commonly found in various plants, such as parsley, celery, and chamomile.[1, 2] Several *in vitro* studies found it to affect various receptors of the CNS. For instance, it inhibited adenosine receptors;[3] acted as an opioid antagonist ($\mu = \kappa > \delta$ in affinity);[4] reduced glutamatergic transmission via inhibition of the *N*-methyl-D-asparate receptor when used to treat cultured cortical neurons;[5] competed with flunitrazepem for the benzodiazepine receptor, which is associated with anxiolytic and sedative effects;[6] and inhibited human monoamine oxidase – an enzyme targeted by antidepressants.[7] A series of *in vitro* studies relating to Alzheimer's disease were also conducted, and its antioxidant properties[8] and ability to suppress proinflammatory mediators and protect against the toxicity induced by $A\beta_{25\text{-}35}$ in brain microvascular endothelial cells in rats assessed.[9]

Due to the very poor water solubility of apigenin, which can be ascribed to intramolecular hydrogen bonding and molecular planarity,[10] its therapeutic utility *in vivo* is negligible. From a pharmacokinetic (PK) standpoint, the poor solubility of apigenin retards the process of dissolution, resulting in a low and delayed absorption. Upon oral administration of a single dose of radiolabeled apigenin to a rat, radioactivity could not be detected in the blood for 24 h, reflecting its poor absorption profile and limited availability to the CNS.[11] To date, efforts to improve the solubility of apigenin have been largely focusing on its formulation. For instance, lipid-based vehicles such as

micelles and liposomes, which encapsulate apigenin in a hydrophilic sphere containing one or more lipid layers, could enhance its oral bioavailability.[12-14] Another, related tactic entailed the use of an auxiliary such as 2-hydroxypropyl-β-cyclodextrin to form an inclusion complex.[15] However, few studies have sought to improve the solubility by way of chemical modifications.

The blood-brain barrier (BBB) is a selectively permeable membrane that segregates the cerebral blood and the extracellular fluid in the CNS, and restricts the entry of more than 98% of all small-molecule drugs. The endothelial cells of capillaries are sewed by numerous tight junctions that render solutes either diffusing across lipid membranes (e.g., water, gases, and small lipophilic molecules) or relying on specific transporters in capillary luminal membranes to enter brain tissues (e.g., glucose and amino acids).[16] Good BBB penetration can be achieved by two approaches: non-specific interaction[17] with the brain tissue; or through screening transporter substrates[18] – but non-specific binding to the brain could result in transporter accumulation in peripheral tissues. An alternative approach to take advantages of transporter substrates is to utilize the prodrug approach, in which a drug is conjugated with a BBB carrier by a cleavable linker.[19]

A carbamate bridge in quercetin-amino acid conjugates was reported to be soluble and stable prodrug of quercetin, a flavonol.[20, 21] To the best of our knowledge, no flavone-amino acid conjugate has been reported to enhance BBB uptake. In this work, apigenin was conjugated with amino acids in order to enhance its solubility and ability to cross the BBB. There are three systems

by which amino acids are transported across the BBB[22-24]: 1) system L1, which transports large, neutral amino acids (LNAA), including phenylalanine, tryptophan, leucine, methionine, isoleucine, tyrosine, histidine, valine, threonine and glutamine; 2) system y^+, which transports cationic amino acids (CAA), including lysine, arginine, and ornithine; and 3) system x^-, which transports anionic amino acids (AAA) such as glutamate and aspartate. We reasoned that the amino acids with the highest affinities for transporters in systems L1 and y^+ would be most suitable for conjugation with apigenin; and so the amino acid K_m values (the concentration at which the transport rate is half of the max transport rate Vmax) were compared. As a result, phenylalanine (system L1; K_m = 170 μmol/L; V_{max} = 41 nmol/min/g) and lysine (system y^+; K_m = 279 μmol/L; = 22 nmol/min/g) were selected.[25] Their lowest K_m values in the respective systems represent the highest affinities for their transporters and advantages in BBB-crossing transportation over their competitors. This choice of phenylalanine and lysine is further validated by their categorization as essential amino acids, reflecting the fact that brain tissues require a steady import of them from the blood.[26] System x- is an inferior transportation mechanism to systems L1 and y+ (the uptake of glutamate and aspartate is much lower - V_{max} = 0.21 and 0.13 nmol/min/g for BBB, respectively)[27] and so the conjugation of apigenin with amino acids transported by system x^- was not investigated in this work. Glycine, which is pumped out brain tissues by abluminal Na^+-dependent channels,[28] without the need for a specialized transportation system, was conjugated with apigenin to serve as a negative control. Synthetic methods for amino acid and apigenin conjugations were developed and their

physical-chemical properties, *in vitro* assays for metabolic stability, and *in vivo* trials for PK profiles were evaluated.

MATERIALS AND METHODS

General. Reagents and solvents for synthesis were reagent grade, and used without further purification. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were acquired on a Bruker-AV-400 (400 MHz). Chemical shifts are referenced to residual solvent peaks in parts per million (δ) ^{1}H δ 2.54, ^{13}C δ 40.5 for d_6-DMSO. Coupling constants (*J*) are given in Hertz (Hz). Splitting patterns are denoted as s (singlet), d (doublet), dd (double doublet), t (triplet), q (quartet), m (multiplet). Mass spectra were acquired using a Burker bioTOF III and reported in m/z. HPLC analyses were performed on Shimadzu LC-20AT with a reverse phase Kanto PPC Mightsil-RP18 (250 mm × 4.6 mm, 5 μm) column and UV-Vis detector SPD-M20A (General conditions for chemical synthesis are in the Supplemental).

Synthesis of *tert*-Butyl ((((5-hydroxy-2-(4-hydroxyphenyl)-4-oxo-4H-chromen-7-yl)oxy)carbonyl)amino)-ethanoate (3). Bis-(4-nitrophenyl) carbonate (608 mg, 2.00 mmol) and DIPEA (0.70 mL, 4.0 mmol) were added to a solution of *tert*-butyl glycinate (**2**, 334 mg, 2.00 mmol) in DMF (3 mL). After being stirred for 12 h at rt, the mixture was concentrated *in vacuo* to give a pale yellow oil, which was added without further purification to a solution of apigenin (540 mg, 2.00 mmol) and DIPEA (1.57

mL, 9.00 mmol) in THF (40 mL). The mixture was stirred for another 12 h and concentrated *in vacuo* to give a residue which was purified by column chromatography (silica gel, tetrahydrofuran/hexane = 4/5, R_f = 0.44) to give **3** (383 mg, 45%) as a white solid; ^{1}H-NMR (d_6-DMSO, 200 MHz) δ 12.87 (s, 1H), 8.31 (t, 1H, *J* = 6.0 Hz), 8.14 (d, 2H, *J* = 8.6 Hz), 7.35 (d, 2H, *J* = 8.6 Hz), 6.99 (s, 1H), 6.55 (d, 1H, *J* = 1.7 Hz), 6.25 (d, 1H, *J* = 1.7 Hz), 3.80 (d, 2H, *J* = 6.0 Hz), 1.47 (s, 9H); ^{13}C-NMR (d_6-DMSO, 50 MHz) δ 128.8, 169.8, 165.4, 163.6, 162.4, 158.4, 155.2, 154.7, 128.9, 128.5, 123.1, 106.0, 104.9, 100.0, 95.1, 82.0, 43.9, 28.7; HRMS (ESI-TOF) calcd. for $C_{22}H_{22}NO_8$ $[M+H]^+$: 428.1345, found m/z 428.1355.

Synthesis of ((((5-hydroxy-2-(4-hydroxyphenyl)-4-oxo-4H-chromen-7-yl)oxy)carbonyl)amino)ethanoic acid (4). 3 (250 mg, 0.58 mmol) was dissolved in TFA (6 mL) at 0 °C, and the mixture was stirred for 3 h. After concentration *in vacuo*, the residue was recrystallized from acetone to give **4** (198 mg, 88%; R_f value = 0.32, THF/hexane = 4/5) as a pale yellow solid. ^{1}H-NMR (d_6-DMSO, 200 MHz) δ 12.88 (s, 1H), 8.31 (t, 1H, *J* = 6.0 Hz), 8.14 (d, 2H, *J* = 8.6 Hz), 7.35 (d, 2H, *J* = 8.6 Hz), 6.99 (s, 1H), 6.56 (d, 1H), 6.26 (d, 1H), 3.83 (d, 2H, *J* = 6.0 Hz); ^{13}C-NMR (d_6-DMSO, 50 MHz) δ 182.9, 172.2, 165.4, 163.7, 162.5, 158.5, 155.2, 154.8, 128.9, 128.5, 123.2, 106.0, 104.9, 100.1, 95.1, 43.3; HRMS (ESI-TOF) calcd. for $C_{18}H_{13}NO_8$ $[M+H]^+$: 372.0719, found m/z 372.0732. The HPLC purity of 4 was 96.4% (t_R =5.97 min, eluted with 0.5% formic acid aqueous solution and methanol, 0 min 35/65 → 9 min 5/95 → 15 min 5/95 → 18 min 50/50).

Synthesis of (*S*)-*tert*-Butyl 2-((((5-hydroxy-2-(4-hydroxyphenyl)-4-oxo-4H-chromen-7-yl)oxy)carbonyl)amino)-3-phenylpropanoate (6). A solution of L-phenylalanine *tert*-butyl ester hydrochloride (**5**, 2.89 g, 11.2 mmol) and DIPEA (19.5 mL, 11.2 mmol) in DMF (10 mL) was stirred for 10 min at r.t., and then added via cannula into solution of bis-(4-nitrophenyl) carbonate (3.40 g, 16.7 mmol) in THF (50 mL) at 0 °C. An additional equivalent of DIPEA (19.5 mL, 11.2 mmol) was added dropwise to the mixture over 2 min. The mixture was stirred at 0 °C for 2 h. TLC analysis showed a major product with an Rf value of 0.44 (THF/hexane = 1/2). The resulting mixture was transferred to a dropping funnel and added to a solution of apigenin (3.02 g, 11.2 mmol) and DIPEA (8.80 mL, 50.4 mmol) in DMF/THF (7 mL/38mL) dropwise, over 1 h. After being stirred for 6 h at rt, the mixture was concentrated *in vacuo* to give a residue which was purified by column chromatography (silica gel, THF/hexane = 4/7, with 0.5% acetic acid, R_f value = 0.32) followed to afford **6** (2.94 g, 51%; 92 % yield based on recovered starting material, 1.36 g of **1**) as a pale yellow solid. ^{1}H-NMR (d_6-DMSO, 400 MHz) δ 12.87 (s, 1H), 8.67 (d, 1H, J = 8.0 Hz), 8.12 (d, 2H, J = 8.8 Hz), 7.38-7.26 (m, 5H), 7.25 (d, 2H, J = 8.8 Hz), 6.98 (s, 1H), 6.55 (d, 1H, J = 2.0 Hz), 6.26 (d, 1H, J = 2.0 Hz), 4.29-4.22 (m, 1H, J_1 =9.6 Hz, J_2 = 8.0 Hz, J_3 = 5.8 Hz), 3.13-3.08 (dd, 1H, J_1 = 13.7 Hz, J_2 = 5.8 Hz), 3.01-2.96 (dd, 1H, J_1 = 13.7 Hz, J_2 = 9.6 Hz), 1.40 (s, 9H); ^{13}C-NMR (d_6-acetone, 100 MHz) δ 183.8, 172.0, 165.9, 164.8, 164.1, 159.6, 155.8, 155.2, 138.9, 131.0, 130.0, 129.6, 129.3, 128.3, 127.6, 123.7, 117.3, 109.1, 106.7, 106.7, 106.2, 100.6, 95.7, 95.6, 82.9, 68.3, 58.0, 57.9, 39.1, 28.8, 25.3; HRMS (ESI-TOF)

calcd. for $C_{29}H_{28}NO_8$ $[M+H]^+$: 518.1809, found m/z 518.1809.

Synthesis of

***(S)*-2-((((5-hydroxy-2-(4-hydroxyphenyl)-4-oxo-4H-chromen-7-yl)oxy)carbonyl)amino)-3-phenylpropanoic acid (7)**. **6 (**268 mg, 0.518 mmol) was dissolved in TFA (5 mL) at 0 °C, and the mixture was stirred at 0 °C for 3 h. The mixture was diluted with toluene (1 mL), then concentrated *in vacuo* to give a residue which was purified by column chromatography (silica gel, THF/hexane = 5/7, 0.5% acetic acid, R_f value = 0.25) to afford **7** (215 mg, 90 %) as a pale yellow solid. ^{1}H-NMR (d_6-DMSO, 400 MHz) δ12.88 (s, 1H), 8.42 (d, 1H, J = 8.4 Hz), 8.10 (d, 2H, J = 8.8 Hz), 7.39-7.28 (m, 5H), 7.22 (d, 2H, J = 8.8 Hz), 6.98 (s, 1H), 6.55 (d, 1H, J = 2.0 Hz), 6.25 (d, 1H, J = 2.0 Hz), 4.33-4.27 (m, 1H, J_1 = 10.7 Hz, J_2 = 8.4 Hz, J_3 = 4.3 Hz), 3.13-3.08 (m, 1H, J_1 = 13.8 Hz, J_2 = 4.3 Hz), 3.01-2.96 (m, 1H, J_1 = 13.8 Hz, J_2 = 10.7 Hz), 1.40 (s, 9H); ^{13}C-NMR (d_6-DMSO, 100MHz) δ 182.9, 173.9, 165.4, 163.6, 162.5, 158.4, 154.7, 138.7, 130.3, 130.2, 129.3, 128.9, 128.8, 128.4, 127.6, 123.0, 106.0, 104.9, 100.0, 95.1, 56.7, 37.5; HRMS (ESI-TOF) calcd. for $C_{25}H_{18}NO_8$ $[M-H]^+$: 460.1027, found m/z 460.1025. The HPLC purity of **7** was 99.4% (t_R = 4.22 min, eluted with 0.5% formic acid aqueous solution and methanol, 10/90 for 18 min).

Synthesis of

(*S*)-6-((*tert*-Butyloxycarbonyl)amino)-2-((((5-hydroxy-2-(4-hydroxyphenyl)-4H-chrome-7yl)oxy)carbonyl)amino)hexanoic acid (9). A solution of Ne-Boc-L-lysine *tert*-butyl ester hydrochloride (**8**, 332 mg, 0.987 mmol) and DIPEA (181 μL, 1.03 mmol) in DMF (4 mL) was stirred for 10 min,

148 then added via cannula into a cold (0 °C) solution of bis-(4-nitrophenyl) carbonate (313 mg, 1.03
149 mmol) in THF (10 mL). An additional equivalent of DIPEA (181 μL, 1.03 mmol) was added
150 dropwise to the mixture over 2 min. The mixture was stirred at 0 °C for 2 h. The resulting mixture
151 was transferred to a dropping funnel and added to a solution of apigenin (266 mg, 0.987 mmol) and
152 DIPEA (773 μL, 4.44 mmol) in DMF/THF (3 mL/15mL) dropwise, over 1 h. After being stirred for
153 6 h at rt, the mixture was concentrated *in vacuo* to give a residue which was purified by column
154 chromatography (silica gel, THF/hexane = 1/2, then DCM/MeOH = 100/2) followed to afford the
155 apigenin-lysine(Boc)-OBn conjugate **11** (R_f value = 0.27, THF/hexane = 2/3) as pale yellow liquid.
156 Then, 20% Pd/C (60 mg) was added to a solution of **11** in THF (15 mL), and the resulting slurry
157 was stirred under positive pressure of H_2 for 30 h. After the filtration through a pad of Celite with
158 THF) and purification by column chromatography (silica gel, THF/hexane = 1/1, 1% AcOH), **9**
159 (282 mg, 0.520 mmol, 53% overall) was obtained as clear liquid. ^{1}H-NMR (d_6-DMSO, 400 MHz) δ
160 12.89 (s, 1H), 8.30 (d,1H, *J* = 7.8 Hz), 8.14 (d, 2H, *J* = 8.8 Hz), 7.35 (d, 2H, *J* = 8.7 Hz), 7.00 (s,
161 1H), 6.87 (t, 1H, *J* = 5.4 Hz), 6.56 (d, 1H, *J* = 1.9 Hz), 6.26 (d, 1H, *J* = 2.0 Hz), 4.03-3.98 (m, 1H),
162 3.87-3.77 (m, 1H), 3.63 (t, 3H, *J* = 6.7 Hz), 2.96 (d, 2H, *J* = 4.6 Hz), 1.82-1.68 (m, 6H), 1.41 (s,
163 9H) ; ^{13}C-NMR (d_6-DMSO, 100 MHz) δ 182.9, 174.5, 165.4, 163.7, 162.5, 158.4, 156.6, 154.8,
164 129.5, 128.9, 128.4, 123.1, 117.0, 108.0, 106.0, 104.9, 100.0, 95.1, 78.4, 68.0, 67.62, 55.1, 31.4,
165 30.1, 29.9, 26.1, 24.6, 23.9; HRMS (ESI-TOF) calcd. for $C_{27}H_{31}N_2O_{10}$ $[M+H]^+$: 543.1973, found
166 m/z 543.1982.

Synthesis of (*S*)-6-amino-2-((((5-hydroxy-2-(4-hydroxyphenyl)-4H-chrome-7yl)oxy)carbonyl)amino) hexanoic acid hydrochloride (10). The mixture was diluted with toluene (1 mL), then concentrated *in vacuo* to give a residue which was purified by column chromatography (silica gel, THF/hexane = 5/7, 0.5% acetic acid, R_f value = 0.25) to afford **7** (215 mg, 90 %) as a pale yellow solid. **9** (190 mg, 0.35 mmol) was dissolved in TFA (3 mL) at 0 ^oC, and the mixture was stirred for 3 h. The mixture was diluted with toluene (1 mL) and saturated ammonium chloride solution (0.5 mL), then concentrated *in vacuo* to give a residue which was recrystallized under methanol to afford **10** (139 mg, 90 %) as a pale brown solid. NMR data refers to the TFA salt. ^{1}H-NMR (d_6-DMSO/d-TFA = 10/1, 400 MHz) δ 8.31 (d,1H, J = 7.8 Hz), 8.13 (d, 2H, J = 8.7 Hz), 7.34 (d, 2H, J = 8.7 Hz), 7.00 (s, 1H), 6.55 (s, 1H), 6.27 (s, 1H), 4.06-4.03 (m, 1H), 3.87-3.78 (m, 3H), 2.92-2.76 (m, 2H), 2.17-1.18 (m, 6H); ^{13}C-NMR (d_6-DMSO/d-TFA = 10/1, 100 MHz) δ 182.9, 174.3, 165.3, 163.6, 162.3, 158.5, 154.8, 128.9, 128.5, 123.1, 108.0, 106.8, 106.0, 104.9, 67.6, 54.8, 31.1, 29.7, 27.4, 24.3, 24.2, 23.5; HRMS (ESI-TOF) calcd. for $C_{22}H_{23}N_2O_8$ $[M\text{-}Cl]^+$: 443.1449, found m/z 443.1448. The HPLC purity of **10** was 97.2% (t_R = 4.43 min, eluted with 0.5% formic acid aqueous solution and methanol, 0 min 40/60 → 18 min 50/50).

Measurement of solubility. Saturated solutions of apigenin derivatives in 1.0 mL ddH_2O were equilibrated by shaking for 24 h and then centrifuged at 14000 g for 10 min. The supernatants were filtered through 0.22 μm PTFE membranes into 12 × 32 mm vials. Standard curves were established

starting from 5 mg/mL stock solutions of Gly-apigenin carbamate **4**, Phe-apigenin carbamate **7**, and Lys-apigenin carbamate **10** in THF, which diluted into concentrations of 0.5, 0.1, 0.05, 0.025, 0.0125, and 0.00625 mg/mL. An aliquot of 1 μL sample solution was injected to HPLC each time. Areas of corresponding peaks (λ = 254 nm) were plotted against concentrations.

Measurement of log P. The oil-water partition coefficient P (log P) is the ratio of the equilibrium concentration of a compound dissolved in a two-phase system consisting of two immiscible solvents. The log P value is related to the log k value in a reverse phase chromatography by the following equation:[29]

$$\log P = a \log k + b$$

$$k = \frac{(t_R - t_0)}{t_0}$$

where k is the capacity factor, t_0 is the dead time of $NaNO_2$, which is unretained by the stationary phase, and t_R is the retention time of the corresponding compound. The analytical standards of reference compounds, including benzyl alcohol, phenol, thiophene, benzene, and toluene, were prepared at a concentration of 1 mg/mL in methanol. An aliquot of 1 μL was injected each time, isocratically eluted with 0.5% formic acid aqueous solution and methanol (30/70, v/v). Generally, the log P is linearly related to log k. By plotting log P values of the reference standards versus their log k, the regression equation was acquired as: log P = 1.6712 log k + 1.7904 (R^2 = 0.9621).

Human Liver S9 Stability Assay. The suspension for the S9 assay was prepared as follows: 2.2

mg/mL human liver S9 fraction (20-donor pool, mixed gender, Corning® Gentest™), 1.1 mM EDTA, 3.67 mM $MgCl_2$ and NADPH regenerating system (1.43 mM NADP, 3.67 mM glucose-6-phosphate and 0.44 U/mL glucose-6-phosphate dehydrogenase) at a total volume of 90 μL. After the suspension was pre-incubated at 37 °C for 10 min, the substrate (Gly-apigenin carbamate **4,** Phe-apigenin carbamate **7,** or Lys-apigenin carbamate **10,** 0.5 mM in 10 μL PBS) was added to the suspension and incubated for 0, 5, 30, and 60 min, after which experiments were halted by dilution with 100 μL of a mixture of methanol/acetonitrile (25/75, v/v) at 0 °C. Followed by vortexing and centrifugation at 14000 *g* for 10 min, supernatants were filtered through 0.22 μm PTFE membranes into 12x32 mm vials. An aliquot of 10 μL the sample solution was injected to HPLC each time, isocratically eluted with 0.5% formic acid aqueous solution and methanol (30/70, v/v). Areas of corresponding peaks (λ = 254 nm) were plotted against concentrations.

Drug administration, sampling, extraction, and sample preparation. Eight-week-old male, wild-type C56BL/6 (C57BL/6JNarl) mice were purchased from the National Laboratory Animal Center (Nangang, Taiwan), kept at the laboratory animal center of National Taiwan University with *ad libitum* access to water and food, and acclimated for at least 3-7 days. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC no. 20150014). In following PK experiments, all suspensions were injected intraperitoneally. Mice were sacrificed under anesthesia using 2,2,2-tribromoethanol (Avertin®, MilliporeSigma, Brulington, MA, USA, 0.25 mg/g body weight by 2 min in advance) at the following time points: 15 min and 1.5 h after

one bolus of Gly-apigenin carbamate **4**, Phe-apigenin carbamate **7**, and Lys-apigenin carbamate **10** (0.4 mg/g wt.; 0.2 mg/μL in PBS); 15 min, 0.5, 1, 1.5, 2, 4, 6, and 12 h after one bolus of apigenin **1** (0.23 mg/g wt.; 0.115 mg/μL in PBS) or Lys-apigenin carbamate **10** (0.4 mg/g wt.; 0.2 mg/μL in PBS). Blood samples were collected via cardiac puncture by syringes preloaded with 100 μL 20% EDTA-K_2 solution, followed by centrifugation at 6000 *g* for 10 min. The entire dissected intracranial contents were placed into round bottom, two milliliter microcentrifuge tubes with 1 mL extraction buffer (T-PER®, ThermoFisher Scientific, Waltham, MA, USA) and 7 mm stainless steel beads, then homogenized by Tissuelyser II® (QIAGEN, Hilden, Germany) at 30 Hz for 3 min. All of the containers were maintained at 0-5 °C throughout the processes. All samples were stored at -30 °C and analyzed within 12 h. Samples of 100 μL plasma and 200 μL brain crude suspension were extracted with 200 μL and 600 μL of a 5 mM solution of ammonium acetate in a mixture of THF/methanol, respectively. After centrifugation at 14000 *g* for 15 min, supernatants were filtered through 0.22 μm PTFE membranes into 12 × 32 mm vials for UPLC-MS/MS analyses.

UPLC-MS/MS analyses. Analyses were performed on a Waters ACQUITY UPLC system and a Waters Quattro Premier XE triple-quadrupole mass spectrometer (Waters, Milford, MA, USA). The reverse phase BEH C18 (100 mm × 2.1 mm, 1.7 μm, Waters) and VanGuard BEH C18 (5 mm×2.1 mm, 1.7 μm, Waters) pre-columns were used to separate the analytes. All data were acquired by MassLynx V4.1. The mobile phase consisted of a 5 mM aqueous solution of ammonium acetate (A) and methanol (B), set as follows: 0.00 min 70% A → 0.50 min 70% A → 4.00 min 10% A → 5.00

241 min 10% A → 5.50 min 70% A → 8.50 min 70% A; at a rate of 0.3 mL/min for 8.50 min with 10
242 μL per injection. The column oven was maintained at 60 °C. Multiple reaction monitoring (MRM)
243 method was applied in quantification. Mass spectrometer parameters were: capillary voltage, 3.0
244 kV/ 3.0 kV respectively for positive/negative ion mode; ion source temperature, 120 °C; desolvation
245 temperature, 450 °C; cone gas flow (N_2), 50 L/h; desolvation gas flow (N_2), 700 L/h; multiplier, 650
246 V; collision gas pressure (Ar), 3-4 × 10^{-3} mbar. Other parameters are listed in Table 1**.**

247 **Calculation of the concentration in brain tissues.** Brains were not perfused to wash out the blood
248 because this procedure would have altered the chemical equilibrium between the vascular
249 compartment and the whole brain.[30] The concentration of compound in brain tissues was calculated
250 by the literature reported equation with modification.[31]

251

$$\frac{(suspension\ volume\ \times suspension\ concentration)\ -(CBV\ \times\ blood\ concentration)}{weight\ of\ entire\ intracranial\ content}$$

252

253 The 'suspension' refers to the entire intracranial contents, homogenized in extraction buffer; the
254 average volume of which was 1.8 mL. The concentration in the numerator was determined by the
255 peak area of the m/z transition from a precursor ion to its corresponding product ion for
256 quantification (quantifiers, as shown in Table 1). The cerebral blood volume (CBV) was 35 μL per
257 gram of brain.[32] The concentration in brain tissues was obtained by subtracting the amount of
258 compound in the cerebral blood vessels from that in the suspension, then divided by the weight of
259 entire intracranial content, expressed in μg/g. The contribution of cerebral blood to the weight of the

whole brain is neglected in the calculation.

PK and statistical analyses. WinNonlin^{TM} (version 5.2, Pharsight, MO, USA) was used to process the plasma concentration versus time data. C_{max} was the observed maximum concentration, and T_{max} corresponding to the time when acquired. $AUC_{0\rightarrow t}$ represented the area under the plasma concentration versus time curve from time zero to the time of the last quantifiable concentration, which was calculated by trapezoidal rule. λ_z was estimated by linear regression with the last three points on the semi-log plot of the plasma concentration versus time curve. Terminal half-life ($t_{1/2}$) was derived from ln 2 over λ_z. $AUC_{0\rightarrow\infty}$ was the sum of $AUC_{0\rightarrow t}$ and the extrapolation term (as calculated by the last quantifiable concentration over $\lambda_{z)}$. Apparent clearance (CL/F) was derived from the dose over $AUC_{0\rightarrow\infty}$, further divided by λ_z for apparent volume of distribution (V/F). Mean residue time (MRT) was acquired by using a non-compartment method.[33]

RESULTS AND DISCUSSION

In our synthetic strategy, a carbamate linkage is used to bridge the amino groups of the amino acids and the 7-OH of apigenin **1** (Figure 2). The conjugation process entails formation of the corresponding *p*-nitrophenyl carbamates of the amino acids using bis-(*p*-nitrophenyl) carbonate under basic conditions, followed by substitution of apigenin **1** for the remaining *p*-nitrophenyl group. Bis-(*p*-nitrophenyl) carbonate is a relatively mild, selective coupling reagent compared with triphosgen or *p*-nitrophenyl chloroformate, which are more reactive and possess higher reactivity

precipitating the formation of ureas via double nucleophilic substitution of amino acids. The *p*-nitrophenyl carbamates, in contrast, undergo only a single nucleophilic substitution of amino acids. These carbamates are stable, and the introduction of the *p*-phenyl moiety permits the use of THF/DMF in the subsequent step. By performing the reaction in THF/DMF, complete conversion to the *p*-nitrophenyl carbamate intermediate could be observed via TLC. In contrast, the use of aprotic solvents such as DCM, dioxane, THF and acetonitrile resulted in much lower conversion, perhaps due to the incompatibility of these solvents with the hydrochloride salts and freebases of the amino acids.

In the second step, *N,N*-diisopropylethylamine deprotonates the 7-OH of apigenin **1** (the most acidic phenol hydroxyl group of apigenin **1** with an estimated p*K*a of 6.6),[34] to give the conjugate base of apigenin **1**, which reacts with the *p*-nitrophenyl carbamate to afford the corresponding amino acid conjugate of apigenin **1**. The fair yield (45-51% over two steps) could not be improved by increasing the ratio of *p*-nitrophenyl carbamates to apigenin **1** from 1:1 to 3:1, but no 7,4′-adduct side-products were observed, and up to 90% of the unconverted apigenin **1** could be recycled. Palladium-catalyzed hydrogenation to deprotect the benzyl group (Bn) of **8** gave **9**, very cleanly, by TLC. The yield was 53% over three steps, much similar to those of **2** → **3** (45%) and **5** → **6** (51%). Removal of *tert*-butyloxycarbonyl (Boc) group proceeded smoothly using TFA and three desired products- Gly-apigenin carbamate **4**, Phe-apigenin carbamate **7**, Lys-apigenin carbamate 1**0** were obtained. This route obviates the need to protect and deprotect the 4'- and 5- OH groups, or

298 deprotect the 7-OH group prior to coupling, saving several steps (Figure 2).

299 To evaluate the metabolic stability of amino acid conjugates of apigenin **1** *in vitro*, a human
300 liver S9 stability assay containing phase I and phase II enzymes was conducted. As shown in Figure
301 3, the remaining ratios after 12 h are at least 90%. After 24 h, the conversion of Gly-apigenin
302 carbamate **4** and Lys-apigenin carbamate **10** was 23%; and that of Phe-apigenin carbamate **7** was
303 7%. This result suggests that the carbamate groups of these conjugates can largely withstand
304 hydrolysis, and that the apigenin moiety is modestly susceptible to oxidation in phase I (e.g., at C6,
305 C8, or C3') and to conjugation in phase II (e.g., glucuronidation at 5- or 4'-OH). Gastrointestinal
306 stability, however, cannot be inferred from the liver S9 assay, and so the effect of these carbamates
307 when administered orally is not known; however, it has been reported that flavonoids undergo ring
308 fission into phenolic acids after oral administration because of the susceptibility to intestinal
309 microflora.[35]

310 In an effort to facilitate entire analyses and to optimize the qualities of signals, conditions of
311 UPLC-MS/MS and sample preparation were tuned in advance. For UPLC-MS/MS analysis, a
312 gradient elution of methanol and water from 3:7 to 9:1 within 4 min was found able to distinctly
313 separate apigenin **1**, Gly-apigenin carbamate **4**, Phe-apigenin carbamate **7**, and Lys-apigenin
314 carbamate **10**. A 5 mM aqueous solution of ammonium acetate was added in the mobile phase, to
315 suppress the formation of metal ion salt adducts (e.g. with NaCl), which resulted in unidentifiable
316 collision fragmentations. For sample preparation, a combination of THF and methanol (1:1,

optimized) was found to ensure complete extraction: the capability of the former to solvate apigenin **1** and its amino acid conjugates far exceeds that of other organic solvents; the latter could compensate for the mediocre ability of the former to precipitate proteins, which facilitates the filtration of supernatant after samples centrifuged and diminishes matrix effects.

A preliminary *in vivo* assay with two time points (15 min and 90 min, n = 1) was performed prior to a multiple time point assay. Both Gly-apigenin carbamate **4** and Phe-apigenin carbamate **7** were barely detectable in blood at 15 min after intraperitoneal injection; the signal-to-noise ratios were lower than 10:1 for quantification. Neither were detected in blood samples at 90 min and in brain samples at any time point. However, significant amounts of Lys-apigenin carbamate **10** was in blood samples at both time points and in brain samples at 90 min. These results can be explained by reference to its physical-chemical properties. Firstly, the presumed superior solubility of the Lys-apigenin carbamate **10** salt compared to those of the other two non-salt carbamates contributed to the absorption of **10** in the peritoneal cavity, enabling its detection. As shown in Table 2, the solubility of Lys-apigenin carbamate **10** is 0.13 mg/mL, 8-fold and 7-fold higher that of Gly-apigenin carbamate **4** and Phe-apigenin carbamate **7**, respectively; and over 100-fold that of apigenin **1**. This trend is consistent with the aqueous solubility of unconjugated lysine, alanine, glycine and apigenin **1**, which is lysine > alanine > glycine > apigenin **1**, and can rationalize our observation that traces of aggregation of Gly-apigenin carbamate **4** and Phe-apigenin carbamate **7** were observed on the fringes of the visceral organs during dissection. Secondly, log P values of

these carbamates were compared (Table 2). The higher the value, the higher the lipophilicity, which would be expected to endow a compound with a superior passive permeability across the BBB. Since the optimal value of log P for BBB penetration was suggested to be 2-5,[36] Gly-apigenin carbamate **4**, Phe-apigenin carbamate **7**, and Lys-apigenin carbamate **10** were predicted to be less capable of diffusing across the BBB. Although Lys-apigenin carbamate **10** had an even lower log P value (= 0.96), suggesting its passage through the BBB by diffusion to be unlikely, it was nonetheless detected in the brain sample. Thus, the alternative mechanism – transportation via an amino acid transporter – was assumed to account for its presence in brain.[37,38] Given results in this preliminary *in vivo* assay showing that only Lys-apigenin carbamate **10** could be detected at a later time point, we therefore chose to further explore its PK and distribution in mice brain.

A PK profile of Lys-apigenin carbamate **10** was determined at a single dose of 0.4 mg per gram of body weight of mouse (Figure 4). The parameters acquired are listed in Table 3 as mean ± S.D.; the apparent volume of distribution (V/F) of Lys-apigenin carbamate **10** is 0.41 ± 0.13 (L/kg), higher than circulating blood volume (0.06-0.08 L/kg), implying that a portion of Lys-apigenin carbamate **10** was distributed into the peripheral tissues. The maximum observed blood concentration (C_{max}) is 49.44 ± 8.74 μg/mL at 1.33 ± 0.29 h (T_{max}), where an equilibrating process across the BBB leads to a delay of 1-2 h between the C_{max} in the blood and the brain. Therefore, the actual highest brain concentration would be slightly higher than that at 1.5-h as illustrated in Figure 5. The estimate of the concentration of Lys-apigenin carbamate **10** in mice brain using the entire

dissected intracranial contents may be significantly influenced by the presence of residual blood. We applied a correction by subtracting the amount of Lys-apigenin carbamate **10** in the intravascular blood present in the brain.[30] The peak concentration in brain of Lys-apigenin carbamate **10** was 0.82 ± 0.18 μg/g of tissue at 1.5 h (Figure 5, Table S13 in the supplemental). Since the carbamate moiety of Lys-apigenin carbamate **10** was cleavable, serum and brain concentrations of apigenin **1** were also detected, and found to peak at concentrations of 2.63-2.81 ug/mL and 0.42±0.11 ug/g, respectively at 1-1.5 h (Figure 4 & 5, and Table S9 & S14 in the supplemental). The **10**/**1** ratio of peak concentration in blood and brain were around 17 and 2, respectively. It seemed that the cleavage apigenin **1** from Lys-apigenin carbamate **10** was immediately distributed to brain tissue, due to higher log P of apigenin **1**. In an attempt to study the PK profile of apigenin **1,** IP injection of suspension apigenin **1** in a concentration of 0.23 mg per gram of body weight of mouse (which equal to molar concentration of Lys-apigenin carbamate **10** in above mentioned assay) in PBS was conducted. The maximal serum concentration of apigenin **1** analyzed was 0.12 ± 0.04 ug/mL at 1 h (see Table S12 in the supplemental), and the concentration of **1** after 4 h were under detection of limit (6.3 ng/mL). Due to the low concentration of apigenin **1** detected in serum, its brain concentration was below the detection of limit. The mean residue time (MRT) and the systematic clearance (CL/F) of Lys-apigenin carbamate **10** were 4.87 ± 2.42(h) and 0.072 ± 0.012 (L/h/kg), respectively (Table 3).

Lys-apigenin carbamate **10** was observed to have a sedative effect on all of the mice who were

given it –within 5 mins after its administration, their activity began to decrease, and they tended to curl up along the boundary of cage. All recovered from this state after 4-6 h. The sedative effect of Lys-apigenin carbamate **10** will be subjected to further investigation in due course.

In conclusion, amino acid conjugates of apigenin have been designed and synthesized; concisely, and with acceptable yields. Optimization of UPLC elution and MS/MS conditions were found to be necessary, especially for the salt Lys-apigenin carbamate **10**, the ionization of which was hampered by chloride ions. In a human liver S9 stability assay, the conjugates were at least 77% intact after incubation with the S9 fraction for 24 h. An *in vivo* assay showed that only Lys-apigenin carbamate **10** could access brain tissues, presumably due to its superior solubility and the assistance of cationic amino acid transporters. The brain concentration of Lys-apigenin carbamate **10** in mice was 0.82 ± 0.18 microgram per gram tissues at 1.5 h after administration, and a sedative effect was observed within 5 min. The development of apigenin conjugates that can cross the BBB and desirably perturb the CNS can inform the future development of BBB-crossing flavonoids.

ASSOCIATED CONTENT

Supplementing Information

The Supplementary is available free of charge on the ACS Publications website at DOI:

The detailed UPLC-Mass analyses to quantify serum and brain concentrations of compound **1** and

393 **10**, UPLC-Mass spectra, and 1D NMR spectra for compounds **3-4**, **6-10** are provided. (PDF)

394

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400 **Author Contributions**

401 Design of the experiments, T.-Y. W, P.-H. L.; synthesis of compounds, T.-T. W.; animal experiments,

402 T.-Y. W., M.-S. T., L.-C. H., S.-W. L.; HPLC analysis and PK analysis, T.-Y. W; wrote, read, and

403 approved the manuscript, T.-Y. W, M.-S. T., L.-C. H., S.-W. L., P.-H. L.

404

405 **Notes**

406 The authors declare no competing financial interest.

407 **6. REFERENCES**

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504

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510

Figure Captions

Figure 1. Apigenin (**1**)

Figure 2. The synthesis of Gly-apigenin carbamate **4**, Phe-apigenin carbamate **7**, and Lys-apigenin carbamate **10.** (a) (i) bis-(*p*-nitrophenyl) carbonate, DIPEA, DMF, 12 h, rt; (ii) **1**, DIPEA, THF, 12 h, rt; (b) TFA, 3h, 0 °C; (c) (i) bis-(*p*-nitrophenyl) carbonate, DIPEA, THF/DMF, 12 h, rt; (ii) apigenin **1**, DIPEA, THF/DMF, 6 h, rt; (d) TFA, 3 h, 0 °C; (e) (i) bis-(*p*-nitrophenyl) carbonate, DIPEA, DMF, 2 h, rt; (ii) apigenin **1**, DIPEA, THF, 6 h, rt; (iii) H_2, Pd/C, THF, 30 h; (f) TFA, 3 h, 0 °C.

Figure. 3. The human liver S9 stability assay: Gly-apigenin carbamate **4**, Phe-apigenin carbamate **7**, and Lys-apigenin carbamate **10** incubated with S9 fraction and a NADPH regenerating system for 0, 6, 12, and 24 h. (n = 1)

Figure 4. The concentration of Lys-apigenin carbamate **10** and apigenin **1** in the blood (n = 3 per time point) after Lys-apigenin carbamate **10** was intraperitoneally injected into mice at 0.4 mg per gram of body weight of mouse. The absorption phase was estimated to be 1.5 hour, according to C_{max} observed at the fourth time point. Data with asterisks are significantly increased from concentration of **1** at the same time ($p < 0.01$, ***; $p < 0.05$, **).

530 **Figure 5.** The concentration of Lys-apigenin carbamate **10** and apigenin **1** in brain tissues (n = 3 per
531 time point) after Lys-apigenin carbamate **10** was intraperitoneally injected into mice at 0.4 mg per
532 gram of body weight of mouse. Data with asterisks are significantly increased from concentration of
533 **1** at the same time ($p < 0.01$, ***; $p < 0.05$, **).

Tables

Table 1. The Retention Times of Gly-apigenin Carbamate **4**, Phe-apigenin Carbamate **7**, and Lys-apigenin Carbamate **10** in UPLC and Their Optimized MS/MS Parameters

	1	**4**	**7**	**10**
Retention time (min)	3.22	2.85	1.14	2.36
Precursor ion (m/z)	269.0, $[M-H]^-$	370.4, $[M-H]^-$	460.2, $[M-H]^-$	443.2, $[M+H]^+$
Product ion (m/z) (quantifier/ qualifier)	116.0/150.8	269.2/116.9	269.2/116.7	271.1/152.9
Declustering potential (V)	55	10	15	30
Entrance potential (V)	50	50	50	50
Collision energy (eV)	35/25	5/35	15/60	20/50
Collision cell exit potential (V)	50	50	50	50

Table 2. Solubilities and Log P Values of Apigenin **1**, Gly-apigenin Carbamate **4**, Phe-apigenin Carbamate **7**, and Lys-apigenin Carbamate **10**.

	1	**4**	**7**	**10**
Solubility (mg/mL)	< 0.001	0.017	0.018	0.13
Log P	1.71	1.51	1.61	0.96

Table 3. The PK Parameters in Mice after Single IP Administration of Lys-apigenin Carbamate **10**. (n=3, Acquired by WinNonlin^{TM})

	$AUC_{0\rightarrow t}$ (μg * h/mL)	$AUC_{0\rightarrow \infty}$ (μg * h/mL)	C_{max} in blood (μg/mL)	T_{max} (h)	$t_{1/2}$ (h)	MRT (h)	CL/F (L/h/kg)	V/F (L/kg)
10	149.24 ± 9.29	168.85 ± 29.97	49.44 ± 8.74	1.33 ± 0.29	4.20 ± 2.07	4.87 ± 2.42	0.072 ± 0.012	0.41 ± 0.13

Figures

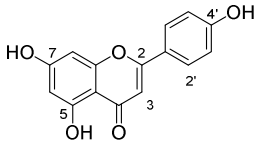

Figure 1.

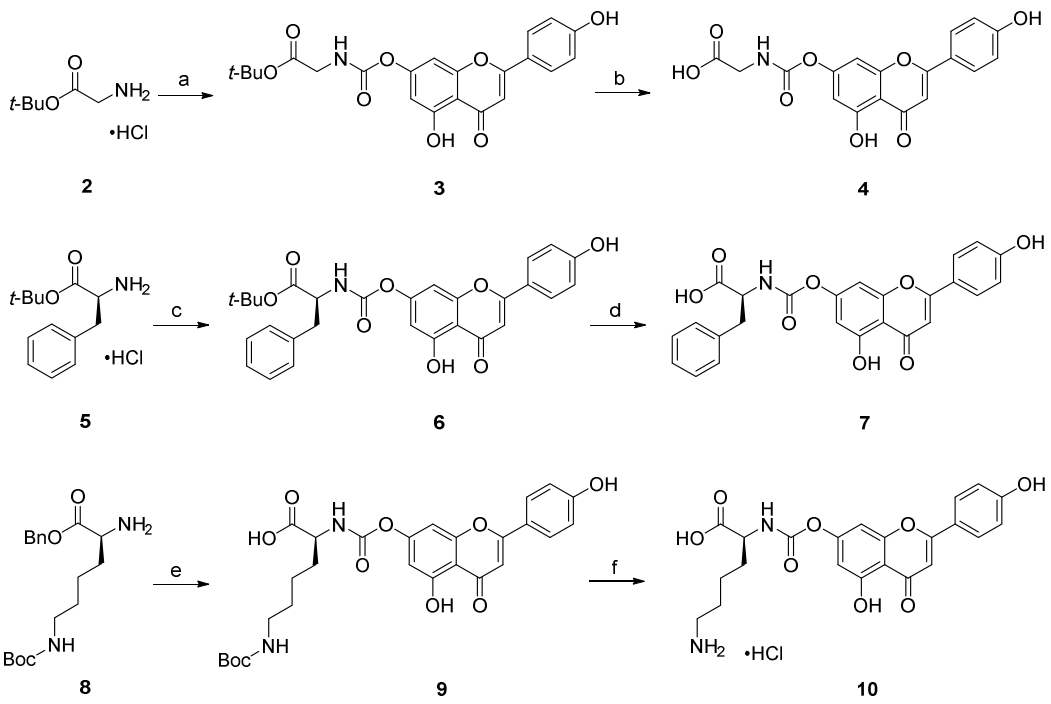

Figure 2.

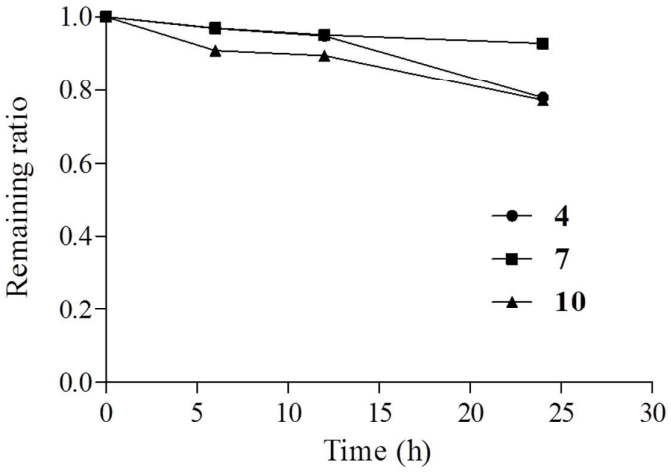

Figure. 3.

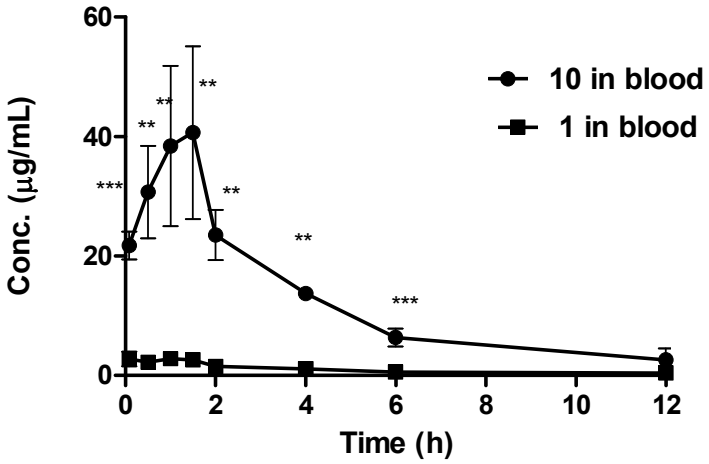

Figure 4.

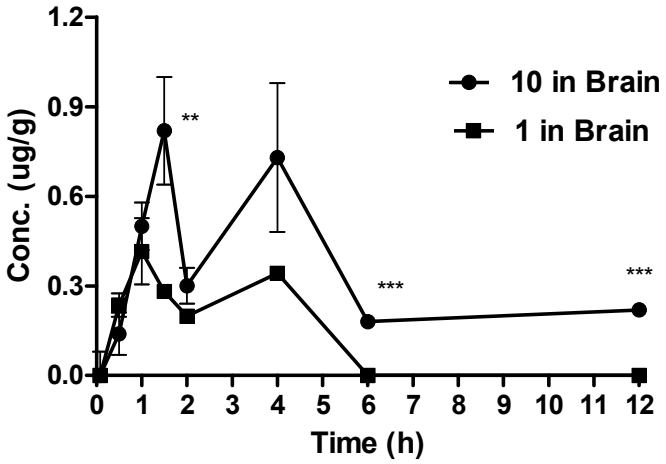
1.2
0.9
0.6
0.3
0.0
Conc. (ug/g)
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (h)
10 in Brain
1 in Brain
**
***
***

Figure 5.

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