Received Date : 03-Apr-2016

Revised Date : 13-Jun-2016

Accepted Date : 11-Jul-2016

Article type : Research Article

Discovery of neurotrophic agents based on hydroxycinnamic acid scaffold

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Abstract:
The number of people affected by neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease is rapidly increasing owing to the global increase in life expectancy. Small molecules with neurotrophic effects have great potential for management of these neurological disorders. In this study, different (C1-C12) alkyl ester derivatives of hydroxycinnamic acids (HCAs) were synthesized (a total of 30 compounds). The neurotrophic capacity of the test compounds was examined by measuring promotion of survival in serum-deprived conditions and enhancement of nerve growth factor (NGF)-induced neurite outgrowth in PC12 neuronal cells. p-Coumaric, ferulic and sinapic acids and their esters did not alter cell survival, while caffeic acid and all its alkyl esters, especially decyl and dodecyl caffeate, significantly promoted neuronal survival at 25 μM. Methyl, ethyl, propyl and butyl caffeate esters also significantly enhanced NGF-induced neurite outgrowth, among which the most effective ones were propyl and butyl esters, which at 5 μM led to 25- and 22-fold increases in the number of neurites, respectively. The findings of the docking study suggested phosphatidylinositol 3-kinase (PI3K) as the potential molecular target. In conclusion, our findings demonstrate that alkyl esters of caffeic acid could be potentially useful for discovery of therapeutic agents for neurodegenerative diseases.

Keywords:

Neurodegenerative diseases, hydroxycinnamic acids, alkyl ester, neurotrophic, synthesis

Introduction

Neurodegenerative diseases are most common in the elderly and with the increase in life expectancy in recent decades, these diseases have begun to impose a huge health burden worldwide(1, 2). No effective disease-modifying treatment is yet available for the most common neurodegenerative disorders, including Alzheimer's disease and Parkinson's disease, therefore, there is a pressing and unmet need for new therapies that stop or reverse the progression of these pathologies. Many investigations have targeted the accumulation of toxic proteins in the central nervous system, including amyloid-β(3-5) and tau(5) in Alzheimer's disease and α-synuclein in Parkinson's disease(6). However, these strategies have failed to deliver an effective disease-modifying treatment so far(5, 7, 8).

Neurotrophic factors of the nervous system, such as nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF), play important roles in neuronal differentiation and survival and are critical for the development and maintenance of the nervous system(9, 10). The binding of neurotrophic factors to their receptors (tropomyosin receptor kinase, Trk receptors) triggers signaling pathways that promote neuronal differentiation and survival(11, 12). Preclinical and clinical findings suggest that neurotrophins could be a promising therapy for neurodegenerative diseases like Alzheimer's disease(11, 13) and Parkinson's disease(11, 14). However, neurotrophin peptides lack a suitable pharmacokinetic profile and hardly cross the blood-brain barrier; therefore, efforts have been devoted to the search for small molecule neurotrophic agents that mimic the action of neurotrophins or potentiate their effect(15-17).

Several investigators have become increasingly interested in polyphenols over the past decades, because of the abundance of these compounds in diet and their possible role in the prevention of several pathologies such as cardiovascular diseases, cancer and neurodegeneration(18). In particular, in the context of neurodegenerative diseases, several polyphenols have been reported to induce neuronal differentiation and neurite outgrowth(19-23). Hydroxycinnamic acids (HCAs) represent an important class of polyphenolic compounds(24), which are widely distributed in plants and abundantly found in fruits and vegetables and possess several biological activities(25-27). Several studies have shown that phenolic compounds may exert their neurotrophic effect by the activation of neurotrophic signalings such as Ras/MAPK/ERK or phosphatidylinositol 3-kinase (PI3K)/Akt pathways(23). A number of studies have shown that PI3K and its downstream effector Akt play an important role in mediating the neuronal survival and increased neurite outgrowth, as well as other neurotrophic effects of polyphenols(28-31).

In continuation of our interest in the synthesis and evaluation of the biological activities of HCAs(32-35), we have synthesized 30 HCA derivatives consisting of short and long alkyl esters of *p*-coumaric acid, ferulic acid, sinapic acid and caffeic acid, and have tested their ability to promote cell survival and induction of differentiation in neuronal cells. We further performed a molecular docking simulation study in order to find out the binding potential of active derivatives with PI3K as a putative molecular target.

Materials and methods

Reagents

Dimethyl sulfoxide, methanol and propanol were obtained from Merck (Darmstadt, Germany). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as well as all reagents needed for chemical synthesis were obtained from Sigma-Aldrich (Lisbon, PT). All reagents and solvents were pro analysis and were used without further purification. Fetal bovine serum (FBS) and horse serum were acquired from Invitrogen (San Diego, CA), while penicillin/streptomycin, RPMI 1640, sterile phosphate-buffered saline (PBS), and trypsin EDTA 0.05% were purchased from Biosera (Ringmer, UK). Deionized water was used in all the experiments.

Chemistry

Apparatus

^{1}H and ^{13}C NMR were acquired at room temperature on a Brüker Biospin GmbH 400 spectrometer operating at 400 and 101 MHz, respectively. Chemical shifts were expressed in δ (ppm) values relative to tetramethylsilane (TMS) as internal reference; coupling constants (*J*) were given in Hz. Assignments were also made from DEPT (Distortionless Enhancement by Polarization Transfer) (underlined values). Microwave-assisted synthesis was performed in Biotage® Initiator Microwave Synthesizer.

Synthesis of alkylcinnamates

Method A - Synthesis of short chain alkyl cinnamates

The cinnamic acid (0.5 g), the appropriate alcohol (15 mmol) and 2 drops of concentrated sulphuric acid were added to a glass vial (2-5 mL) and sealed. The system was heated to 20ºC above the boiling point of the alcohol for 5 min(36). After cooling to room temperature, the crude product was purified by flash chromatography using silica gel as stationary phase and dichloromethane or ethyl acetate as eluent. Compounds **CAF1-4**, **FER 1,2** and **4**, **SIN1-4** have been synthesized as previously described and the structural data was in accordance with the previously reported data(32, 35).

Trans*-Methyl-3-(4-hydroxyphenyl)propenoate (PCC1).** Yield: 74%**.** ***^{1}H NMR(DMSO-d6): 3.70 (3H, *s*,CH_3), 6.40 (1H, *d*, *J*=16.0 H(2)), 6.80 (2H, *m*, H(3''), H(5'')), 7.50 (3H, *m*, H(2'), H(6'), H(3)), 10.05 (1H, *s*, COOH). ***^{13}C NMR(DMSO-d6):***51.3 (CH_3), 113.9 (C(3''), C(5'')), 115.8 (C(2)), 125.1 (C(1')), 130.0 (C(2''), C(6'')), 144.8 (C(3)), 159.9 (C(4'')), 167.1 (CO). ***MS/EI m/z:*** 178 ($M^{+\bullet}$, 24), 147 (100).

Trans*-Ethyl-3-(4-hydroxyphenyl)propenoate (PCC2).** Yield: 63%.^{1}H NMR(DMSO-d6):*** 1.25 (3H, *t*, *J*=7.1, CH_3), 4.16 (2H, *t*, *J*=7.1 Hz, CH_2), 6.39 (1H, *d*, *J*=15.9, H(2)), 6.81 (2H, *m*, H(3''), H(5'')), 7.56 (3H, *m*, H(2'), H(6'), H(3)), 10.04 (1H, *s*, COOH). ***^{13}C NMR(DMSO-d6):***14.8 (CH_3), 59.5 (CH_2), 114.3 (C(3''), C(5'')), 115.8 (C(2)), 125.1 (C(1')), 130.3 (C(2''), C(6'')), 144.6 (C(3)), 159.9 (C(4'')), 166.7 (CO). ***MS/EI m/z:*** 192 ($M^{+\bullet}$, 76), 147 (100).

Trans*-Hexyl-3-(4-hydroxy-3-methoxyphenyl)propenoate (FER5).** Yield: 80%. ***^{1}H NMR($CDCl_3$): 0.87 (3H, *t*, *J*=8.0, H(6')), 1.32 (6H, *bs*, H(3')-H(5')), 1.65 (2H, *m*, H(2')), 3.82 (3H, *s*, OCH_3), 4.16 (2H, *t*, *J*=8.0 H(1')), 6.26 (1H, *d*, *J*=16.0 H(2)), 6.87 (1H, *d*, *J*=8.0 H(6'')), 6.99 (2H, *m*, H(2''), H(5'')), 7.58 (1H, *d*, *J*=16.0, H(3)). ***^{13}C NMR($CDCl_3$):***14.0 (C(6')), 22.5 (C(5')), 25.6 (C(4')), 28.7 (C(3')), 31.5 (C(2')), 55.8 (OCH_3), 64.6 (C(1')), 109.6 (C(2'')), 115.0 (C(5'')), 115.3 (C(2)), 122.9 (C(6'')), 126.9 (C(1'')), 144.9 (C(3)), 147.1 (C(4'')), 148.2 (C(3'')), 167.6 (CO). ***MS/EI m/z:*** 278 ($M^{+\bullet}$, 93), 194 (100).

Method B- Synthesis of long chain alkyl cinnamates

Part B1-Synthesis of malonic acid half esters

Equimolar quantities of Meldrum's acid and of the appropriate alcohols were refluxed in toluene (5 mL) for 4 h. After cooling the reaction to room temperature a solution of saturated $NaHCO_3$ was added to the mixture. The compounds were extracted with diethyl ether; the organic layer was washed with water and dried with Na_2SO_4. The solvents were evaporated to yield the malonic acid half esters. The compounds were used in the next reaction without further purification.

Monohexyl malonate.*^{1}H NMR($CDCl_3$):* 0.89 (3H, *t*, *J*=8.0 H(6')), 1.33 (6H, *bs*, H(3')-H(5')), 1.65 (2H, *m*, H(2')), 3.43 (2H, *s*, H(2)), 4.16 (2H, *t*, *J*=8.0 H(1')).

Monooctyl malonate. *^{1}H NMR($CDCl_3$):* 0.89 (3H, *t*, *J*=8.0 H(8')), 1.30 (10H, *bs*, H(3')-H(7')), 1.65 (2H, *m*, H(2')), 3.43 (2H, *s*, H(2)), 4.16 (2H, *t*, *J*=8.0 H(1')).

Monodecyl malonate. ***^{1}H NMR($CDCl_3$):*** 0.88 (3H, *t*, *J*=8.0 H(10')), 1.30 (14H, *bs*, H(3')-H(9')), 1.65 (2H, *m*, H(2')), 3.43 (2H, *s*, H(2)), 4.16 (2H, *t*, *J*=8.0 H(1')).

Monododecyl malonate. ***^{1}H NMR($CDCl_3$):*** 0.89 (3H, *t*, *J*=8.0 H(12')), 1.31 (18H, *bs*, H(3')-H(11')), 1.68 (2H, *m*, H(2')), 3.45 (2H, *s*, H(2)), 4.19 (2H, *t*, *J*=8.0 H(1')).

Monohexadecyl malonate. ***^{1}H NMR($CDCl_3$):*** 0.89 (3H, *t*, *J*=8.0 H(16')), 1.30 (26H, *bs*, H(3')-H(15')), 1.67 (2H, *m*, H(2')), 3.45 (2H, *s*, H(2)), 4.19 (2H, *t*, *J*=8.0 H(1')).

Part B2-Synthesis of alkyl chain cinnamates via Knoevenagel condensation

Equimolar quantities of half ester of malonic acid (1-5) and the appropriate benzaldehyde derivative were added to cyclohexane (3-5 mL), and then anhydrous pyridine (1 equiv.) and aniline (1.6 equiv.) were added to the mixture, and the mixture was refluxed for 6-10 h. After cooling, the mixture was added in an ice bath concentrated HCl. After pH neutralization, the mixture was extracted with diethyl ether and the organic layer was died with Na_2SO_4. The solvent was evaporated under vacuum obtaining the crude product. The compounds were purified by flash chromatography using gradient elution (petroleum ether/ ethyl acetate).

Trans*-Hexyl-3-(4-hydroxyphenyl)propenoate (PCC5).** Yield 45%. ***^{1}H NMR($CDCl_3$): 0.90 (3H, *t*, *J*=8.0 H(6')), 1.35 (6H, *bs*, H(3')-H(5')), 1.70 (2H, *m*, H(2')), 4.20 (2H, *t*, *J*=8.0 H(1')), 6.30 (1H, *d*, *J*=16.0 H(2)), 6.87 (2H, *d*, *J*=8.0 H(3''), H(5'')), 7.41 (2H, *d*, *J*=8.0

H(2''), H(6'')), 7.63 (1H, *d*, *J*=16.0 H(3)). **^{13}C*NMR(CDCl$_3$):***14.0 (C(6')), 22.7 (C(5')), 25.7 (C(4')), 28.7 (C(3')), 31.5 (C(2')), 65.0 (C(1')), 115.3 (C(2)), 116.0 (C(3''), C(5'')), 126.9 (C(1'')), 130.0 (C(2''), C(6'')), 144.9 (C(3)), 158.2 (C(4')), 168.2 (CO). ***MS/EI m/z: MS/EI m/z:*** 248 ($M^{+\bullet}$, 35), 147 (100).

Trans*-Octyl-3-(4-hydroxyphenyl)propenoate (PCC6).** Yield 40%.^{1}H NMR(CDCl$_3$):***0.92 (3H, *t*, *J*=8.0 H(8')), 1.35 (10H, *bs*, H(3')-H(7')), 1.73 (2H, *m*, H(2')), 4.25 (2H, *t*, *J*=8.0 H(1')), 6.33 (1H, *d*, *J*=16.0 H(2)), 6,95 (2H, *d*, *J*=8.0 H(3''), H(5'')), 7.43 (2H, *d*, *J*=8.0 H(2''), H(6'')), 7.68 (1H, *d*, *J*=16.0 H(3)). **^{13}C *NMR(CDCl$_3$):***14.1 (C(8')), 22.7 (C(7')), 26.0 (C(6')), 28.7 (C(5')), 29.2 (C(4')), 29.3 (C(3')), 31.8 (C(2')), 65.2 (C(1')), 114.7 (C(2)), 116.1 (C(3''), C(5'')), 126.5 (C(1'')), 130.2 (C(2''), C(6'')), 145.5 (C(3)), 158.8 (C(4)), 168.8 (CO). ***MS/EI m/z:*** 276 ($M^{+\bullet}$, 21), 147 (100).

Trans*-Decyl-3-(4-hydroxyphenyl)propenoate (PCC7).** Yield34%. ***^{1}H NMR(CDCl$_3$):0.88 (3H, *t*, *J*=8.0 H(10')), 1.32 (14H, *bs*, H(3')-H(9')), 1.70 (2H, *m*, H(2')), 4.20 (2H, *t*, *J*=8.0 H(1')), 6.30 (2H, *d*, *J*=16.0 H(2)), 6.86 (2H, *d*, *J*=8.0 H(3''), H(5'')), 7.42 (2H, *d*, *J*=8.0 H(2''), H(6'')), 7.63 (1H, *d*, *J*=16.0 H(3)). **^{13}C *NMR(CDCl$_3$):***14.1 (C(10')), 22.7 (C(9')), 26.0 (C(8')), 28.8 (C(7')), 29.3 (C(6'), C(5')), 29.6 (C(4'), C(3')), 31.9 (C(2')), 64.9 (C(1')), 115.5 (C(2)), 115.9 (C(3''), C(5'')), 127.1 (C(1'')), 130.0 (C(2''), C(6'')), 144.6 (C(3)), 158.0 (C(4'')), 168.0 (CO). ***MS/EI m/z:*** 304 ($M^{+\bullet}$, 34), 147 (100).

Trans*-Dodecyl-3-(4-hydroxyphenyl)propenoate (PCC8).** Yield 30%. ***^{1}H NMR(CDCl$_3$):0.90 (3H, *t*, *J*=8.0 H(12')), 1.33 (18H, H(3')-H(11')), 1.72 (2H, *m*, H(2')), 4.22

(2H, *t*, *J*=8.0 H(1')), 6.32 (2H, *d*, *J*=16.0 H(2)), 6.89 (2H, *d*, *J*=8.0 H(3''), H(5'')), 7.44 (2H, *d*, *J*=8.0 H(2''), H(6'')), 7.65 (1H, *d*, *J*=16.0 H(3)). **^{13}C *NMR(CDCl$_3$):*** 15.0 (C(12')), 23.6 (C(11')), 26.9 (C(10')), 29.6 (C(9')), 30.2 (C(8')), 30.3 (C(7')), 30.4 (C(6')), 30.5 (C(5')), 30.6 (C(4'), C(3')), 32.8 (C(2')), 65.8 (C(1')), 116.2 (C(2)), 116.9 (C(3''), C(5'')), 127.8 (C(1'')), 130.9 (C(2''), C(6'')), 145.8 (C(3)), 159.2 (C(4'')), 169.1 (CO). ***MS/EI m/z:*** 332 ($M^{+\bullet}$, 40), 147 (100).

Trans*-Hexyl-3-(3,4-dihydroxyphenyl)propenoate (CAF5).** Yield 58%. ***^{1}H NMR(DMSO-d$_6$): 0.87 (3H, *t*, *J*=8.0 H(6')), 1.31 (6H, *bs*, H(3')-H(5')), 1.62 (2H, *m*, H(2')), 4.10 (2H, *t*, *J*=8.0 H(1')), 6.26 (1H, *d*, *J*=16.0 H(2)), 6.76 (1H, *d*, *J*=8.0 H(5'')), 7.02 (2H, *m*, H(2''), H(6'')), 7.47 (1H, *d*, *J*=16.0 H(3)), 9.12 (1H, *s*, OH), 9.57 (1H, *s*, OH). **^{13}C *NMR(DMSO-d$_6$):*** 14.4 (C(6')), 22.5 (C(5')), 25.6 (C(4')), 28.7 (C(3')), 31.4 (C(2')), 64.2 (C(2')), 114.5 (C(2'')), 115.3 (C(2)), 116.2 (C(5'')), 121.8 (C(6'')), 126.0 (C(1'')), 145.5 (C(3)), 146.0 (C(3'')), 148.8 (C(4'')), 167.1 (CO). ***MS/EI m/z:*** 264 ($M^{+\bullet}$, 80), 180 (100).

Trans*-Octyl-3-(3,4-dihydroxyphenyl)propenoate (CAF6).** Yield 53%. ***^{1}H NMR(DMSO-d$_6$): 0.86 (3H, *t*, *J*=8.0 H(8')), 1.30 (10H, *bs*, H(3')-H(7')), 1.62 (2H, *m*, H(2')), 4.10 (2H, *t*, *J*=8.0 H(1')), 6.26 (1H, *d*, *J*=16.0 H(2)), 6.76 (1H, *d*, *J*=8.0 H(5'')), 7.02 (2H, *m*, H(2''), H(6'')), 7.47 (1H, *d*, *J*=16.0 H(3)), 9.40 (2H, *bs*, 2xOH). **^{13}C *NMR(DMSO-d$_6$):*** 15.3 (C(8')), 23.5 (C(7')), 26.8 (C(6')), 29.7 (C(5')), 30.0 (C(4'), C(3')), 32.6 (C(2')), 65.1 (C(1')), 115.4 (C(2'')), 116.2 (C(2)), 117.1 (C(5'')), 122.7 (C(6'')), 126.7 (C(1'')), 146.4 (C(3)), 146.9 (C(3'')), 149.7 (C(4'')), 168.0 (CO). ***MS/EI m/z:*** 292 ($M^{+\bullet}$, 50), 180 (100).

Trans*-Decyl-3-(3,4-dihydroxyphenyl)propenoate (CAF7).** Yield 42%. ***^{1}H NMR(CDCl$_3$): 0.88 (3H, *t*, *J*=8.0 H(10')), 1.32 (14H, *bs*, H(3')-H(9')), 1.70 (2H, *m*, H(2')), 4.20 (2H, *t*, *J*=8.0 H(1')), 6.27 (1H, *d*, *J*=16.0 H(2)), 6.88 (1H, *d*, *J*=8.0 H(5'')), 7.01 (1H, *dd*, *J*=8.0; 4.0 H(6'')), 7.12 (1H, *d*, *J*=4.0 H(2'')), 7.59 (1H, *d*, *J*=16.0 H(3)). **^{13}C *NMR(CDCl$_3$):*** 14.1 (C(10')), 22.7 (C(9'')), 26.0 (C(8'')), 28.7 (C(7'')), 29.3 (C(6''), C(5'')), 29.6 (C(4''), C(3'')), 31.9 (C(2'')), 65.0 (C(1'')), 114.5 (C(2'')), 115.5 (C(5''), 115.6 (C(2)), 122.4 (C(6'')), 127.5 (C(1'')), 143.9 (C(3'')), 145.0 (C(3)), 146.4 (C(4'')), 168.1 (CO). ***MS/EI m/z:***320 ($M^{+\bullet}$, 28), 180 (100).

Trans*-Dodecyl-3-(3,4-dihydroxyphenyl)propenoate (CAF8).** Yield 50%. ***^{1}H NMR(DMSO- d$_6$): 0.85 (3H, *t*, *J*=8.0 H(12')), 1.24 (18H, *bs*, H(3')-H(11')), 1.61 (2H, *m*, H(2')), 4.10 (2H, *t*, *J*=8.0 H(1')), 6.25 (1H, *d*, *J*=16.0 H(2)), 6.76 (1H, *d*, *J*=8.0 H(5'')), 7.00 (2H, *m*, H(2''), H(6'')), 7.46 (1H, *d*, *J*=16.0 H(3)), 9.20 (1H, *bs*, OH), 9.60 (1H, *bs*, OH). **^{13}C *NMR(DMSO-d$_6$):*** 15.3 (C(12')), 23.5 (C(11')), 26.8 (C(10')), 29.6 (C(9')), 30.0 (C(8')), 30.1 (C(7')), 30.3 (C(6'), C(5')), 30.4 (C(4'), C(3')), 32.7 (C(2')), 65.1 (C(1')), 115.4 (C(2'')), 116.2 (C(2)), 117.1 (C(5'')), 122.7 (C(6'')), 126.9 (C(1'')), 146.4 (C(3)), 146.9 (C(3'')), 149.7 (C(4'')), 168.0 (CO). ***MS/EI m/z:*** 348 ($M^{+\bullet}$, 45), 180 (100).

Trans*-Decyl-3-(4-hydroxy-3-methoxyphenyl)propenoate (FER7).** Yield 34%. ***^{1}H NMR(CDCl$_3$): 0.90 (3H, *t*, *J*=8.0 H(10')), 1.35 (14H, *bs*, H(3')-H(9')), 1.71 (2H, *m*, H(2')), 3.93 (3H, *s*, OCH_3), 4.21 (2H, *t*, *J*=8.0 H(1')), 6.31 (1H, *d*, *J*=16.0 H(2)), 6.93 (1H, *d*, *J*=8.0 H(5'')), 7.07 (2H, *m*, H(2''), H(6'')), 7.63 (1H, *d*, *J*=16.0 H(3)). **^{13}C *NMR(CDCl$_3$):***14.1 (C(10')), 22.7 (C(9')), 26.0 (C(8')), 28.8 (C(7')), 29.3 (C(6'), C(5')), 29.6 (C(4'), C(3')), 31.9 (C(2')), 56.0(OCH_3), 64.7 (C(1')), 109.4 (C(2'')), 114.8 (C(5'')), 115.7 (C(2)), 123.0

(C(6'')), 127.1 (C(1'')), 144.7 (C(3)), 146.8 (C(4'')), 148.0 (C(3'')), 167.4 (CO). ***MS/EI m/z:*** 334 ($M^{+\bullet}$, 79), 194 (100).

Trans*-Dodecyl-3-(4-hydroxy-3-methoxyphenyl)propenoate (FER8).** Yield 27%. ***^{1}H NMR($CDCl_3$): 0.90 (3H, *t*, *J*=8.0 H(12')), 1.33 (18H, *bs*, H(3')-H(11')), 1.72 (2H, *m*, H(2')), 3.95 (3H, *s*, OCH_3), 4.21 (2H, *t*, *J*=8.0 H(1')), 6.31 (1H, *d*, *J*=16.0 H(2)), 6.94 (1H, *d*, *J*=8.0 H(5'')), 7.07 (2H, *m*, H(2''), H(6'')), 7.63 (1H, *d*, *J*=16.0 H(3)). **^{13}C *NMR($CDCl_3$):*** 14.3 (C(12')), 22.9 (C(11')), 26.2 (C(10')), 29.0 (C(9')), 29.5 (C(8'), C(7')), 29.7 (C(6'), C(5')), 29.8 (C(4'), C(3')), 32.1 (C(2')), 56.1 (OCH_3), 64.8 (C(1')), 109.5 (C(2'')), 114.9 (C(5'')), 115.9 (C(2)), 123.2 (C(6'')), 127.2 (C(1'')), 144.8 (C(3)), 146.9 (C(4'')), 148.1 (C(3'')), 167.6 (CO). ***MS/EI m/z:*** 362 ($M^{+\bullet}$, 99), 194 (100).

Trans*-Octyl-3-(4-hydroxy-3,5-dimethoxyphenyl)propenoate (SIN6).** Yield 48%. ***^{1}H NMR($CDCl_3$): 0.91 (3H, *t*, *J*=8.0 H(8')), 1.36 (10H, *bs*, H(3')-H(7')), 1.72 (2H, *m*, H(2')), 3.94 (6H, *s*, 2xOCH_3), 4.22 (2H, *t*, *J*=8.0 H(1')), 6.33 (1H, *d*, *J*=16.0 H(2)), 6.80 (2H, *s*, H(2''), H(6'')), 7.61 (1H, *d*, *J*=16.0 H(3)). **^{13}C *NMR($CDCl_3$):*** 14.1 (C(8')), 22.7 (C(7')), 26.0 (C(6')), 28.8 (C(5')), 29.2 (C(4'), C(3')), 31.8 (C(2')), 56.4 (2xOCH_3), 64.7 (C(1')), 105.1 (C(2''), C(6'')), 116.1 (C(2')), 126.2 (C(1'')), 137.1 (C(4'')), 144.8 (C(3)), 147.2 (C(3''), C(5'')), 167.3 (CO). ***MS/EI m/z:*** 336($M^{+\bullet}$, 100)

Trans*-Decyl-3-(4-hydroxy-3,5-dimethoxyphenyl)propenoate (SIN7).** Yield 37%. ***^{1}H NMR($CDCl_3$): 0.88 (3H, *t*, *J*=8.0 H(10')), 1.32 (14H, *bs*, H(3')-H(9')),1.70 (2H, *m*, H(2')), 3.91(6H, *s*, 2xOCH_3), 4.19 (2H, *t*, *J*=8.0 H(1')), 6.31 (1H, *d*, *J*=16.0 H(2)), 6.77 (2H, *s*,

H(2''), H(6'')), 7.59 (1H, *d*, *J*=16.0 H(3)). **^{13}C *NMR(CDCl$_3$):*** 14.1 (C(10'), 23.0 (C(9')), 26.0 (C(8')), 28.8 (C(7')), 29.3 (C(6'), C(5')), 29.6 (C(4'), C(3')), 31.9 (C(2')), 56.3 (2xOCH$_3$), 64.7 (C(1')), 105.1 (C(2''), C(6'')), 116.1 (C(2)), 126.0 (C(1'')), 137.1 (C(4'')), 144.8 (C(3)), 147.2 (C(3''), C(5'')), 167.3 (CO). ***MS/EI m/z***: 365 ($M^{+\bullet}$, 100).

Trans*-Dodecyl-3-(4-hydroxy-3,5-dimethoxyphenyl)propenoate (SIN8).** Yield 30%. ***^{1}H NMR(CDCl$_3$): 0.90 ((3H, *t*, *J*=8.0 H(12')), 1.34 (18H, *bs*, H(3')-H(11')), 1.71 ((2H, *m*, H(2')), 3.94(6H, *s*, 2xOCH$_3$), 4.21 (2H, *t*, *J*=8.0 H(1')), 6.33 (1H, *d*, *J*=16.0 H(2)), 6.79 (2H, *s*, H(2''), H(6'')), 7.61 (1H, *d*, *J*=16.0 H(3)). **^{13}C *NMR(CDCl$_3$):***15.0 (C(12')), 23.6 (C(11')), 26.9 (C(10')), 29.7 (C(9')), 30.2 (C(8')), 30.3 (C(7')), 30.5 (C(6'), C(5')), 30.6 (C(4'), C(3')), 32.8 (C(2')), 57.2 (2xOCH$_3$), 65.6 (C(1')), 105.9 (C(2''), C(6'')), 117.0 (C(2)), 126.9 (C(1'')), 138.0 (C(4'')), 145.7 (C(3)), 148.1 (C(3''), C(5'')), 168.2 (CO). ***MS/EI m/z***: 392 ($M^{+\bullet}$, 100).

Cell Culture

PC12 cells (rat pheochromocytoma) were a generous gift from Professor Lloyd A. Greene (Department of Pathology and Cell Biology, Columbia University, New York, NY, USA). Cells were grown in RPMI 1640 medium, supplemented with 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum, 100 U/mL penicillin G, and 100 mg/mL streptomycin. Cells were cultured on plates coated with collagen obtained from rat tail and were grown at 37 ºC in humidified air containing 5% CO_2. Two-thirds of the growing medium was changed every 2–3 days and the cells were subcultured once a week.

Cell viability assay

The effect of synthesized cinnamic acid derivatives on the viability of the PC12 cells was determined by the MTT reduction assay, which is based on the reduction of a tetrazolium salt by mitochondrial dehydrogenase in viable cells(37, 38). The cells were seeded in collagen coated 96-well microplates at a density of 6×10^5 cells/mL (80 µL per well) in serum free medium. Ten µL of NGF dissolved in RPMI at 5 or 50 ng/ml or the test compounds at the final concentrations of 1, 5 and 25 µM were added in triplicate. Test compound were first dissolved in DMSO and then diluted in growth medium at least 1000 times. After incubation of cells at 37 ◦C for 48 h, the media were removed from the wells and 20 µL of MTT solution (0.5 mg/mL) was added to each well and incubated for another 90 min. DMSO (200 µL/well) was added to each well and plates were further incubated at 37 ◦C for 60 min and then placed on a shaker for another 30 min. The absorbance was measured with a microplate reader (Model 680, Bio-Rad) at 570 nm with background correction at 650 nm.

Quantification of neurite outgrowth in PC12 cells

PC12 cells were seeded in collagen-coated 12-well plates in RPMI medium supplemented with 2% horse serum and 1% fetal bovine serum at a density of 5×10^4 cells/mL (1 mL per well). After 24 h of incubation, different concentrations of the test compounds (1, 5 and 25 µM) with or without NGF (50 and 5 ng/mL) were added to the wells. After 3 days of incubation, the growth medium was refreshed with the same concentrations of the test compounds and NGF. The numbers of neurites were counted in at least five randomly selected microscopic fields by visual examination after 8 days. Neuronal processes with a length equal to or longer than a cell body were counted as neurites. The total numbers of neurites as well as those equal or longer than 100 µm were counted separately. Microscopic

observations were made with an inverted Nikon microscope (model eclipse TS100-F) using phase contrast objectives. Each experiment was repeated at least 3 times.

Molecular docking study

Molecular docking study was performed using Autodock 4.2 and Auto Dock Tools 1.5.4 (ADT). For this purpose, the X-ray crystal structure of PI3K carrying wortmannin as cognate ligand in binding site (PDB code: 1E7U, downloaded from protein data bank, http://www.rcsb.org) was used for molecular docking study and investigation of biding interactions of **CAF** analogues with PI3K active site as their possible target was performed. The PDB file of protein was regenerated by removing water molecules and cognate ligand, adding polar hydrogens and computing Collman charges. The 3D structures of **CAF** and **CAF1-8** were created using Chem3D Ultra 12.0 software. Each ligand was separately submitted to a molecular mechanics conformational search (MM^+ force field) and the geometry of the most stable conformer was further optimized by semi-empirical method (AM1) in Hyperchem software. The Gasteiger charges were added and the degrees of torsions were defined to generate PDBQT format of the ligands. The grid parameter file was generated using AutoGrid. Numbers of points in x, y, z dimensions were set to 60*60*60 with 0.375 A ° grid spacing. Rigid macromolecule was chosen to define the docking parameter file using Lamarckian Genetic Algorithm (LGA), with 100 GA runs and 25000000 energy evaluations while the other parameters were left as default.

Statistical analysis

Values were expressed as mean ± S.E.M. from 3-4 independent determinations. Statistical analyses were carried out by analysis of variance (ANOVA) followed by appropriate post hoc tests including multiple comparison tests (LSD). All analyses were made using the SPSS statistical software package (Version 11.5 for Windows). Differences with probability values of less than 0.05 were considered as statistically significant.

Results

Chemistry

In order to evaluate the influence of esterification on the neurotrophic activity, a set of alkyl cinnamate derivatives based on *p*-coumaric acid, ferulic acid, sinapic acid and caffeic acid were synthesized (Figure 1). Short chain length alkyl esters were obtained following a microwave-assisted Fischer esterification of the acid with the appropriate alcohol, as previously described (Scheme 1A)(39). Long alkyl chain ester derivatives were prepared by a Knoevenagel-Doebner condensation between the benzaldehyde with the appropriate aromatic pattern and the previously prepared malonate half ester with the desired tether length (Scheme 1B)(40). The compounds were obtained in moderate to high yields and fully characterized by magnetic resonance spectroscopy (^{1}HRMN, ^{13}CRMN and DEPT135) and mass spectrometry.

Hydroxycinnamic acids and their alkyl esters promote survival in serum deprived PC12 neuronal cells

Survival of serum-deprived PC12 cells exposed to synthesized compounds was evaluated using (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) (MTT) assay. As expected, NGF at the concentrations of 5 and 50 ng/mL significantly promoted PC12 cells survival as compared to untreated serum-deprived control cells (Figure 2). On the other hand, non-esterified *p*-coumaric acid, ferulic acid and sinapic acid at the concentrations of 1, 5 and 25 μM did not have any effect on the survival. Similarly, exposure to *p*-coumarate, ferulate, and sinapate esters at the same concentrations did not enhance viability. However, caffeic acid and its esters (**CAF1**, **CAF2**, **CAF3**, **CAF4**, **CAF5**, **CAF6**, **CAF7**, **CAF8**) at the concentration of 25 μM, but not at 1 and 5 μM, significantly promoted the survival of PC12 cells. **CAF7** and **CAF8** were the most active compounds and could enhance the cell survival up to more than 250% compared to the control. Interestingly, the effects of these last 2 esters were stronger than the effect of nerve growth factor (NGF) at the concentrations of 5 and 50 ng/mL.

Caffeate ester derivatives enhance neurite outgrowth induced by NGF in PC12 cells

The effect of caffeate esters on the NGF-induced neurite outgrowth in PC12 cells was examined. Treatment of cells with NGF alone (5 and 50 ng/mL) induced the outgrowth of neurites in the cells (Figures 3 and Table 1). When PC12 cells were exposed to NGF 5 ng/mL co-treated with methyl (**CAF1**) and ethyl caffeate (**CAF2**) at the concentrations of 5 and 25 μM, and also propyl (**CAF3**) and butyl caffeate (**CAF4**) at the concentration of 5 μM, the total number of neurites significantly increased compared to the cells that were exposed to NGF 5 ng/mL alone. Similarly, the number of longer neurites (longer than 100 μm)

significantly increased in cells co-treated with methyl, ethyl, propyl and butyl caffetaes at 5 μM (Figures 3 and Table 1). The concomitant treatment of cells with NGF and the rest of the caffeate esters including hexyl (**CAF5**), octyl (**CAF6**), decyl (**CAF7**) and doceyl (**CAF8**) esters as well as caffeic acid did not significantly change the number of neurites compared to NGF treatment alone (data not shown).

Molecular docking study of CAFs with PI3K

According to previous reports, PI3K might serve as a probable molecular target of polyphenolic compounds underlying the neuroprotective potential of these structures(41, 42). In order to gain insight on binding interactions of **CAFs** with PI3K active site, we performed a molecular docking study using Autodock 4.2 software with flexible ligand/rigid macromolecule procedure based on the crystal structure of PI3K (PDB ID: 1E7U) with its cognate ligand, wortmannin. Docking optimization and validation was performed by redocking of the co-crystallized conformation of cognate ligand into the PI3K binding site. Consequently, RMSD (root mean square deviation) of the best-docked conformation of the native ligand from the experimental one was used for validation of the docking study(43). The best-docked and experimental conformation of wotmannin correlated quite well with an RMSD of 1.73Å and binding free energy of -8.96 Kcal/mol (Table 2).

Docking results of different **CAF** analogues are illustrated in Table 3. Based on the obtained data, the introduction and elongation of alkyl side chains into caffeic acid structure resulted in an enhanced binding interaction of **CAF** alkyl esters with PI3K active site (ΔG_b= -5.05 to -7.33 Kcal/mol). Moreover, phenolic hydroxyl substitutes were involved in key hydrogen binding with Asp841 and Asp964 of the active site in all alkyl ester derivatives of caffeic acid and elongation of the alkyl ester pendant indicated further binding interactions with Met953

and Glu880 of catalytic site in most cases. The binding mode of **CAF7** with PI3K binding site is depicted in Figure 4. **CAF7** was well accommodated in the active site and was involved in key hydrogen bond interactions with Val882 Asp841, and Asp964 (respective binding distances were 1.70, 1.33 and 1.35 Å) and hydrophobic interactions with residues of the catalytic site including Phe961, Met953, Val882 and Trp812.

Discussion

In this study, 30 derivatives of HCAs including *p*-coumaric acid, ferulic acid, sinapic acid and caffeic acid and their C1-C12 alkyl esters were synthesized and their neurotrophic capacities were examined in PC12 neuronal cells. Caffeic acid esters were the most efficient neurotrophic compounds.

We first screened the protective effects of the synthesized compounds against serum deprivation in PC12 cells by MTT assay. Several studies have used serum deprivation and MTT assay to test the neurotrophic effect of various compounds (44-46). Our findings showed that caffeic acid and its esters significantly enhanced cell viability and inhibited cell death induced by serum deprivation at the concentration of 25 µM. Decyl and dodecyl caffeates exhibited the strongest neuroprotective activities, followed by shorter esters C1-C4. On the other hand, *p*-coumaric acid, ferulic acid and sinapic acid ester derivatives did not show any significant neuroprotective activity.

Furthermore, the influence of caffeate esters on the neurite outgrowth of PC12 cells was also examined and it was observed that they intensely enhanced the effect of low dose NGF. The most prominent effects belonged to **CAF3** and **CAF4** derivatives, which at 5 µM considerably increased the total number of neurites (25- and 22-fold increases, respectively)

and the number of neurites longer than 100 µm (36- and 34-fold increases, respectively) compared to NGF alone. Although long alkyl chain caffeate esters (**CAF7** and **CAF8** esters) were the most active agents in promotion of neuronal survival, they did not show any significant effect on neuritogeneis. Other studies have similarly shown that phenolic compounds such as green tea polyphenols(47), isorhamnetin, a flavonol(48), and genistein, an isoflavone(49), potentiated the action of NGF and induced neuritogenesis in PC12 cells.

We had previously reported that short alkyl esters of caffeic acid significantly inhibited hydrogen peroxide-induced neuronal PC12 cell death, but ferulate esters could not reduce oxidative stress-induced cell damage(35). Lipophilicity is an important physicochemical property of phenolic compounds and several lines of evidence have shown that esterification of phenolic compounds can increase the lipophilicity and therefore their pharmacological activity (33, 34, 50, 51).

The neuroprotective effect of caffeic acid phenetyl ester (CAPE) has been shown in rat organotypic midbrain slice cultures and in an animal model of neurodegeneration induced by LPS and 6-hydroxydopamine(52). Furthermore, a recent study has shown that CAPE induces neurite outgrowth in PC12 cells and protects these cells from MPP(+) neurotoxin-induced damage(53).

HCA derivatives have been reported to have diverse biological activities including anticancer effect (54) by different mechanism including inhibition of 17β-hydroxysteroid dehydrogenases (55). In this study, only caffeic acid ester derivatives containing catechol moiety in their structures exhibited the biological effect and other HCAs did not show significant activities. This structural feature may guide the discovery of selective agents.

The neuroprotective properties of NGF and its potential implication in treatment of neurodegenerative diseases are well established(56, 57); however, pharmacokinetic limitations and side effects such as neuropathic pain have hindered the therapeutic application of NGF peptide(58). Hence, small molecular weight neurotrophic agents that are devoid of the limitations of endogenous neurotrophins could be of therapeutic value in neurodegenerative diseases.

Several studies have shown that phenolic compounds may exert their neurotrophic effect by the activation of neurotrophic signaling pathways such as PI3K/Akt and ERK1/2 (23). PI3K/Akt and ERK1/2 signaling pathways can promote cell survival in the nervous system as well as other tissues(59, 60). Various studies have shown that PI3K and its downstream effector Akt, are involved in neuronal survival and increasing neurite outgrowth as well as other neurotrophic effects of polyphenols(28-31).

Possible mechanisms that might take place at a molecular level underling the neurotrophic effects of HCAs might be comparable to those associated with compounds with similar structures: Caffeoylserotonin has shown protective effects against oxidative stress-induced cell death in keratinocytes through the activation of PI3K/Akt pathway and subsequent activation of Nrf2(61). In another study, caffeic acid ester fraction (a mixture of different esters of caffeic acid mainly consisting of quinic acid esters) has shown protective effects in primary rat astrocytes against hypoxia through the production and release of neurotrophins by stimulation of CREB and Akt signaling pathways(62). Protective effects of caffeic acid and CAPE against acrolein-induced neurotoxicity in mouse hippocampal neuronal cells has also been shown to be mediated by MAPKs and Akt signaling(63).

On the other hand, although ferulic acid and its derivatives did not show significant activities in our neuronal cell model, other investigators applying different methodologies have reported that ferulic acid exerts cytoprotective effects against oxidative stress in umbilical vein endothelial cells by stimulation of PI3K and ERK pathways(42). Another study on ferulic acid conjugated to tacrine, has also shown neuroprotective effects in attenuation of 6-hydroxydopamine-induced apoptosis in PC12 cells through the stimulation of Akt pathway(64). Furthermore, it has been reported that sodium ferulate exerts protective effects against glutamate toxicity in cortical neurons by modulation of PI3K and the MEK/ERK signaling pathways(65) and against amyloid-β-induced neurotoxicity by activation of Akt in rat hippocampal neurons(66).

We performed a docking study to assess the interaction of caffeate esters with PI3K, as a potential target. The findings of the docking study clearly showed that caffeate esters had a reasonable interaction with the active site of PI3K and the binding energies of the ester derivatives correlated well with the results of the survival promotion assay.

In conclusion, our findings demonstrate the neurotrophic effects of caffeic acid ester derivatives, among which methyl, ethyl, propyl and butyl esters have the most prominent capacities. The findings of the docking study also suggest that PI3K may be a molecular target that mediates the neurotrophic action of these compounds. These derivatives could form the basis for the rational design of neurotrophic agents with potential applications for management of neurodegenerative diseases.

Acknowledgements

The authors wish to thank the Iranian National Science Foundation (Grant number: 92024294) and the Natural Antimicrobials Centre of Excellence for the financial supports of this project. We also thank the Foundation for Science and Technology (FCT) of Portugal (PEst-C/QUI/UI0081/2015) and QREN (FCUP-CIQ-UP-NORTE-07-0124-FEDER- 000065) for financial support and grant of T. Silva (SFRH/BD/79671/2011). This study was part of the PhD thesis of Razieh Hosseini.

Conflict of interests

The authors declare that they have no conflicts of interest.

Figure captions:

Scheme 1. Synthetic strategies used for the synthesis of short chain (**1A)** and long chain alkyl cinnamates (**1B) (i)** Meldrum's acid, toluene, reflux, 4h; **(ii)** benzaldehyde derivative, aniline, cyclohexane, reflux, 6-10h.

Figure 1. Chemical structure of hydroxycinnamic acids and the alkyl ester derivatives under study.

Figure 2: Hydroxycinnamic acids and their alkyl esters promote survival in serum deprived PC12 neuronal cells.

PC12 cells were seeded in 96-well plates deprived of serum and were immediately treated with NGF (5 or 50 ng/mL) or hydroxycinnamic acids and their alkyl esters [*p*-coumarate (A), ferulate (B), sinapate (C) and caffeate esters (D)] at different concentrations. After 48 h, cell viability was measured by the MTT assay. The results are plotted as the percentage of cell survival compared to untreated cells and expressed as mean ± S.E.M of 3-4 independent experiments. * Significantly different from untreated control cells ($P<0.05$).

Figure 3: Caffeic acid and its alkyl esters enhance NGF-induced neurite outgrowth in PC12 cells.

PC12 cells were seeded in 12-well plates and treated with either NGF alone or in concomitant incubation with caffeic acid and caffeate alkyl esters (**CAF**, **CAF1-12**) at the concentrations of 1, 5 and 25 μM. The images were taken after 8 days of incubation. Representative images are shown for the most active caffeate alkyl esters (**CAF1**, **CAF2**, **CAF3** and **CAF4**).

Figure 4. Best docking pose of CAF7 into the active site of PI3K.

The ligand is depicted in stick mode and the interaction residues are demonstrated in line style. Hydrogen bond interactions are depicted as green lines.

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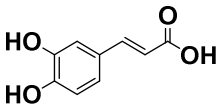
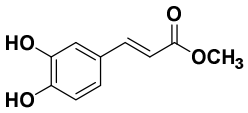
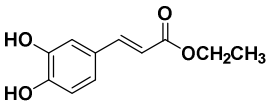
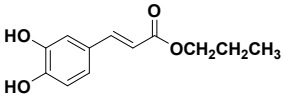
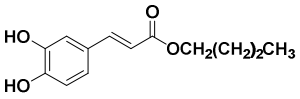
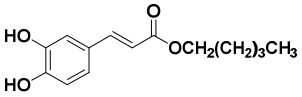
Table 1. Caffeic acid and its alkyl esters enhance NGF-induced neurite outgrowth in PC12 cells.

Compound	Code	Concentration (μM)*	Number of neurites	Number of neurites (d ≥ 100 μm)
	**CAF**	1	2.5 ± 2.5	0.5 ± 0.5
		5	10.3 ± 10.3	0.3 ± 0.5
		25	11.7 ± 7.5	1.2 ± 0.4
	**CAF1**	1	6.0 ± 3.0	1.2 ± 0.2
		5	50.1[ab] ± 18.5	9.0[a] ± 4.2
		25	38.3[a] ± 9.3	1.8 ± 1.2
	**CAF2**	1	11.9 ± 0.1	2.1 ± 0.1
		5	66.9[ab] ± 37.6	13.7[ab] ± 8.7
		25	36.7[a] ± 18.8	2.0 ± 2.0
	**CAF3**	1	21.4 ± 3.4	4.7 ± 1.3
		5	88.1[ab] ± 38.9	17.7[ab] ± 7.8
		25	26.2 ± 9.8	2.3 ± 1.2
	**CAF4**	1	21.3 ± 4.3	7.3 ± 1.6
		5	78.0[ab] ± 18.8	17.0[ab] ± 3.0
		25	15.9 ± 4.1	3.8 ± 1.1
	**CAF5**	1	6.5 ± 1.5	0.5 ± 0.5
		5	14.2 ± 2.9	2.3 ± 0.7
		25	3.0 ± 3.0	0.7 ± 0.0
	NGF	5 ng/mL	0.5 ± 0.3	0.5 ± 0.1
	NGF	50 ng/mL	16.3 ± 0.8	2.6 ± 0.2

* NGF 5 ng/mL was used in each assay. [a] Significantly different from cells treated with NGF 5 ng/mL ($P<0.05$). [b] Significantly different from cells treated with NGF 50 ng/mL ($P<0.05$)

Table 2. Docking validation results for PDB structure of PI3K (1E7U) using AutoDock 4.2

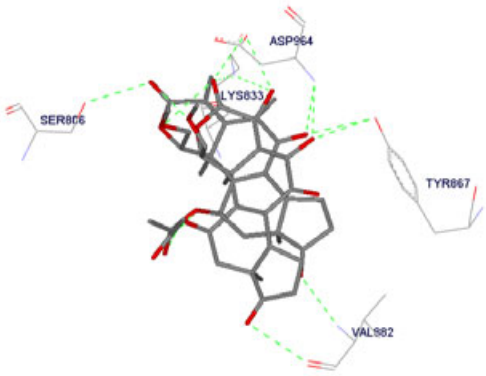
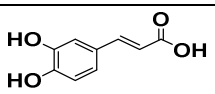
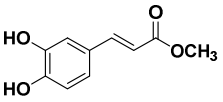
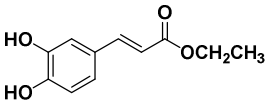
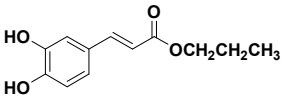
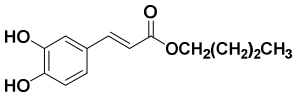
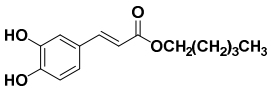
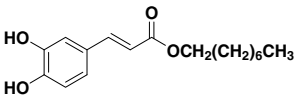
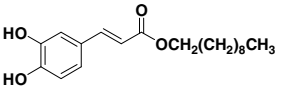
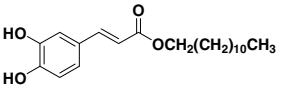
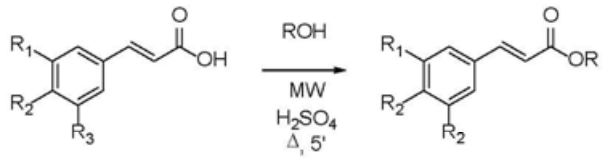
PDB code	Binding mode of wortmannin with PI3K active site	No. GA runs	Population in the optimum cluster (%)	RMSD from reference structure(°A)	Estimated free energy of binding (kcal/Mol)
1E7U		100	80	1.74	-8.96

Table 3. Binding interactions of caffeic acid and its ester derivatives into the PI3K active site calculated by the docking study.

Compound	Structure	ΔG_b (Kcal/Mol)	Ki (μM)	Atom of the ligand	Amino acid
CAF		-5.05	199.83	3-OH 4-OH	Glu 880 Lys 833
CAF1		-5.91	46.47	3-OH 4-OH C=O	Asp 841 Glu 880 Val 882
CAF2		-6.23	27.29	C=O 3-OH 4-OH	Val 882 Asp 841 ASP 964
CAF3		-6.55	15.94	C=O 3-OH 4-OH	Val 882 Asp 841 ASP 964
CAF4		-6.81	10.13	C=O 3-OH 4-OH	Val 882 Asp 841 ASP 964
CAF5		-7.02	7.21	C=O 3-OH 4-OH	Val 882 Asp 841 ASP 964
CAF6		-7.12	6.06	C=O 3-OH 4-OH	Val 882 Asp 841 ASP 964
CAF7		-7.33	4.24	C=O 3-OH 4-OH	Val 882 Asp 841 ASP 964
CAF8		-7.03	7.07	C=O 3-OH 4-OH	Val 882 Asp 841 ASP 964

Scheme 1

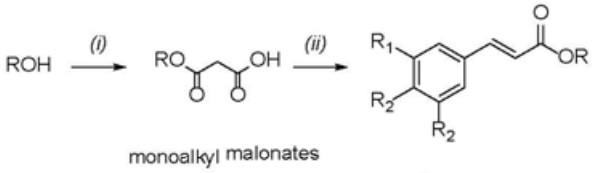
A



PCC: R_1=H, R_2=OH, R_3=H
CAF: R_1=OH, R_2=OH, R_3=H
FER: R_1=OCH_3, R_2=OH, R_3=H
SIN: R_1=OCH_3, R_2=OH, R_3=OCH_3

1: R=CH_3
2: R=CH_2CH_3
3: R=$(CH_2)_2CH_3$
4: R=$(CH_2)_3CH_3$

B



monoalkyl malonates

5: R=$(CH_2)_5CH_3$
6: R=$(CH_2)_7CH_3$
7: R=$(CH_2)_9CH_3$
8: R=$(CH_2)_{11}CH_3$

Figure 1

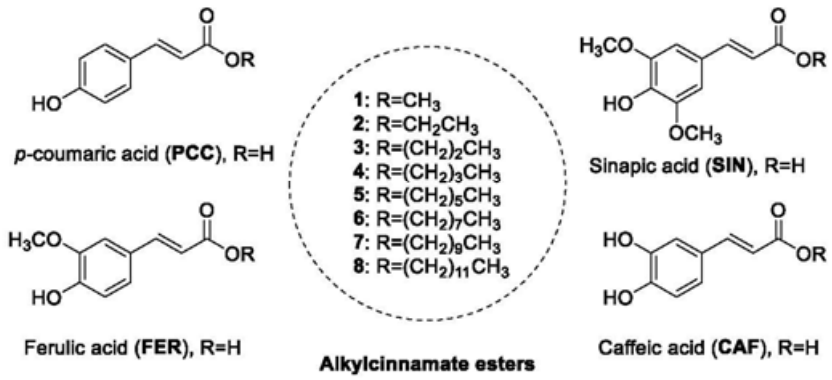


Figure 2

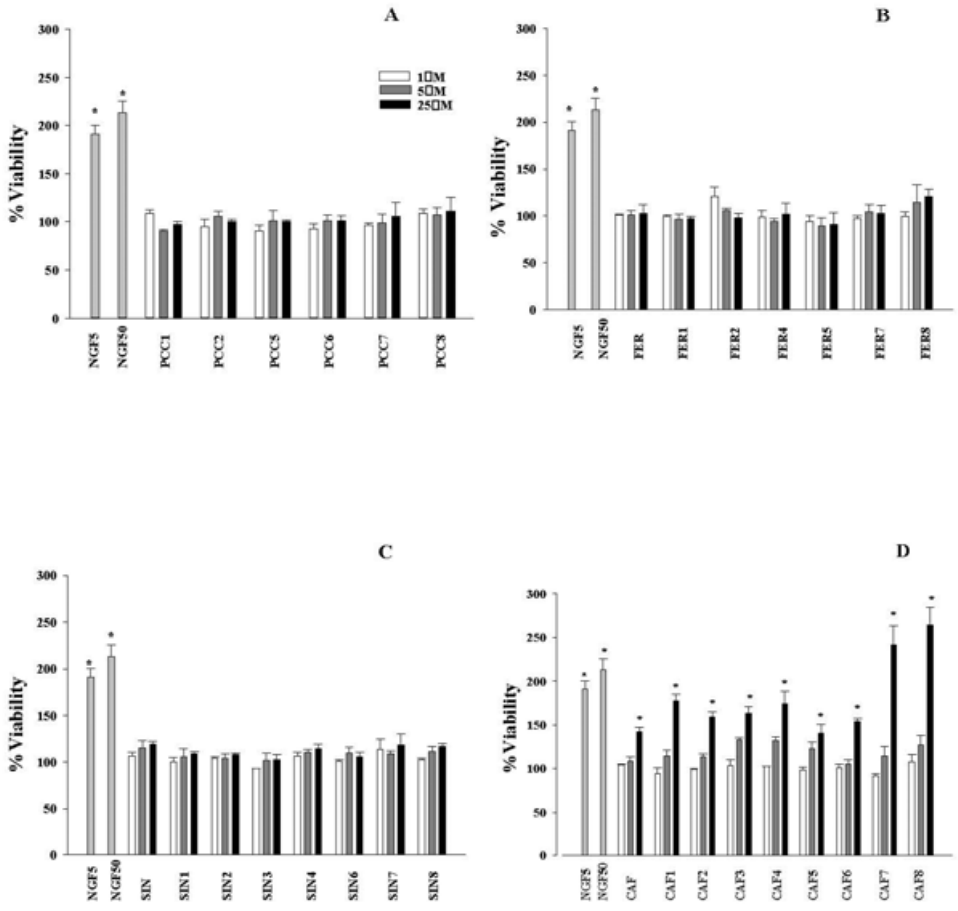


Figure 3

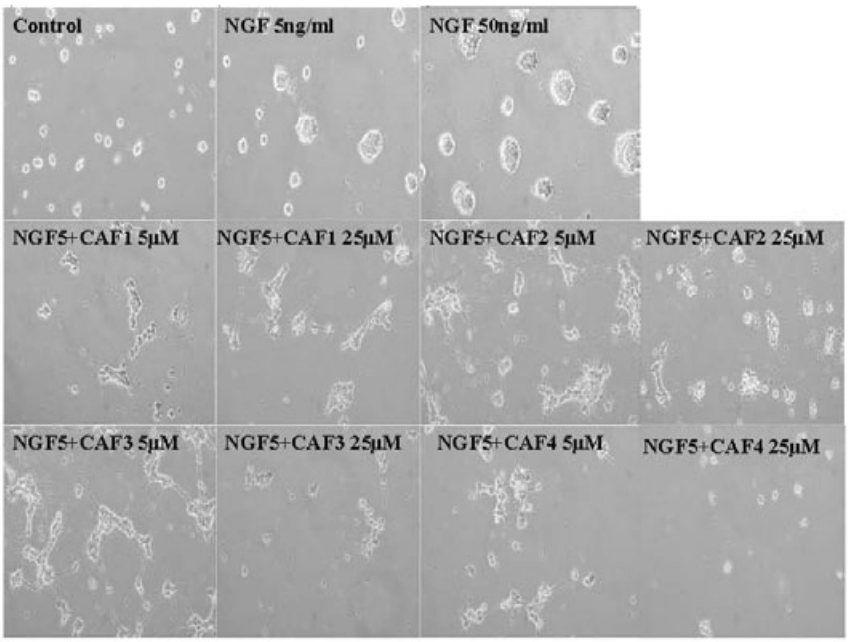


Figure 4