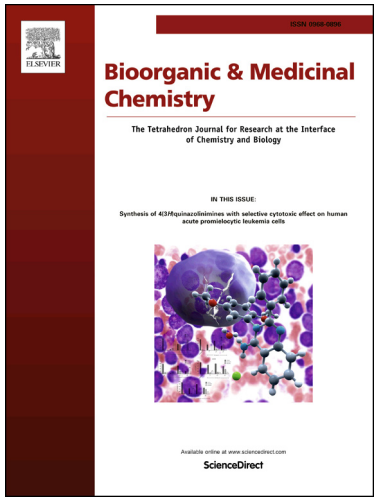

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Derivatives of caffeic acid, a natural antioxidant, as the basis for the discovery of novel nonpeptidic neurotrophic agents

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Abstract

Neurodegenerative disorders, such as Parkinson's disease and Alzheimer's disease, threaten the lives of millions of people and the number of affected patients is constantly growing with the increase of the aging population. Small molecule neurotrophic agents represent promising therapeutics for the pharmacological management of neurodegenerative diseases. In this study, a series of caffeic acid amide analogues with variable alkyl chain lengths, including **ACAF3** (C3), **ACAF4** (C4), **ACAF6** (C6), **ACAF8** (C8) and **ACAF12** (C12) were synthesized and their neurotrophic activity was examined by different methods in PC12 neuronal cells. We found that all caffeic acid amide derivatives significantly increased survival in PC12 neuronal cells in serum-deprived conditions at 25 μM, as measured by the MTT assay. **ACAF4**, **ACAF6** and **ACAF8** at 5 μM also significantly enhanced the effect of nerve growth factor (NGF) in inducing neurite outgrowth, a sign of neuronal differentiation. The neurotrophic effects of amide derivatives did not seem to be mediated by direct activation of tropomyosin receptor kinase A (TrkA) receptor, since K252a, a potent TrkA antagonist, did not block the neuronal survival enhancement effect. Similarly, the active compounds did not activate TrkA as measured by immunoblotting with anti-phosphoTrkA antibody. We also examined the effect of amide derivatives on signaling pathways involved in survival and differentiation by immunoblotting. **ACAF4** and **ACAF12** induced ERK1/2 phosphorylation in PC12 cells at 5 and 25 μM, while **ACAF12** was also able to significantly increase AKT phosphorylation at 5 and 25 μM. Molecular docking studies indicated that compared to the parental compound caffeic acid, **ACAF12** exhibited higher binding energy with phosphoinositide 3-kinase (PI3K) as a possible molecular target. Based on Lipinski's rule of five, all of the compounds obeyed 3 molecular descriptors (HBD, HBA and MM) in drug-likeness test. Taken together, these findings show for the first time that caffeic amides possess strong neurotrophic effects exerted via modulation of ERK1/2 and AKT signaling pathways presumably by activation of PI3K and thus represent promising agents for the discovery of neurotrophic compounds for management of neurodegenerative diseases.

Keywords: Caffeic amides, neurotrophic agents, cell signaling, ERK1/2, AKT

Running title: Neurotrophic effects of caffeic acid amide derivatives

1. Introduction

Age related neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease represent heavy health burdens that result in the loss of millions of human lives every year and impose huge direct and indirect socioeconomic costs [1]. There is currently no disease-modifying treatment available for these pathologies and finding novel therapeutic strategies is a pressing and unmet clinical need. Nerve growth factor (NGF) is a neurotrophin that has been shown to prevent neuronal cell damage, and also to promote neuronal regeneration in several *in vitro* and *in vivo* models [2]. Its actions are mediated through tropomyosin receptor kinase A (TrkA) and the major neurotrophic signaling pathways: phosphoinositide 3-kinase (PI3K)/AKT, mitogen-activated protein kinase (MAPK)/MEK/ERK and phospholipase C-γ1 pathways [3]. NGF may provide an attractive therapeutic choice for neurodegenerative disorders [4,5]; however, it has poor pharmacokinetic profile including poor stability in plasma, no oral bioavailability and poor blood–brain barrier penetration, and may also cause adverse effects such as severe pain [4,6]. These limitations have led to the exploration of small molecules, which activate the TrkA receptor or potentiate the neurotrophic effects of limited amounts of endogenous NGF [2].

Caffeic acid (**CA**) and its related compounds such as caffeic acid phenethyl ester (CAPE), rosmarinic acid, chlorogenic acid and ferulic acid are well-known for their diverse biological actions including antioxidant and neuroprotective effects [7,8]. The neuroprotective action of these compounds has been shown in various studies; for instance, CAPE has shown to protect dopaminergic neurons from IFN-g/LPS-induced injury by upregulation of brain-derived neurotrophic factor (BDNF) in rat midbrain slice cultures [9]. CAPE also induced neurite outgrowth in PC12 cells associated with increased expression of differentiation markers [10]. Recently, it has been shown that some **CA** derivatives are able to potentiate neuroprotection and induce neurogenesis via interactions with neuronal intracellular signaling pathways [11,12]. Nevertheless, the cellular mechanisms by which these compounds induce neurotrophic effects are not completely understood.

Following our line of research based on the development of new chemical entities derived from naturally occurring scaffolds, we focused our efforts on the discovery of novel neurotrophic agents based on the hydroxycinnamic acid scaffold. In this context, we synthesized a series of caffeic amide analogues with variable chain lengths and assessed the effect of these derivatives on the survival and induction of neurite outgrowth in PC12 cells. Moreover, the effects of these

compounds on TrkA receptor activation and alteration of its downstream signaling pathways, including ERK1/2 and AKT pathways, were also examined.

2. Materials and methods

2.1. Reagents

Fetal bovine serum (FBS), horse serum (HS), penicillin/streptomycin, RPMI 1640, sterile phosphate-buffered saline (PBS) and trypsin EDTA 0.25% were purchased from Biosera (Ringmer, UK), while rat recombinant NGF-β and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St Louis, MO). Methanol was obtained from Merck (Darmstadt, Germany). K252a was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-TrkA, anti-phospho-AKT and anti-phospho-MAP kinase ERK1/2 (pThr202/Tyr204), anti-MAP kinase ERK1/2, as well as rabbit anti-mouse and mouse anti-rabbit HRP-conjugated secondary antibodies were obtained from Cell Signaling (Danvers, MA), while anti-TrkA and anti-AKT were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Solvents were pro analysis grade and were acquired from Merck (Lisbon, Portugal). All the other reagents used for synthesis were purchased from Sigma-Aldrich (Sintra, Portugal). Deionized water was used in all the experiments.

2.2. Chemistry

Thin layer chromatography (TLC) was performed on pre-coated silica gel 60 F254 acquired from Merck (Darmstadt, Germany) and spots were detected using a UV lamp at 254 nm. Reaction progress was monitored using TLC (dichloromethane/methanol/formic acid, 9:1:0.01). Following the extraction step, subsequent work up of the organic layers included drying over anhydrous sodium sulphate, filtration and evaporation of solvents under reduced pressure. Column chromatography was carried out with silica gel 60A acquired from Carlo-Erba Reactifs (SDS, France). The crude products were purified by flash column chromatography. The fractions containing the desired product were gathered, concentrated and the product was recrystallized. Solvents were evaporated with a Buchi rotary evaporator.

The purity of the final products (>97% purity) was verified by high-performance liquid chromatography (HPLC) equipped with a UV detector. Chromatograms were obtained in an

HPLC/DAD system, a Jasco instrument (pumps model 880-PU and solvent mixing model 880-30, Tokyo, Japan), equipped with a commercially prepacked Nucleosil RP-18 analytical column (250 mm x 4.6 mm, 5 μm, Macherey-Nagel, Duren, Germany), and UV detection (Jasco model 875-UV) at the maximum wavelength of 254 nm. The mobile phase consisted of a methanol/water or acetonitrile/water (gradient mode, room temperature) at a flow rate of 1 mL/min. The chromatographic data was processed in a Compaq computer, fitted with CSW 1.7 software (DataApex, Czech Republic).

^{1}H, ^{13}C NMR and DEPT135 data were acquired at room temperature on a Brüker AMX 400 spectrometer operating at 400 and 100 MHz, respectively. Chemical shifts were expressed in δ (ppm) values relative to tetramethylsilane (TMS) as internal reference and coupling constants (*J*) were given in Hz. Carbon signals present in DEPT135 (Distortionless Enhancement by Polarization Transfer) spectra were underlined. Mass spectra were obtained on a VG AutoSpec instrument. The data were reported as m/z (% of relative intensity of the most important fragments).

2.3. General procedure for PyBOP-assisted amidation

Caffeic acid (2.78 mmol) was dissolved in diisopropylethylamine (0.48 mL) and dimethylformamide (4 mL). The reaction was placed on ice and PyBOP (2.78 mmol) in dichloromethane (5 mL) was added. The mixture was stirred for 30 minutes. Then, the amine (2.78 mmol) was added, the mixture was allowed to reach room temperature and stirred for an additional 6 hours. The solvents were removed under reduced pressure and the residue was dissolved in ethyl acetate (30 mL). The solution was washed with water (3 x 10 mL), HCl 1M (3 x 10 mL) and brine (10 mL). The compounds were purified by column chromatography in dichloromethane/methanol (95:5 until 90:10) and recrystallized from dichloromethane/n-hexane. The procedure was adapted from Gaspar et al. 2011 [13].

2.3.1. (*E*)-3-(3,4-dihydroxyphenyl)-*N*-propylacrylamide (ACAF3)

Yield (%): 12.7. ^{1}H NMR (MeOD, 400 MHz) δ (ppm) 7.40 (d, *J*=15.7 Hz, 1H, Hα), 7.02 (d, *J*=2.0 Hz, 1H, H2), 6.92 (dd, *J*=8.2, 2.0 Hz, 1H, H5), 6.79 (dd, *J*=8.0 Hz, 1H, H6), 6.38 (d, *J*=15.7 Hz, 1H, Hβ), 3.26 (t, *J*=7.1, 2H, CH_2), 1.59 (m, 2H, CH_2), 0.97 (t, *J*=7.4 Hz, 3H, CH_3).

^{13}C NMR (MeOD, 100 MHz): 167.9 (CONH), 147.3 (C4), 145.3 (C3), 140.7 (Cβ), 120.6 (C6), 117.1 (Cα), 115.1 (C5), 113.7 (C2), 41.0 ($NHCH_2$), 22.3 (CH_2), 10.4 (CH_3). EI-MS *m/z* 221 ($M^{\bullet+}$, 8), 178 (40), 163 (100), 145 (18), 134 (16), 117 (10), 89 (20), 77 (11).

2.3.2. (*E*)-3-(3,4-dihydroxyphenyl)-*N*-butylacrylamide (ACAF4)

Yield (%): 50. ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm) 7.54 (d, *J*=15.5 Hz, 1H, Hβ), 7.13 (d, *J*=2.0 Hz, 1H, H2), 6.97 (dd, *J*=8.3, 2.0 Hz, 1H, H6), 6.86 (d, *J*=8.2 Hz, 1H, H5), 6.20 (d, *J*=15.5 Hz, 1H, Hα), 5.70 (t, *J*=5.5 Hz, 1H, CONH), 3.37 (m, 2H, CH_2), 1.54 (m, 2H, CH_2), 1.38 (m, 2H, CH_2), 0.94 (t, *J*=6.9 Hz, 3H, CH_3). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm) 167.9 (CONH), 147.3 (C4), 145.3 (C3), 140.8 (Cβ), 120.7 (C6), 117.0 (Cα), 115.1 (C5), 113.7 (C2), 38.9 ($NHCH_2$), 31.2 (CH_2), 19.7 (CH_2), 12.7 (CH_3). EI-MS *m/z* 235 ($M^{\bullet+}$, 6), 178 (35), 163 (100), 145 (15), 134 (14), 117 (12), 89 (16), 77 (10).

2.3.3. (*E*)-3-(3,4-dihydroxyphenyl)-*N*-hexylacrylamide (ACAF6)

Yield (%): 59. ^{1}H NMR (DMSO-*d6*, 400 MHz) δ (ppm) 9.37 (1H, s, OH), 9.14 (1H, s, OH), 7.96 (t, *J*=5.5 Hz, 1H, CONH), 7.22 (d, *J*=15.7 Hz, 1H, Hβ), 6.92 (d, *J*=1.8 Hz, 1H, H2), 6.83 (dd, *J*=8.1, 1.8 Hz, 1H, H6), 6.74 (d, *J*=8.1 Hz, 1H, H5), 6.32 (d, *J*=15.7 Hz, 1H, Hα), 3.14 (m, 2H, CH_2), 1.36 (m, 8H, $(CH_2)_4$), 0.87 (t, *J*=6.7 Hz, 3H, CH_3). ^{13}C NMR (DMSO-*d6*, 100 MHz) δ (ppm) 165.4 (CONH), 147.4 (C4), 145.7 (C3), 139.0 (Cβ), 126.6 (C1), 120.5 (C6), 118.8 (Cα), 115.9 (C5), 113.9 (C2), 38.6 ($NHCH_2$), 31.1 (CH_2), 29.3 (CH_2), 26.3 (CH_2), 22.2 (CH_2), 14.1 (CH_3). EI-MS *m/z* (%) 263 ($M^{\bullet+}$, 5), 178 (36), 163 (100), 145 (14), 134 (14), 117 (12), 89 (18), 77 (10).

2.3.4. (*E*)-3-(3,4-dihydroxyphenyl)-*N*-octylacrylamide (ACAF8)

Yield (%): 45. ^{1}H NMR (DMSO-*d6*, 400 MHz) δ (ppm) 9.19 (s, 2H, 2xOH), 7.93 (t, *J*=5.5 Hz, 1H, CONH), 7.22 (d, *J*=15.7 Hz, 1H, Hβ), 6.94 (d, *J*=1.8 Hz, 1H, H2), 6.83 (dd, *J*=8.3, 1.8 Hz, 1H, H6), 6.74 (d, *J*=8.1 Hz, 1H, H5), 6.32 (d, *J*=15.7 Hz, 1H, Hα), 3.14 (m, 2H, CH_2), 1.43 (m, 2H, CH_2), 1.26 (m, 2H, $(CH_2)_5$), 0.86 (t, *J*=6.7 Hz, 3H, CH_3). ^{13}C NMR (DMSO-*d6*, 100 MHz) 166.6 (CONH), 148.6 (C4), 146.9 (C3), 140.2 (Cβ), 127.8 (C1), 121.7 (C6), 120.0 (Cα), 117.1 (C5), 115.1 (C2), 39.9 ($NHCH_2$), 32.6 (CH_2), 30.6 (CH_2), 30.1 (CH_2), 30.0 (CH_2), 27.9 (CH_2),

23.5 (CH_2), 15.3 (CH_3). EI-MS *m/z* 291 ($M^{\bullet+}$, 10), 178 (34), 163 (100), 145 (13), 134 (15), 117 (13), 89 (18), 77 (8).

2.3.5. (*E*)-3-(3,4-dihydroxyphenyl)-*N*-decylacrylamide (ACAF10)

Yield (%): 55.4. ^{1}H NMR (DMSO-*d6*, 400 MHz) δ (ppm) 9.20 (s, 2H, 2xOH), 7.93 (t, *J*=5.4 Hz, 1H, CONH), 7.22 (d, *J*=15.7 Hz, 1H, Hβ), 6.94 (d, *J*=1.6 Hz, 1H, H2), 6.83 (dd, *J*=8.1, 1.6 Hz, 1H, H6), 6.74 (d, *J*=8.3 Hz, 1H, H5), 6.32 (d, *J*=15.7 Hz, 1H, Hα), 1.33 (m, 16H, $(CH_2)_8$), 0.88 (t, *J*=6.9 Hz, 3H, CH_3). ^{13}C NMR (DMSO-*d6*, 100 MHz) 165.7 (CONH), 147.7 (C4), 145.9 (C3), 139.3 (Cβ), 126.9 (C1), 120.7 (C6), 119.1 (Cα), 116.2 (C5), 114.3 (C2), 39.1 ($NHCH_2$), 31.8 (CH_2), 29.7 (CH_2), 29.5 (CH_2), 29.4 (CH_2), 29.2 (CH_2), 29.1 (CH_2), 26.9 (CH_2), 22.6 (CH_2), 14.4 (CH_3). EI-MS *m/z* 319 ($M^{\bullet+}$, 8), 178 (40), 163 (100), 145 (14), 134 (15), 117 (11), 89 (17), 77 (10).

2.3.6. (*E*)-3-(3,4-dihydroxyphenyl)-*N*-dodecylacrylamide (ACAF12)

Yield (%): 55.4. ^{1}H NMR (DMSO-*d6*, 400 MHz) δ (ppm) 9.21 (s, 2H, 2xOH), 7.93 (t, *J*=5.5 Hz, 1H, CONH), 7.22 (d, *J*=15.7 Hz, 1H, Hβ), 6.94 (d, *J*=1.6 Hz, 1H, H2), 6.83 (dd, *J*=8.1, 1.7 Hz, 1H, H6), 6.74 (d, *J*=8.3 Hz, 1H, H5), 6.32 (d, *J*=15.7 Hz, 1H, Hα), 3.14 (m, 2H, CH_2), 1.30 (m, 20H, $(CH_2)_{10}$), 0.85 (t, *J*=6.7 Hz, 3H, CH_3). ^{13}C NMR (DMSO-*d6*, 100 MHz) 165.7 (CONH), 147.7 (C4), 145.9 (C3), 139.3 (Cβ), 126.9 (C1), 120.7 (C6), 119.1 (Cα), 116.2 (C5), 114.3 (C2), 46.3 ($NHCH_2$), 39.1 (CH_2), 31.8 (CH_2), 29.7 (CH_2), 29.5 (CH_2), 29.4 (2xCH_2), 29.2 (CH_2), 29.1 (CH_2), 26.9 (CH_2), 22.6 (CH_2), 14.5 (CH_2). EI-MS *m/z* 347.1 ($M^{\bullet+}$ EI-MS *m/z* (%) 263 ($M^{\bullet+}$, 5), 178 (36), 163 (100), 145 (20), 134 (18), 117 (10), 89 (17), 77 (7).

2.4. Cell Culture

PC12 cells were a kind gift from Professor Lloyd A. Greene (Department of Pathology and Cell Biology, Columbia University, New York, NY, USA)[14]. They were seeded in plates coated with collagen obtained from rat tail and grown in RPMI 1640 medium, supplemented with 10% heat-inactivated HS, 5% heat-inactivated FBS, 100 U/ml penicillin G, and 100 mg/ml streptomycin in a 5% CO_2 incubator at 37 ºC. Two-thirds of the growing medium was changed every 2–3 days

and cell passages were carried out in collagen coated dishes using trypsin 0.25% to detach cells once a week.

2.5. Cell viability assay

PC12 cells (6×10^5 cells/mL) were seeded in 96-well plates in serum-free RPMI-1640 medium and treated with the synthesized derivatives at the final concentrations of 1, 5 and 25 µM or NGF at 5 or 50 ng/mL. After 48 h, cell viability was measured by the MTT reduction assay as previously describe [15]. Briefly, the media were carefully removed from the wells after centrifugation at 500g for 10 min. Then cells were incubated with MTT solution (0.5 mg/mL final concentration) for 90 min at 37 ◦C. Formazan crystals, which are formed due to the reduction of MTT by dehydrogenase enzymes in viable cells, were dissolved in 200 µL DMSO for 90 min. The absorbance was measured at 570 nm with background correction at 650 nm using a microplate reader (Bio-Rad model). The increase in viability was calculated by dividing the absorbance of treated cells by the absorbance of untreated control cells.

For evaluation of the role of TrkA signaling in the promotion of survival induced by caffeic acid amides, a selective TrkA inhibitor, K252a, was co-incubated with the test compounds at 50 and 100 nM and the survival rates were compared to the cells treated with the synthesized derivatives alone.

PC12 cells (6×10^5 cells/mL) were seeded in collagen coated 96-well plates in serum-free medium (80 µL per well). Ten µL of NGF diluted in RPMI-1640 at the final concentrations of 5 or 50 ng/ml or the test compounds at the final concentrations of 1, 5 and 25 µM were added in triplicate. Synthesized derivatives were first dissolved in dimethyl sulphoxide and then diluted to the appropriate concentration in growth medium before use and added to the culture medium. The final concentration of DMSO did not exceed 0.25%, which had no effect on the PC12 cells (data not shown). After 48 h, cell viability was measured by the MTT reduction assay as previously describe [15]. Briefly, the media were carefully removed from the wells after centrifugation at 500g for 10 min. Then cells were incubated with 20 µL MTT solution (0.5 mg/mL final concentration) for 90 min at 37 ◦C. Formazan crystals, which are formed due to the reduction of MTT by dehydrogenase enzymes in viable cells, were dissolved in 200 µL DMSO for 90 min. The absorbance was measured at 570 nm with background correction at 650 nm

using a microplate reader (Bio-Rad model). The increase in viability was calculated by dividing the absorbance of treated cells by the absorbance of untreated control cells.

For evaluation of the role of TrkA signaling in promotion of survival induced by caffeic acid amides, 50 and 100 nM K252a (a selective TrkA inhibitor), dissolved in DMSO, was added to the culture 1 h prior to the addition of caffeic acid amides (25 µM) and NGF (5 or 50 ng/ml). Then, MTT assay was performed as described above. The survival rates were compared to the cells treated with the synthesized derivatives and NGF alone.

2.6. Differentiation analysis

PC12 cells (5×10^4 cells/mL) were cultured on collagen-coated 12-well plates in low serum RPMI medium (2% HS and 1% FBS). After overnight growth, cells were treated with the synthesized amides (5 and 25 µM) with or without NGF (5 and 50 ng/mL). After 3 days of incubation, the growth medium was replaced with fresh low serum medium with the same concentrations of the test compounds and NGF. Neurite outgrowth was quantified after 8 days of incubation, as the proportion of neurites greater than or equal to the length of one cell body. These determinations were made with an inverted microscope (model eclipse TS100-F). The total number of neurites, as well as the neurites that were equal to or more than 100 µm, were counted. The number of neurites was counted in at least five randomly selected microscopic fields (magnification power of 20X). Each experiment was repeated at least 5 times.

2.7. Western blotting

PC12 cells (6×10^5cells/mL) were seeded in collagen-coated 35 mm dishes in normal serum medium for 48h, then shifted to low serum medium (2% HS and 1% FBS) for 24 h prior to exposure to test compounds (5 and 25 µM) and NGF (5 ng/mL) for the indicated time intervals. Cells were first washed with PBS, and then scraped in ice cold lysis buffer solution (20 mM Tris base, 150 mM NaCl, 1% Np40, 1 mM EDTA, 5% sodium deoxycholate and 0.1% SDS, pH 8.0) containing phenylmethylsulfonyl fluoride 1 µM, $Na_4O_7P_2$ 10 mM, Na_3VO_4 2 mM, and incubated on ice for 15 min. The cellular debris was removed by centrifugation (12,000 g for 20 min) at

4°C, and the cell lysate were carefully transferred to micro-centrifuge tubes. The protein concentration was measured by BCA assay kit using bovine serum albumin as a standard. The cell lysate (25 µg) was separated on 7.5% SDS−PAGE and transferred onto the PVDF membrane at 60 V for 3h. Nonspecific binding sites on the membranes were blocked with 4% BSA dissolved in Tris buffer saline containing 0.1% Tween-20 (TBST) for 50 min at room temperature. Blots were incubated with the appropriate antibodies; anti-p44/p42 MAPK (ERK1/2, 1:1000), anti-phospho-p44/p42 MAPK (p-ERK1/2, Thr202/Tyr204, 1:1500), anti-TrkA (1:250), anti-AKT (1:250), anti-phospho-AKT (ser473, 1:1500) and anti-phospho-TrkA (1:1000) overnight at 4°C. After three washes with TBST, the blots were incubated with appropriate horseradish peroxidase-conjugated rabbit anti-mouse or mouse anti-rabbit secondary antibodies (1:1000) at room temperature for 1 h. The blots were washed 3 times with TBST, and the proteins were detected by enhanced chemiluminescence (ECL) (GE Healthcare, Buckinghamshire, UK) and images were obtained with G: Box chemi-XR5 GeneSys image analyzer. The intensities of bands were calculated with Software Gene Tools SyneGene for Windows.

2.8. Statistical analysis

All data are presented as mean ± S.E.M based on at least three independent experiments. All statistical analyses were performed by ANOVA followed by appropriate post hoc test using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). P Values of less than 0.05 were considered significant.

2.9. Molecular docking study

Molecular docking study of **CA** and the promising compound of this study **ACAF12** with PI3K as conceivable molecular target was carried out using AutoDock 4.2 and AutoDock Tools 1.5.4 program. The 3D crystal structure of target protein co-crystallized with wortmannin, as cognate ligand (PDB code: 1E7U) was downloaded from RSCB protein data bank (http://www.pdb.org). The pre-treatment process was performed to prepare both the ligand and protein for docking

steps. The PDB file of protein was refined as following steps: 1) removing water molecules and crystallographic ligand 2) polar hydrogens addition 3) calculation of collman charges 4) generation of PDBQT file. The 3D structure of **CA** and **ACAF12** was constructed using Chem3D Ultra 12.0 software. Generated structures of ligands were submitted to a molecular mechanic conformational search (MM^+ force felid) and the geometry of the most stable conformer of each ligand was energetically optimized by semi-empirical method (AM1) in Hyperchem 7 software. In the next step, the optimized structures were transferred to ADT 1.5.4; the Gasteiger charges were added and the degree of torsions was defined to generate PDBT format.

The active site of protein was selected based on the ligand binding domain of wortmannin and the grid parameter file (gpf) was created using AutoGrid. Numbers of points in x, y, z dimensions were set to 60*60*60 with 0.375 Å grid spacing and grid center was set as 23.57, 61.04, 21.72 in each dimension, respectively. Consequently, docking parameter file was defined based on rigid macromolecule docking procedure using Lamarckian Genetic Algorithm (LGA), with 100 runs and 2500000 energy evaluations, other parameters were left as default. The most favorable docked pose was selected according to the minimum free energy of protein-ligand complex and was used for further exploration of binding interaction.

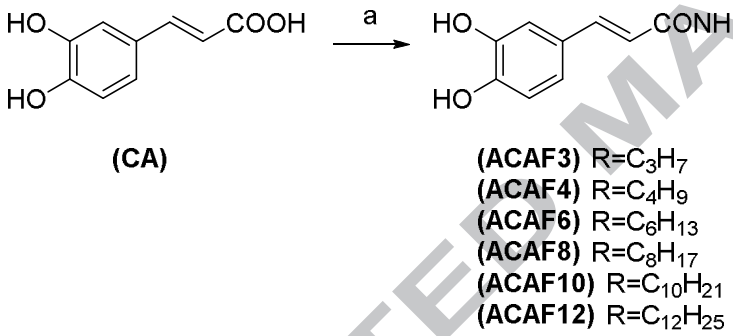
2.10. Drug-likeness properties study

The theoretical study of drug-like properties of target compounds was performed in the Molinspirations chemiinformatic on-line program (http://www.molinspiration.com). The 'drug-likeness' of all synthesized compounds was assessed applying Lipinski's 'rule of five'.

3. Results

3.1. Chemistry

The structures and synthetic strategy pursued to obtain caffeic acid amide derivatives is depicted in Scheme **1**. Carboxamide derivatives were obtained by a benzotriazol-1-yloxytripyrolidinophosphonium hexafluorophosphate (PyBOP) assisted amidation reaction between the caffeic acid and the appropriate alkylamine. With the exception of **ACAF3**, the compounds were obtained in moderate yields. The structural identity of all compounds was confirmed by nuclear magnetic resonance (NMR) spectroscopy (1H NMR, 13C NMR, and DEPT135) and electronic impact mass spectrometry (EI-MS).



Scheme 1. Synthetic strategy pursued to obtain caffeic acid amide derivatives. (a) 1. DIPEA, DMF, PyBOP, dichloromethane, 0 °C, 30 min; 2. appropriate amine, rt, 6h.

3.2. Effects of caffeic acid amide derivatives on survival of PC12 cells in serum-deprived conditions

Neuroprotective activity of caffeic acid amide derivatives against serum deprivation-induced cell death was assessed by MTT assay. NGF (5, 50 ng/ml) served as a positive control in the experiments. Cells treated with caffeic acid amides at the concentration of 25 μM, showed a significant increase in survival compared to untreated cells. The cell viability increased to 157.6%, 201.6%, 183.1%, 157.8%, 192.6% and 230.5% for the cells treated with 25 μM of

ACAF3, **ACAF4**, **ACAF6**, **ACAF8**, **ACAF10** and **ACAF12**, respectively. **ACAF12** was the most active compound (Figure **1**).

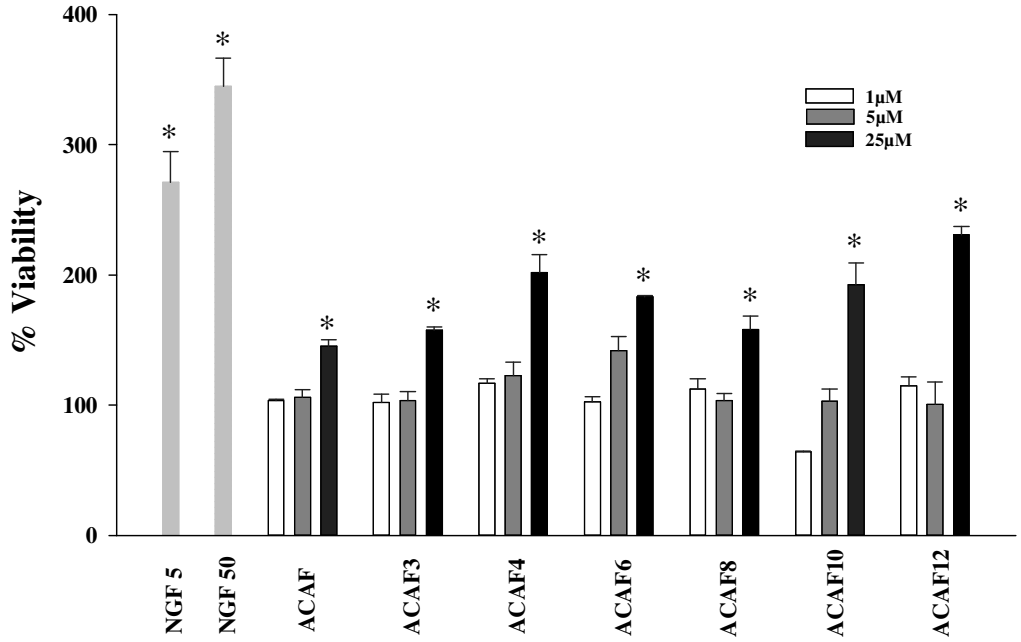


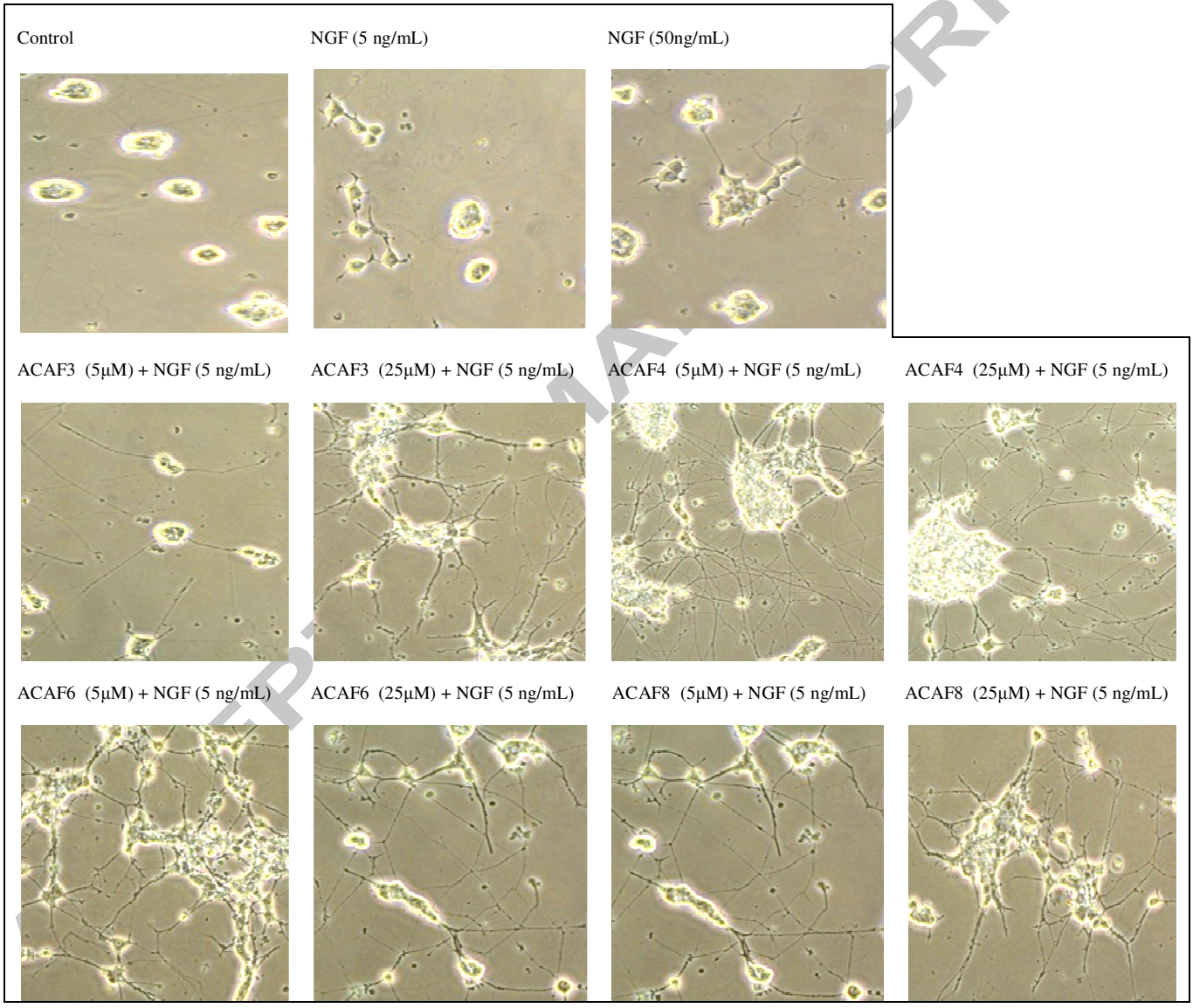
Figure 1: Effects of caffeic amide derivatives on survival of PC12 cells. PC12 cells were seeded in 96-well plates in serum-free medium and were induced with synthesized caffeic acid amide derivatives at the final concentrations of 1, 5 and 25 µM or NGF at 5 or 50 ng/ml. After 48 h, cell viability was measured by the MTT assay. The results are plotted as the percentage cell survival in treated cells relative to untreated cells. The data are presented as the mean ± S.E.M of 3-5 independent experiments. * $p < 0.05$.

3.3. Caffeic acid amides potentiate NGF-induced neuronal differentiation in PC12 cells

We next studied the effect of caffeic acid amides on the neurite outgrowth of PC12 cells. As it is shown in Figure **2A**-**C**, incubation of neuronal cells with NGF 5 and 50 ng/ml induced neurite outgrowth. The incubation of cells with the synthesized compounds alone did not promote neurite outgrowth (data not shown). On the other hand, the concomitant incubation of some caffeic acid amides at the concentrations of 5 µM (**ACAF4**, **ACAF6** and **ACAF8**) and 25 µM (**ACAF3**, **ACAF4**, **ACAF6** and **ACAF8**) with low dose NGF (5 ng/mL), significantly increased the total number of neurites and also the number of neurites longer than 100 µM, compared to

NGF treatment alone (Figure **2B** and **C**). Caffeic acid amides at 1 µM did not potentiate neuritogenesis induced by NGF (data not shown). Co-treatment of cells with NGF and long alkyl chain amide derivatives (**ACAF10** and **ACAF12**) failed to potentiate neuritogenesis induced by NGF.

A



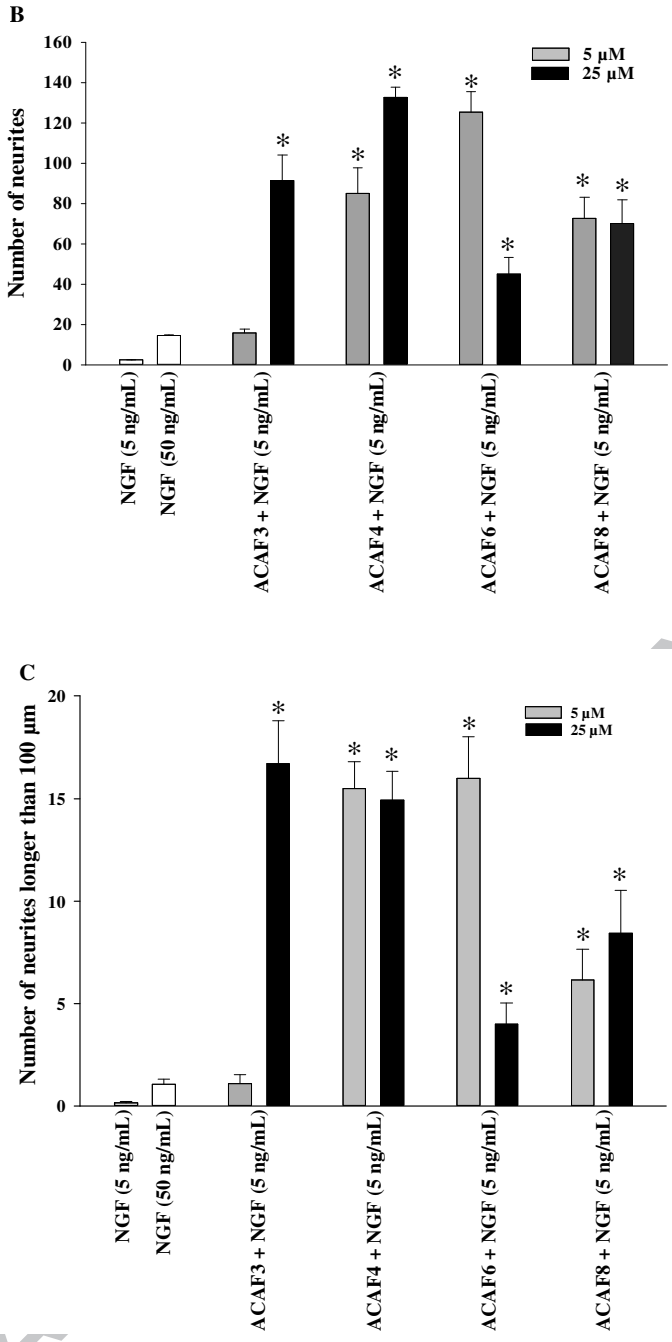


Figure 2: Caffeic acid amides potentiate NGF-induced neuronal differentiationin PC12 cells. PC12 cells were seeded at a low density (5×10^4 cells/well) and exposed to thecaffeic acid amides at the concentrations of 5 and 25 µM with or without NGF (5 ng/mL) or NGF alone (5 and 50 ng/mL). After 8 days, neurite outgrowth was quantified as the proportion of neurites greater than or equal to the length of one cell body.A: morphological changes in PC12 cells treated with indicated agents. B: The total number of neurites were counted with a phasecontrast microscope in

five fields. C: The neurites that were equal to or more than 100 μm were counted. Results (mean ± S.E.M.) are representative of five independent experiments (n=5). * p<0.05.

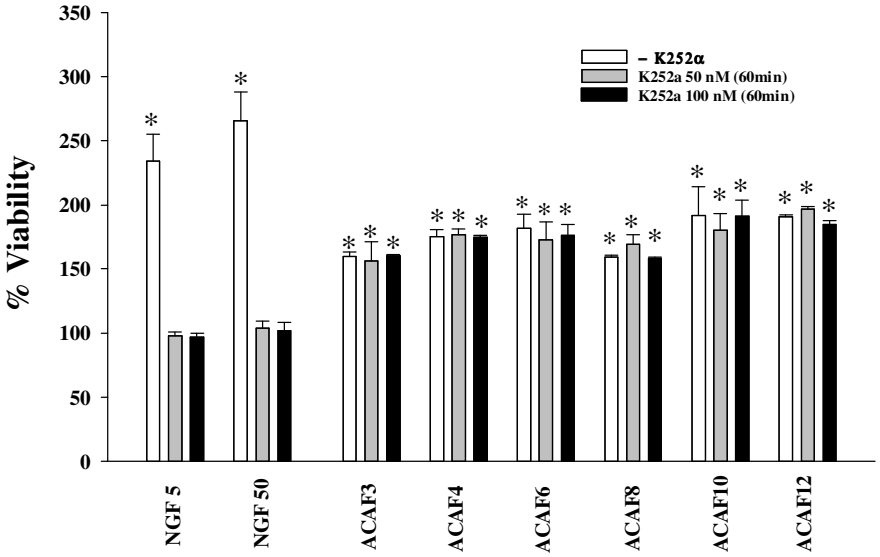
3.4. Caffeic acid amides prompted neuroprotective effects in a TrkA-independent manner

To evaluate the relation between the neuronal survival enhancement effect of caffeic acid amide derivatives with TrkA receptor agonism, the effect of K252a, a selective TrkA inhibitor, was examined on caffeic acid amide-promoted rescue of PC12 cells in serum-free conditions.

In these experiments, serum-free PC12 cells were treated with 25 μM of synthesized derivatives in the presence or absence of 50 and 100 nM K252a. As expected, K252a was able to inhibit NGF-induced rescue of PC12 cells, however, it did not significantly alter the neurotrophic effect of the caffeic acid amides (Figure 3A). This observation indicates that TrkA signaling is not essential for the promotion of neuronal cell survival induced by caffeic acid amide derivatives.

These results were validated by western blot analyses. Two caffeic acid amides with short and long alkyl chain amide derivatives (**ACAF4** and **ACAF12**, respectively) were tested for their ability to activate TrkA receptor. To investigate whether caffeic acid amides can activate the TrkA receptor, PC12 cells were treated with **ACAF4** and **ACAF12** (5 and 25 μM) and NGF 5 ng/ml as positive control for 60 min, and the cell lysates were immunoblotted for detection of TrkA and phospho-TrkA. NGF 5 ng/mL triggered TrkA phosphorylation in PC12 cells (Figure 3B); however, none of the test compounds could cause any detectable phosphorylation in TrkA receptor. **ACAF4** and **ACAF12** were also tested for their ability to potentiate NGF-induced TrkA phosphorylation, but their concomitant incubation with NGF did not induce any alteration in the phosphorylation level of TrkA compared to cells treated with NGF alone (Figure 3C).

A



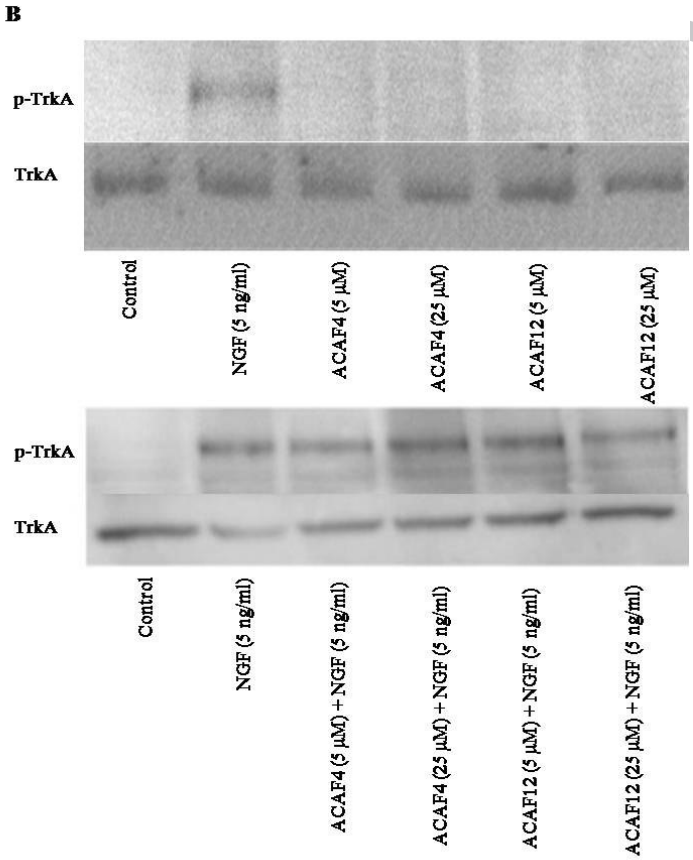


Figure 3: Caffeic acid amides prompted neuroprotective effects in a TrkA independent manner. A: Serum-deprived PC12 cells in 96-well plates were treated for 60 min without or with K252a (50 and 100 nM) and then were either untreated or stimulated with caffeic acid amides (25 μM) and NGF (5 or 50 ng/ml). Subsequently, cell viability was measured by MTT assay. Data are plotted as the percentage cell survival in treated cells relative to untreated cells and are representative as mean ± S.E.M of three independent experiments (n=3). * p<0.05.B: Cultured PC12 cells were stimulated with ACAF4 and ACAF12 alone, NGF alone, or a combination of both for 1h. Phosphorylation of TRKA were detected by immunoblotting with antibodies to p-TRKA, TRKA. Data are expressed as mean ± SEM (n=3, p<0.05).

3.5. Caffeic acid amides induce ERK1/2 activation

We next examined the effects of a short (**ACAF4)** and a long chain caffeic acid amide (**ACAF12)** added alone or concomitant with low dose NGF to the PC12 cells on ERK1/2 signaling. As shown in Figure 4, NGF (5 ng/ml) induced, as expected, an intense phosphorylation of ERK1/2. Also **ACAF4** and **ACAF12** alone significantly induced ERK1/2 phosphorylation in a dose-dependent manner. **ACAF12** was more effective than **ACAF4** to induce ERK1/2 activation (Figure 4A-B). Interestingly, when the cells were treated with the test compounds and low dose NGF, **ACAF12** significantly increased NGF-induced ERK1/2 phosphorylation. Similarly, **ACAF4** potentiated NGF-induced ERK1/2 activation, but to a lower extent compared to **ACAF12** (Figure 4A-B). These results suggest that the long chain alkyl amide derivative was more effective than short chain alkyl amide derivate in activation of ERK1/2 pathway.

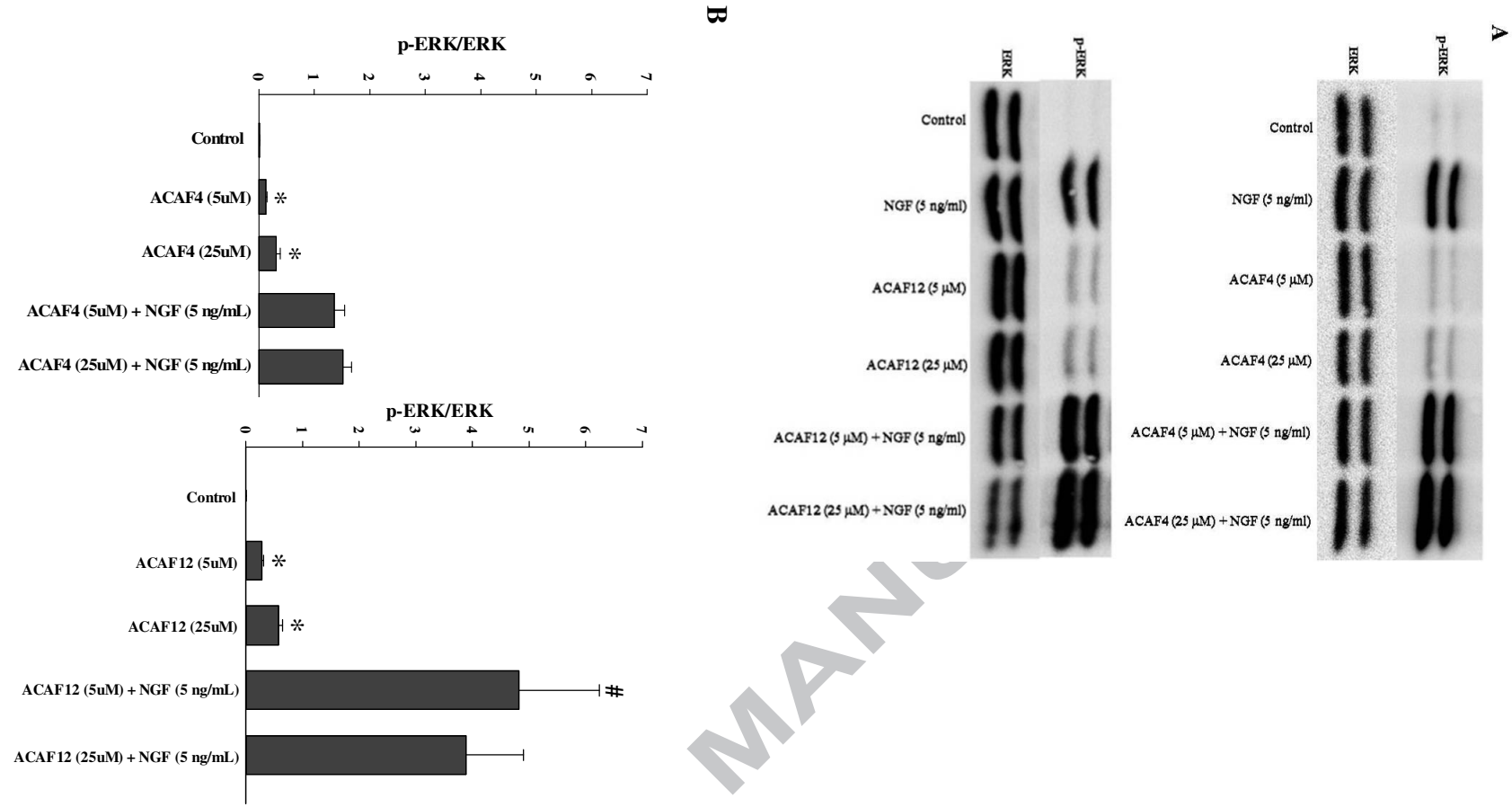


Figure 4: Caffeic acid amides induce the activation of ERK1/2. PC12 cells were stimulated with 5 ng/ml NGF alone and **ACAF4** and **ACAF12** (5 and 25 μM) alone, and with a combination of both for 2h. A: Phosphorylated form and total ERK were detected by immunoblotting. B: The band density of the phosphorylated ERK1/2 was normalized to the band density of the total ERK1/2 present in the same lane and then each value of the ratio p-ERK/ERK were divided by p-ERK/ERK of NGF (5 ng/ml). Each value of caffeic acid amides(5 and 25 μM) alone was expressed relative to control. The results are shown as mean ± SEM ($n=4$, $p < 0.05$).

3.6. ACAF12 induces the phosphorylation of AKT

We also tested whether or not the neurotrophic effects of **ACAF4** and **ACAF12** (5 and 25 μM) are linked to AKT signaling pathway. PC12 cells were treated with **ACAF4** and **ACAF12** (5 and 25 μM) either alone or concomitant with low dose NGF for 2h (Figure 5). **ACAF12** at 5 and 25 μM significantly increased AKT phosphorylation. On the other hand, **ACAF4** did not increase AKT phosphorylation. Furthermore, co-incubation of caffeic acid amide derivatives with NGF did not increase the AKT phosphorylation to a degree stronger than that induced by NGF alone (Figure5A-B).

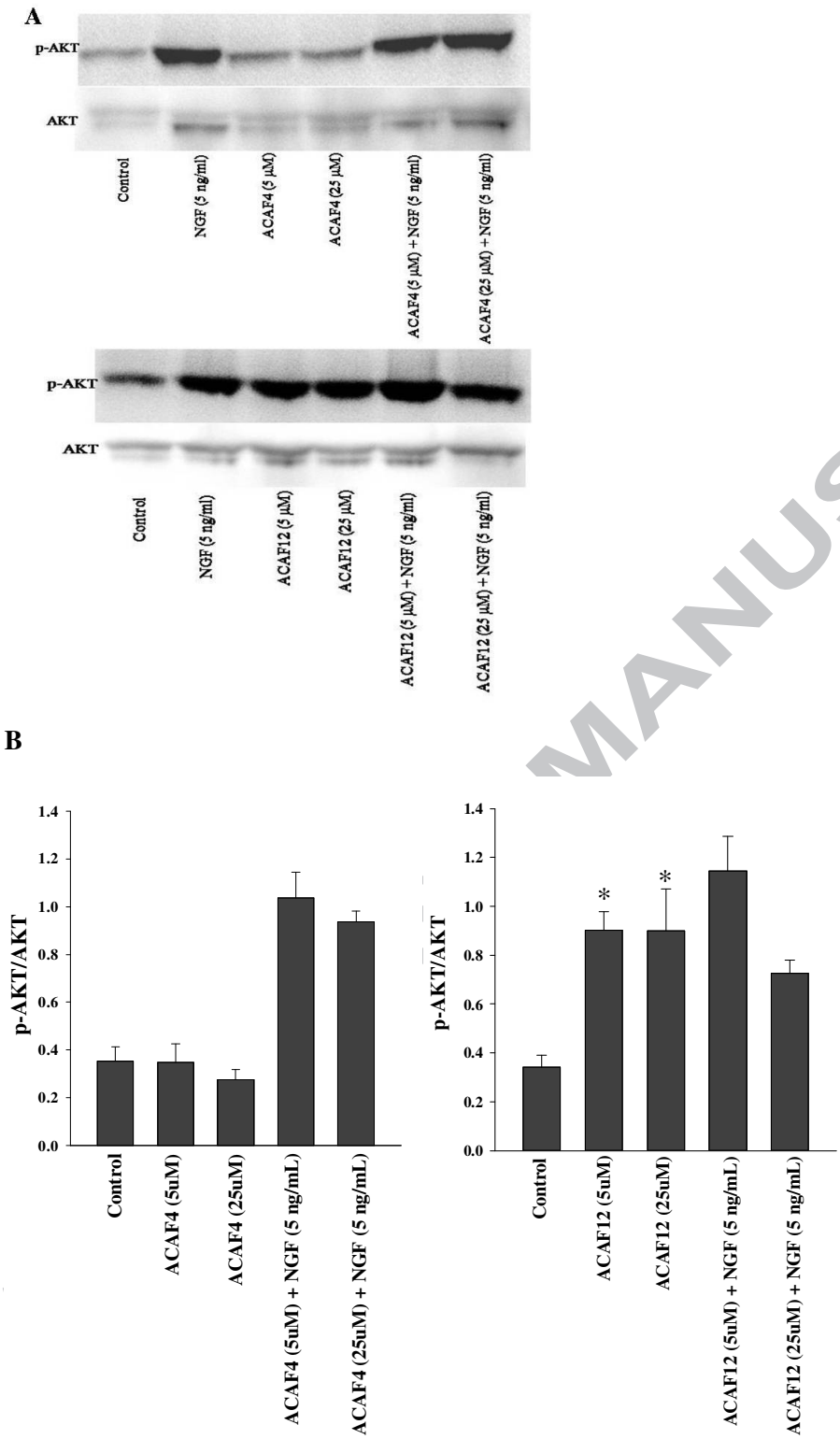


Figure 5: ACAF12 induces the activation of AKT. PC12 cells were incubated with NGF (5 ng/ml) alone and ACAF4 and ACAF12 (5 and 25 µM) alone, and with a combination of both for 2h. A: Phosphorylated form and total AKT were detected by western blot analysis.B: Activation of AKT was determined by normalization of the

band density from the phosphorylated form (p) with that of the total form and then each value of the ratio p-AKT/AKT were divided by p-AKT/AKT of NGF (5 ng/ml). Values obtained with caffeic acid amides(5 and 25 μM) alone were compared with control. Data are shown as mean ± SEM (n=4, $p < 0.05$).

3.7. *In silico* docking simulation of compounds CA and ACAF12 with PI3K

Several studies have suggested that PI3K and its downstream pathways are involved in the regulation of neuronal survival and neurogenesis both *in vitro* and *in vivo* [16-18]. PI3K can activate downstream kinases such as AKT, PKC and ERK family members [19]. Therefore, molecular docking study was carried out to investigate the binding and mode of interaction of **CA** and its promising amide derivative (**ACAF12**) with PI3K as a possible molecular target responsible for neurotrophic activity of synthesized compounds [20]. To this end, flexible ligand/rigid macromolecule docking was performed using crystal structure of PI3K (PDB ID: 1E7U) with its cognate ligand, wortmannin. Docking procedure was further optimized and validated via re-docking of the co-crystallized conformation of cognate ligand (wortmannin) 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. The best-docked and experimental conformation of wortmannin correlated quite well with an RMSD of 1.73Å and binding free energy of −8.96 Kcal/mol (Table 1). Validated docking protocol was used for molecular docking study of test compounds under investigation. The results are presented in Table 1. The binding profile of **CA** and **ACAF12** docked with PI3K (PDB code: 1E7U) is depicted in Figure 6. **ACAF12** and **CA** demonstrated different binding orientations in PI3K active site. In the case of **ACAF12**, both of phenolic hydroxyl groups were involved in H-bond interaction with Asp841 and Asp964 of PI3K active site. In return, orientation of phenolic part of **CA** resulted to only one H-bond interaction between *meta*-phenolic hydroxyl moiety and Glu880. In addition, carbonyl group of dodecyl amide derivative (**ACAF12**) was involved in H-bond network interaction with Ile-881 and Val882 of N-terminal lobe; while carbonyl of acidic derivative (**CA**) interacted with Lys833 of binding site. It is evident from Figure 6 that additional dodecylamide pendant of **ACAF12** got directed toward C-terminal lobe covered by hydrophobic residues such as Met953, Phe961 and Ile963 and produced hydrophobic interactions with this hydrophobic cavity. Such particular induced binding pose of

ACAF12 into the PI3K active site could justify its superior binding free energy (ΔG_b=-6.70 Kcal/mol) and observed neuroprotective effect against serum deprivation-induced cell death compared to its acidic counterpart **CAF** (ΔG_b=-5.05 Kcal/mol) and sustenance PI3K as its probable molecular target underlying the neurotrophic effect observed by amide derivatives of HCAs.

Table 1. Binding interactions and results of molecular docking study of caffeic acid (CA), caffeic acid amide analogues (ACAF) and wortmannin.

Compound	ΔG_b (Kcal/mol)	Ki (μm)	Atom of the ligand	Amino acid
CA	-5.05	199.83	3-OH	Glu 880
			C=O	Lys 833
ACAF3	-7.29	4.57	3-OH	Asp841
			4-OH	Asp964
			C=O	Val882
ACAF4	-7.43	3.59	3-OH	Asp841
			4-OH	Asp964
			C=O	Val882
ACAF6	-7.52	3.06	3-OH	Asp841
			4-OH	Asp964
			C=O	Val882
ACAF8	-6.41	19.87	4-OH	Asp964
			C=O	Val882
ACAF10	-6.14	31.78	3-OH	Asp841
			4-OH	Asp964
ACAF12	-6.70	12.28	3-OH	Asp841
			4-OH	Asp964
			C=O	Ile-881
			C=O	Val882
Wortmannin*	-8.96	0.28	---	**---**

*The best-docked and experimental conformation of wortmannin correlated quite well with an RMSD of 1.73Å.

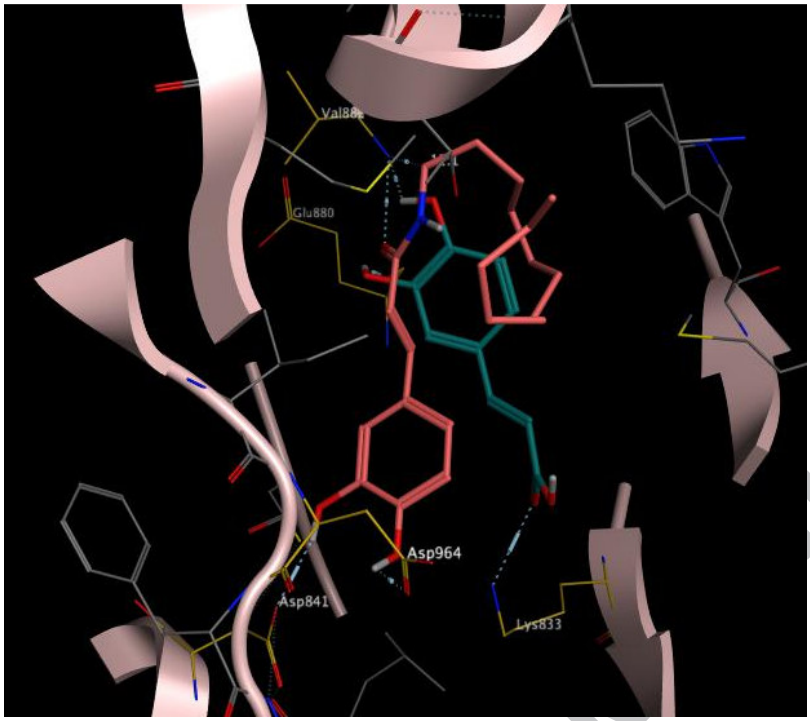


Figure 6. Comparative binding mode of CA (ΔGb=-5.05 Kcal/mol) and ACAF12 (ΔGb=-6.70Kcal/mol) (backbones were colored in green and orange, respectively) in PI3K active site. H-bond interactions were demonstrated as light blue lines.

3.8. Drug-likeness

In order to achieve insight about the structural features that cause the observed experimental results, we evaluated the molecular properties important for drug accessibility to its molecular target. Based on the Lipinski's rule of five, molecular properties of the active compounds must pass at least 3 of the 4 rules in order to consider them as suitable drug candidates: H-bond donors (HBD) ≤ 5, H-bond acceptors (HDA) ≤ 10, molecular mass (MM) ≤ 500, and the calculated logP (clogP) ≤ 5 [21]. Calculated molecular descriptors are presented in Table 2. The results indicated that values of three molecular descriptors (HBD, HBA and MM) were within the acceptable limits of Lipinski's rule for **CA** and its alkyl amide derivatives.

Table 2. Assessment of the molecular descriptors of drug-likeness applying Lipinski's rule of five.

Compound	cLogP≤5	NH/OH≤5	N/O≤10	MW≤500
ACAF3	1.68	4	3	221.26
ACAF4	2.24	4	3	235.28
ACAF6	3.25	4	3	263.34
ACAF8	4.26	4	3	291.39
ACAF10	5.27	4	3	319.44
ACAF12	6.28	4	3	347.50
CAF	0.94	4	3	180.16

cLogP, Calculated decimal logarithm of octanol/water partition coefficient. NH/OH, Number of H-bond donors. N/O, Number of H-bond acceptors. MW, Molecular weight.

4. Discussion

In this study, we synthesized a series of caffeic acid amide analogues with variable alkyl chain lengths and tested their neurotrophic action in PC12 neuronal cells. We found that these compounds are capable of increasing cell survival in serum-deprived conditions and also enhancing the differentiation induced by NGF. We also found that the neurotrophic effects of caffeic acid amides are not mediated by direct agonistic action on TrkA receptors, but rather through the activation of ERK1/2 and AKT signaling pathways.

CA is a phenolic compound widely distributed in some species of the plant kingdom and abundantly present in the human diet [22]. Previous studies have revealed neuroprotective effects of **CA** and its derivatives against oxidative stress and ischemic injury [23,24]. For example, it has been shown that caffeic acid ester fraction from the plant *Erigeron breviscapus*, increases neuronal cell viability in rat primary cultured microglia [25]. Furthermore, CAPE has been shown to block 6-hydroxydopamine-inducedneurotoxicity in cerebellar granule neurons [26] and also to be effective in induction of differentiation in PC12 cells [10].

A previous study of our group recently showed that caffeic acid esters possess neurotrophic activities [20]. As an improved stability is generally considered advantageous *in vivo,* in order to increase the stability of caffeic acid esters we synthetized caffeic acid amide derivatives with variable alkyl chain lengths (C3-C12). The amide analogues have higher hydrolytic energies of activation and are able to avoid enzymatic hydrolysis by esterases [27].

We first examined the effects of caffeic acid amides in protection of neuronal cells against serum deprivation-induced cell death. It was found that pretreatment with caffeic acid amides at the concentration of 25 μM increased neruonal survival compared to untreated cells. Another **CA** derivative, CAPE, has been previously demonstrated to have neuroprotective effects against H_2O_2-induced cytotoxicity in PC12 cells [28] and also against IFN-γ/LPS-induced injury in nigral dopaminergic neurons [9]. Moreover, several other polyphenolic compounds have been reported to enhance neuronal survival in serum deprived neuronal cells. For instance, Lin and colleagues have shown that in PC12 cells, luteolin, a flavonoid compound, decreases serum withdrawal-induced cytotoxicity [29]. Other examples of polyphenolic neuroprotective compounds that stimulate proliferation of PC12 cells include epigallocatechin-3-gallate [30], 5-hydroxy-3,6,7,8,3',4'-hexamethoxyflavone and nobiletin [31].

This study also revealed that co-incubation of some of the synthesized caffeic acid amides (**ACAF3**, **ACAF4**, **ACAF6** and **ACAF8**) with low dose NGF (5 ng/ml), increased the total number of neurites and also those longer than 100 µm compared to NGF alone. Although long alkyl chain caffeic acid amides (**ACAF10** and **ACAF12** amides) were active compounds in enhancement of neuronal survival, they did not show any significant effect on neurite outgrowth. Since the incubation time in the neurite assay was significantly longer than survival assay (8 vs. 2 days) longer chain amides may exert toxic effects in a longer incubation time. Correlation of the length of the alkyl chain with toxicity has been previously shown [32].

Different studies have shown that age-related disorders such as Alzheimer's disease are associated with NGF deficiency [33]. Hence, the NGF-potentiating action of caffeic acid amide derivatives could be considered of therapeutic value in neurodegenerative diseases related to NGF insufficiency. Several studies have identified a variety of polyphenols that potentiate neurotrophins' (NGF and BDNF) effect in neurite outgrowth induction. For instance, green tea polyphenols potentiate the neuritogenic effect of NGF and BDNF in PC12 cells [34,35]. Genistein, a polyphenol compound belonging to the class of flavonoids has also the potential to enhance NGF-induced neurite outgrowth in PC12 cells [36]. Another compound, isorhamnetin, a flavonol isolated mainly from *G. biloba* plant, has also been shown to enhance the neuronal differentiation induced by NGF in cultured PC12 cells [37].

We also demonstrated that K252a, an inhibitor of TrkA receptor, did not significantly alter the effect of caffeic acid amide derivatives on cell survival. Additionally, western blot experiments showed that **ACAF4** and **ACAF12** (5 and 25 µM) did not induce TrkA phosphorylation. Further, none of the test compounds increased NGF-induced TrkA phosphorylation. Previous studies have shown that inhibition of TrkA receptors block most of the effects prompted by NGF in PC12 cells, including rescue from cell death induced by serum withdrawal [38]. Our findings suggest that the effects of caffeic acid amides in rescuing cells from serum deprivation-induced death and promoting NGF-induced neuronal differentiation are regulated via pathways that are independent of direct activation of TrkA receptor, and probably act through downstream pathways.

Other studies have also shown that neurotrophic action of phenolic compounds such as 5-hydroxy-3, 6, 7, 8, 3', 4'-hexamethoxyflavone [31] and nobiletin [39] are not associated with TrkA activation in PC12 cells. There are indeed only few compounds that directly activate Trk

receptors, while several compounds stimulate the more downstream pathways that lead to the neurotrophic effects [8]. Therefore, we sought to assess the effect of test compounds on downstream neurotrophic pathways, and our results showed that **ACAF12** could directly stimulate ERK1/2 and AKT signaling pathways, while **ACAF4** elevated only the level of phosphorylated ERK1/2. We also co-treated the synthesized compounds with NGF (5 ng/ml) to test possible potentiating effect of these compounds on the NGF-induced activation of ERK1/2 and AKT signaling. Co-treatment with **ACAF12** significantly increased the NGF-induced phosphorylation of ERK1/2, while **ACAF4** lacked such an effect. Furthermore, when co-incubated with NGF, neither **ACAF4** nor **ACAF12** increased NGF-induced AKT activation.

The superior effect of **ACAF12** compared to **ACAF4** could be explained by its enhanced lipophilicity due the longer alkyl chain in **ACAF12** ($cLogP_{ACAF12}$=6.36 >$cLogP_{ACAF4}$=2.13). Previous observations of our groups and also others' have clearly shown that the addition of longer alkyl chain to phenolic acid molecules increases their lipophilicity, which consequently enhances permeability through the cell membrane and improves the biological activity [40-42].

Previous studies have reported that the activation of ERK and AKT signaling by natural or synthetic compounds is associated with neuronal survival and differentiation, and that the inhibition of these pathways blocks the neurotrophic effects induced by the test compounds [16,43]. For instance, carnosic acid and rosmarinic acid, compounds that are structurally related to **CA**, exhibited neurotrophic effects in PC12 cells by activation of ERK1/2 signaling pathway [11]. Another study demonstrated that luteolin-induced neurite outgrowth was inhibited by ERK 1/2 inhibitors [44]. Similarly, the involvement of the ERK signaling pathways in the neuritogenic action in PC12 cells has been demonstrated in response to curcuminoids [45]. Sagara and co-workers have reported that ERK1/2, but not the PI3K/AKT pathway, is involved in fisetin-induced PC12 cell differentiation [46]. ERK1/2 and PI3K/AKT are both important signaling pathways involved in neuronal survival and differentiation, therefore, small molecule compounds that activate these important pathways could be of therapeutic value in neurodegenerative diseases [47].

CA and **ACAF12** compounds were subjected to molecular docking and drug likeness studies. Results of docking studies predict that **CA** and promising neuroprotective agent, **ACAF12**, docked with PI3K as a potential target. **ACAF12** has higher binding affinity towards PI3K than the **CA** and the binding energies of these compounds correlated well with the results of the cell

survival promotion activity. In our study, Lipinski's rule of five (molecular weight < 500 Da, HB donor < 5, HB acceptor < 10 and QPlogPo/w (octanol/water partition coefficient) < 5) for all caffeic acid and its promising amidated derivative were also generated. All the analogues except **ACAF10** and **ACAF12** satisfied Lipinski's rule of five. Considering the cLogP values as a major criteria of lipophilicity, it could be concluded that the superior neuroprotective potency of alkyl amide derivatives, especially **ACAF10** and **ACAF12** (cLogP = 5.27 and 6.28, respectively) compared to their acidic counterpart **CA** (cLogP= 0.94), might be partly attributed to their higher lipophilicity and their capacity in crossing cell membranes and accessing their molecular target.

In conclusion, the present findings have revealed that alkyl amides of **CA** have remarkable neurotrophic effects, which are not mediated by direct TrkA activation, but rather via activation of ERK1/2 and AKT signaling pathways. We have also found that caffeic acid amides with longer alkyl chains have higher activities compared to those with shorter chains. Long alkyl chain amide derivatives (**ACAF6**, **ACAF8**) potentiate neuritogenesis induced by NGF when compared to long alkyl chain ester compounds, however, caffeic acid amides was found to be similarly neuroprotective to caffeic acid esters against serum deprivation-induced cell death. These compounds with considerable neurotrophic capacities may hold great promise for the development of novel therapeutics for neurodegenerative diseases.

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Highlights

A series of Caffeic amides with variable alkyl chain lengths were synthesized.

Synthesized amides increased cell survival and NGF-induced neurite outgrowth in PC12.

The neurotrophic effects were mediated by activation of ERK1/2 and AKT signaling.

Interaction with PI3K and drug likeness were studied.

Graphical abstracts

Synthesized alkyl amides of caffeic acid induce cell differentiation and increase survival in PC12 neuronal cells at 25 µM. These neurotrophic effects seem to be mediated by activation of ERK1/2 and AKT signaling pathways, but not by direct TrkA phosphorylation.