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

Fatemeh Moosavi [a,b], Razieh Hosseini [a,b], Hamid Rajaian [b], Tiago Silva [c], Diogo Magalhães e Silva [c], Luciano Saso [d], Najmeh Edraki [a], Ramin Miri [a], Fernanda Borges [c,*], Omidreza Firuzi [a,*]

[a] Medicinal and Natural Products Chemistry Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
[b] Department of Pharmacology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran
[c] CIQUP/Department of Chemistry and Biochemistry, Faculty of Sciences, University of Porto, 4169-007 Porto, Portugal
[d] Department of Physiology and Pharmacology "Vittorio Erspamer", Sapienza University of Rome, Italy

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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 putative molecular target. Based on Lipinski's rule of five, all of the compounds obeyed three 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.

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.[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 finding of small molecules, which activate the TrkA receptor or

* Corresponding authors.
E-mail addresses: fborges@fc.up.pt (F. Borges), firuzio@sums.ac.ir (O. Firuzi).

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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 × 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 min. Then, the amine (2.78 mmol) was added, the reaction was allowed to reach room temperature and stirred for an additional 6 h. The solvents were removed under reduced pressure and the residue was dissolved in ethyl acetate (30 mL). The solution was washed with water (3 × 10 mL), HCl 1M (3 × 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 ($M^{\cdot+}$, 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 ($M^{\cdot+}$, 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-d_6, 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,