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Effects of a verbenachalcone derivative on neurite outgrowth, inhibition of caspase induction and gene expression

Li-An Yeh,[a] Deppa Padmanaban,[a] Pei Ho,[a] Xeuchao Xing,[a] Patricia Rowley,[b] Lee Jae Morse,[b] Roderick V. Jensen[b,c] and Gregory D. Cuny[a,*]

[a]*Laboratory for Drug Discovery in Neurodegeneration, Harvard Center for Neurodegeneration and Repair, Brigham & Women's Hospital and Harvard Medical School, 65 Landsdowne Street, Cambridge, MA 02139, USA*
[b]*Center for Neurologic Diseases, Brigham & Women's Hospital and Harvard Medical School, 65 Landsdowne Street, Cambridge, MA 02139, USA*
[c]*Department of Physics, Wesleyan University, Middletown, CT 06459, USA*

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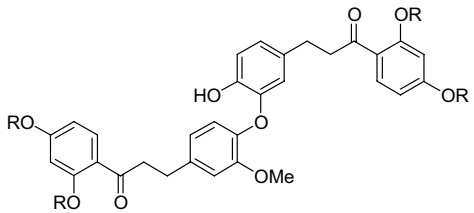
Abstract—A verbenachalcone derivative was synthesized and shown to protect N2a cells from caspase induction caused by serum starvation and to enhance the effect of NGF on neurite outgrowth in PC12 cells. As an initial investigation of the compound's mechanism(s) of action, we performed differential gene expression profiling in PC12 cells using oligonucleotide (~10,000 gene probes) microarrays. Gene expression patterns were compared in the presence of NGF (2 and 50 ng/mL) and NGF (2 ng/mL) plus the verbenachalcone derivative. Ten genes were significantly ($\geqslant$2-fold; $p \leqslant 0.05$) up-regulated and seven genes were significantly down-regulated in the presence of the compound. These results were independently validated by quantitative real-time PCR for a subset of genes (cathepsin L, sigma-1 receptor and protein tyrosine phosphatase receptor type R). These genes or their protein products may represent useful therapeutic targets for treating neurodegeneration, such as Alzheimer's disease.

Among the suspected causes of neurodegeneration in Alzheimer's disease (AD) is a decrease in neurotrophic support, including nerve growth factor (NGF). NGF belongs to a class of small basic proteins whose effects are almost exclusively on the central and peripheral nervous systems.[1] NGF binds to two classes of cell surface receptors on cholinergic axon terminals, the high-affinity TrkA receptor with catalytic tyrosine kinase activity and the lower-affinity p75 neurotrophin receptor ($p75^{NTR}$), followed by retrograde transport to the neuron cell body. Like other neurotrophins, it serves a vital role in the development and homeostasis of the nervous system. Receptor activation can result in an array of cellular events, including enhanced survival, differentiation, neuritogenesis and neurotransmitter synthesis, and secretion.[2,3]

A considerable amount of in vitro and in vivo data has been generated that demonstrates NGF's neuroprotective role.[4,5] However, delivery of exogenous NGF has failed to demonstrate efficacy in several human clinical trials.[6] In addition, direct in vivo administration of NGF is hampered by numerous factors, including the inherent problems frequently associated with peptide therapeutics (e.g., inadequate blood–brain-barrier penetration and metabolic stability).

Interestingly, NGF protein and mRNA levels remain either unchanged or are increased in the hippocampus and cerebral cortex of AD patients and only decreased in the nucleus basalis.[4] However, TrkA receptor



1 R = H
2 R = Me

* Corresponding author. Tel.: +1 617 768 8640; fax: +1 617 768 8606; e-mail: gcuny@rics.bwh.harvard.edu

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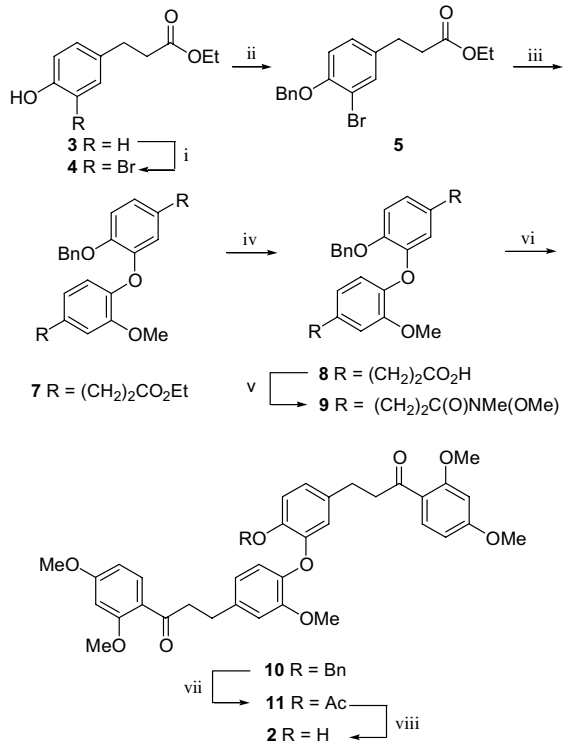
expression was reduced, while $p75^{NTR}$ receptor expression levels remain unclear.[7] Therefore, AD patho-physiology may in part result from inadequate NGF signal transduction.[8–10] Consequently, molecules that augment NGF signaling pathways may slow and/or prevent neurodegeneration in patients suffering from AD and other dementias.

Small organic molecules (MW < 500 amu) that possess NGF-like properties can generally be classified into three mechanistic categories: (i) agonists for the TrkA receptor, (ii) stimulators of NGF synthesis or secretion, and (iii) enhancers of NGF (i.e., increase the cellular response to NGF, but lack intrinsic activity in the absence of NGF). Recently the natural product verbenachalcone, **1**, isolated from *Verbena littoralis* was reported to enhance NGF's ability to stimulate neurite outgrowths from PC12D cells and to be devoid of this activity in the absence of the neurotrophin.[11]

Herein we describe the synthesis of the verbenachalcone derivative **2** that has increased hydrophobicity and report its effects on neurite outgrowth, its ability to inhibit caspase induction caused by serum starvation and the results of differential gene expression profiling as an initial investigation of it's mechanism(s) of action.

The synthesis of **2** began by first converting ethyl 3-(4-hydroxyphenyl)propionate, **3**, to the aryl bromide **4** by treatment with bromine in acetic acid (Scheme 1).[12] Benzylation of the phenol was accomplished with benzyl bromide in refluxing ethanol in the presence of potassium carbonate to give **5**. Next, the aryl bromide **5** was coupled to ethyl 3-(4-hydroxy-3-methoxyphenyl)-propionate, **6**, which was readily prepared from commercially available 3-(4-hydroxy-3-methoxyphenyl)-propionic acid, to give **7** in 78% yield (based on recovered **5**) utilizing 5 mol% $(CuOTf)_2PhMe$. The esters were hydrolyzed with sodium hydroxide in a mixture of THF and methanol (2:1) to give **8**. This material was converted to its corresponding Weinreb amide **9** utilizing the coupling reagent HBTU in dichloromethane (DCM) in the presence of diisopropyl ethylamine (DIEA). Treatment of the amide with 4 equiv of 1-lithio-2,4-dimethoxybenzene (generated in situ from 1-bromo-2,4-dimethoxybenzene and *n*-BuLi in THF at −78 °C for 1.5 h) followed by the addition of hydrochloric acid (1 N) yielded **10**. Debenzylation of **10** in the presence of acetic anhydride, hydrogen (1 atm), 10% Pd/C, and sodium acetate in benzene gave **11**. Interestingly, hydrogenation in the absence of acetic anhydride resulted in concomitant removal of the benzyl group and reduction of the ketones to methylene groups. Finally, hydrolysis of acetate **11** gave **2** in quantitative yield.

The ability of **2** to enhance NGF's effects for stimulating neurite outgrowths in PC12 cells was accessed utilizing a morphological assay previous reported.[13,14] In order to increase the sensitivity of this assay, PC12 cells were exposed to **2** for 48 h, followed by a 72 h incubation in the absence of the test compound. A significant dose-dependent enhancement of NGF's effects was demonstrated



Scheme 1. Reagents and conditions: (i) Br_2, AcOH, 85%; (ii) BnBr, EtOH, K_2CO_3, Δ, 85%; (iii) **6**, 5 mol% $(CuOTf)_2PhMe$, Cs_2CO_3, py, 110 °C, 78%; (iv) 8 N NaOH, THF/MeOH (2:1), rt, 95%; (v) MeNHOMe·HCl, HBTU, DIEA, DCM, 94%; (vi) 1-Li-2,4-$(OMe)_2$Ph, THF, −78 °C then 1 N HCl, 87%; (vii) 1 atm H_2, 10% Pd/C, Ac_2O, NaOAc, benzene, 2 h, 81%; (viii) KOH, MeOH/H_2O (2:1), rt, 100%.

with **2** (Fig. 1). For example, in the presence of NGF (2 ng/mL) plus **2** (6 μM) a 5-fold increase in the number of neurites-bearing cells was observed compared with the NGF (2 ng/mL) control. The compound induced longer neurite processes in virtually all cells. Compound **2** was devoid of this activity in the absence of NGF. Increasing the concentration of **2** did not further increase the extent of neurite outgrowth, possibly due to

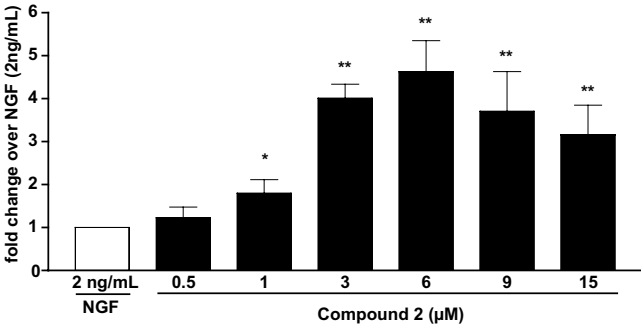


Figure 1. Enhancement of NGF's effects for stimulating neurite outgrowths in PC12 cells with NGF (2 ng/mL) and with NGF (2 ng/mL) in the presence of **2** (0.5–15 μM) for 48 h, followed by 72 h incubation in the absence of the compound. The neurite-bearing cell to total cell ratio was determined and expressed as a fold-change compared to NGF (2 ng/mL)±standard deviation ($N = 6$). The data were analyzed by Student unpaired *t*-test. * Indicates a significant difference ($p < 0.002$) versus 2 ng/mL NGF. ** Indicates a significant difference ($p < 0.0008$) versus 2 ng/mL NGF.

compound toxicity. In addition, these experiments illustrate that the hydroxyl groups on the terminal aromatic rings of verbenachacone are not necessary for the enhancement of NGF's effects on neurite outgrowth.

As previously mentioned, neurotrophin signaling is important for neuron survival. Since **2** has demonstrated significant enhancement of NGF's effects on neurite outgrowth, its ability to inhibit caspase induction caused by neurotrophin withdrawl has been evaluated in N2a cells. The end point of these experiments was caspase-3/7 induction after a 48 h treatment. This time point corresponded to the maximum induction of caspase-3/7. After 48 h, caspase-3/7 decreased due to cell death. Cells were grown in the presence of 10% fetal bovine serum, in the absence of serum, or in the absence of serum but in the presence of **2** (0.1–15 μM). As can be seen in Figure 2, compound **2** significantly and dose-dependently protected serum-deprived cells from caspase-3/7 induction with an $EC_{50} \sim 1$ μM.[15] PDTC, an inhibitor of NF-κB activation, was utilized as a positive control and had an $EC_{50} < 5$ μM in this model. In addition, 15 μM **2** resulted in 80% inhibition of caspase induction compared with the serum free control.

Our initial foray into the mechanism(s) of action for **2** utilized a chemical genetics approach. Differential gene expression profiles of PC12 cells grown in the absence of NGF, in the presence of NGF (2 and 50 ng/mL) and in the presence of NGF (2 ng/mL) plus **2** (15 μM) were compared after 48 h exposure.[16] The gene expression profiles were obtained utilizing Amersham CodeLink™ oligonucleotide arrays (~10,000 genes represented).

The differential gene expression profile of NGF (2 ng/mL) versus NGF (2 ng/mL) plus **2** (15 μM) showed that the expression levels of relatively few genes were altered upon treatment with **2**. Only 10 genes (~0.10%) were significantly up-regulated and seven genes (~0.07%) were down-regulated by a factor >2 ($p < 0.05$, $N = 2$) with

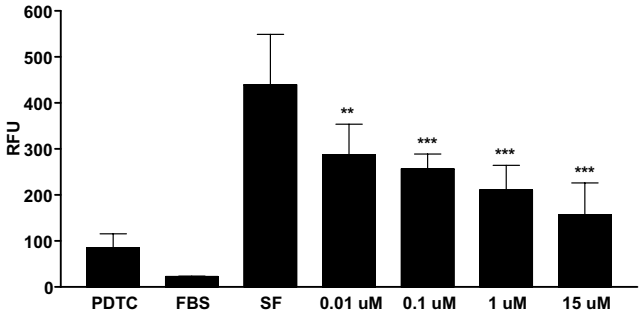


Figure 2. Evaluation of **2** for inhibition of caspase induction caused by serum starvation utilizing N2a cells. Following 48 h cell incubation with fetal bovine serum (FBS), without serum (SF) or, without serum but in the presence of various concentrations of **2** (0.1–15 μM) or PDTC (50 μM), caspase activity was determined (reported as RFU). Each sample represents the average of six replicates. The data were analyzed by Student unpaired *t*-test. *** Indicates a significant difference ($p < 0.0001$) versus SF. ** Indicates a significant difference ($p < 0.001$) versus SF.

Table 1. Genes significantly up-regulated and down-regulated by **2**

Gene	Fold + or −	*p*-value
Glycoprotein transmembrane NMB	+7.15	0.002
Transcriptional repressor CREM	+3.10	0.030
Salt-inducible protein kinase	+2.94	0.006
Kruppel-like factor 4	+2.91	0.014
Kinase-deficient TNF-beta	+2.81	0.003
B-cell translocation gene 2	+2.55	0.025
Cathepsin L	+2.38	0.012
Ceruloplasmin	+2.27	0.032
Sigma-1 receptor	+2.25	0.007
Gankyrin homologue	+2.06	0.044
Adenosine A_{2A} receptor	−2.00	0.019
Neuronatin	−2.07	0.043
Interferon regulatory factor 1	−2.07	0.045
Transmembrane superfamily member 4	−2.11	0.009
Protein kinase C-binding protein zeta 1	−2.25	0.017
Neuroserpin	−2.38	0.005
Protein tyrosine phosphatase receptor type R (Ptprr)	−2.67	0.013

treatment of 15 μM **2** plus 2 ng/mL NGF compared with 2 ng/mL of NGF only. The genes significantly altered by **2** are shown in Table 1. Several of the observed gene changes in response to NGF, such as sigma-1 receptor,[17] cathepsin L,[18] adenosine A_{2A} receptor,[19] neuronatin,[20] neuroserpin,[21] and protein tyrosine phosphatase receptor type R (Ptprr)[22] are in agreement with literature reports.

In order to validate the microarray results, quantitative RT-PCR was performed on a representative subset of genes (cathepsin L, sigma-1 receptor, and Ptprr). In all cases the microarray results and the quantitative RT-PCR results were in close agreement (Table 2). In the case of the Ptprr gene, exposure to NGF (2 or 50 ng/mL) did not result in significant changes in expression compared to untreated controls after 48 h. However, in the presence of NGF (2 ng/mL) and **2** (15 μM) a significant decrease in gene expression was observed as evident utilizing both methods. Cathepsin L and sigma-1 receptor gene expressions, however, were dose-dependent on NGF. Moreover, a further increase in gene expression for these two genes was observed in the presence of a combination of NGF (2 ng/mL) and **2** (15 μM). In fact, the response exceeded that observed with 50 ng/mL NGF.

The up-regulation of the sigma-1 receptor is an intriguing finding. The biochemical and physiological role of this receptor remains poorly understood. A host of cellular events have been linked to this receptor include modulation of NGF-induced neurite sprouting, neurotransmitter release, and neuronal firing. It is currently believed that agonism of the sigma-1 receptor results in the amplification of other concurrent signal transduction processes.[23] In the context of the observations reported herein for **2**, up-regulation of the sigma-1 receptor could serve to amplify NGF-signaling pathways leading to enhancement of neurite outgrowth.

The down-regulation of the Ptprr gene after a 48 h exposure to NGF is another interesting finding. This gene

Table 2. Validation of microarray results by quantitative RT-PCR

Gene	Gene expression (fold change relative to untreated, $N = 2$)					
	NGF (2 ng/mL) —		NGF (50 ng/mL) —		NGF (2 ng/mL) +**2** (15 μM)	
	RT-PCR	Micro-array	RT-PCR	Micro-array	RT-PCR	Micro-array
Ptprr	0.91 ± 0.01	0.90 ± 0.11	0.64 ± 0.20	1.04 ± 0.24	0.39 ± 0.09	0.37 ± 0.09
Cathepsin L	1.50 ± 0.90	1.77 ± 0.15	2.31 ± 0.10	2.75 ± 0.25	3.40 ± 0.25	3.88 ± 0.82
Sigma-1 receptor	1.21 ± 0.01	1.37 ± 0.11	1.77 ± 0.11	2.41 ± 0.11	2.93 ± 0.11	3.21 ± 0.24

encodes for two protein tyrosine phosphatases, PTP-SL, and PTP-BR7.[24] PTP-SL has been shown to down-regulate the extracellular signal-related protein kinase 5 (ERK5) pathway and to impede translocation of ERK5 to the nucleus.[25] However, endocytosed neurotrophin receptors (e.g., TrkA receptor) activated the ERK5 pathway leading to both enhanced nuclear translocation of ERK5 and neuronal survival.[26] Thus, the down-regulation of the Ptprr gene by **2** may result in reduced levels of PTP-SL and enhancement of the ERK5 pathway. Other molecules that either down-regulate Ptprr gene expression or directly inhibit PTP-SL phosphatase activity may be found to augment the ERK5 pathway resulting in enhanced neuronal survival.

In summary, the verbenachalcone derivative **2** enhances NGF's effects on neurite outgrowths and inhibits caspase induction caused by serum starvation. Differential gene expression profiles of NGF compared to NGF plus **2** revealed a small subset of genes whose expression was significantly altered. Further studies are needed to delineate the precise mechanism(s) responsible for these two observed cellular responses to **2**. In addition, further refinement of the structure–activity relationship of verbenachalcone could provide more pharmacologically tractable molecules enabling in vivo evaluation.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2004.12.001.

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15. (a) Some cell loss (10–20%) was observed in the presence of **2** (1 and 15 μM), but this did not account for the significant decrease (50–60%) in caspase induction (see supplementary data); (b) No direct inhibition of caspase-3/7 enzymatic activity was observed in the presence of **2** (15 μM).
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