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A synthetic analog of verbenachalcone potentiates NGF-induced neurite outgrowth and enhances cell survival in neuronal cell models

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Abstract

This study uses NeuroScreen-1 (NS-1) cells, a derivative of pheochromocytoma (PC12) cells, to examine neurite outgrowth induced by a novel synthetic verbenachalcone derivative, DSRB20-022 (C22). We treated NS-1 cells with varying concentrations of C22 in the presence of 2ng/mL nerve growth factor (NGF). A dose dependent effect of C22 was observed at concentrations of 2 μ M and above, resulting in significant enhancement of NGF-dependent neurite outgrowth in NS-1 cells. C22 did not exhibit neurotogenic activity in the absence of NGF, but promoted a concentration-dependent increase in neurite-bearing cells without inducing cytotoxicity. Cell viability assays showed that C22 and the parent compound verbenachalcone (VC) are neuroprotective and enhanced survival of NS-1, PC12, and the murine neuro-2A (N2a) cell lines under conditions of serum deprivation. The results show that augmentation of NGF-induced neurite outgrowth by C22 in NS-1 was dependent on MAP kinase. Furthermore, the neuroprotective function of C22 and VC was accompanied by suppression of caspase-3/7 activation. However, C22 and VC exerted their antagonistic effects on caspase 3/7 activation through potentially different mechanisms of action.

Keywords

Neuroscreen-1; Neurite elongation; Verbenachalcone; Synthetic analog; Potentiate; Neurite outgrowth

Nerve growth factor (NGF), a member of the neurotrophin family, plays an important role in differentiation, survival and maintenance of neurons in the central nervous system (CNS) and is known to stimulate neurite outgrowth in neuronal cells [1,2]. It is widely reported that natural products and synthetic compounds potentiate NGF's ability to stimulate neurite outgrowths from neuronal cell models by potentiating NGF dependent activity [3–7]. Previous studies identified verbenachalcone (VC) as the active component of *Verbena littoralis* responsible for

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the NGF enhancing effects [4,5]. Using the core structure of VC as a template, we synthesized a novel VC congener, DSRB20-022 (C22) in which we replaced the hydroxyl groups of VC with fluorines (Fig. 1). Since fluorine substitution is known to affect the behavior of biologically active molecules [8], we wanted to study the effect of replacing VC's hydrogen bond donating/accepting hydroxyl groups with fluorine groups that only accept hydrogen bond. In this study, we used NS-1 cells to examine the ability of C22 to potentiate NGF-induced neurite outgrowth and to protect against cell death caused by serum deprivation in neuronal cells.

VC and the fluorinated analog C22 were synthesized as reported by Xing et al. [9]. The compounds were structurally characterized using a Varian 300 MHz NMR and their mass determined with Agilent LC/MS. Reagents were purchased from the following vendors: NS-1 cells (Cellomics Inc., Pittsburgh, PA), PC12 and N2a (ATCC, Manassas, VA.), rabbit anti-caspase3 (8G10) (Cell Signaling Technology, Boston, MA.), anti-rabbit GAPDH (AnaSpec, San Jose, CA.), mouse NGF (2.5S) (Millipore, Billerica, MA.), active caspase-3 (Biovision Inc., Mountain View, CA.), caspase 3/7 substrate (AnaSpec, San Jose, CA.) and caspase-3 inhibitor (Chemicon, Anderson, CA). RPMI 1640, fetal bovine serum (FBS), and horse serum (HS) were purchased from Biowhittaker (Walkersville, MD). Stock solution of C22 was prepared in DMSO at a concentration of 10 mM.

NS-1 cells were maintained in growth media (RPMI-1640 supplemented with 10% FBS and 100 µg/mL Penicillin-streptomycin) at 37°C in 5% CO₂ and 90% humidity. PC-12 cells were maintained in DMEM containing 5% FBS and 10% HS. N2a cells were cultured in EMEM with 10% FBS. Cells were plated overnight (O/N) in growth media at a density of 5×10³ cells/well in 96 well collagen-coated plates. After O/N incubation, the growth media was replaced with treatment media (RPMI, or DMEM, 1% FBS, 2% HS) containing NGF (2ng/mL) alone or NGF with C22. The final DMSO concentration in the assay was 0.2%. After 48 h in a humidified CO₂ incubator the treatment media was removed and replaced with treatment media without NGF or C22. The cells were returned to the incubator for an additional 48h.

The morphology of treated cells was analyzed for the presence of neurite bearing cells using an Olympus IX51 inverted phase-contrast microscope (Olympus, PA). Cells with neurite processes equal to or greater than twice the diameter of the cell body were scored as neurite-bearing. The ratio of neurite-bearing cells to total cells was determined and expressed as a percentage by scoring at least 100 cells per field in triplicate per treatment group. The data was statistically analyzed by ANOVA and Tukey's multiple comparison tests.

Cell viability was assessed using MTT assay kit. Caspase-3/7-like activity was measured using Promega Apo-ONE homogeneous caspase-3/7 assay kit and fluorescence measured (λ_{exc} 485nm/ λ_{em} 535nm) utilizing a PheraStar Plus plate reader (BMG Labtech). Protein electrophoresis and western blots were performed using standard procedures. All data were presented as means ± S.E.M. of at least three experiments. Statistical analysis was performed by one-way ANOVA and Tukey's test.

The chemical synthesis of VC and its analogs has been described [9,10]. Yeh and coworkers reported that VC enhancement of NGF-induced neurite outgrowth was not dependent on the terminal aromatic hydroxyl groups [10]. Their conclusion was based on biological data from a VC analog in which the hydroxyl groups were substituted with acetate [9]. This compound, VC pentaacetate, appeared to have a slightly improved neurite inducing property as compared to VC. To further explore the relevance of the terminal aromatic ring positions in enhancing NGF induced neurite outgrowth, we synthesized C22, a VC analog with fluorine substitution on the terminal benzene rings. The presence of fluorine is reported to modulate the behavior of biologically active molecules [8]. When positioned strategically in active molecules, fluorine

can provide improvements in affinity, metabolic stability, bioavailability, and lipophilicity of a compound. Therefore, we examined the effect of fluorine substitution in promoting neurite elongation by treating NS-1 cells with NGF (2ng/mL) with or without C22 (2 μ M). After 48h incubation at 37°C in a humidified incubator, the treatment media was exchanged with fresh low serum media (without C22 or NGF) for an additional 48h. Morphological analysis of the cells showed that a small population of naive NS-1 cells exhibited spontaneous neurite-like processes of approximately the same length or less than the diameter of the cell body. In control cells exposed to suboptimal concentrations of NGF (2ng/mL), changes in neurite length were observed in approximately 10% to 15% of the cell population. However, when C22 (2 μ M) was added to the treatment media in the presence of NGF (2ng/mL) the compound induced longer neurite processes with pronounced increase in the number of neurite bearing cells (Fig. 2A, Right panel). In contrast, C22 alone had no effect on neurite outgrowth in the absence of NGF (Fig. 2A, Left panel).

To assess the dose effect of C22 on neurite elongation, NS-1 cells were incubated with C22 at concentrations ranging from 2–8 μ M in combination with NGF at 2ng/mL. Under these conditions, C22 exerted a concentration dependent increase in neurite bearing cells compared to control cells (Fig. 2B). The mean values expressed as percent of neurite bearing cells in samples treated with 2-, 4-, and 8 μ M of C22 and NGF (2ng/mL) were 28.8%, 34.1%, and 42.2%, respectively. The mean value of neurite bearing cells in NGF treated control cells was 14.9%. Co-treatment of cells with 2ng/mL NGF and C22 resulted in a 3-fold increase in neurite-bearing cells compared to cultures treated with NGF (2ng/mL) alone. These results indicate that C22 enhanced the neurite outgrowth inducing properties of NGF in NS-1 cells.

Extracellular signal-regulated protein kinase (ERK) activity plays a prominent role in NGF induced neuronal differentiation and neurite outgrowth [11–13]. To investigate whether ERK activity was involved in the mechanism by which C22 potentiates NGF-induced neurite outgrowth, cells were treated as previously described for C22 neurite outgrowth assay with and without the MEK inhibitor U0126. The potentiation of neurite outgrowth in NS-1 cells by NGF and the synergistic actions of C22 in the presence of NGF was significantly inhibited by 1 μ M of U0126 (Fig. 3). The blockade of NGF and C22-dependent potentiation of neurite elongation by U0126 supports literature findings of a key role for MEK involvement in neurite outgrowth and suggests that C22 possibly mediates its synergistic actions through the NGF receptor or NGF-like signaling pathway. Experiments to further elucidate the molecular mechanisms of C22 potentiation of NGF activity are currently ongoing.

A role for VC in the enhancement of NGF-induced neurite outgrowth has been previously reported [14]. Substitution of the hydroxyl moieties of VC with either acetoxy [9] or methoxy [10] groups did not impair the ability of the analogs to enhance NGF-induced neurite outgrowth. However, analogs in which the ketones of the α -carbonyl groups were disconnected failed to enhance NGF effects. These findings suggest that the hydroxyl groups of VC are not essential for neurite outgrowth activity. Our data with C2 supports this finding as fluorine substitution promoted neurite outgrowth. In general, substitution of fluorine improves parameters such as lipophilicity, hydrolytic stability, affinity, structural conformation, and bioavailability of a compound. Although C22 did not appear to substantially improve upon the biological efficacy of VC in the neurite outgrowth assay, it is conceivable that the threshold for neurite induction in NS-1 is higher than in PC12 cells. NS-1 is a clonal derivative of the rat pheochromocytoma PC12 cell, a model system for neurons. NS-1 cells exhibit substantial improvement over PC12, especially in sensitivity and response to NGF treatment, growth profile, and diminished tendency towards aggregation. A comparison of the effects of C22 and VC on NGF-assisted neurite outgrowth in NS1 and PC12 is in progress.

VC potentiates NGF induced neurite outgrowth but there are no reports describing its role in neuroprotection. Therefore, we examined the role of C22 and VC in cell survival using the serum deprivation model. NS-1, PC12, and N2a cells were treated with concentrations of C22 or VC ranging from 0.025 μM to 4 μM for 48h in serum deprived conditions. The data demonstrated that C22 and VC significantly enhanced cell survival in a dose-dependent manner in all three neuronal cell lines (Fig. 4). C22 significantly inhibited serum-induced cell death in NS-1 and PC12 cells at doses $\geq 0.05\mu\text{M}$ (Fig. 4A). However, its protective effect in N2a cells was much weaker, with significant protection achieved only at concentrations of 2 μM and above. Similar to C22, VC concentrations $\geq 0.05\mu\text{M}$ significantly reduced neuronal cell death in serum deprived conditions (Fig 4B). Interestingly, 0.05 μM of VC produced maximal protective effect in the three neuronal cell models compared to C22. Concentrations of VC $>0.05\mu\text{M}$ appeared to induce a slight cytotoxic related decrease in N2a cell viability but a cytotoxic effect was not observed in NS-1 or PC12 cells at concentrations of VC up to 4 μM (Fig 4B). The divergent cellular responses of N2a and NS-1/PC12 to C22 and VC appears to be compound specific, as both molecules produced different responses in the cell lines. This is the first report to show that VC affects neuronal cells in different ways.

Diverse cellular phenotypes can be induced in neurons by chemical molecules. A fungal alkaloid reported to produce neurite outgrowth in PC12 induced apoptosis in N2a by activating the same pathways but in different time frames [15]. In contrast, glendamyacin treatment caused neurite outgrowth in N2a cells by ERK activation but triggered apoptosis in PC12 by activating JNK and down regulating ERK [16]. These reports demonstrate the potential of a single chemical molecule to activate dissimilar pathways in neuronal cell lineages and lead to different phenotypic outcomes. Therefore, it is likely that VC exerted its differential effects by modulating diverse or analogous signaling circuits that led to differing degrees of sensitivity in N2a and PC12 cells. Future studies will determine the mechanisms of NGF potentiation by VC and C22 and reveal the pathways affected by these compounds.

Apoptosis is tightly linked to activation of the caspase cascade and serum starvation has been shown to induce apoptosis in a variety of cell lines. To examine whether C22 and VC exerted their protective effects by attenuating the caspase cascade, caspase 3/7 activity was analyzed in the cell models after compound treatment in serum deprived conditions. The activity of the enzyme was determined by measuring the ability of cell lysates to cleave a fluorometric caspase-3/7 substrate, Z-DEVD-Rhodamine 110. After 48h exposure to C22 and VC the level of caspase-3/7 activity significantly decreased at concentrations of C22 $>0.05\mu\text{M}$ in NS-1 and parental PC12 cells compared to serum free control (Fig. 5A and 5B). A 44% – 58% reduction in caspase 3/7 activity was observed between 0.1–4 μM of C22 in NS-1 and PC12 cells. In N2a cells, C22 was not nearly as potent an antagonist in the caspase pathway, requiring a 10-fold increase in concentration (0.5 μM) relative to its activity in PC12, in order to produce significant inhibition of caspase activation (Fig. 5A). In contrast, VC exerted a greater protective effect in N2a than either NS-1 or PC12 cells (Fig. 5B). These results imply that C22 and VC confer protection to murine N2a through mechanisms that are potentially dissimilar to those of NS-1 and PC12 cells.

Caspase-3 is synthesized as a precursor protein that undergoes cleavage activation in response to apoptotic stimuli. Western blot analyses to evaluate the level of caspase-3 in NS-1 cells following treatment with C22 revealed a 56% increase in caspase-3 enzyme in treated cells over serum free control cells (Fig 6). Treatment with H_2O_2 was used as a control for cell death. To examine whether C22 and VC function as direct caspase inhibitors, we incubated activated caspase-3 enzyme with a fluorogenic caspase substrate in the presence and absence of C22 and VC. There was no significant inhibition of caspase function by C22 up to 100 μM (Figure 7). In contrast, VC exhibited a dose-dependent reduction of caspase-3/7 substrate cleavage by activated caspase-3, with an EC_{50} of $<0.1\mu\text{M}$. A 68% reduction in caspase-3 activity was

observed at 0.1 μ M of VC, with caspase-3 activity essentially reaching the level obtained with a potent inhibitor of caspase-3 enzyme at 100 μ M VC. C22 differs from VC by the substitution of fluorine in the hydroxyl positions of the resorcinol groups of VC. Therefore, the functional antagonism of caspase 3/7 by VC implies a critical role for the hydroxyl groups in caspase inhibition. These data imply that the neuroprotective effects of C22 and VC are exerted through different mechanisms.

In our present study we have synthesized C22, a fluorinated analog of VC, and investigated its potential to promote NGF-induced neurite outgrowth in NS-1 cells. In addition, we explored the neuroprotective capacity of C22 and VC against serum deprived cell death in NS-1, PC12, and N2a. This study demonstrates that C22 plays a synergistic role with NGF in inducing neurite outgrowth in NS-1 cells. Co-treatment of cells with 2ng/mL NGF in combination with C22 resulted in approximately a 3-fold increase in neurite-bearing cells compared to NGF (2ng/mL) treatment alone. Our data also showed that C22 enhanced cell viability and inhibited apoptosis induced by serum deprivation. C22 markedly suppressed serum-induced apoptosis in NS-1, PC12, and N2a cells, presumably by attenuating the caspase-3 cascade. In addition, C22 markedly enhanced NGF-induced neurite outgrowth through a pathway that involves MEK signaling. Therefore, C22 could form the basis for the rational design of pharmacological agents which restore, protect, or maintain the architecture of cells in the central nervous system.

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References

1. Woodruff RH, Franklin RJ. Growth factors and remyelination in the CNS. *Histol Histopathol* 1997;12:459–466. [PubMed: 9151135]
2. Patrick CW Jr, Kukreti S, McIntire LV. Quantitative effects of peripheral monocytes and nerve growth factor on CNS neural morphometric outgrowth parameters in vitro. *Exp Neurol* 1996;138:277–285. [PubMed: 8620926]
3. Li YS, Matsunaga K, Kato R, Ohizumi Y. Verbenachalcone, a novel dimeric dihydrochalcone with potentiating activity on nerve growth factor-action from *Verbena littoralis*. *J Nat Prod* 2001;64:806–808. [PubMed: 11421751]
4. Li Y, Ishibashi M, Satake M, Chen X, Oshima Y, Ohizumi Y. Sterol and triterpenoid constituents of *Verbena littoralis* with NGF-potentiating activity. *J Nat Prod* 2003;65:696–698. [PubMed: 12762811]
5. Tsang CK, Ina A, Goto T, Kamei Y. Sargachromenol, a novel nerve growth factor-potentiating substance isolated from *Sargassum macrocarpum*, promotes neurite outgrowth and survival via distinct signaling pathways in PC12D cells. *Neuroscience* 2005;132:633–643. [PubMed: 15837125]
6. Middlemiss PJ, Glasky AJ, Rathbone MP, Werstuijk E, Hindley S, Gysbers J. AIT-082, a unique purine derivative, enhances nerve growth factor mediated neurite outgrowth from PC12 cells. *Neurosci Lett* 1995;199:131–134. [PubMed: 8584241]
7. Pradines A, Magazin M, Schiltz P, Le Fur G, Caput D, Ferrara P. Evidence for nerve growth factor-potentiating activities of the nonpeptidic compound SR 57746A in PC12 cells. *J Neurochem* 1995;64:1954–1964. [PubMed: 7722483]
8. Kirk KL. Selective fluorination in drug design and development: an overview of biochemical rationales. *Curr Top Med Chem* 2006;6:1447–1456. [PubMed: 16918460]
9. Xing X, Padmanaban D, Yeh L-A, Cuny G. Utilization of a copper-catalyzed diaryl ether synthesis for the preparation of verbenachalcone. *Tetrahedron* 2002;58:7903–7910.
10. Yeh LA, Padmanaban D, Ho P, Xing X, Rowley P, Morse LJ, Jensen RV, Cuny GD. Effects of a verbenachalcone derivative on neurite outgrowth, inhibition of caspase induction and gene expression. *Bioorg Med Chem Lett* 2005;15:1193–1196. [PubMed: 15686940]

11. Pang L, Sawada T, Decker SJ, Saltiel AR. Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by nerve growth factor. *J Biol Chem* 1995;270:13585–13588. [PubMed: 7775407]
12. Vaudry D, Stork PJ, Lazarovici P, Eiden LE. Signaling pathways for PC12 differentiation: making the right connections. *Science* 2002;31:1648–1649. [PubMed: 12040181]
13. Li P, Yamakuni T, Matsunaga K, Kondo S, Ohizumi Y. Nardosinone enhances nerve growth factor-induced neurite outgrowth in a mitogen-activated protein kinase- and protein kinase C-dependent manner in PC12D cells. *J Pharmacol Sci* 2003;93:122–125. [PubMed: 14501162]
14. Li YS, Matsunga K, Kato R, Ohizumi Y. Verbenachalcone, a novel dimeric hydrochalcone with potentiating activity on nerve growth factor-action from *Verbena littoralis*. *J Nat Prod* 2001;64:806–808. [PubMed: 11421751]
15. Küenzi P, Kiefer S, Koryakina A, Hamburger M. Promotion of cell death or neurite outgrowth in PC-12 and N2a cells by the fungal alkaloid militarinone A depends on basal expression of p53. *Apoptosis* 2008;13:364–376. [PubMed: 18293087]
16. Lopez-Maderuelo MD, Fernandez-Renart M, Moratilla C, Renart J. Opposite effects of the Hsp90 inhibitor Geldanamycin: induction of apoptosis in PC12, and differentiation in N2A cells. *FEBS Letters* 2001;490:23–27. [PubMed: 11172804]

Fig. 1. Chemical structures of Verbenachalcone and the fluorinated analog C22 (DSRB-20-022). The compounds were synthesized using a copper catalyzed Ullmann ether synthesis and characterized for structure and mass using Varian 300 MHz NMR and Agilent LC/MS.

Fig. 2.

Potentiation of NGF induced neurite outgrowth by C22. (A) Phase contrast photomicrographs of NS-1 cells treated with C22 in the presence or absence of 2ng/mL NGF. Cells treated with C22 without NGF (L) and Cells treated with C22 (2μM) in the presence of 2ng/mL NGF (R). (B) C22 concentration response on NGF induced neurite outgrowth in NS-1. The bar graph represents percentage of cells extending neurites after exposure to indicated concentrations of C22, in the presence and absence of NGF (2ng/mL). All experiments were performed in triplicate. The data was analyzed by ANOVA and Tukey's multiple comparison tests. * $p < .01$ and ** $p < .001$ represent significant difference from control value (2ng/mL NGF).

Fig. 3.

The effect of MEK inhibition on C22-dependent enhancement of neurite outgrowth in NS-1 cells. Cells were treated with or without the MEK inhibitor U0126, in the presence or absence of C22. Results represent means $\pm$ S.D. * indicates a significance difference ($p < 0.005$) versus NGF with C22 treatment.

Fig. 4.

C22 and VC protects against cell death induced by serum starvation in a dose dependent manner. Survival of NS-1 (black bars), PC12 (grey bars) and N2a (striped bars) cells was determined following 48h incubation in serum (FBS), without serum (SF), and in the presence of various concentrations of C22 (A) and Verbenachalcone (B) in serum free media. Cell protection is reflected in increased absorbance values. Each sample represents the average of at least three replicates. The data were analyzed by ANOVA and Tukey's multiple comparison tests. * indicates a significant difference ($p < .001$) and ** indicates a significant difference of ($p < 0.01$) versus SF.

Fig. 5. Evaluation of C22 (A) and verbenachalcone (B) on inhibition of caspase 3/7 activity induced by serum starvation in NS-1 (black bars), PC12 (grey bars), N2a (striped bars). Caspase activity in treated and control cells was determined and reported as relative fluorescent units (RFU). Each sample represents the average of at least three replicates. The data were analyzed by ANOVA and Tukey's multiple comparison tests. * indicates a significant difference ($p < .01$) and ** indicates a significant difference ($p < .001$) versus SF.

Fig. 6. Protective effects of C22 on the activation of caspase-3 in NS-1 cells. (A) Western blots of cell lysates were probed with caspase-3 antibody to determine the effects of C22 on caspase-3 protein expression in serum-free conditions. Lane 1, serum-free control. Lane 2, treatment with 2 μ M C22. Lane 3, treatment with 50mM H₂O₂. GAPDH was used as internal control. (B) Densitometry scans of western blot data. Shown are the mean densitometry values ($\pm$ SD) derived from three independent experiments compared to serum starved conditions ($p<0.05$).

Fig. 7.

Activity of human caspase-3 enzyme for the cleavage of fluorogenic caspase 3/7 substrate (Ac-DEVD-AMC) in the presence of various concentrations of C22 or verbenachalcone. Results represent means $\pm$ S.D. * indicates a significance difference ($p < 0.01$) and ** indicates a significance difference ($p < 0.05$) versus caspase 3/7 substrate plus caspase 3 enzyme.