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A novel synthetic activator of Nurr1 induces dopaminergic gene expression and protects against 6-hydroxydopamine neurotoxicity *in vitro*

Sean L. Hammond¹, Stephen Safe², and Ronald B. Tjalkens^{1,*}

¹Center for Environmental Medicine, Department of Environmental and Radiological Health Sciences, Colorado State University, Fort Collins, CO, USA

²Center for Environmental and Genetic Medicine, Institute of Biosciences and Technology, Texas A&M Health Science Center, Houston, TX, and Department of Veterinary Physiology and Pharmacology, Texas A&M University, College Station, TX, USA

Abstract

Degeneration of dopaminergic neurons in Parkinson's disease (PD) is associated with decreased expression of the orphan nuclear receptor Nurr1 (NR4A2), which is critical for both homeostasis and development of dopamine (DA) neurons. The synthetic, phytochemical-based compound, 1,1-bis (3'-indolyl)-1-(p-chlorophenyl) methane (C-DIM12) activates Nurr1 in cancer cells and prevents loss of dopaminergic neurons in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD in mice. In the present study, we examined the capacity of C-DIM12 to induce expression of Nurr1-regulated genes in two dopaminergic neuronal cell lines (N2A, N27) and to protect against 6-hydroxydopamine (6-OHDA) neurotoxicity. C-DIM12 induced expression of Nurr1-regulated genes that was abolished by Nurr1 knockdown. C-DIM12 increased expression of transfected human Nurr1, induced Nurr1 protein expression in primary dopaminergic neurons and enhanced neuronal survival from exposure to 6-OHDA. These data indicate that C-DIM12 stimulates neuroprotective expression Nurr1-regulated genes in DA neurons.

Keywords

Nurr1; NR4A receptors; dopamine; neuroprotection; transcriptional regulation

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder worldwide. There are no disease-modifying therapies for PD and patients become resistant to current symptomatic treatments as loss of dopaminergic neurons progresses [1]. There is

*Corresponding Author: Ronald B. Tjalkens, Ph.D., Professor of Toxicology and Neuroscience, Center for Environmental Medicine, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, 1680 Campus Delivery, Fort Collins, CO 80523-1680, Tel: 970 491 2825, Fax: 970 491 7569, Ron.Tjalkens@colostate.edu.

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considerable interest in the nuclear receptor, NR4A2 (Nurr1), as a promising target for control of PD progression. Nurr1 is a member of the steroid/thyroid hormone nuclear receptor transcription factor superfamily [2] and regulates DA metabolism by inducing expression of tyrosine hydroxylase (TH), vesicular monoamine transporter (VMAT2), and aromatic amino acid decarboxylase (AADC) [3,4]. Additionally, Nurr1 is important for development of DA neurons and can inhibit expression of neuroinflammatory genes in glial cells, suggesting a cell-specific context for the transcriptional regulatory effects of the receptor [5,6]. Ablation of Nurr1 in mature DA neurons recapitulates the progressive pathology of PD, with reduced striatal DA, impaired motor behaviors and dystrophic axon/dendrites [7]. The endogenous ligand for Nurr1 is unknown but selected synthetic lipophilic molecules can enhance the transcriptional activity of Nurr1 *in vitro* [8,9].

We previously demonstrated that one such molecule, 1,1-bis(3'-indolyl)-1-(*p*-chlorophenyl)methane (C-DIM12), has neuroprotective efficacy in the sub acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD [10,11]. C-DIM12 activates Nurr1 in pancreatic cancer and keratinocyte epidermal cells [9] and also enhances expression of Nurr1 in DA neurons *in vivo*, along with the Nurr1-regulated proteins, tyrosine hydroxylase (TH) and the dopamine transporter (DAT) [11]. In the present study, we investigated the capacity of C-DIM12 to regulate expression of Nurr1 and Nurr1-regulated genes in cultured dopaminergic neuronal cell lines and in primary dopaminergic neurons. We found C-DIM12 induced expression of Nurr1-regulated genes in multiple neuronal cell lines and increased Nurr1 expression in TH expressing primary neurons. RNAi studies show these effects were dependent upon expression of Nurr1. Treatment with C-DIM12 also preserved cell viability following exposure to the neurotoxin, 6-hydroxydopamine (6-OHDA). These findings suggest that C-DIM12 is a direct transcriptional activator of Nurr1 in DA neurons.

Materials and Methods

Cell culture and Reagents

Neuro-2a cells (N2A) and MN9D cells were cultured as previously described [12,4]. N27 cells were cultured in RPMI1640 medium (Life Technologies, Carlsbad, CA) supplemented with 10% FBS and 1X-PSN. All cell-lines were undifferentiated with the exception of MN9D cells, which were differentiated by the addition of 1mM sodium butyrate in media (Sigma) for 7 days prior to treatments. Primary dopaminergic neurons were isolated at E18 and cultured as previously described [12].

Quantitative PCR and transfections

qPCR was performed as previously described [13] and total RNA was quantified relative to hypoxanthine-guanine phosphoribosyltransferase (N2A) or β -actin (N2A). The sequences of qPCR primers are listed in Supplementary Table 1. Transfections with DsiRNA oligonucleotides or expression plasmids was performed as previously described [13].

Immunoblotting and immunofluorescence

Immunoblots were performed as described [11] using the following antibodies: rabbit anti-Nurr1 (1:500; Santa Cruz, Dallas, TX), anti-Rabbit HRP (1:5,000; Cell Signaling), mouse anti-Beta Actin (1:1,000; Sigma, St. Louis, MO) and anti-mouse HRP (1:5,000; Cell Signaling, Danvers, MA). For immunofluorescence staining, N2A cells and primary neurons fixed and stained as previously described [12]. Primary antibodies used: rabbit polyclonal anti-Nurr1 (1:250; Santa Cruz, Dallas, TX), chicken polyclonal anti-Tyrosine Hydroxylase (1:500; Abcam, Cambridge, MA), rabbit polyclonal anti-Flag (1:500; Sigma F-7425). Secondary antibodies used: Alexafluor647 (1:500; Invitrogen, Carlsbad, CA) and Alexafluor488 (1:500; Invitrogen). All imaging was performed as previously described [11]

Cell Viability Assays

Differentiated MN9D and undifferentiated N2A cells were grown on 96-well plates for 24 hrs before treatment with 6-OHDA and C-DIM12. After 24 hrs, cells were imaged using the PrestoBlue Cell viability reagent (Life Technologies, Carlsbad, CA) per the manufacturers protocol.

Statistical Analysis

All data are presented as mean $\pm$ SEM. Analyses of multiple experimental groups was performed using a one-way ANOVA with a Tukey *post hoc* test or Dunnett's multiple comparison test. With two group comparisons, an unpaired t-test Welch's correction and two-sided P-value with 95 % comparison interval was used. Statistical significance is represented by $p < 0.05$ (*), $p < 0.001$ (**), $p < 0.001$ ***), and $p < 0.0001$ (****). Statistical analyses were performed using Prism software (version 6.0; Graph Pad Software, San Diego, CA).

Results

Time-dependent expression of Nurr1, TH and VMAT2 was determined in N2A and N27 cells (Figure 1). Treatment with 10 μ M C-DIM12 increased expression of Nurr1 in N2A cells that was maximal at 8 hr (Fig 1A) and remained relatively constant up to 24 hrs, whereas mRNA expression of TH and VMAT2 in N2A cells was maximal at 4 and 8 hr, respectively (Fig 1A). In N27 cells, Nurr1 mRNA level was induced at 8 hr, TH was increased at 8 and 24 hrs and VMAT2 mRNA levels were significantly elevated at 4 hrs (Fig 1B). Dose-dependent expression of Nurr1, TH and VMAT2 was examined in N2A (Fig 1C) and N27 (Fig 1D) cells following treatment with 5 – 10 μ M C-DIM12. Treatment with 10 μ M C-DIM12 increased expression of Nurr1, TH and VMAT2 in N2A cells (Fig 1C), whereas mRNA levels for Nurr1 and VMAT2 were maximally induced by 5 μ M C-DIM12 in N27 cells and TH levels were increased by 10 μ M C-DIM12 in N27 cells (Fig 1D).

To determine whether C-DIM12-induced expression of TH and VMAT2 requires Nurr1, expression of Nurr1 was knocked down using RNA interference with Dicer substrate duplex RNA (DsiRNA) oligonucleotides. Consistent knockdown was observed by immunoblotting in siNurr1-transfected cells compared to siScr-transfected cells (Fig 1E). Cell morphology was unaffected by transfection with RNAi oligonucleotides (Fig 1F), as determined by

differential interface contrast (DIC) imaging. The structure of C-DIM12 is shown in Fig 1G. In N2A cells transfected with siScr control RNA oligonucleotides, C-DIM12 significantly induced expression of Nurr1 and VMAT2 (Fig 1H). Levels of mRNA for Nurr1, TH and VMAT2 were reduced relative to siScr controls in N2A cells transfected with siNurr1 oligonucleotides. Likewise, Nurr1 RNAi largely abolished the capacity of C-DIM12 to increase expression of Nurr1, TH and VMAT2 in N2A cells. A slight increase in C-DIM12-induced expression of VMAT2 was still observed following Nurr1 RNAi in N2A cells, although the overall level of expression VMAT2 was decreased relative to control cells transfected with scrambled RNAi oligonucleotides (Fig 1H).

To determine the capacity of C-DIM12 to enhance levels of exogenously expressed Nurr1, N2A cells were transfected with a plasmid containing FLAG-tagged full length human Nurr1 or Gal4 control vector and treated with C-DIM12 for 24 hrs (Figure 2 A-D). Expression of FLAG was evident 24 hrs after transfection and was localized to the nucleus of N2A cells (Fig 2A), identical to the pattern of Nurr1 expression (Fig 2B). Treatment with C-DIM12 (10 μ M) increased nuclear fluorescence of FLAG (Fig 2C, $p<0.05$) and Nurr1 (Fig 2D, $p<0.0001$) relative to DMSO (0.1%) treated controls. No expression of FLAG was detected in N2A cells transfected with Gal4 control vector.

The effect of C-DIM12 on expression of Nurr1 in primary dopaminergic neurons was examined in Figure 2, E-G. Neurons were cultured for 1 week until morphologically mature and then treated with C-DIM12 (10 μ M) or DMSO (0.1%) vehicle control for 24 hrs. Dopaminergic neurons were identified by expression of TH and Nurr1 expression was determined in TH-positive neurons by immunofluorescence (Figure 2E). C-DIM12 increased expression of both Nurr1 (Fig 2F, $p<0.001$) and TH (Fig 2G, $p<0.0001$) compared to control cells treated with DMSO (0.1%, vehicle control).

To examine the neuroprotective effects of C-DIM12 in both functionally mature neurons and undifferentiated neuronal cells, we compared the response of differentiated MN9D dopaminergic neurons cells to that of N2A cells following exposure to 6-hydroxydopamine (6-OHDA) (Figure 3). After five days of differentiation with sodium butyrate, MN9D neurons responded to a depolarizing K^+ stimulus with robust intracellular Ca^{2+} transients (Fig 3A,B).

Relative changes in intracellular Ca^{2+} were determined by live cell imaging using Fluo-4-AM and compared to the baseline image prior to stimulation with K^+ (F/F_0). We next exposed N2A and differentiated MN9D cells to increasing concentrations of the neurotoxin, 6-hydroxydopamine (6-OHDA), for 24 hrs and determined viability by measuring cellular reducing potential. Treatment with 0.1 – 100 μ M 6-OHDA caused dose-dependent cell death in MN9D and N2A cells, with LD50 values of 100 and 10 μ M, respectively (Fig 3C,E). Exposure to 6-OHDA for 24 hrs in the presence of C-DIM12 (10 μ M) significantly increased viability in both cell lines ($p<0.01$, MN9D; $p<0.05$, N2A. Fig 3D,F). The protective effect was greater in differentiated MN9D cells than in undifferentiated N2A cells.

Discussion

Nurr1 DNA binding sequences regulate the transcriptional activity of genes necessary for DA production and transport, such as TH, the synaptic dopamine transporter (DAT) and the vesicular monoamine transporter (VMAT2) [14,15]. Nurr1 knockout mice fail to develop midbrain dopaminergic neurons and die soon after birth, whereas conditional Nurr1 knockout mice exhibit deficits in the nigro-striatal dopamine system and are more susceptible to alpha-synuclein toxicity [5] [16]. Thus, Nurr1 is thought to regulate both the development and maintenance of DA neurons, as well as protecting DA neurons from neurotoxic insults. Interestingly, transcriptional responses to Nurr1 appear to depend both on cell type and on the constitutive level of Nurr1 expression [17]. For example, Nurr1 strongly induces TH expression in rodent neural precursor and differentiated cells, but the inductive effects on TH in human neural precursor cells are more modest [18] and can even be repressive in human neural stem cells [19]. Such varying transcriptional responses to Nurr1 may depend on the constitutive level of protein expression. In studies that generated a number of neuronal cell lines with graded expression of Nurr1, bioinformatics analysis indicated that many transcripts that were induced at low levels of Nurr1 protein expression were suppressed at high levels of Nurr1 and vice versa [17]. Thus, cell- and concentration-specific effects of Nurr1 likely influence the biological outcome in different cell types.

In the current study, we noticed differences in the pattern of expression of dopaminergic genes between the different neuronal cell lines evaluated, indicated by variance in the time and magnitude of mRNA expression across the time points and concentrations of C-DIM12 evaluated in N2A and N27 cells (Figure 1). C-DIM12 treatment increased expression of Nurr1 and VMAT2 after 8 hrs of treatment in N2A cells, whereas expression of TH maximal at 4hrs, prior to the peak of induction of Nurr1 mRNA (Figure 1A), suggesting that expression of TH and VMAT2 depend on the concentration of Nurr1 or other regulatory factors needed for TH gene transcription. In N27 cells, Nurr1 mRNA levels also were moderately induced by C-DIM12 at 8hrs, whereas VMAT2 mRNA was significantly increased at 4hrs (1B). These temporal patterns in gene induction in response to C-DIM12 could also reflect saturation of Nurr1 binding by the compound. Although differences in mRNA responses were evident between cell lines, C-DIM12-induced expression of the Nurr1-regulated genes TH and VMAT2 was conserved across mouse and rat cells, suggesting a common mechanism of regulation. C-DIM12 directly activates Nurr1, based on transcriptional reporter assays in bladder cancer cells and protein induction studies in epidermal keratinocytes [10] [20] and we recently reported that C-DIM12 induced Nurr1 nuclear translocation and increased protein expression in dopaminergic neurons in the MPTP/probenecid model of PD [11]. Although Nurr1 appears to lack a classic ligand binding pocket, a separate region of the LBD site is thought to possess ligand binding affinity at the co-activator binding site [21]. Computational modeling and binding studies indicated that other C-DIM compounds bind the co-activator binding site of NR4A1 (Nur77), which is highly homologous to Nurr1 [22]. Further studies are now being conducted to investigate whether C-DIM compounds have direct binding affinity for Nurr1 at a similar site in the LBD.

To test the hypothesis that C-DIM12 requires Nurr1 to induce expression of dopaminergic genes in neuronal cultures, the expression of Nurr1 was ablated using RNAi in N2A cells (Figure 1E-H). Loss of Nurr1 expression prevented the capacity of C-DIM12 to induce expression of the Nurr1-mediated mechanism (Figure 1E-H), indicating that Nurr1 is required for the transcriptional activation of these genes by C-DIM12. Even in the absence of C-DIM12, expression of VMAT2 and TH significantly decreased in Nurr1 siRNA cells compared to siRNA control cells, indicating direct regulation of these genes by Nurr1 in N2A cells (Figure 1H). Our current findings support the hypothesis that C-DIM12 directly regulates dopaminergic gene expression of TH and VMAT2 in N2A cells through a Nurr1-dependent mechanism.

Nurr1 is down-regulated in patients with PD and polymorphisms in Nurr1 increase the risk for a rare familial form of the late onset disease [23,24]. Therefore, preservation or increased expression of Nurr1 in neurons is a therapeutically desirable outcome in PD and related neurodegenerative disorders. In this regard, AAV-mediated gene delivery of Nurr1 and the forkhead transcription factor, Foxa2, preserved TH-positive neurons in a mouse model of PD [25]. When we expressed full length human Nurr1 in N2A cells (Fig 2A-D), C-DIM12 treatment increased expression of the Flag-tagged protein, as determined by immunofluorescence labeling for both Flag and Nurr1. Similarly, C-DIM12 increased expression of Nurr1 and TH in primary mouse dopaminergic neurons isolated from the ventral midbrain at E18 (Fig 2E-G). The capacity of C-DIM12 to increase protein levels of both exogenous human Nurr1 as well as to enhance expression of native Nurr1 protein in primary DA neurons suggests that C-DIM12 is a direct regulator of Nurr1 in neurons.

To examine the direct neuroprotective effect of C-DIM12 in functionally mature neurons compared to undifferentiated neuronal cells, we exposed differentiated MN9D cells and undifferentiated N2A cells to increasing concentrations of 6-OHDA in the presence and absence of C-DIM12. Differentiated MN9D neurons were less sensitive to 6-OHDA than undifferentiated N2A cells, with an LD₅₀ of 100 μ M 6-OHDA, compared to 10 μ M 6-OHDA in N2A cells (Figure 3C,E). This is consistent with other studies of 6-OHDA in MN9D cells and with similar studies comparing the response of differentiated and undifferentiated neuronal cells to mitochondrial toxicants [26]. Moreover, undifferentiated N2A cells may share phenotypic similarity with other immortalized cell lines, which may render them less sensitive to the protective effects of C-DIM12 [9]. Concurrent treatment of MN9D and N2A cells with C-DIM12 and 6-OHDA at the LD₅₀ for each cell type significantly increased cell viability, particularly in differentiated MN9D neurons (Figure 3D,F), indicating that C-DIM12 provides direct neuroprotective benefit. This may be due in part to the capacity of C-DIM12 to increase expression of VMAT2, which has been shown to protect against 6-OHDA neurotoxicity in mice through vesicular sequestration of 6-OHDA [27]. Although the underlying mechanisms require further investigation, C-DIM12 may better protect functionally mature MN9D neurons than undifferentiated N2A cells due to a greater responsiveness to Nurr1, similar to the perseveration of DA neurons we reported in the MPTP/probenecid model of PD, where levels of Nurr1 were strongly induced in TH-positive soma in the SNpc [13].

Conclusions

In conclusion, these data demonstrate that the substituted diindolylmethane compound, C-DIM12, activates Nurr1 dependent gene expression in neuronal cell lines, induces exogenously expressed human Nurr1 protein and increases expression of native Nurr1 in primary dopaminergic neurons. These pharmacological effects were mediated by Nurr1, as RNAi knockdown of the protein abolished C-DIM12-dependent gene expression. In addition, C-DIM12 has direct neuroprotective effects against 6-OHDA in functionally mature MN9D neurons, as well as in N2A cells, suggesting that activation of Nurr1 by C-DIM12 confers positive phenotypic and trophic support in dopaminergic neuronal cells. The signaling events that mediate the Nurr1-dependent inductive effects of C-DIM12 on transcription in neurons require further exploration but likely involve distinct protein complexes, similar to the highly specific effects of Nurr1 in glial cells [6] [13]. Pharmacological activators of Nurr1 such as C-DIM12 therefore may hold promise as beneficial therapeutic agents for the treatment of PD and related neurodegenerative disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. C-DIM12 induces expression of Nurr1-regulated genes in dopaminergic cell lines by Nurr1 dependent mechanism

Levels of mRNA for Nurr1, VMAT2 and TH were measured by qPCR in N2A (**A**) and N27 cells (**B**) following treatment with C-DIM12 (10 μM) for 4, 8 and 24 hrs. Dose-dependent changes in mRNA for Nurr1, VMAT2 and TH were measured by qPCR in N2A (**C**) and N27 cells (**D**) following treatment with vehicle control (DMSO) or C-DIM12 (5 and 10 μM) for 4, 8 and 24 hrs. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, $n = 3-4$ biological replicates across 3 independent experiments. (**E**) Protein samples collected from N2A cells transfected with scrambled control sequence (siScr) and Nurr1 siRNA (siNurr1) were examined for expression of Nurr1 and β-actin as a loading control. (**F**) Morphology of N2A cells was determined following transfection with siScr and siNurr1 using differential interference contrast (DIC) imaging. Nuclei were counterstained with DAPI and visualized by fluorescence microscopy. Scale bar = 10 μm. (**G**) Structure of C-DIM12. (**H**) mRNA levels of Nurr1, TH and VMAT2 were measured by qPCR in N2A cells transfected with siScr or siNurr1 in the presence or absence of C-DIM12 for 24 hrs. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, $n = 3-4$ biological replicates across 3 independent experiments.

Figure 2. C-DIM12 induces expression of transfected human Nurr1 in N2A cells and induces expression of TH in primary dopaminergic neurons

N2A cells transfected with FLAG-Nurr1 or vector control and treated with C-DIM12 or DMSO for 24 hrs were fixed and stained with anti-FLAG (yellow) (A) and anti-Nurr1 (green) (B) counterstained with DAPI (blue) and imaged using DIC and fluorescence microscopy. Quantification of FLAG (C) and Nurr1 (D) fluorescence intensity in transfected N2A cells (arbitrary units, AU). * $p < 0.05$, **** $p < 0.0001$. $n = 100-200$ cells from three biological replicates across 3 independent experiments. (E) Primary mouse dopaminergic neurons were treated for 24 hrs with 10 μM C-DIM12 or 0.1% DMSO (vehicle control) and immunostained for Nurr1 (cyan), Tyrosine Hydroxylase (TH, green) or MAP2 (red) and counterstained with DAPI (blue). Fluorescence images were acquired using a 40X air Planapochromat objective with a 1.6X optivar lens (64X total magnification, scale bar = 10 μm). Nurr1 (F) and TH (G) protein levels were quantified based on fluorescence intensity in TH⁺ cells. *** $p < 0.001$, **** $p < 0.0001$ $n = 100 - 200$ cells per group from three biological replicates across 3 independent experiments.

Figure 3. Treatment with C-DIM12 is neuroprotective against 6-hydroxydopamine in MN9D and N2A cells

(A) Pseudocolor images of Ca^{2+} influx pre-treatment (0 seconds) and upon administration of 25mM, 56mM and 75mM KCl (24 seconds) in live differentiated MN9D cells. 20X objective, Scale bars=10 μm . (B) Trace plots of dose dependent KCl induced Ca^{2+} influx over time, 8-16 cells from $n=3$ biological replicates. Arrow= KCl administration. $\frac{1}{2}$ log dosage of 6-OHDA administered to differentiated MN9D (C) and undifferentiated N2A cells (E) for LD₅₀ curve, $n=6$ biological replicates. Cell viability of differentiated MN9D (D) and N2A (F) cells treated +/- 6-OHDA (100 μM) and +/- C-DIM12 (10 μM). * $p<0.05$.