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Research Article

A Small Molecule Induces Oligodendrogenesis Leading to Myelin Sheath Repair

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Abstract

There is an unmet need for remyelinating therapies to treat demyelinating disorders. NDC-1308 is a small molecule that rapidly crosses the blood brain barrier to drive oligodendrogenesis, a process by which mature, myelinating oligodendrocytes repair damaged myelin sheaths in the central nervous system. NDC-1308 was previously shown to stimulate differentiation of mouse Oligodendrocyte Progenitor Cells (OPCs) in culture by causing a dramatic up-regulation of genes in the pathway for OPC differentiation and myelin synthesis. The present study demonstrates that damaged myelin sheaths found in mice demyelinated with cuprizone can be repaired by treatment with NDC-1308. A chronic NDC-1308 treatment resulted in a 44% and 18% increase in hippocampal and cortical myelination, respectively. In addition, an 18% increase in myelination was measured in the pons. Following NDC-1308 treatment, the OPC pool remained intact, demonstrating that it can serve as a renewable source for sustaining oligodendrogenesis and repair. While NDC-1308 is structurally related to estrogen and it functions through the estrogen receptors, it appears safe for either acute or chronic administration because it is not estrogenic, mutagenic or genotoxic. NDC-1308 is a potential first-in-class remyelinating therapy for repairing the myelin sheath in patients suffering from demyelinating disorders.

Keywords: Demyelinating Disorders; Multiple Sclerosis; Myelin; Oligodendrocyte; Remyelination; Repair; Therapeutic

Introduction

Although there has been substantial effort, a therapy that directly induces the remyelination and repair of damaged myelin sheaths in patients with demyelinating or neurodegenerative disorders remains elusive. This process requires penetration of a therapeutic agent into Central Nervous System (CNS) tissues to drive oligodendrogenesis, the process by which Oligodendrocyte Progenitor Cells (OPCs) differentiate into mature myelinating oligodendrocytes. Assuming remyelination of demyelinated (but intact) axons will restore some level of function, achieving this goal in patients could lead to improved quality of life.

The need for a remyelinating therapy is evident when considering a disease like Multiple Sclerosis (MS). MS is a chronic autoimmune disorder where the myelin sheath is under attack by the patient's own immune cells. While oligodendrogenesis and myelin repair could remain intact in some Relapsing-Remitting MS (RRMS) patients, this process will eventually fail in most patients leading to disability [1]. Disease Modifying Therapies (DMTs) for MS patients such as the interferons and glatiramer acetate, along with oral DMTs like fingolimod, teriflunomide and dimethyl fumarate and newer antibody therapies like daclizumab, natalizumab, alemtuzumab and ocrelizumab, modulate the immune system attack. However, most patients eventually progress with deficits in ambulation, fatigue and cognitive impairment [2-4]. Results from two recent clinical trials show the promise of stem cell treatments by either slowing MS disease [5] or halting CNS inflammatory activity and enabling recovery of some neurological functions in patients [6]. Aggressive treatments with newer medications have been effective for slowing disease progression in RRMS patients, but often have significant side effects [2]. A remyelinating therapy could also benefit patients with other demyelinating disorders, such as transverse myelitis [7], neuromyelitis optica [8] and osmotic

demyelination syndrome [9].

There are several reports of remyelinating therapies in development that stimulate OPC differentiation. One such therapy is an antibody that blocks Lingo-1, an inhibitor of oligodendrogenesis found on the extracellular membrane of OPCs [1]. While anti- Lingo-1 treatment of rat OPCs in culture stimulated oligodendrogenesis [10] and led to increased myelin production in the cuprizone [11] and EAE models [12], it failed to significantly impact remyelination in RRMS and SPMS patients during the Phase 2, SYNERGY clinical trial. In this case, anti-Lingo-1 (opicinumab) therapy missed the primary endpoint for measuring an improvement of physical function, cognitive function, and disability [13]. This approach is complicated by the presence of other members of the leucine-rich repeat family within CNS tissues, including Amigo3, which suggests antagonizing Lingo-1 alone may not be sufficient to induce remyelination [14].

A recent screen identified clemastine, a small molecule that stimulates significant OPC differentiation in vitro through the M1 muscarinic receptor [15,16]. Clemastine had positive results for a recent Phase 2 clinical trial that reduced the latency delay in the optic nerve of RRMS patients [17]. Other compounds that stimulate OPC differentiation and remyelination in animal models have been reported such as an ER-β ligand [18], indazole chloride [19], disogenin [20], miconazole and clobestasol [21]. Only clemastine has advanced into the clinic.

We previously reported that the small molecule, NDC-1308, has activity to induce oligodendrogenesis in vitro [22]. NDC-1308 is structurally related to estrogen and functions by activating Estrogen Receptors (ERs) differently than estradiol (E2) to induce genes necessary for OPC differentiation. The studies described here investigate whether a therapeutic treatment with NDC-1308 can induce and sustain remyelination in a mouse model of demyelination.

Materials and Methods

Chemical Synthesis of NDC-1308

The manufacturing method for NDC-1308 was previously described [22]. NDC-1308 made by this method was contaminated with up to 559 ppm of E2. An optimized manufacturing method was developed that incorporates a solvent extraction combined with potassium hydroxide prior to final crystallization which reduces E2 contamination to ≤ 1 ppm. NDC-1308 synthesized with 559 ppm E2 contamination was used in the following in vivo studies: the mouse cuprizone model to evaluate remyelination, measurement of the OPC population size, clinical chemistry analysis after chronic treatment, and evaluation of the CNS absorption/elimination of NDC-1308. Genotoxicity and mutagenicity studies were carried out with NDC-1308 having 340 ppm E2 contamination, while cell-based ER transcription assays were carried out with NDC-1308 having 12 ppm E2 contamination. NDC-1308 having ≤1 ppm E2 contamination was used for mouse uterotrophic assays that measure estrogenicity as well as the in vitro OPC differentiation assays and ion channel assays.

Formulation of NDC-1308

NDC-1308 formulated with N-methyl-2-pyrrolidone (NMP, ISP Technologies, and Waterford Township, MI) plus hydroxypropyl-γ-cyclodextrin (HP-γ-CD, CTD Holdings, Alachua, FL) was used to treat cuprizone demyelinated mice during a 2-week pilot study. An optimized formulation used SBE-β-cyclodextrin (SBE-β-CD, CTD Holdings) in place of the HP-γ-CD. The SBE-β-CD formulation was stably stored at 2°C-8°C and used to treat cuprizone demyelinated mice for the 6-week in vivo remyelination study. Briefly, NDC-1308 was dissolved in a 20% SBE-β-cyclodextrin solution made in water to 6 mg/mL, stirred and then brought to high pH with 10N NaOH. The solution was stirred at room temperature overnight then pH adjusted to 7.5 with H_3PO_4 and filtered through a 0.2 µm Pall acrodisc syringe filter with Supor membrane (Pall Laboratory, USA). The concentration of NDC-1308 was determined by HPLC (Agilent 1100, Agilent Technologies, USA) using a Gemini C18 column, 5µm, 105x4.6mm (Phenomex, Torrence, CA, USA).

Functional activation of ERs by NDC-1308

NDC-1308 dependent activation of ER-α and ER-β was assessed in MDA-MB-231 cells using a dual luciferase transcription assay as previously described [22]. E2 (Sigma-Aldrich, St. Louis, MO) was used as a positive control. The ER antagonist, ICI 182,780 (Tocris Bioscience, Minneapolis, MN), was dissolved in DMSO and added to the assay mixture at the same time as the addition of NDC-1308 or E2. The assay was carried out for 24 h at 37°C. The ratio of firefly luciferase compared to renilla luminescence was calculated for each treatment group. The functional expression of each compound was expressed as a percentage of the E2 ratio (100%) measured at 10 nM.

OPC Differentiation Assay

OPC differentiation assays were performed as previously described with mouse primary OPC cultures isolated from a PLP-EGFP transgenic mouse strain [22]. OPC cultures were treated with NDC-1308, vehicle or a positive control thyroid hormone, Triiodothryonine (T3), for 5 days. Differentiation to oligodendrocytes was measured using fluorescence by counting the number of Enhanced Green Fluorescent Protein (EGFP) expressing cells, and also by counting the number of Myelin Basic Protein (MBP) expressing cells by staining with antibodies to MBP.

Safety Assessment

Mutagenicity studies were carried out using the bacterial

reverse mutation assay in 6-well plates (Bio Reliance, Rockville, MD, USA) with S. typhimurium tester strains TA100, TA1535 and the E coli tester strain WP2uvra. The cells were exposed to vehicle and 8 concentrations of NDC-1308 in duplicate, both in the presence and absence of S9 lysate. Following overlay, the plates were incubated for 48-72 hours at 37°C. Micronucleus assays were employed to determine the level of NDC-1308 genotoxicity (Bio Reliance) using TK6 cells (ATCC, Manassas, VA). In this assay, TK6 cells were treated with either vehicle or NDC-1308 for 4 hours in the presence of S9 lysate, and with vehicle or NDC-1308 for 27 hours in the absence of S9 lysate. After incubation, cells were swollen with hypotonic solution, fixed and store overnight at 2-8°C and then stained with acridine orange to identify micronuclei.

Proarrhythmia was evaluated for NDC-1308 using the Fast Patch Channel Panel Assay (Charles River Laboratories, Wilmington, MA). Cloned L-type calcium channel (hCav 1.2), potassium channel (hKvLQT1/hminK) and sodium channel (hNav1.5) were expressed in CHO cells while potassium channels (hKir2.1, hERG) were expressed in HEK293 cells. The in vitro effects of NDC-1308 were evaluated with a dose range of 0.15-5.0 μM at room temperature for 3-6 minutes using the QPatch HT (Sophion Bioscience A/S, Denmark). Uterotrophic assays were modeled after the U.S. Environmental Protection Agency guidelines for measuring suspected estrogens (EPA 2011). NDC-1308 (0.06, 0.6, 6.0 and 60.0 mg/Kg, i.p., q.d.) and positive control E2 (0.01, 0.1, 1.0 mg/Kg, i.p., q.d.) were formulated in SBE-β-cyclodextrin and administered to ovariectomized C57BL/6 mice for 4 days, after which the uterus was harvested, blotted dry and weighed.

Pharmacokinetic Analysis

A single injection of NDC-1308 (75 mg/Kg, i.p.) formulated in SBE-β-CD was administered to female C57BL/6 mice with plasma and CNS samples (brain and spinal cord) collected at 10, 30, 60 minutes, 2 and 6 hours post-injection (Hooke Laboratories, Lawrence, MA). The level of NDC-1308 was detected in samples by mass spectrophotometry (Climax Laboratories, San Jose, CA, USA).

Assessment of Remyelination Using Mouse Models

For in vivo remyelination studies, 8-week-old male C57BL/6J mice were demyelinated by placing them on rodent chow containing 0.3% cuprizone along with rapamycin treatment (10 mg/Kg, i.p., q.d.) for 12 weeks [23]. Following the demyelination phase, the animals were returned to standard chow and treated with NDC-1308 (68 mg/Kg, i.p. q.d.), vehicle or E2 (implanted pellets, 80 mg/Kg/day release) for either 3- or 6-weeks. Animals treated with cuprizone/rapamycin were smaller than age-matched control animals, but did not display any behavioral abnormalities. Following the 6-week remyelination phase, plasma was isolated from each animal and subjected to a complete clinical chemistry panel analysis (Marshfield Clinic, Marshfield, WI). For analysis of the hippocampus and cortex, mouse brains were isolated, 30 μm coronal slices made consecutively and stained for myelin using anti-Proteolipid Protein (PLP) antibody (Cleveland Clinic Hybridoma Core, Cleveland, OH) as previously described [24]. Images were acquired with an Axioscan high resolution slide scanner equipped with a 20× objective (Zeiss). Specific regions of interest were analyzed using ImageJ software (NIH, Bethesda, MD).

For analysis of the pons, 30 μm serial coronal sections were taken at 200μm intervals and stained for myelin using anti-PLP primary antibody. Light microscopy was used to determine areas of significant demyelination. Images were imported into NIH Image J software for analysis. The parabrachial nucleus (Pb) and the periaqueductal grey matter (Pa) were two regions clearly demyelinated, while other regions of the pons appeared to have less robust PLP staining. Once Regions of Interest (ROI) had been outlined, color images were converted into 8- bit grayscale images and thresholds made based on intensity. The threshold masks and ROIs were measured to calculate percentage area occupied by PLP staining. For quality control, the threshold mask was overlaid on the original image to confirm accuracy. The results from 2 sections per mouse were averaged. To quantify the extent of the OPC population in the hippocampus and cortex, brain sections were stained with antibodies to Platelet-Derived Growth Factor Receptor Alpha (PDGFRα), a marker for OPCs. After image analysis, automated counting was performed with a custom algorithm (Renovo Neural, Cleveland, OH).

Statistical Analysis

For analysis of cell-based transcription assays, Tukey's multiple comparisons test was first conducted, followed by Dunnett's multiple comparisons test versus vehicle or E2 and then Sidak's multiple comparisons test of NDC-1308 treatment at each dose versus the same dose of NDC-1308 in combination with ICI 182,780. For the mouse oligodendrocyte differentiation studies, ANOVA was used for comparison between the different treatment groups. Comparisons were then made to vehicle using Student's t-test. For analysis of NDC-1308 absorption into CNS tissues, comparisons were made to the first collection time point (10 min) using Student's t- test. For the in vivo studies using the curpizone model, Sigma Plot/Sigma Stat was used to conduct statistical analysis of remyelination (%PLP). All data was tested for normal distribution by Kolmogorov-Smirnov or Shapiro-Wilk test. The normally distributed data were subjected to Student's t-test or one-way ANOVA followed by Dunnett's multiple comparison tests. The data that did not pass normality or equal variance test was compared using the Mann-Whitney Rank Sum test. ANOVA with Dunnett's multiple comparison was used for statistical analysis of OPC population data in the hippocampus and cortex. For the uterotrophic assay, Student's t-test was used to show statistically significant differences between a treatment group and vehicle

without consideration for multiple comparisons.

Results

ER Agonist Activity of NDC-1308

A cell-based, Estrogen Receptor (ER)-specific gene transcription assay was employed to ascertain whether NDC-1308 functionally activates the ERs (Figure 1). MDA-MB-231 cells that lack significant levels of endogenous ERs [25] were co-transfected with expression plasmids for either ER-α or ER-β and an estrogen-responsive luciferase reporter plasmid. Similar to E2, NDC-1308 treatment drives luciferase production from both ER-α (Figure 1A) and ER-β (Figure 1B) expressing cells in a dose-dependent manner. The agonist activity of NDC-1308 is blocked at both ER-α and ER-β when co-treating the cells with the ER antagonist ICI 182,780 (Figure 1 A, B).

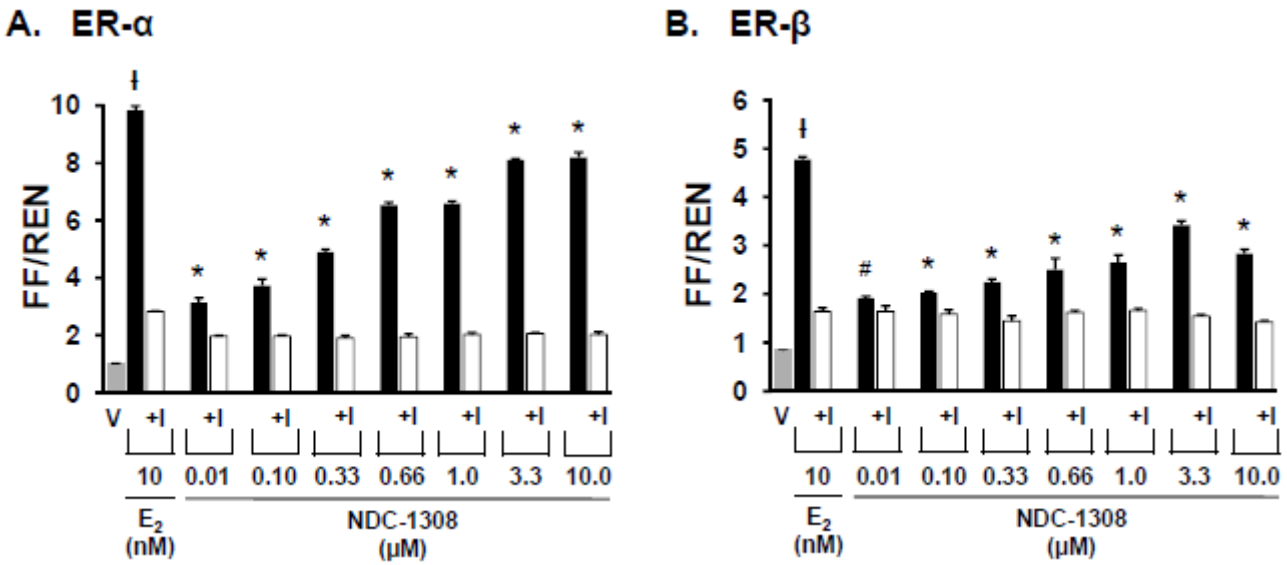

Figure 1: Functional Activation of ER-α and ER-ß by NDC-1308 Using a Cell-Based Transcription Assay. MDA-MB-231 cells co-transfected with an ER plasmid, ERE-firefly luciferase reporter plasmid and renilla plasmid (internal control) were treated with NDC-1308, E2 (positive control) or vehicle. The ratio of firefly luciferase (FF) to renilla (REN) is plotted on the Y- axis and represents the level of ER activation with either NDC-1308 or E2 (black histograms) compared to vehicle (V, grey histograms). The level of ER activation with either NDC-1308 or E2 in the presence of the ER antagonist, ICI 182,780 (I), is also plotted (white histograms). Functional activation of either ER-α (A) or ER-β (B) is shown. Mean values were plotted with S.D., n=3. $P < 0.0001$ of NDC-1308 vs. E2, vehicle or NDC-1308 + ICI 182,780 treated is denoted by '*', $P < 0.0001$ of E2 vs. vehicle is denoted by 'ł', $P < 0.05$ of NDC-1308 vs. NDC-1308 + ICI 182,780 treated is denoted by '#'.

Induction of Oligodendrogenesis by NDC-1308

NDC-1308 was previously shown to induce oligodendrogenesis using mouse OPCs isolated from PLP-EGFP transgenic mice [22]. Figure 2 shows the results of a dose response study to determine the EC_{50} for NDC-1308 in the OPC assay. Following 5 days in culture, the maximal response was measured with 10 μM NDC-1308. In this case, there was a 2.0 to 2.5-fold increase in cells expressing either EGFP (Figure 2A) or MBP (Figure 2B), respectively. This corresponds to approximately 30-35% of the culture differentiating into mature, myelinating oligodendrocytes. The majority of mature oligodendrocytes co-express both EGFP and MBP (Figure 2C). The EC_{50} for NDC-1308 in this assay was calculated to be 3.8 μM.

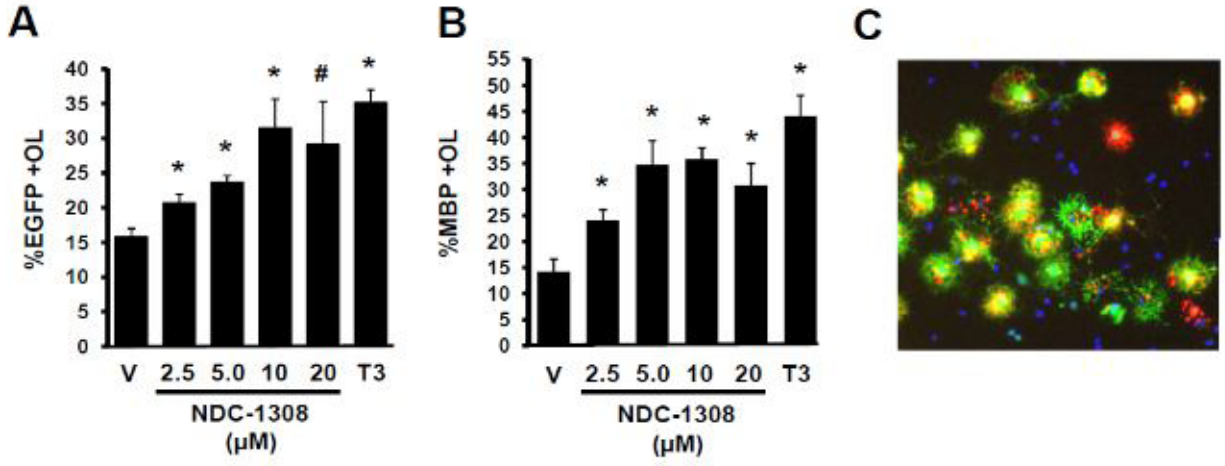

Figure 2: Differentiation of Mouse OPCs in Culture with NDC-1308. OPCs were isolated from PLP- EGFP transgenic mice and treated with NDC-1308, vehicle or a thyroid hormone, triiodothyronine (T3) for 5 days. (A) After treatment, cells were fixed and mature oligodendrocytes (EGFP expressing) detected by luminescence. (B) The same cells were counterstained with antibodies for Myelin Basic Protein (MBP), a major component of the myelin sheath. The percentage of EGFP positive oligodendroctyes (%EGFP+OL) and the percentage of MBP positive oligodendrocytes (%MBP+OL) were calculated with imaging software from 20 fields for each well. Mean values were plotted with S. D., n=6, P< 0. 0001vs. Vehicles denoted by'*', P< 0. 005 are denoted by '#'. (C) Representative image of cells expressing EGFP (green), MBP (red) or co-expressing both EGFP and MBP (yellow) are visualized.

CNS Tissue Absorption of NDC-1308

In order to test whether NDC-1308 effectively traverses the blood brain barrier and is absorbed into CNS tissues, it was formulated with SBE-β-cyclodextrin. Following a single, intraperitoneal injection of NDC-1308 (75 mg/Kg), peak absorption of NDC-1308 into mouse brain and spinal cord was detected by mass spectrophotometry within 10 minutes. Intraperitoneal injection was chosen for this study because it was the route of administration used for efficacy studies. NDC-1308 was essentially eliminated after 6 hours from both brain and spinal cord (Figure 3A). A similar pattern of absorption and elimination for NDC-1308 was found in the plasma (Figure 3B), although the concentration of NDC-1308 detected in plasma is about 6-fold higher than brain levels (Table 1).

Table 1: Absorption of NDC-1308 into the Mouse CNS Tissue and Plasma.

NDC-1308 Injected (mg/kg)	NDC-1308 Detected				NDC-1308 Brain: Plasma[a]
	Brain		Plasma		
	mg	μM	mg	μM	
75. 0	[b]0. 0006	[b,c]4. 2	[d]0. 011	27. 8	0. 151

[a]Brain to plasma ratio is calculated by dividing the concentration of NDC-1308 estimated in brain by the concentration estimated in plasma. Plasma concentration of NDC-1308 (ng/mL) is measured directly by mass spectrophotometry and converted to μM using 386. 57 g/mole as the MW for NDC-1308. Brain and plasma samples were collected 10 minutes after injection.
[b]To calculate the amount of NDC-1308 in brain, the average brain weight used for a 20g mouse is 0. 4 g.
[c]To calculate the concentration of NDC-1308 in brain, the average brain volume used is 0. 4 mL.
[d]To calculate the amount of NDC-1308 in plasma, the average volume of plasma used is 1 mL.

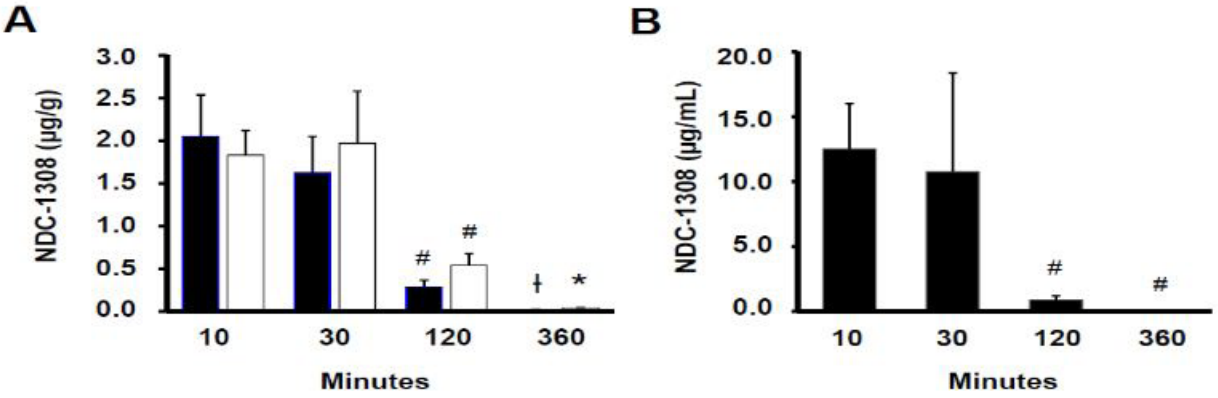

Figure 3: Absorption and Elimination of NDC-1308 into CNS Tissues and Plasma. C57BL/6 mice were treated with a single injection of NDC-1308 (75 mg/Kg, i. p) formulated in SBE-β- cyclodextrin. Brain, spinal cord and plasma were collected over time and the level of NDC-1308 measured by mass spectrophotometry. (A) The elimination pattern of NDC-1308 (µg/g) from brain (black histograms) and spinal cord (white histograms) is plotted over a period of 6 hours (x-axis). (B) A similar pattern is measured for NDC-1308 (µg/mL) in plasma. Mean values were plotted with S. D. n=5, P< 0. 0005 versus the 10-minute time point is denoted by '*', P< 0. 001 versus the 10-minute time point is denoted by 'ł', P< 0. 005 versus the10 minute time point is denoted by '#'.

The concentration of NDC-1308 measured in mouse brain tissues 10 minutes after injection was estimated to be 4.2 µM. This is similar to the EC_{50} measured in the mouse OPC assay (Figure 2).

Remyelinating Activity of NDC-1308

The ability of NDC-1308 to induce remyelination was tested using the cuprizone mouse model of demyelination [23]. After the demyelination phase, and when cuprizone and rapamycin are removed from the diet, the mouse can spontaneously remyelinate damaged myelin sheaths. Cuprizone is toxic to mature oligodendrocytes, but not the OPCs or newly formed oligodendrocytes [23]. Therefore, a successful remyelinating agent induces myelin synthesis and repair above and beyond the spontaneous level of remyelination. In a 2-week pilot study using cuprizone demyelinated mice, administration of NDC-1308 (50 mg/Kg, i.p., q.d.) formulated in NMP/HP-γ-CD was found to induce hippocampal myelination (Figure 4A) and cause OPCs to differentiate into both pre-myelinating and mature, myelinating oligodendrocytes (Figure 4B). Comparing the level of proteolipid protein (PLP, a major component of myelin) staining in the NDC-1308 treatment group to the spontaneous level of remyelination (vehicle), it was found that NDC-1308 enhanced pre-myelinating oligodendrocyte formation by 34% (Figure 4C) and enhanced mature myelinating oligodendrocyte formation by 20% (Figure 4D).

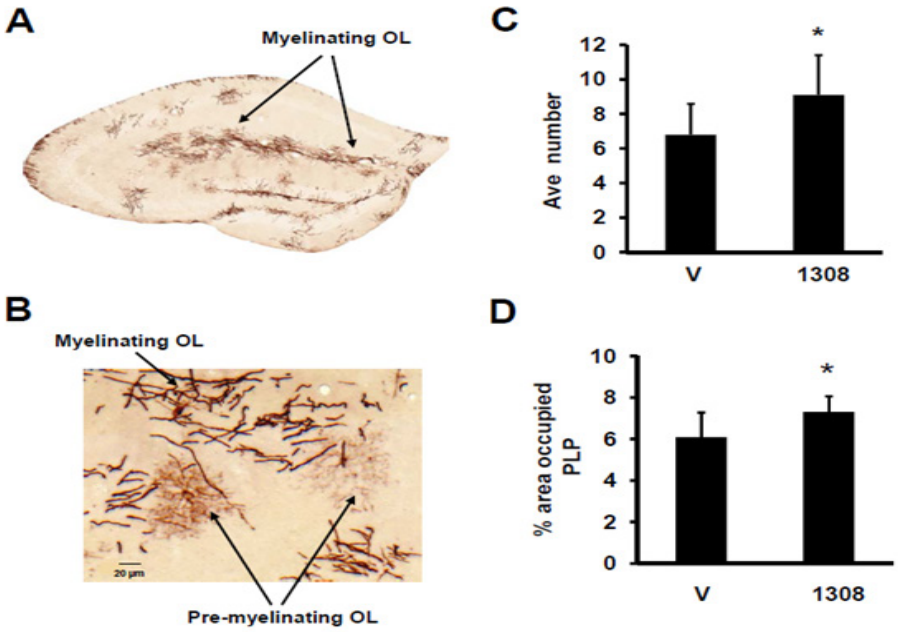

Figure 4: Preliminary Assessment of NDC-1308 Remyelinating Activity in the Hippocampus of Cuprizone Demyelinated Mice. Mice were demyelinated with a cuprizone/rapamycin treatment for 12 weeks, switched to normal diet and then treated for 2 weeks with either vehicle (V) or NDC-1308 (50 mg/Kg, i. p.q. d) formulated in NMP/HP-γ-CD. To assess remyelination in the hippocampus, brain tissue was isolated, sectioned and stained with antibodies against Proteolipid Protein (PLP). (A) Dark brown staining of oligodendrocytes (OL, arrows) shows the pattern of myelin formation in a representative hippocampal section. (B) A magnified view of a representative hippocampal section shows both light-stained pre- myelinating oligodendrocytes and dark-stained mature, myelinating oligodendrocytes forming around axons. (C) The number of pre-myelinating oligodendrocytes was compared in hippocampal sections from vehicle (V) or NDC-1308 (1308) treated mice. (D) The number of mature, myelinating oligodendrocytes was compared in hippocampal sections from Vehicle (V) or NDC-1308 (1308) treated mice, and expressed as average percent area occupied by PLP staining (y-axis). Mean values were plotted with S. D. n= 12 for vehicle, n=11 for NDC-1308, $P < 0.001$ vs. vehicle is denoted by '*'.

Cuprizone demyelinated mice were next treated with NDC-1308 (68 mg/Kg, i.p.) formulated in SBE-β-CD daily for either 3- or 6-weeks. After 3-weeks of treatment, there was no effect in the cortex, but there was a significant 16% increase in remyelination compared to vehicle in the hippocampus (Figure 5A). After 6-weeks of treatment, NDC-1308 induced a 44% increase in remyelination in the hippocampus and an 18% increase in remyelination of the cortex compared to vehicle treated mice (Figure 5B).

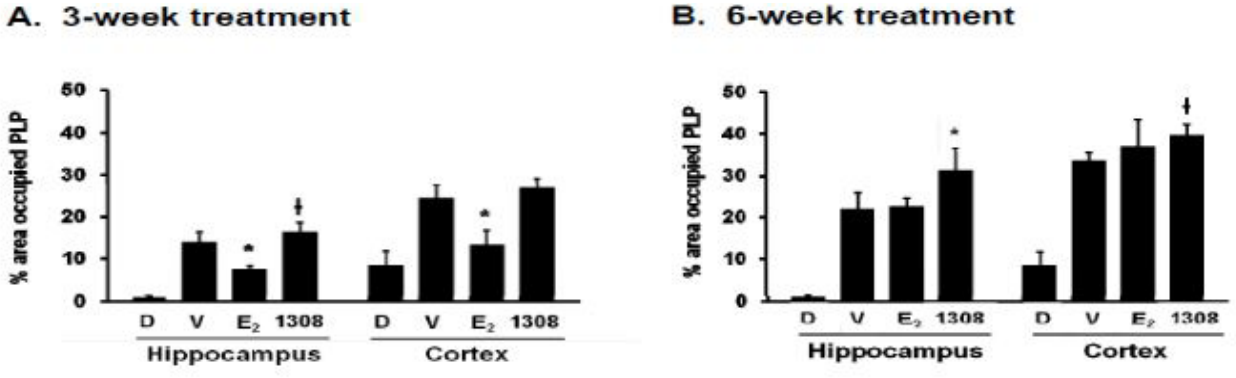

Figure 5: Assessment of NDC-1308 Remyelinating Activity in the Hippocampus and Cortex of Cuprizone Demyelinated Mice After 3- and 6-weeks of Treatment. Mice were demyelinated (D) with a cuprizone/rapamycin treatment for 12 weeks. NDC-1308 (68 mg/Kg, i. p. , q. d.), estradiol (E2) or vehicle (V) was then administered for 3-weeks (A) or 6-weeks (B). NDC-1308 was formulated in SBE-β-CD while E2 was administered as a subcutaneous pellet. Myelin synthesis in hippocampal and cortical tissues was detected by staining with antibodies to PLP and quantified as average percent area occupied by PLP staining

(y-axis). Mean values were plotted with S. D, n=9-13, except for E2 where n=7-8. P<0. 0001 vs. vehicle is denoted by '*', P< 0. 01 vs. vehicle is denoted by 'ł'.

The discrepancy in the level of NDC-1308 specific remyelination measured for hippocampus compared to cortex is explained by a faster rate of spontaneous remyelination (vehicle) in the cortex. A comparator group receiving E2 did not induce remyelination in either the hippocampus or cortex, which is consistent with the inability of E2 to induce OPC differentiation in vitro [22]. An increase in remyelination was not detected in the corpus callosum at either 3- or 6-weeks of treatment (data not shown).

To address whether NDC-1308 can enhance remyelination in white matter regions of the brain, such as the pons, a subset of brain tissue was randomly selected and analyzed from cuprizone demyelinated mice following treatment with NDC-1308 (68 mg/Kg, i.p., q.d.). As shown in Figure 6A, the parabrachial (pb) and periaqueductal (pa) were two demyelinated regions identified in the pons. The level of remyelination was determined in NDC-1308 treated animals and compared to Age-Matched (AM), Demyelinated (D) and spontaneously remyelinated (vehicle,V). Figure 6B shows magnified views from representative sections of the parabrachial region quantified for each treatment group. After 3-weeks of NDC-1308 treatment, an 18% increase in myelination was measured in the parabrachial region of the pons, but not the periaqueductal region (Figure 6C).

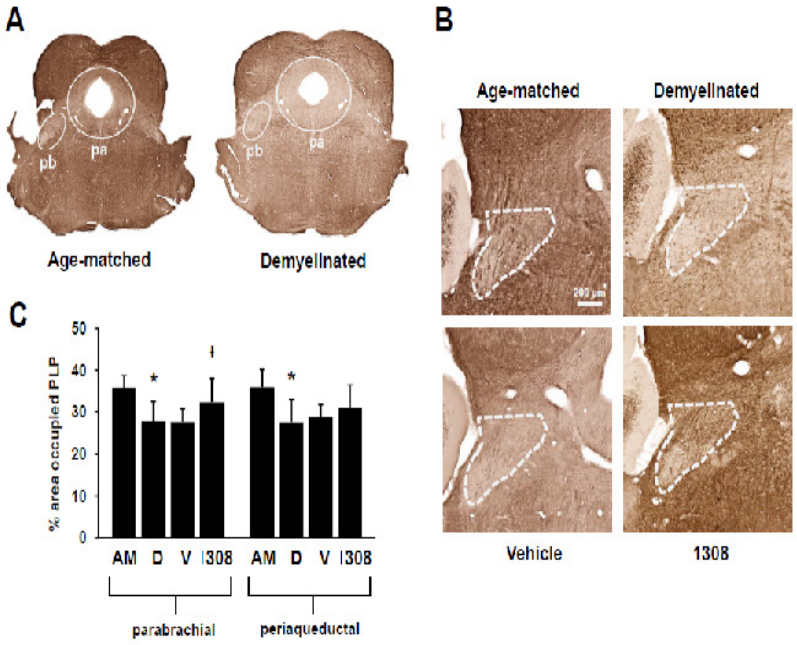

Figure 6: Assessment of NDC-1308 Remyelinating Activity in the Pons of Cuprizone Demyelinated Mice. Mice were demyelinated then administered NDC-1308 (68 mg/Kg, i. p. , q. d.) or vehicle (V) for 3 weeks. (A) Representative images of PLP-stained whole brain (pa, periaqueductal region; pb, parabrachial). (B) Magnified view of PLP-stained parabrachial sections from age-matched (AM, n=7), demyelinated (D, n=10), vehicle (V, n=15) and NDC-1308 treated (1308, n=11) are shown. (C) Myelin synthesis in the parabrachial or periaqueductal regions was quantified as the average percent area occupied by PLP staining (y-axis). Mean values were plotted with S. D., P< 0. 0001 vs. age-matched is denoted by '*', P<0. 05 versus vehicle is denoted by 'ł'.

There was no difference measured in either region after 6- weeks of NDC-1308 treatment (data not shown).

Safety Assessment of NDC-1308

To assess whether a chronic treatment with NDC-1308 depletes the endogenous OPC pool, hippocampal and cortical tissue samples were randomly selected from mice treated with NDC- 1308 for 6-weeks and counterstained with antibody to PDGFRα, a marker for detecting OPCs [26].

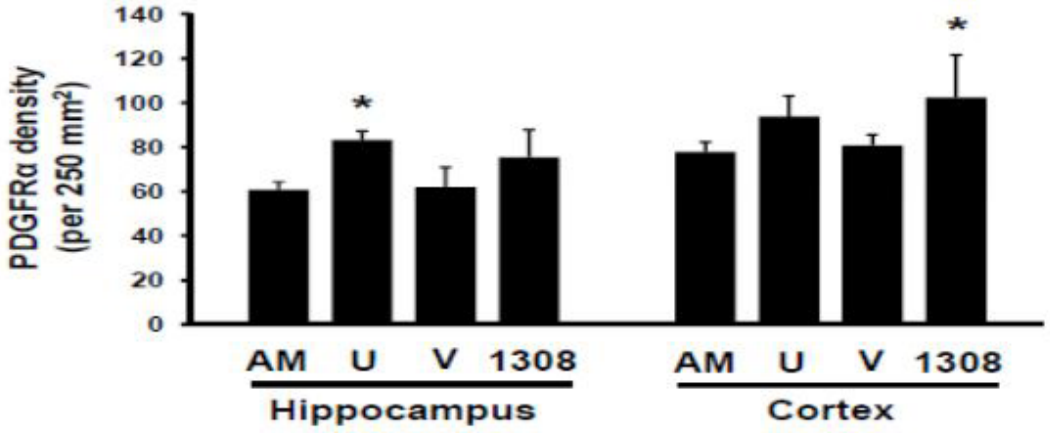

Figure 7: Intactness of the OPC Population in the Hippocampus and Cortex Following Chronic NDC-1308 Treatment. . Cuprizone demyelinated mice were treated with NDC-1308 (68 mg/Kg, i. p.q. d.) or Vehicle (V) for 6 weeks. Age-matched, normal mice (AM) and demyelinated but untreated mice (U) were used as comparators. (A) Hippocampal and (B) cortical tissues were isolated and stained with antibodies to platelet derived growth factor receptor α antibodies PDGFRα), a marker for OPCs. The level of OPC is quantified as PDGFRα density per 250 mm2 (y-axis). Note that these were the same tissue used to determine the level of remyelination (Figure 5). Mean values were plotted with S. D, n=8 except for E2 where n=6. P < 0. 05 versus. Age-matched is denoted by '*'

Figure 7 shows that the NDC-1308 treatment group had a similar number of OPCs compared to the Age-Matched (AM), demyelinated and Untreated (U) and Vehicle (V) treatment groups. This means that the OPC population remained intact in both the hippocampus and cortex following either the demyelination phase or a chronic exposure of mice to NDC-1308. Because NDC-1308 is structurally related to estrogen, an uterotrophic assay was employed to determine whether NDC-1308 is estrogenic. This in vivo assay was modeled after the Environmental Protection Agency's (EPA) test used to detect estrogens in suspected compounds [27]. It relies on the highly proliferative nature of uterine cells following ovariectomy and subsequent exposure to an estrogen. Ovariectomized mice were treated daily for 4 days with intraperitoneal administration of NDC-1308 and compared to E2 treatment as a positive control. Figure 8 shows that NDC-1308 has no significant estrogenic activity up to 60 mg/Kg.

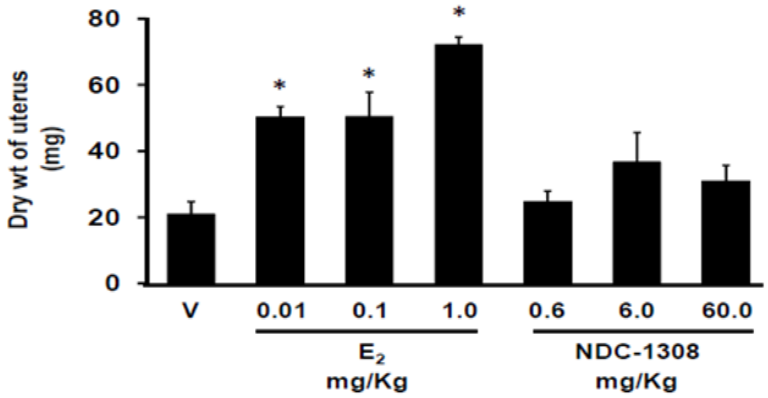

Figure 8: Estrogenicity Assessment Using the Mouse Uterotrophic Assay. Ovariectomized C57BL/6 mice were administered either E2 or NDC-1308 by i. p. injection for 4 days. Both E2 and NDC-1308 were formulated with the SBE-β-CD formulation. Mice were euthanized 6 hours after the last dose, after which each uterus was removed, punctured and dried by blotting. Mean values were plotted with S. D, n=6; P< 0001 vs. vehicle is denoted by '*'.

Estrogens are suspected mutagens and genotoxins [28]. NDC-1308 was tested in a bacterial reverse mutation assay (Ames test) and a micronucleus assay and was not found to be mutagenic (Table 2) or genotoxic (Table 3) compared to standard controls.

Table 2: NDC-1308 Mutagenicity Assessed by Bacterial Reverse Mutation Assay.

Tester Strain	Treatment	µg/ well	Without S9 lysate		With S9 lysate	
			Average Revertant±SD	Fold Induction[a]	Average Revertant±SD	Fold Induction[a]
TA100[b]	DMSO	-	19±1		22±1	
	NDC-1308	0. 3	20±5	1. 1	26±8	1. 2
		1	12±0	0. 6	28±4	1. 3
		3	17±3	0. 9	15±1	0. 7
		10	15±1	0. 8	19±4	0. 9
		30	23±4	1. 2	19±2	0. 9
		100	17±2	0. 9	17±4	0. 8
		300	20±1	1. 1	11±0	0. 5
		1000	21±5	1. 1	18±4	0. 8
	Sodium Azide	0. 2	113±4	6. 1		
	2-aminoanthracene	0. 4	-		184±17	8. 6
TA1535[c]	DMSO		1±1		4±1	
	NDC-1308	0. 3	5±4		6±5	
		1	5±1		2±1	
		3	3±0		4±1	
		10	2±0		5±1	
		30	3±2		3±1	
		100	1±1		2±1	
		300	1±0		6±2	
		1000	3±1		8±4	2
	Sodium Azide	0. 2	170±1	170		
	2-aminoanthracene	0. 4			41±6	10. 1
WP2uvrA[d]	DMSO		2±1		5±1	
	NDC-1308	0. 3	4±1		7±1	1. 4
		1	3±1		8±1	1. 5
		3	6±6	3	6±2	
		10	5±0		9±2	1. 7
		30	3±0		3±3	
		100	2±1		5±1	
		300	4±1		4±4	
		1000	6±1	3	8±4	1. 6
	Methyl methane sulfonate	200	35±4	17. 3		
	2-aminoanthracene	3.0			96±2	19. 1

[a]Fold induction is the average number of test article revertants/average number of vehicle control revertants, only presented if accompanied with at least 6 revertants.
[b]TA100, S. typhimurium tester strain.
[c]TA1535, S. typhimurium tester strain.
[d]WP2uvr, E coli tester strain.

Table 3: In vitro Micronucleus Assay Using Human TK6 Cells.

Time (Hours)	Treatment	Concentration(μg/mL)	Population Doubling	Cytotoxicity(%)	%MN[a]
4	DMSO		1. 6	0	0. 5
	NDC-1308	10. 9	1. 2	22	0. 6
	NDC-1308	31. 9	1. 1	34	0. 5
	NDC-1308	65	0. 7	55	0. 6
	Cyclophosphamide	3	0. 8	51	1. 8*
27	DMSO		1. 9	0	0. 5
	NDC-1308	5. 4	1. 7	8	0. 4
	NDC-1308	10. 9	1. 5	19	0. 4
	NDC-1308	22. 3	0. 8	55	0. 6
	Vinblastin	0. 012	1. 2	36	1. 4

*P≤0.05 vs DMSO
[a]Micronucleus frequency in mononucleate cells

To determine if NDC-1308 is proarrhythmic, the level of activity of NDC-1308 was measured in an in vitro Fast Patch Channel Panel Assay (Table 4). While NDC-1308 inhibits hERG2 at >10 μM, this is countered by inhibition of Cav 1. 2 at 8. 8 μM. In addition, NDC-1308 does not selectively block Ikr current and thus has a low risk for causing arrhythmia in vivo.

Table 4: NDC-1308 Effect on Ion Channel Currents in Human Cell Lines.

Ion Channel	IC_{50} (μM)
hERG[a]	>10
Cav 1. 2[b]	8. 829
Nav 1. 5 (tonic inhibition)[c]	>5
Nav 1. 5 (clonic inhibition)[c]	>5
KvLQT1/mink[d]	>5
hKir2. 1[e]	>5

[a]hERG, human ether-à-go-go-related potassium ion channel.
[b]Cav 1. 2, voltage-dependent, L-type alpha 1C subunit calcium ion channel.
[c]Nav 1. 5, integral membrane protein and tetrodotoxin reistant voltage gated sodium ion channel subunit.
[d]KvLQT1/mink, potassium ion channel.
[e]hKir2. 1, inward-rectifier potassium ion channel.

Finally, to assess the overall health of organ systems for demyelinated mice treated chronically with NDC-1308 (68 mg/Kg, i. p. q. d), plasma was collected at termination and analyzed using a complete clinical chemistry panel (Table 5).

Table 5: Assessment of Clinical Chemistries in Mice Following NDC-1308 Treatment.

Treatment Group	AST	ALT	ALKP	TBIL	BUN	CREAT	GLU	ALB	NA	K	CL	PHOS	CA
	U/L	U/L	U/L	mg/dL	mg/dL	mg/dL	mg/dL	g/dL	mmol/L	mmol/L	mmol/L	mg/dL	mg/dL
Age matched[a]	84. 3	28. 3	43. 3	0. 2	28. 7	0. 2	369	2. 6	146	7. 5	111	8. 6	8. 9
	±26. 5	±2. 9	±3. 3	±0. 0	±1. 7	±0. 0	±29. 2	±0. 08	±0. 0	±0. 6	±0. 0	±0. 2	±0. 1
Demyelinated[b]	59. 2	6. 1	52	0. 2	28	0. 2	328. 1	2. 6	147. 7	7. 2	115. 2	7. 2	8. 4
	±3. 9	±0. 3	±1. 9	±0. 0	±1. 5	±0. 0	±16. 9	±0. 0	±0. 6	±0. 1	±0. 5	±0. 2	±0. 1

Untreated[c]	63. 2	32. 6	61. 2	0. 2	27. 8	0. 2	328. 8	2. 6	148	6. 8	113. 4	9. 1	8. 7
	±8. 5	±1. 7	±1. 8	±0. 0	±0. 9	±0. 0	±29. 4	±0. 1	±0. 7	±0. 3	±0. 5	±0. 3	±0. 1
Vehicle[d]	54. 4	30. 8	61. 2	0. 1	28. 8	0. 2	350. 8	2. 5	146. 2	5. 2	112. 4	6. 8	8. 5
	±11. 8	±2. 3	±1. 5	±0. 0	±1. 0	±0. 0	±10. 9	±0. 0	±0. 4	±0. 2	±0. 6	±0. 3	±0. 1
E2[e]	83	41. 5	466. 5	0. 2	25. 5	0. 1	251	2. 5	151. 5	6. 1	113. 5	7. 2	8. 8
	±16	±4. 5	±54. 5	±0. 0	±1. 5	±0. 0	±40. 0	±0. 0	±0. 5	±0. 1	±0. 5	±0. 9	±0. 2
NDC-1308[f]	54. 6	22. 2	60	0. 2	27. 6	0. 2	262	2. 6	149. 6	5. 7	113. 6	6. 8	8. 7
	±5. 6	±0. 9	±3. 0	±0. 0	±0. 9	±0. 0	±24. 3	±0. 0	±0. 6	±0. 1	±0. 5	±0. 4	±0. 1

[a]Age-Matched are normal, untreated mice at the same age as the other mice.
[b]Demyelinated are mice treated with cuprizone and rapamycin for 12 weeks and then terminated.
[c]Untreated are rats treated with cuprizone and rapamycin for 12 weeks and then allowed to spontaneously recovery for 6 weeks without treatment.
[d,e,f]Vehicle, E2 and NDC-1308 are demyelinated mice that are treated with vehicle, E2 and NDC-1308, respectively for 6 weeks.

Compared to age- matched controls, mice demyelinated with cuprizone show signs of liver damage (ALT, ALP) along with an elevation in chloride ions. These anomalies disappear once cuprizone is removed from the diet as observed in untreated demyelinated mice and the vehicle treated group, although in the vehicle group low levels of potassium and phosphate were detected. For the most part, the NDC-1308 treatment group appears to have clinical chemistries at, or near, the normal range.

Discussion

NDC-1308 is a small molecule possessing many attributes of a CNS therapeutic necessary for treating demyelinating disorders and potentially neurodegenerative diseases. The studies presented here demonstrate that a chronic administration of NDC-1308 to mice demyelinated with cuprizone can lead to significant repair of the myelin sheath in a number of different brain regions. At least in the hippocampus, the effect of NDC-1308 on oligodendrogenesis can be detected as early as 2-weeks after treatment, with an additive effect seen after a longer 3- or 6-weeks of treatment.

There are several lines of evidence that NDC-1308 functions through the nuclear ERs. First, NDC-1308 selectively binds to the nuclear ERs [22]. Second, NDC-1308 does not require GPR30 (an intracellular seven transmembrane G protein-coupled estrogen receptor) to function since the MDA-MB-231 cells used in the cell-based expression system are devoid of GPR30 [29]. Third, the NDC-1308 activity in the cell-based expression system can be blocked with the ER antagonist ICI 182,780. Fourth, preliminary data suggests that in vitro OPC differentiation can be partially blocked with ICI 182,780 (not shown).

NDC-1308 is an ER agonist, yet it possesses a very different biological activity than the estrogens. NDC-1308 can induce oligodendrogenesis from OPCs maintained in culture while related estrogens, such as E2 and estriol, do not have this activity [22]. In addition, the studies reported here show that NDC-1308 induces oligodendrogenesis and remyelination in the mouse cuprizone model, but it does not possess the side-effects associated with estrogens, including estrogenicity, mutagenicity and genotoxicity, and it is not predicted to be proarrhythmic. How can this be? The answer may be found in the specific gene expression profiles elicited by treatment of cells with NDC-1308. NDC-1308 binds to nuclear ERs which then form active dimers that associate with transcription complexes to regulate gene expression of numerous intracellular pathways [22]. Since the structure of an ER ligand can impact the composition of the transcription complex, by altering both the shape of the ligand binding domain and the conformation of the ERs [30], it is likely that the alkoxyalkyl tail substitution on the C-6 position of the B-ring of NDC-1308 alters its biological activity leading to the up-regulation of genes for OPC differentiation and myelin synthesis [22].

NDC-1308 possesses many of the qualities desirable in a CNS therapeutic and appears to be well-tolerated after chronic administration. NDC-1308 is highly lipophilic by virtue of its long alkoxyalkyl tail and when administered systemically it rapidly traverses the blood brain barrier and is absorbed into CNS tissues at efficacious levels. From a safety perspective, NDC- 1308 displays favorable pharmacokinetics. It is mostly eliminated from the CNS tissues and plasma after 6 hours and does not accumulate with chronic treatment. Metabolic studies using hepatic cells treated with NDC-1308 indicates that it is glucuronidated at the hydroxyls found on both the A and D ring of the steroidal structure (data not shown). This would be consistent with NDC-1308 being deactivated and eliminated from the periphery first-pass by the hepatic system. After 6-weeks of NDC-1308 treatment, there were no major side-effects detected after chronic dosing by gross necropsy (data not shown) or clinical chemistry analysis (Table 5) as most of the clinical chemistry results were at, or near, the

normal range. Induction of remyelination appears to be additive as mice terminated after 3-weeks of NDC-1308 treatment had a significant, but lower, amount of remyelination (16%) in the hippocampus compared to the 44% induction of remyelination measured after 6-weeks of daily dosing. The OPC population was found to be intact over the long course of NDC-1308 treatment (Figure 7) and this is consistent with a chronic NDC-1308 therapy potentially leading to greater myelin sheath repair in patients. That NDC-1308 administered chronically to rodents is safe also bodes well for chronic administration in patients, especially since the timing for oligodendrogenesis is expected to be longer in humans compared to rodents [31].

NDC-1308 is a potential remeylinating therapy that could be used to treat diseases, like MS, that are impacted either directly, or indirectly, by demyelination. While newer and more aggressive therapies for treating chronic demyelinating disorders are available that substantially slow disease progression in relapsing-remitting MS patients, the relentless attack on myelin in these patients inevitably leads to disease progression, increased disability and poor quality of life. In these patients, NDC-1308 could be envisioned to repair damaged myelin sheath in combination with current drugs that halt the persistent immune system attack on myelin. Since multiple cellular targets are likely involved in the pathophysiology of demyelinating disorders, NDC-1308 may have a therapeutic advantage over antibody therapies that affect only a single cellular target.

Besides MS, Osmotic Demyelination Syndrome (ODS) is an acute demyelinating disorder of the pons and extrapontine brain regions of the brain resulting from over-correction of hyponatremia and with no existing therapeutic interventions tested in controlled clinical trials [32]. In the cuprizone model, NDC-1308 enhanced remyelination of the parabrachial region of the pons by 18%. The ability of NDC-1308 to remyelinate the demyelinated white region could benefit patients with ODS. For patients with Transverse Myelitis (TM) or Neuromyelitis Optica (NMO), in which spinal cord lesions (TM) or spinal cord plus optic Nerve Lesions (NMO) lead to functional losses and/or functional losses plus visual deficits, respectively, there is currently no treatment except for supportive care [7,8]. The ability of NDC-1308 to induce remyelination within multiple regions of the CNS suggests NDC-1308 could benefit these patients as well. For Alzheimer's Disease (AD) patients, white matter loss through normal ageing is typically accelerated and along with lesion formation could lead to mild cognitive impairment [33,34]. One study suggests that Aβ plaque-associated focal demyelination in the cortical grey matter could lead to processing deficits in AD patients [34]. In cuprizone demyelinated mice, NDC-1308 therapy enhanced remyelination of both white matter (pons) and grey matter (hippocampus, cortex) and likewise could benefit this group of patients.

In summary, there continues to be an unmet need for a remyelinating therapy to treat patients with demyelinating and neurodegenerative diseases. As a remyelinating therapy, the ability of NDC-1308 to induce oligodendrogenesis and remyelination has direct implications for treating patients with demyelinating disorders, like MS, TM, NMO or ODS, or for treating patients with neurodegenerative disorders, like AD, who suffer from mild cognitive impairment.

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