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**14. ABSTRACT**

Environmental exposures are closely linked to the development of Parkinson's disease (PD). A number of environmental compounds have been implicated as risk factors for PD, including heavy metals, pesticides and anti-cholinergic chemical warfare agents. A central mechanism of action of many of these agents is the capacity to inhibit mitochondrial energy production in cells, leading to several common pathways of cellular dysfunction associated with PD, including protein misfolding, inflammation and injury to nerve cells in the brain that regulate movement. It is now known that veterans of the 1991 and more recent Gulf wars were widely exposed to a number of these agents, particularly pesticides, that has increased the prevalence of PD and PD-like disorders amongst Gulf war service veterans. It is therefore critical to identify both the cellular mechanisms driving this pathology as well as key signaling pathways that could be therapeutically targeted to interdict further neurological decline in individuals diagnosed with parkinsonism related to environmental exposures.

**15. SUBJECT TERMS**

(i) Environmental exposures and gene - environment interactions at prodromal or clinically diagnosed PD; (ii) Translational outcome measures and animal models

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## 1. Introduction

Neurotoxic environmental exposures in the etiology of Parkinson's disease. Environmental exposures are closely linked to the development of Parkinson's disease (PD). A number of environmental compounds have been implicated as risk factors for PD, including heavy metals and pesticides, as well as anti-cholinergic insecticides and chemical warfare agents. A central mechanism of action of many of these agents is the capacity to inhibit mitochondrial function, leading to several common pathways of cellular dysfunction associated with PD, including enhanced protein aggregation of alpha-synuclein, inhibition of autophagy, inflammatory activation of microglia and astrocytes, decreased production of dopamine and, ultimately, progressive loss of dopaminergic neurons in the basal midbrain. Anti-cholinergic insecticides, in particular, have been well studied for their detrimental effects on dopaminergic neurons. It is now known that veterans of the 1991 and more recent Gulf wars were widely exposed to a number of these agents, particularly insecticides, that has increased the prevalence of PD and PD-like disorders amongst Gulf war service veterans suffering from Gulf War Syndrome. It is therefore critical to identify both the cellular mechanisms driving this pathology as well as key signaling pathways that could be therapeutically targeted to interdict further neurological decline in individuals diagnosed with parkinsonism related to environmental exposures. Disruption of mitochondrial function in dopaminergic neurons by environmental toxicants is directly linked to neuroinflammation in surrounding glial cells, which can continue to damage neurons and worsen neurological decline in PD long after the initial insult. Neuroinflammation in PD occurs prior to spread of Lewy Bodies and precedes loss of dopaminergic neurons. However, there are no available therapeutics to treat neuroinflammation in PD or any other neurodegenerative disease, highlighting the urgent need for new targets to improve patient outcomes and prevent neurological decline. It was demonstrated that the nuclear receptor, NR4A2/Nurr1, functions as a critical regulator of inflammatory signaling pathways in the brain to protect dopaminergic neurons from stress and injury, highlighting the possibility of targeting this pathway to mitigate the damaging effects of neuroinflammation in PD. The rationale for targeting these receptors in the proposed studies is presented in the following sections. The overall goal of this work is to identify mechanisms to reduce neurotoxic injury in PD.

## 2. Keywords

Parkinson's Disease – PD
Substantia Nigra – SN
Neuroinflammation
Nuclear receptor 4A1 – NR4A1/Nur77
Nuclear receptor 4A2 – NR4A2/Nurr1
Nuclear receptor 4A3 – NR4A3/Nor1
Nuclear factor kappaB – NFκB
N-methyl-D-aspartate receptor – NMDA receptor
NGF1-β response element – NBRE
Nur-responsive element – NuRE
1,1-bis(3'-indolyl)-1-(p-chlorophenyl)methane – CDIM12
Bis-indolyl substituted methane – DIM
Chemokine ligand 5 – Ccl5
Tumor necrosis factor ligand superfamily member 10 – Tnsf10
Tumor necrosis factor receptor-associated factor 6 – Traf6
Complement C3 – C3
B-cell lymphoma related gene 2 - Bcl2
Early growth factor 1 – Egr1
Synaptotagmin 1 – Syt1
Isothermal titration calorimetry – ITC

## 3. Accomplishments

***What were the major goals of the project?***

*The goals of this project in Year 1 were as follows:*

**Aim 1, Major Task 1: Identify the second-generation CDIM analogs with optimal molecular binding characteristics as dual receptor ligands for NR4A1 and NR4A2**

*Subtask 1 – Synthesis of 3,5-disubstituted analogs of DIM and DIM8*. The bis-indole analogs will be synthesized in the laboratory by condensation of indole for each compound as previously described.
Milestone value: ≥ 25 mg of each compound

*Subtask 2 - Recombinant NR4A1 and NR4A2 LBD*.
Milestone value: ≥10 mg pure recombinant protein

*Subtask 3 – Direct binding assays/Fluorescence spectroscopy*. Tryptophan fluorescence spectra will be obtained using histidine-tagged ligand binding domain (LBD) of NR4A1/2 at a final concentration of 0.5 μmol/L.
Milestone value: KD ≤ 15 μmol/L

*Subtask 4 - Isothermal titration calorimetry (ITC) binding assays*. ITC is used to determine the ligand binding constant (Kd) to NR4A1 or NR4A2 utilizing Affinity ITC.
Milestone value: KD ≤ 50 nmol/L

*Subtask 5 - CDIM-receptor molecular modeling in silico*. Molecular docking studies will be conducted with 3D protein crystal structures (Protein Data Bank) to calculate energy minimization values for ligand binding.
Milestone value: Docking score (energy) ≤ -5.0 Kcal/mol

*Subtask 6 - Ranking of flavonoid potencies as NR4A1/2 ligands*. Dose-response data will be used to derive point-of-departure (POD; e.g.: IC50 or 1 SD) values which will then be integrated and visualized using the Toxicological Priority Index Graphical User Interface.
Milestone value: ToxPi POD score ≥ 0.5

**Aim 1, Major Task 2: Determine the capacity of high affinity CDIM analogs to modulate PD-relevant cellular targets in glia and neurons**.

*Subtask 7 - Transactivation assays (neurons) - NuRE/NBRE*. Human LUHMES cells will be transfected with NuRE and NBRE reporters to identify ligand potencies as transcriptional inducers of NR4A1/2.
Milestone value: 50% reporter activation ≤ 15 μmol/L

*Subtask 8 - Anti-inflammatory NFκB reporter assays in glial cells*. DIM compounds will be evaluated for potency in suppressing inflammation-induced NFκB-GFP-luciferase reporter signal in primary mixed glial cultures from transgenic mice.
Milestone value: 50% reduction in qPCR signal ≤ 15 μmol/L

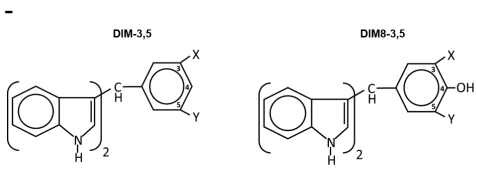
*Subtask 9 - Quantitative PCR assessment of inflammatory gene expression*. Profiling of the inflammatory phenotype of microglia and astrocytes will be performed by real-time qPCR following treatment with TNF, LPS or polyI:C in the presence of DIM compounds.
Milestone value: 50% reduction in qPCR signal ≤ 15 μmol/L

*Subtask 10 - Glial conditioned media experiments to examine neuroprotective efficacy in vitro*. Glial-conditioned media (GCM) from inflammation treatments of glial (+/-) DIM compounds will be placed on immortalized human dopaminergic neuronal (LUHMES) cells and apoptosis evaluated by flow cytometry and live-cell imaging.
Milestone value: ≤ 25% apoptotic neurons at 15 μm

***What was accomplished under these goals?***

**Aim 1, Major Task 1: Identify the second-generation CDIM analogs with optimal molecular binding characteristics as dual receptor ligands for NR4A1 and NR4A2**

*Subtask 1 – Synthesis of 3,5-disubstituted analogs of DIM and DIM8*. The bis-indole analogs will be synthesized in the laboratory by condensation of indole for each compound as previously described.
Milestone value: ≥ 25 mg of each compound




DIM-3,5-$Cl_2$ (X=Y=Cl)
DIM-3,5-$Br_2$ (X=Y=Br)
DIM-3,5-$F_2$ (X=Y=F)
DIM-3,5-$(CH_3)_2$ (X=Y=$CH_3$)
DIM-3,5-$(OCH_3)_2$ (X=Y=$OCH_3$)
DIM-3-Br-5-$CF_3$ (X=Br ; Y=$CF_3$)
DIM-3-Cl-5-$CF_3$ (X=Cl ; Y=$CF_3$)
DIM-3-F-5-$CF_3$ (X=F ; Y=$CF_3$)
DIM-3-Cl-5-$OCF_3$ (X=Cl ; Y=$OCF_3$)
DIM-3-F-5-$OCF_3$ (X=F ; Y=$OCF_3$)
DIM-3-Br-5-$OCF_3$ (X=Br ; Y=$OCF_3$)
DIM-3-Br-5-$OCH_3$ (X=Br ; Y=$OCH_3$)
DIM-3-Cl-5-$OCH_3$ (X=Cl ; Y=$OCH_3$)

DIM8-3,5-$Cl_2$ (X=Y=Cl)
DIM8-3,5-$Br_2$ (X=Y=Br)
DIM-3,5-$(tBu)_2$ (X=Y=tBu)
DIM8-3,5-$(CH_3)_2$ (X=Y=$CH_3$)
DIM8-3,5-$(OCH_3)_2$ (X=Y=$OCH_3$)
DIM-3-Cl-5-F (X=Cl ; Y=F)
DIM-3-Br-5-F (X=Cl ; Y=Br)
DIM8-3-Br-5-$OCH_3$ (X=Br ; Y=$OCH_3$)
DIM-3-Cl-5-$OCH_3$ (X=Cl ; Y=$OCH_3$)

**Figure 1. Structure of DIM-3,5 and Dim8-3,5 and their analogs used in this study.**

*Milestone achieved*: ≥ 25 mg of each compound has been synthesized for use in studies. Descriptions and structures of each CDIM analog used in these studies is depicted in **Figures 1 and 2**.

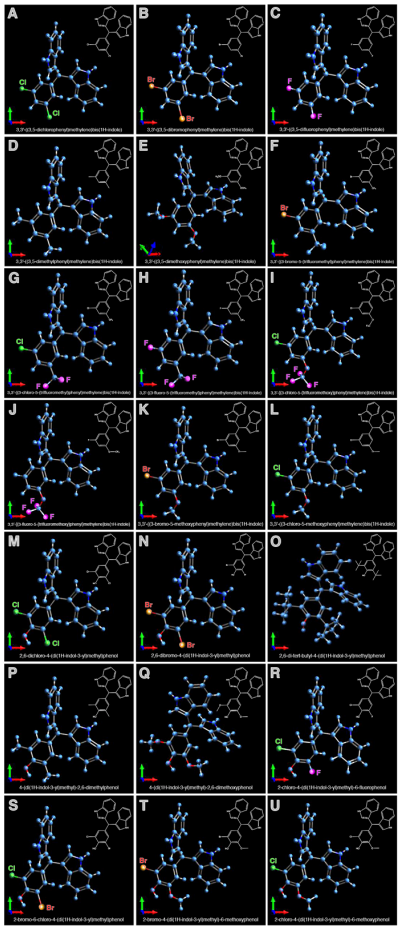



**Figure 2. Three-dimensional structural rendering of CDIM compounds.** Three-dimensional structural renderings and accompanying line formulas of 3,3'-((3,5-dichlorophenyl)methylene)bis(1H-indole) (**A**), 3,3'-((3,5-dibromophenyl)methylene)bis(1H-indole) (**B**), 3,3'-((3,5-difluorophenyl)methylene)bis)1H-indole (**C**), 3,3'-((3,5-dimethylphenyl)methylene)bis(1H-indole) (**D**), 3,3'-((3,5-dimethyoxyphenyl)methylene)bis(1H-indole) (**E**), 3,3'-((3-bromo-5-(trifluromethyl)phenyl)methylene)bis(1H-indole) (**F**), 3,3'-((3-chloro-5-(trifluromethyl)phenyl)methylene)bis(1H-indole) (**G**), 3,3'-((3-fluoro-5-(trifluromethyl)phenyl)methylene)bis(1H-indole) (**H**), 3,3'-((3-bromo-5-(trifluromethoxy)phenyl)methylene)bis(1H-indole) (**I**), 3,3'-((3-fluoro-5-(trifluromethoxy)phenyl)methylene)bis(1H-indole) (**J**), 3,3'-((3-bromo-5-methooxyphenyl)methylene)bis(1H-indole) (**K**), 3,3'-((3-chloro-5-methoxyphenyl)methylene)bis(1H-indole) (**L**), 2,6-dichloro-4-(di(1H-indol-3-yl)methyl)phenol (**M**), 2,6-dibromo-4-(di(1H-indol-3-yl)methyl)phenol (**N**), 2,6-di-tert-butyl-4-(di(1H-indol-3-yl)methyl)phenol (**O**), 4-(di(1H-indol-3-yl)methyl)-2,6-dimethylphenol (**P**), 4-(di(1H-indol-3-yl)methyl)-2,6-dimethoxyphenol (**Q**), 2-chloro-4-(di(1H-indol-3-yl)methyl)-6-fluorophenol (**R**), 2-bromo-6-chloro-4-(di(1H-indol-3-yl)methyl)phenol (**S**), 2-bromo-4-(di(1H-indol-3-yl)methyl)-6-methoxyphenol (**T**), and 2-choloro-4-(di(1H-indol-3-yl)methyl)-6-methoxyphenol (**U**) where carbon (light blue), chlorine (green), bromine (orange), fluorine (purple), and double bonds to nitrogen (dark blue) and oxygen (red) are depicted.

*Subtask 2 - Recombinant NR4A1 and NR4A2 LBD*.
Milestone value: ≥10 mg pure recombinant protein

*Milestone achieved*: NR4A1 and NR4A2 LBD protein has been purified and used in the following studies.

*Subtask 3 – Direct binding assays/Fluorescence spectroscopy*. Tryptophan fluorescence spectra will be obtained using histidine-tagged ligand binding domain (LBD) of NR4A1/2 at a final concentration of 0.5 µmol/L.
Milestone value: KD ≤ 15 µmol/L

*Milestone achieved*: Direct binding assays by fluorescence microscopy have been completed (**Figure 3**). Fig. 3 summarizes the direct binding of 22 analogs to the LBD of NR4A1 and NR4A2 using a fluorescence quenching assay. The results show that each of these compounds bind both NR4A1 and NR4A2, with respective KD values for binding NR4A1 ranging from 1.3-133 µM and for binding NR4A2 the range was 2.2-79 µM. Among the DIM-3,5 and DIM8-3,5 analogs, the compound with the highest KD values for binding NR4A1 and NR4A2 using the fluorescence assay was DIM-3,5-(CH3)2. For the remaining compounds there was not an apparent structure-binding relationship for KD values associated with their binding to NR4A1 and NR4A2 or for their corresponding KD(NR4A1)/KD(NR4A2) ratios.

| Compound | $K_D$ (NR4A1) (µM) | $K_D$ (NR4A2) (µM) | Compound | $K_D$ (NR4A1) (µM) | $K_D$ (NR4A2) (µM) |
|---|---|---|---|---|---|
| DIM-3,5-$Cl_2$ | 7.7 | 12.0 | DIM8-3,5-$Cl_2$ | 4.1 | 13.9 |
| DIM-3,5-$Br_2$ | 6.5 | 12.2 | DIM8-3,5-$Br_2$ | 1.3 | 7.4 |
| DIM-3,5-$F_2$ | 17.8 | | DIM8-3,5-(tBu)$_2$ | 7.9 | |
| DIM-3,5-$(CH_3)_2$ | 133 | 79 | DIM8-3,5-$(CH_3)_2$ | 24.5 | 10.7 |
| DIM-3,5-$(OCH_3)_2$ | 15.8 | | DIM8-3,5-$(OCH_3)_2$ | 4.0 | 14.3 |
| DIM-3-Br-5-$CF_3$ | 4.8 | 4.9 | DIM8-3,5-Cl-F | 1.5 | 4.2 |
| DIM-3-Cl-5-$CF_3$ | 3.1 | 5.5 | DIM8-3-Cl-5-Br | 4.3 | 4.3 |
| DIM-3-F-5-$CF_3$ | 7.3 | 8.1 | DIM8-3-Br-5-$OCH_3$ | 6.7 | 7.3 |
| DIM-3-Br-5-$OCF_3$ | 2.0 | 4.5 | DIM8-3-Cl-5-$OCH_3$ | 6.6 | 2.2 |
| DIM-3-Cl-5-$OCF_3$ | 2.3 | 3.5 | | | |
| DIM-3-F-5-$OCF_3$ | 17.1 | 33.6 | | | |
| DIM-3-Br-5-$OCH_3$ | 1.3 | 3.5 | | | |
| DIM-3-Cl-5-$OCH_3$ | 60.3 | 5.2 | | | |

**Figure 3. Direct Binding of DIM-3,5- and DIM8-3,5- compounds to the LBD of NR4A1 and NR4A2.**

*Subtask 4 - Isothermal titration calorimetry (ITC) binding assays*. ITC is used to determine the ligand binding constant (Kd) to NR4A1 or NR4A2 utilizing Affinity ITC.
Milestone value: KD ≤ 50 nmol/L

*Milestone achieved*: The same set of compounds in Fig. 3 was used to determine their KD values for binding NR4A1 and NR4A2 using isothermal titration calorimetry (ITC) which measure the heat lost or absorbed during the ligand-receptor (LBD) interaction (**Figure 4**). The KD values for binding NR4A1 and NR4A2 were highly variable using the ITC assay and some of these compounds including DIM-3,5-(OCH3)2, DIM-3-Br-5-CF3, DIM-3-Cl-5-OCH3, DIM8-3,5-(OCH3)2 and DIM-3,5-Cl2 exhibited KD values in the low nM range. There was considerable variability in the KD values obtained with the fluorescence and ITC assays and some of the low KD values observed in the latter assay may be due to ligand interactions outside the binding pockets of NR4A1 and NR4A2.

| Compound | $K_D$ (NR4A1) (μM) | $K_D$ (NR4A2) (μM) | Compound | $K_D$ (NR4A1) (μM) | $K_D$ (NR4A2) (μM) |
|---|---|---|---|---|---|
| DIM-3,5-$Cl_2$ | 12.3 | 2.04 | DIM8-3,5-$Cl_2$ | 0.001 | 0.0059 |
| DIM-3,5-$Br_2$ | 5.30 | 0.30 | DIM8-3,5-$Br_2$ | 2.1 | 2.62 |
| DIM-3,5-$F_2$ | 0.05 | 1.88 | DIM8-3,5-$(tBu)_2$ | 4.31 | 1.47 |
| DIM-3,5-$(CH_3)_2$ | | 2.33 | DIM8-3,5-$(CH_3)_2$ | 2.21 | 0.0024 |
| DIM-3,5-$(OCH_3)_2$ | 0.001 | 1.67 | DIM8-3,5-$(OCH_3)_2$ | 0.0012 | 1.44 |
| DIM-3-Br-5-$CF_3$ | 0.26 | 2.39 | DIM8-3-Cl-5-F | 0.001 | 2.25 |
| DIM-3-Cl-5-$CF_3$ | 2.72 | 2.58 | DIM8-3-Cl-5-Br | 52.5 | 28.5 |
| DIM-3-F-5-$CF_3$ | 2.14 | 0.0011 | DIM8-3-Br-5-$OCH_3$ | 25.4 | 18.1 |
| DIM-3-Br-5-$OCF_3$ | 2.40 | 6.01 | DIM8-3-Cl-5-$OCH_3$ | 20.7 | 2.35 |
| DIM-3-Cl-5-$OCF_3$ | 19.6 | 2.30 | | | |
| DIM-3-F-5-$OCF_3$ | 5.02 | 2.44 | | | |
| DIM-3-Br-5-$OCH_3$ | 2.64 | 2.34 | | | |
| DIM-3-Cl-5-$OCH_3$ | 0.001 | 0.23 | | | |

**Figure 4. Direct Binding of DIM-3,5- and DIM8-3,5- compounds to the LBD of NR4A1 and NR4A2.**

*Subtask 5 - CDIM-receptor molecular modeling in silico*. Molecular docking studies will be conducted with 3D protein crystal structures (Protein Data Bank) to calculate energy minimization values for ligand binding.
Milestone value: Docking score (energy) ≤ -5.0 Kcal/mol

*Milestone achieved*: Molecular docking studies have been completed to examine the relative predicted binding affinities of each compound to NR4A1 and NR4A2 (**Figure 5**). Maestro/Schrodinger software was used in molecular modeling studies to investigate interactions between the DIM-3,5 and DIM8-3,5 analogs with the TMY301 and TMY302 binding sites previously identified in the LBD of NR4A1 (30): Among the DIM-3,5 analogs only the DIM-3,5-Br2, DIM-3-Br-5-CF3 and DIM-3-Cl-5-CF3 did not exhibit a lower docking score for the TMY301 site whereas for the DIM8-3,5 analogs all compounds except DIM-3,5-(tBu)2 exhibited a lower docking score for the TMY301 site in NR4A1. Supplemental Figures 1 summarize interactions between individual DIM-3,5 and DIM8-3,5 analogs with the ligand binding domain of NR4A1 (TMY301 and TMY302) which are close to the surface of the ligand binding pocket.

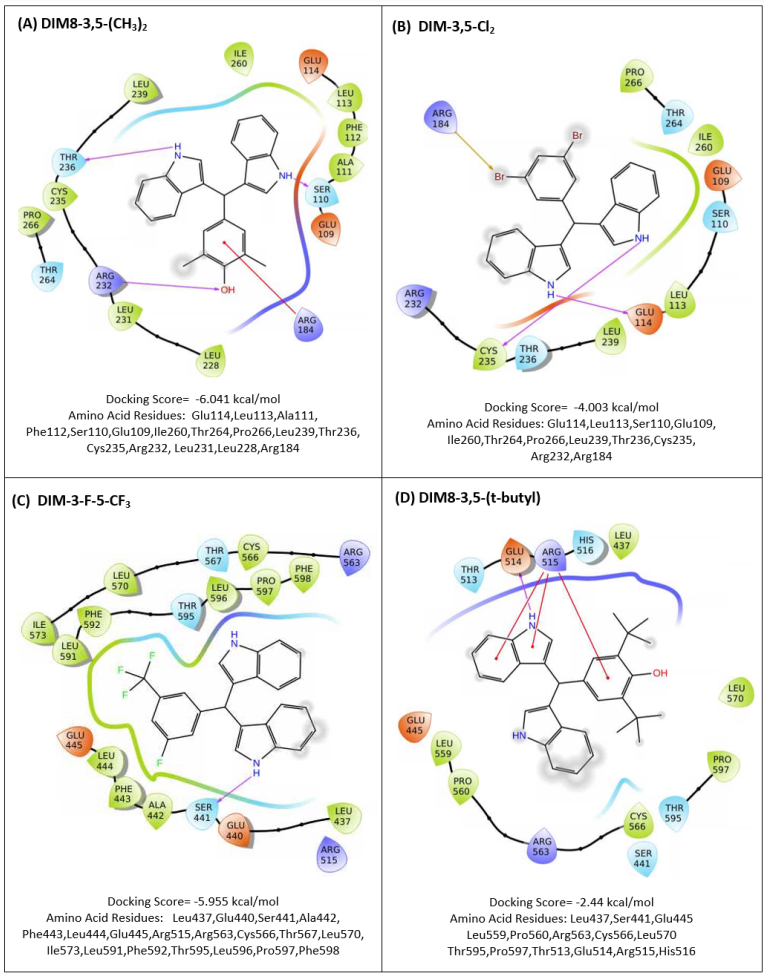



**Figure 5. Direct Binding of DIM-3,5- and DIM8-3,5- compounds to the LBD of NR4A1 and NR4A2.**

Amino acids involved in ligand-LBD interactions are somewhat variable and Figures 2A and 2B illustrate specific interactions between DIM8-3,5-(CH3)2 and DIM-3,5-Br2 which have large differences in docking scores of -6.04 and -4.00 kcal/mole respectively. Results of the modeling studies show that both compounds interact with Glu114, Leu113, Ser110, Glu109, Ile260, Thr264, Pro266, Leu239, Thr236, Cys235, Arg232 and Arg184 and these are the only amino acids associated with DIM-3,5-Br2-LBD interactions. In contrast, DIM8-3,5-(CH3)2 with the more favorable docking score (-6.04 kcal/mol) also interacts with amino acids Ala111, Phe112, Leu231 and Leu228, and presumably these amino acids contribute to the lower docking score for this compound. These scores are currently being combined with binding data (Figures 3 and 4) to generate overall ranking scores for binding in Subtask 6 (below).

*Subtask 6 - Ranking of flavonoid potencies as NR4A1/2 ligands*. Dose-response data will be used to derive point-of-departure (POD; e.g.: IC50 or 1 SD) values which will then be integrated and visualized using the Toxicological Priority Index Graphical User Interface.
Milestone value: ToxPi POD score ≥ 0.5

*Milestone achieved*: Tox/POD analysis of each compound is ongoing and should be completed by Q4 2023.

**Aim 1, Major Task 2: Determine the capacity of high affinity CDIM analogs to modulate PD-relevant cellular targets in glia and neurons**.

*Subtask 7 - Transactivation assays - NuRE/NBRE*. Human LUHMES cells will be transfected with NuRE and NBRE reporters to identify ligand potencies as transcriptional inducers of NR4A1/2.
Milestone value: 50% reporter activation ≤ 15 µmol/L

*Milestone achieved*: The effects of the DIM-3,5 and DIM8-3,5 analogs on transactivation was determined using U87G human glioblastoma cells transfected with GAL4-NR4A1 (LBD) or GAL4-NR4A2 (LBD) and the UAS-luc reporter gene which contains 5 tandem GAL4-DNA binding sites (**Figure 6**). These neural cells were selected because they transfect more easily than human dopaminergic LUHMES cells. However, they invoke the same molecular signaling pathways in response to NR4A1/2 ligands, and both LUHMES and N2A neuronal cells are currently being examined as well. The U87G cell line has previously been used with DIM compounds and expresses both NR4A1 and NR4A2. The results obtained after treatment of U87G cells with 7.5 and 15 µM of DIM-3,5 compounds for 24 hours showed that all ligands decreased transactivation in cells transfected with GAL4-NR4A1 or GAL4-NR4A2 (Figs. 3A and 3B). These results complement the binding of the DIM-3,5 analogs to NR4A1 and NR4A2 and further confirm that these compounds are dual receptor ligands. Preliminary studies with the series of DIM8-3,5 analogs showed minimal effects on luciferase activity after treatment for 24 hours. However, after incubation for 48 hours, these compounds also decreased NR4A1- and NR4A2-dependent transactivation in U87G cells (Figs. 3C & 3D). The potencies for most of the DIM8-3,5 analogs were less than observed for the DIM-3,5-compounds and this correlated with their reported differences in vivo potencies as inhibitors of mammary tumor growth in an orthotopic athymic nude mouse xenograft model. The DIM8-3,5 analogs appeared to be most postent in transfection assays, similar to the binding assays.

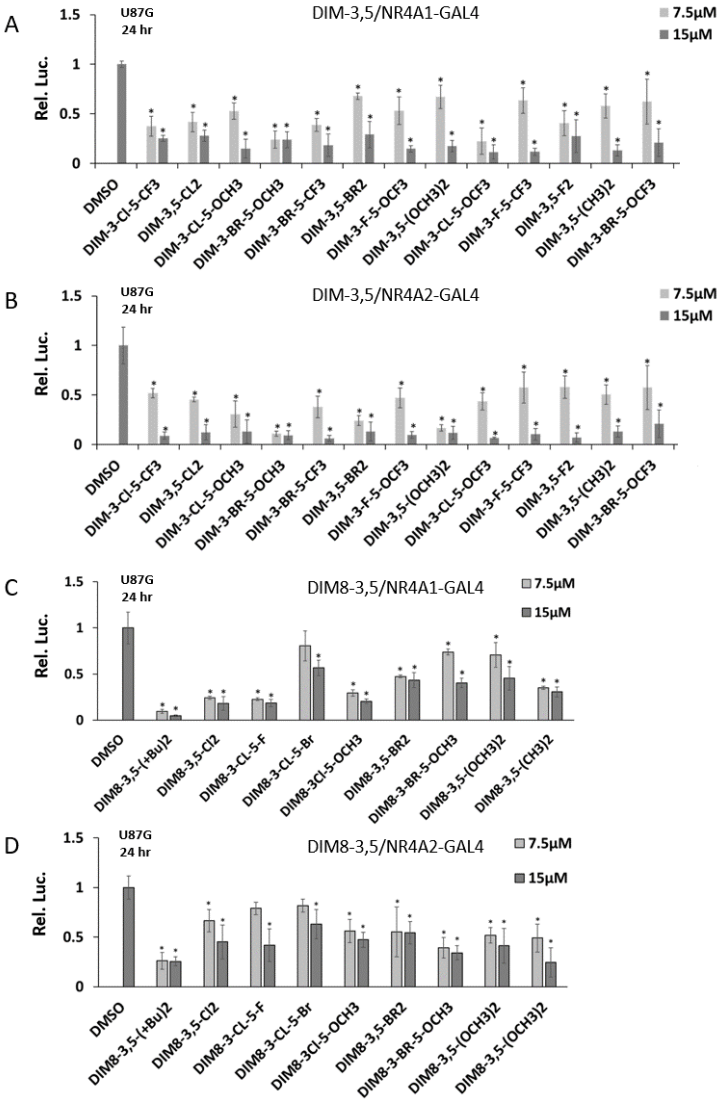



**Figure 6. Activation of NR4A1 and NR4A2 by DIM-3,5 and DIM8-3,5 analogs.** U87G cells were transfected with UAS-Luc and GAL4-NR4A1 (A) or GAL4-NR4A2 (B), treated with DIM-3,5 compounds (7.5 and 15 µM) and luciferase activity was determined as outlined in the Methods. The same protocol was used for activation of NR4A1- (C) and NR4A2-dependent (D) activities by DIM8-3,5 compounds. Results are expressed as means ± SD for at least three replicate determinations and significant ($p < 0.05$) changes compared to DMSO (control) are indicated (*).

*Subtask 8 - Anti-inflammatory NFκB reporter assays in glial cells*. DIM compounds will be evaluated for potency in suppressing inflammation-induced NFκB-GFP-luciferase reporter signal in primary mixed glial cultures from transgenic mice.
Milestone value: 50% reduction in qPCR signal ≤ 15 µmol/L

*Milestone achieved*: Dose-response curves for inhibition of NFkB reporter activity in primary astrocytes isolated from mouse brain is depicted in **Figure 7**. Primary astrocytes isolated from NFkB-EGFP-Luciferase reporter mice were stimulated with 1 ng/ml TNF + 10 pg/ml IFN for 6 hours in the absence or presence of each CDIM analog. In general, 3,5-substituted DIM analogs achieved lower IC50 values for inhibition of inflammatory signaling than did DIM-8-3,5-substituted analogs (data not shown for brevity). Values were: DIM-3-5-Cl2 (IC50=23.87µM; A), DIM-3-5-Br2 (IC50=5.432µM; B), DIM-3-Cl-5-CF3 (IC50=3.096µM; C), and DIM-3-Br-5-CF3 (IC50=13.71µM; D). The lowest IC50 value observed was for DIM-3-Cl-5-CF3 (IC50=3.096µM), making this one of the top candidates for animal studies in Year 2 of the project. This coincides with the low Kd for binding both NR4A1 and NR4A2 for this compound in Isothermal titration calorimetry (ITC) binding assays (**Figure 4**). Several compounds met criteria for the milestone of >50% inhibition of reporter activity at <15 uM, with the top amongst these to be selected for advancement into animal studies.

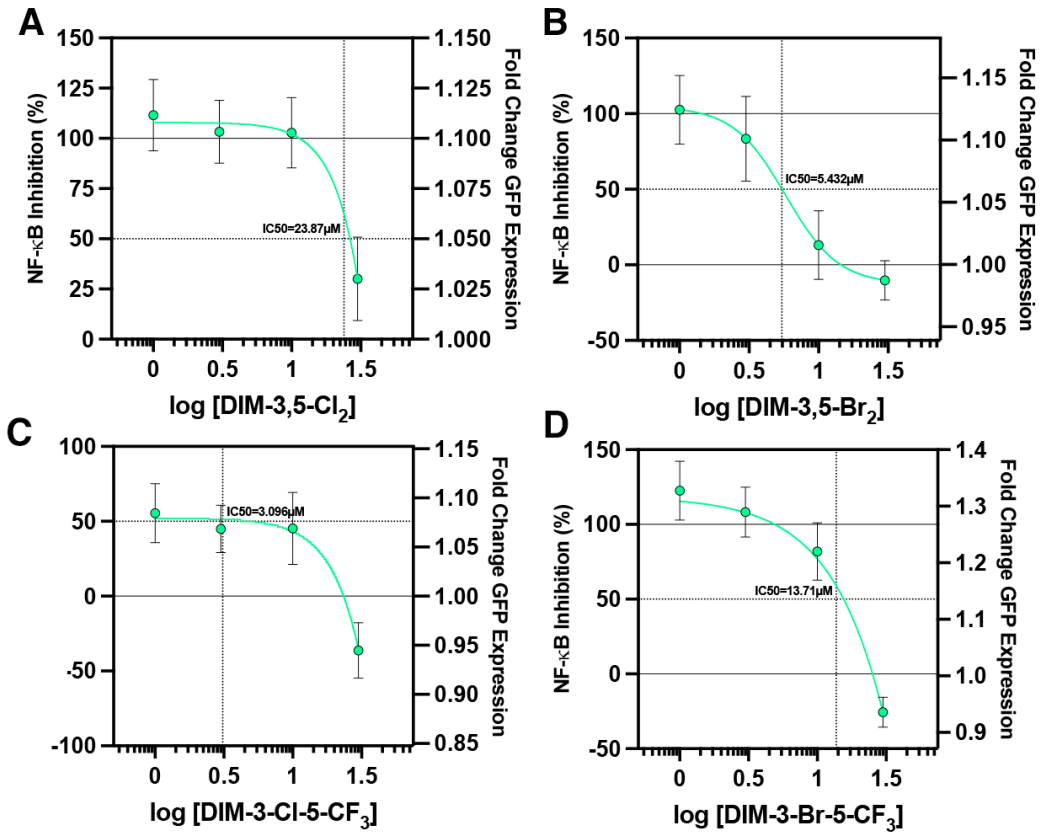



**Figure 7. Dose response curves of CDIM molecules needed to inhibit NF-kappaB activation in primary glial cells.** Quantitative fluorescence measurement of NF-kappaB expression in dual reporter primary glial cells reveals concentration and CDIM dependent inhibition of NF-kappaB activation with DIM-3-5-Cl2 (IC50=23.87µM; A), DIM-3-5-Br2 (IC50=5.432µM; B), DIM-3-Cl-5-CF3 (IC50=3.096µM; C), and DIM-3-Br-5-CF3 (IC50=13.71µM; D).

*Subtask 9 - Quantitative PCR assessment of inflammatory gene expression*. Profiling of the inflammatory phenotype of microglia and astrocytes will be performed by real-time qPCR following treatment with TNF, LPS or polyI:C in the presence of DIM compounds.
Milestone value: 50% reduction in qPCR signal ≤ 15 µmol/L

*Milestone achieved*: Expression of mRNA for selected inflammatory genes is currently being evaluated for each compound, with data still being analyzed. In preparation for these pharmalogic dose-response studies, we first conducted cell viability assays in primary astrocyte cultures to determine the relative cytoxicity of each compound in order to establish appropriate dose ranges for qPCR experiments (**Figure 8**). Viability was evaluated by uptake of the cell-impermeant DNA intercalating dye, 7-AAD, relative to the number of cells per well (as determined by co-incubation with Hoecsht 33342), normalized to vehicle-treated control wells. None of the CDIM analogs evaluated displayed cytotoxicity at the concentrations in which we observed >50% inhibition of NFkB-luciferase reporter signaling (Figure 7). Based on this finding, we are proceeding with mRNA expression studies to determine the relative efficacy of each compound in mitigating inflammatory gene expression in astrocytes.

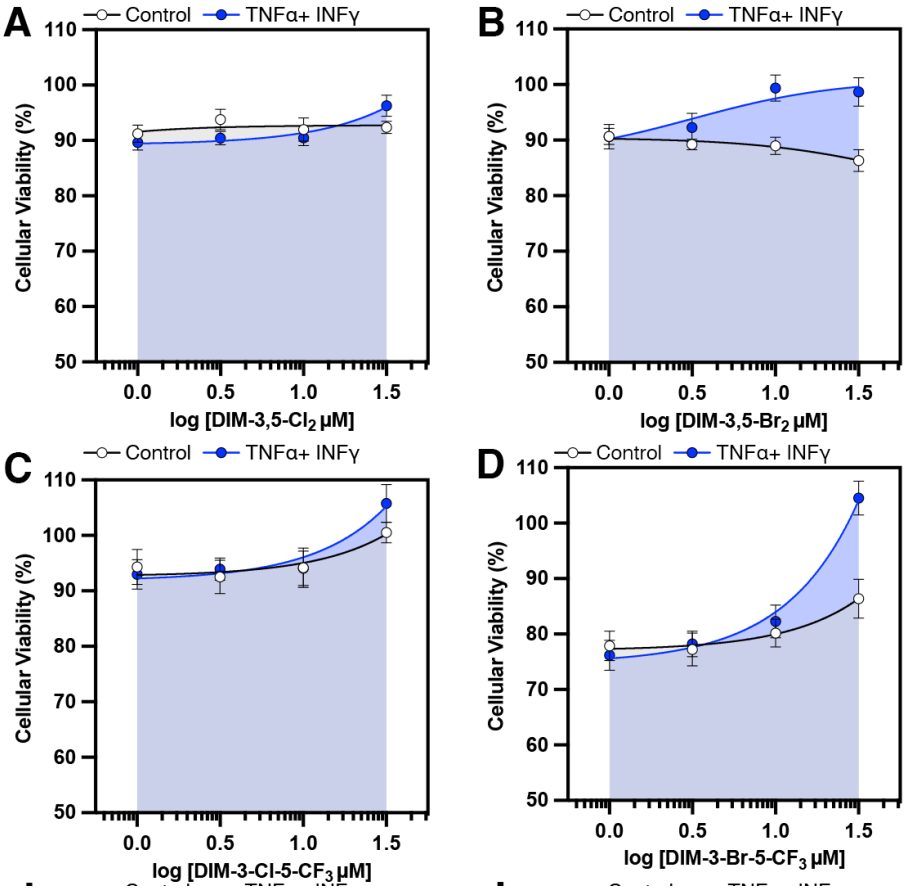



**Figure 8. CDIM compounds do not induce toxicity within primary glial cell cultures in the presence or absence of inflammatory stimulus**. Normalized cellular viability to primary glial cells that were not treated with CDIM compounds are depicted for DIM-3-5-Cl2 (A), DIM-3-5-Br2 (B), DIM-3-Cl-5-CF3 (C), and DIM-3-Br-5-CF3 (D) alone (white data points) at half log fold concentrations, or prior to addition of inflammatory stimulus (blue data points).

*Subtask 10 - Glial conditioned media experiments to examine neuroprotective efficacy in vitro*. Glial-conditioned media (GCM) from inflammation treatments of glial (+/-) DIM compounds will be placed on immortalized human dopaminergic neuronal cells and apoptosis evaluated by flow cytometry and live-cell imaging.
Milestone value: ≤ 25% apoptotic neurons at 15 µm

*Milestone achieved*: Glial-neuronal co-culture and conditioned media studies for evaluating neuroprotectice efficacy of each CDIM compound *in vitro* are ongoing and should be completed by Q4 of 2023. This will inform the final selection of compounds for animal studies in Year 2 of the project.

***What opportunities for training and professional development has the project provided?***

Not part of this project/Nothing to report

***How were the results disseminated to communities of interest?***

Nothing to report

***What do you plan to do during the next reporting period to accomplish the goals?***

Per the original Scope of Work for this proposal, we are now beginning work on the Major Tasks for Specific Aim 2 of the project, which will move the top compounds from Aim 1 into animal studies in Aim 2:

Specific Aim 2: Characterize the pharmacokinetic properties and the *in vivo* neuroprotective efficacy of the CDIM analogs displaying the most favorable molecular binding and cytoprotective activity.

Aim 2, Major Task 1: Characterize the pharmacokinetic properties and oral bioavailability in mice of the CDIM analogs from Specific Aim 1 displaying the optimal molecular and cellular activities toward NR4A receptors.

Aim 2, Major Task 2: Determine the in vivo neuroprotective efficacy of the CDIM analogs displaying the most favorable pharmacokinetic distribution to brain

*To complete the studies in Aim 2, several of the top compounds emerging from the studies in Specific Aim 1 will be evaluated for pharmacokinetic behavior in mice and then the best 1 – 2 compounds will be used in in vivo neuroprotection studies using the rotenone model of Parkinson's disease*.

## 4. Impact

***What was the impact on the development of the principal discipline(s) of the project?***

Nothing to report

***What was the impact on other disciplines?***

Nothing to report

***What was the impact on technology transfer?***

Nothing to report

***What was the impact on society beyond science and technology?***

Nothing to report

## 5. Changes/Problems

None

## 6. Products

Manuscripts in preparation or submitted:

## 7. Participants & Other Collaborating Organizations

| | |
|---|---|
| Name: | Ronald Tjalkens |
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| Nearest person month worked: | 1 |
| Funding support: | DoD/NETP W81XWH2210663 |

| | |
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| Nearest person month worked: | 1 |
| Funding support: | DoD/NETP W81XWH2210663 |

| | |
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| Nearest person month worked: | 12 |
| Funding support: | DoD/NETP W81XWH2210663 |

| | |
|---|---|
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| Project Role: | Graduate Research Assistant |
| Researcher Identifier: | No ORCID ID |
| Nearest person month worked: | 6 |
| Funding support: | DoD/NETP W81XWH2210663 |

## 8. Special Reporting Requirements

None

## 9. Appendices

None