The overall aim of this current proposal is to explore diverse triterpenoids that will enable careful dissection of the Nrf2-dependent and independent pathways mediating triterpenoid neuroprotection and will define a novel class of potent and effective therapeutic and chemo preventive agents for multiple sclerosis. In our earlier report we reported the successful large scale (in gram quantity) isolation of Bryonolic acid from zucchini plant (Cucurbita pepo L.) roots and characterized its activity. We also applied Diversity Oriented Synthesis to Bryonolic acid to rearrange skeletal structure and analyzed the specificity and activity of skeletally diverse triterpenoids in vivo and in vitro including its potential role in suppression of disease symptoms in EAE (Experimental Autoimmune Encephalomyelitis). In the current report we: 1) characterize the Nrf2-targeted antioxidant properties of the natural triterpenoid Celastrol, isolated from the root extracts of Tripterygium wilfordii (Thunder god vine); 2) standardize an imaging technique used to capture T cell/APC interaction and infiltration in CNS during the disease course of EAE; and finally 3) characterize the potential benefit of CDDO-DFEA treatment in EAE by influencing T cell/APC interactions.
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Introduction:

The overall aim of this proposal and the funded effort is to explore diverse triterpenoids that will enable careful dissection of the Nrf2-dependent and Nrf2-independent pathways mediating triterpenoid neuroprotection. An important and anticipated outcome of the effort is the description and characterization of a novel class of potent and effective therapeutic and chemopreventive agents that will have utility as agents that can prevent and ameliorate symptoms associated with multiple sclerosis (MS). In our first annual report we described the successful large scale (in gram quantity) isolation of Bryonolic acid from zucchini plant (*Cucurbita pepo* L.) roots and characterized the activities of Byronolic acid in a series of *in vitro* and *in vivo* assays. We also applied Diversity Oriented Synthesis to Bryonolic acid, thereby rearranging the skeletal structure and subsequently we analyzed the specificity and activity of these skeletally diverse triterpenoids *in vivo* and *in vitro* including their potential to suppress disease symptoms in EAE. In current report we first characterized the Nrf2 targeted antioxidant properties of Celastrol (a pentacyclic triterpenoid compound), isolated from the root extracts of *Tripterygium wilfordii* (Thunder god vine), extracted from *Tripterygium wilfordii* Hook F., a plant used to treat rheumatic disease for many years. Celastrol is reported as a potent inhibitor of the NF-κB signaling pathway via IKK-β targeting\(^1\)\(^2\) and has shown real therapeutic potential in many inflammation-related diseases, such as allergic asthma,\(^3\) amyotrophic lateral sclerosis, rheumatoid arthritis\(^4\) and skin inflammation.\(^5\) Further it has been reported that Celastrol can inhibit inflammatory reactions between lymphocytes and endothelial cells in blood vessels, maintain blood integrity, and exert neuro-protective effects by reducing CNS inflammation.\(^6\)\(^7\) Moreover, Celastrol is fat-soluble and thus easily diffuses into the central nervous system. Due to these known properties of this compound we performed a large scale of isolation of this compound and first tested its anti-inflammatory properties *in vitro*. In the body of this report, we detail results from these studies and describe planned efforts that will build on the data generated in this funding period.
**Overall Project Summary:**

*In vitro* Characterization of anti-inflammatory properties of Celastrol:

![Chemical structure of Celastrol](image)

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**Figure 1.** Celastrol suppresses iNOS expression and NO production in a dose and time dependent manner.

Shown above, *Figure 1a* depicts the chemical structure of Celastrol. Firstly, in order to analyze the anti-inflammatory properties of isolated Celastrol, we analyzed the time and dose dependent effect of Celastrol on inflammatory markers in LPS treated RAW 264.7 cells. As shown in *Figure 1b* RAW 264.7 cells activated with LPS and treated simultaneously with increasing concentrations of Celastrol (10-750 nM) for 16 hours showed a dose dependent decline in secreted nitrite in media,
(Averages represent triplicate treatments +/- SEM). We further analyzed the protein expression of iNOS expression in lysates from RAW 264.7 cells activated with LPS and treated simultaneously with increasing concentrations of Celastrol (6.25-1000 nM) for 16 hours. **Figure 1c** shows the Western blot result of the same which correlates with earlier observation of nitrite secretion. Celastrol was able to suppress iNOS protein in cells in dose dependent manner. Based on these observations we chose 500 nM as the effective dose of Celastrol to be used in our experiments. We next tested the effect of this dose in a time dependent manner on iNOS expression. As shown in **Figure 1d** we observed that in RAW 264.7 cells activated with LPS Celastrol was able to suppress iNOS protein level as early as 4 hrs and remained active up to 24 hrs. Next, in order to reconfirm our observation we harvested mouse peritoneal macrophages and activated them with LPS in presence or absence of increasing concentrations of Celastrol for 24 hours. Western blot results shown in **Figure 1e** reconfirm that Celastrol can inhibit LPS induced iNOS protein expression in inflammatory macrophages in dose dependent manner.

**Analyzing the anti-inflammatory properties of Celastrol on LPS induced inflammation in vivo:**

The purpose of the next set of experiments is twofold. One is to analyze the *in vivo* anti-inflammatory effects of Celastrol in mice and second to prepare an edible formulation of this compound, which is effective and nontoxic. To achieve this goal we performed a series of experiments to analyze the efficacy and safety of Celastrol in mice *in vivo*. We did not observe any side effect or toxicity in mice even at very high doses of Celastrol (up to gram quantities). Based on a series of observations and on available literature, we prepared a Celastrol-containing rodent diet which contained either 12.5 ppm Celastrol or 31.25 ppm Celastrol (effective doses roughly = 2 and 5 mg/kg per day, respectively, for a 30 g mouse consuming 5 g diet per day).

As shown in **Figure 2a** (below), C57BL/6 mice were maintained on either control diet or Celastrol-containing diet beginning at 8 weeks of age. At 7 months of age, mice received LPS or vehicle, administered via intra-peritoneal injection and then were euthanized 6 hours later. As depicted in **Figure 2b**, mice receiving Celastrol diet showed significantly lower concentrations of
nitric oxide, as reflected by the combined concentration of nitrite and nitrate in sera as compared to mice fed control diet after LPS treatment. We next tested the gene induction profile of different inflammatory markers such as iNOS, COX-2, IFN-γ, IL1β, IL-6 and TNF-α. In Figure 2c and d, we show a significant decline in expression of these genes in the liver of Celastrol fed animals after LPS treatment compared to control mice as measured by RT-PCR. Results are the mean +/- SEM. **P<0.01, *P<0.05. In all cases, the effect of 2mg/kg B.W. dose of Celastrol didn’t show any significant effect, however exposure to the 2mg/kg B.W. dose of Celastrol led to a significant declined in the profile of inflammatory genes in all mice used in this study.

![Figure 2: Celastrol attenuates inflammatory response to LPS in vivo.](image)

Identifying the molecular mechanisms of action of Celastrol: We next analyzed the potential molecular mechanisms of action of Celastrol, utilizing our mouse model system that harbors a targeted deletion in the Nrf2 gene. Since the other triterpenoids with similar chemical structures (as mentioned in previous report) exert their function in an Nrf2 dependent manner, we tested the hypothesis that Celastrol will also exert its anti-inflammatory properties by activating the antioxidant pathway via Nrf2 activation. As shown in Figure 3a (below), HO-1 expression in lysates from RAW 264.7 cells activated with LPS and treated simultaneously with increasing concentrations of
Celastrol for 16 hours, shows a significant increase in dose dependent manner. Figure 3b depicts the time course of this study where, HO-1 expression in lysates from RAW 264.7 cells activated with LPS were analyzed in the presence or absence of 500 nM Celastrol. These results indicate that the 500 nM dose of Celastrol was sufficient to activate HO-1 as early as 4 hrs, however this effect starts to decline from 16 hrs and remains insignificantly active by 24 hrs. To specifically test our hypothesis if this effect was Nrf2 dependent we next harvested bone marrow-derived macrophages from Nrf2+/+ and Nrf2-/– mice and treated them with LPS in presence or absence of various doses of Celastrol. As clearly shown in Figure 3c, Celastrol was able to induce HO-1 expression in Nrf2+/+ macrophages, however this effect was completely lost in macrophages derived from the Nrf2-/– mouse indicating that, like other triterpenoids, some of the anti-inflammatory properties of Celastrol are truly Nrf2-dependent. We next tested the cytoprotective target mRNA expression in lysates from mouse peritoneal macrophages (derived from Nrf2+/+ and Nrf2-/– mice) and treated with 750 nM Celastrol for 16 hours. As shown in Figure 3d, the induction of genes such as HO-1, NQO1, and catalase by Celastrol significantly depends on the activity of Nrf2 acting through the anti-oxidant response element (ARE) in their gene promoters. In all experiments n = 5 experiments +/- SEM. *P<0.05, **P<0.01. In the next phase of our study we will be analyzing the efficacy of Celastrol in the inhibition of disease pathogenesis in EAE.

Figure 3: Celastrol induces cytoprotective targets in an Nrf2-dependent manner, in vitro.
The role of antigen presenting cell (APC) and T cell interaction during the EAE pathogenesis.

CD11c+ dendritic cells (DCs) are the most potent and effective antigen presenting cell,\(^8\) defined as Ly6C\(^{hi}\)/CD45\(^{hi}\)/Iba-1\(^-\). DCs are bone marrow derived and are virtually non-existent in the CNS parenchyma under non-inflamed conditions.\(^9\) It has also been shown that MHCII-restricted to infiltrating dendritic cells are required for induction and progression of disease in EAE.\(^{10}\) There are 2 critical times within the induction of EAE where DCs play a pivotal role: first during the activation of T cells in the periphery, and second during reactivation of T cells right before entry into the parenchyma. To date, the trafficking and specific interactions of these APCs and T cells within the CNS have not been clearly and fully elucidated. In other tissues during inflammation, T cells interact with selectins, adhesion molecules and chemokines expressed on endothelial cells to home to the site of inflammation. In the CNS, it is thought that T cells require interaction with an APC before entering the parenchyma. The idea that an APC needs to ‘license’ the T cell to enter the CNS is born from the observation that in a chimera model where peripheral cells lack MHCII and microglia have functional MHCII, transferred cells are unable to infiltrate the CNS, whereas in a chimera where microglia lack MHCII and peripheral cells have functional MHCII, these mice develop EAE normally. In addition, transferred activated T cells still require 3 or more days to gain access to the parenchyma even when the recipient is irradiated to cause openings in the BBB. The role of APCs in EAE is still controversial. In contrast to the role of DCs described above, other studies have shown that infiltrating macrophages using the CCL2-CCR2 axis are required for disease.\(^{11}\) Once in the CSF the reactivation of T cells occurs in locations in contact with CSF during inflammation, where the presentation of CNS myelin peptides by meningeal macrophages is postulated to play an important role during the induction of EAE.\(^{12}\) Additionally, it has been shown that increase in circulating inflammatory monocytes correlates with relapses in EAE.\(^{13}\) Finally, the activation of resident microglia has been shown to play a critical role in the destruction of axons in EAE.\(^{14}\) In the studies pursued through this funding period, we aim to understand the mechanism of APC/T cell interaction by standardizing the available mouse model and imaging techniques in our lab, and to analyze whether the protective effects of selected triterpenoids may be attributed to their ability to
influence or modulate this interaction. In order to answer these questions, we first standardized the protocol and techniques to capture this interaction both in vivo and in vitro and later tested the efficacy of new class of triterpenoids in treatment of EAE by potentially disrupting this interaction.

Development of relevant mouse models to assess APC/T cell interaction during EAE: Six- to 12-week old syngeneic female C57BL/6, B6.129P Cx3cr1 GFP/GFP (stock #005582) and B6.Cg-Tg Thy-1-YFP-H (stock #003782) mice (H-2 b ), B6.FVB-Tg CD11c-DTR/GFP mice (stock #004509), C57BL/6-Tg 2D2 mice (stock #006912), B6.129 (ICR)-Tg actin beta CFP mice (stock #004218), and C57BL/6-Tg ubiquitin GFP mice (#004353) were obtained from the Jackson Laboratory (Bar Harbor, ME). B6.129S6 OTII mice (stock #1896) were obtained from Taconic. 2D2 mice expressing the TCR transgene specific for MOG35-55 peptide / I-Ab were crossed with actin beta CFP mice to derive mice expressing 2D2-CFP cells for in vivo tracking by TPM. Similarly, OTII mice expressing the TCR transgene specific for OVA323–339 peptide / I-Ab were crossed with ubiquitin GFP mice to derive OTII-GFP cells for in vivo TPM tracking. Cx3cr1 +/-GFP reporter mice have one Cx3cr1 allele replaced with the gene encoding GFP, and are derived by crossing Cx3cr1 GFP/GFP with C57BL/6. CX3CR1 (fractalkine receptor) is almost exclusively expressed in the microglia population in the CNS of these mice, while NK cells, activated CD8+ T cells, dendritic cells and a subset of monocytes also express the GFP marker in the peripheral tissues. Thy-1-YFP-H mice harbor yellow fluorescent protein (YFP) expression in a subset of neurons in the dorsal root ganglion and cortical layers. Thy-1-YFP-H mice were crossed with Cx3cr1 GFP/GFP mice to obtain double transgenic mice, Thy-1-YFP-H x Cx3cr1 +/-GFP. Animals were housed, bred and handled in the Animal Resource Center facilities at Case Western Reserve University according to approved protocols. Similarly, all animal experiments were executed with strict adherence to active experimental animal protocols approved by Case Western Reserve University Institutional Animal Care and Use Committee. In order to induce passive EAE in these mice, 3x10^6 naïve splenic 2D2 CFP CD4 T cells, isolated by negative depletion using Dynal beads (Life Technologies, Grand Island, NY, USA), were injected into recipient mice. 24 hours later, an emulsion with 200 µg myelin oligodendrocyte
glycoprotein (MOG) peptide 35–55 (MOG35–55; Anaspec, San Jose, CA, USA) with 8 mg/ml H37RA and incomplete Freund’s adjuvant was injected subcutaneously (s.c.) bilaterally on the lower back of the recipient mouse (Hooke Laboratories, Lawrence, MA, USA). Control mice were injected s.c. bilaterally with an emulsion of PBS, 8 mg/ml H37RA and incomplete Freund’s adjuvant. Pertussis toxin (100 ng) was administered by intraperitoneal (i.p.) injection on days 0, 1 and 2. To determine whether myelin specific T cells require antigen-specific APCs for recruitment to the CNS, EAE was actively induced using a combination of two antigenic peptides in 8-12 week old female recipient mice. 3x10^6 naïve 2D2-CFP and 3x10^6 naïve OTII-GFP CD4+ T cells were isolated separately by negative depletion using Dynal beads and co-injected into naïve recipient mice. Next, 24 hours later, an emulsion of either 200 µg MOG35-55 only, 10 µg OVA323-339 only, or of 200 µg MOG35-55 and 10 µg OVA323-339 combined (Anaspec, San Jose, CA, USA) with 8 mg/mL H37RA (Difco Laboratories, Detroit, MI, USA) and incomplete Freund’s adjuvant (Difco Laboratories, Detroit, MI, USA) was injected s.c. bilaterally on the lower back of the recipient mice. Control mice were injected s.c. bilaterally with an emulsion of PBS, 8 mg/mL H37RA and incomplete Freund’s adjuvant. 100 ng Pertussis toxin (List Biological Laboratories, Inc, Campbell, CA, USA) was administered i.p. on days 0, 1 and 2 to induce EAE.

Presentation of myelin antigens by CNS APCs enhances activated myelin specific T cell entry and retention in EAE: We first investigated the timing and antigen requirement of 2D2 cell recruitment and behavior within the CNS (Figure 4). We co-injected equal number of differentially labeled naïve OTII and 2D2 cells into recipient mice followed by immunization with bothOVA323-339 and MOG35-55 peptides in the presence of CFA and PTx (Figure 4a). Significantly more 2D2 cells began to infiltrate the brain parenchyma as early as day 3 and day 6, even though both T cell populations were found in equivalent numbers within the peripheral lymph nodes (Figure. 4c). The majority of the OTII cells found within the CNS remain in the perivascular location near sites of previous vessel leaks, migrated with a reduced speed as compared with2D2 cells (Figure. 4b), and remain in the CNS only transiently (data not shown). However, when mice infused with naïve OTII
and 2D2 cells were immunized only with OVA323-339, we saw a near complete absence of infiltrating 2D2 or OTII cells in either the meninges or the CNS parenchyma. This absence of activated myelin-specific T cells disrupts this intricate cellular and structural collaboration, resulting in a complete absence of effector cell infiltration and disease induction (data not shown), even though vessel leaks, phagocytic APC accumulation and robust activation of non-CNS specific T cells were all present within the host. Taken together, our data imply that the cooperation between PTx-induced leaky vessels, resident and infiltrating APCs, the availability of CNS-derived antigens and activated myelin-specific T cells are preconditions for MOG-specific T cell entry into the CNS, which in turn amplifies other cellular infiltration later in the disease progression.

Triterpenoids (CDDO-DFEA) effectively suppress EAE clinical symptoms if administered during the course of APC/T cell interaction: We know that the interaction between dendritic cells (DCs) and T cells is required for initiation of disease pathogenesis in the EAE model. As discussed above, we now know that APC/T cell interaction happens usually within 5 to 7 days after EAE induction by MOG-35 immunization, although mice start typically to exhibit clinical symptoms of the
disease around 3 weeks later. In order to assess
the efficacy of triterpenoids in altering APC/T cell
interactions, we treated mice with one of our most
effective triterpenoid (CDDO-DFEA) within the first
week of EAE induction.

As shown in Figure 5 and Figure 6 we
found that CDDO-DFEA not only increased the
survival of mice after EAE induction but also
ameliorated the clinical symptoms of EAE, if mice
were treated during the course of APC/T cell
interaction i.e. within the first week of EAE
induction. Interestingly, CDDO-DFEA
also increased survival and
protected against clinical pathology
of EAE in Nrf2 -/- mice. This
observation supports the concept
that these compounds are
multifunctional and that the observed
therapeutic effects are in part
exerted via Nrf2-independent
mechanisms triggered by CDDO-
DFEA during this process. We next
queried whether the triterpenoids
merely impaired the T cell/APC
interaction, and /or influenced
antigen presentation by APC, and/or
suppressed antigen-induced T cell

Figure 5: CDDO-DFEA treatment increased
the survival rate in the EAE model. Mice (8-12
weeks old; n=7/group) were immunized
with MOG and treated with CDDO-DFEA or
PBS from day 5 for one week. The survival
rate of mice recorded, daily (Kaplan-Meier
survival curve) for 1 month.

Figure 6: CDDO-DFEA reduced the severity of EAE. 7
mice (8-12 weeks old) of each group were induced EAE by
subcutaneous immunization on both flanks with Complete
Fruend’s adjuvant. Pertussis toxin was injected by intra-
peritoneal (i.p.) at the time and after 48 hours later. CDDO-
DFEA groups were daily treated 200 nM CDDO-DFEA
from day 5 for a week. Mice were daily monitored for
clinical scores of EAE: 0-no sign of disease; 1-limp tail; 2-
moderate hindlimb weakness; 3-severe hindlimb weakness; 4-
complete hindlimb paralysis; 5- quadriplegia or moribund state; 6-death.
proliferation. As shown in Figure 7, the effects we have observed thus far are not a consequence of impaired dendritic cell activation as evidenced by unaltered expression of co-stimulatory molecules, such as CD80, CD86, and MHCII in bone marrow derived dendritic cells (BMDCs) after exposure to LPS ± triterpenoids.

![Figure 7: CDDO-DFEA has no effect on co-stimulatory factor expression in LPS treatment DCs. BMDCs were incubated with LPS (1 µg/ml) for 24 hrs after CDDO-DFEA (200 nM) pretreatment for 1 hr. Co-stimulatory factors are expressed on cell membranes during DC activation by LPS treatment. CD80, CD86, and MHC II were measured by Flow Cytometry.](image)

**Key research accomplishments:**

Within the first two year of this project period we have accomplished the following milestones:

- Developed a method to isolate large quantities of Bryonolic acid (BA) and Celastrol
- Established the biological activity of purified BA and Celastrol both in vivo and in vitro
- Development of potent/active compounds by applying DOS chemistry on parent/natural triterpenoids
- Establishing the Nrf2-mediated mode of action of the selected triterpenoids
- Establishing the relevant EAE mouse models and methods to study the effects of triterpenoids on T cell/APC interactions
- Demonstrated the efficacy of selected triterpenoids in preclinical mouse models of MS

**Conclusions:**

- Both BA and Celastrol are very well tolerated in mouse models and are less toxic, natural triterpenoids.
- Diversification of the triterpenoid skeletal structure through DOS resulted in molecules with a wide spectrum of anti-inflammatory and antioxidant activity.
• The core skeleton structure of triterpenoids dictates their activity and skeletal rearrangement of the carbocyclic triterpenoid in combination with optimization of the A-ring resulted in the identification of effective and structurally diverse Nrf2 activators.

• We have identified a few potent compounds 14f- BA, 26f-BA, Celastrol and CDDO-DFEA which we will use for the remainder of our studies.

• APCs begin to infiltrate the brain parenchyma as early as day 3 and day 6 after EAE induction.

• Cooperation between PTx-induced leaky vessels, resident and infiltrating APCs, the availability of CNS-derived antigens and activated myelin-specific T cells are preconditions for MOG-specific T cell entry into the CNS, which in turn amplifies other cellular infiltration later in the disease progression.

• Treatment with CDDO-DFEA during APC/T Cell interaction period of EAE disease course is very effective in suppressing disease pathology. However, APC activation as measured by up-regulation of cell surface expression of co-stimulatory molecules is not influenced by exposure to the either the natural triterpenoid Celastrol or to synthetic triterpenoids such as CDDO-DFEA.

• The protective effect of CDDO-DFEA is not solely dependent on Nrf2 signaling and it may act through other alternative signaling pathways.

Publications, Abstracts, and Presentations:

The following publications are related to the information presented in this report and/or are included to provide additional information regarding relevant research activity in the laboratories of project co-investigators:


**Inventions, Patents, and Licenses:** Nothing to report.

**Reportable outcomes:**

The strong collaboration among Letterio, Tochtrop and Huang laboratories have resulted in the development of new triterpenoids, mouse imaging techniques and biochemistry and chemical library construction. For example, work pursued in the Huang lab is leading to the development of new experimental CNS imaging techniques that will enhance our capacity to image the key steps in EAE pathogenesis and understand how small molecules like triterpenoids act to disrupt and prevent disease. An example published by the Huang lab in Nature Protocols can be viewed at this link: [http://www.nature.com/nprot/journal/v5/n2/full/nprot.2009.222.html](http://www.nature.com/nprot/journal/v5/n2/full/nprot.2009.222.html)

Related references from the Huang Lab:


Our labs are independently interested in triterpenoids: the Tochtrop lab from the point of view of the chemistry, biochemistry, and biodiversity, the Letterio lab from the point of view of immune modulation, preclinical disease models, and disease therapy and prevention and Huang lab in cellular immunology. With the outstanding leadership provided by Senior Research Scientists for both the chemical (Yong Han) and biological (Tej Pareek) aspects of the proposal, we have a very strong research team to complete the outlined studies.

**Other Achievements:**

Collaboration around this project resulted in the successful completion of the PhD theses of three graduate students in each lab (Tonibelle Gatbonton-Schwager, Ph.D., Emily Barker, Ph.D. in the Departments of Pharmacology and Chemistry, working the Tochtrop and Letterio laboratories, and Deborah, Barkauskas, Ph.D., Department of Biomedical Engineering, working in the Huang Laboratory) committed to joint projects. The bulk of the work presented in this annual report reflects the accomplishments presented in the PhD theses of Emily Barker & Deborah Barkauskas).

**References:**


Appendices: None