

Implications of Withaferin A in neurological disorders

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Extract from the medicinal herb *Withania somnifera* (WS), commonly known as Ashwagandha, has therapeutic implications in various conditions such as infection, inflammation, cancer, stress, neurodegenerative diseases, heart disease, diabetes, and others. Withaferin A (WA), a relatively nontoxic steroidal lactone isolated from WS extract, has often been tested for its anti-tumor properties (Dar et al., 2015; Marlow et al., 2017). It has been demonstrated that WA may also serve as a neuroprotectant in a number of neurological conditions, including Parkinson's disease (PD), cerebral infarctions (CI), reactive gliosis, amyotrophic lateral sclerosis (ALS), and human immunodeficiency virus-associated neurological dysfunctions (HIV-AND).

One of the major challenges in finding treatments for brain-related diseases is the inability of potential therapeutics to pass through the blood-brain barrier (BBB). Interestingly, data from multiple studies demonstrate that WA has the ability to penetrate the BBB. WA has also been successfully administered both orally and intraperitoneally in various animal models (Dar et al., 2015). Furthermore, a recent study revealed that orally administered WA was well-tolerated by patients in a phase I clinical trial for treatment of osteosarcoma (Pires et al., 2020). Based on these findings, WA is a drug candidate worthy of further investigation for the treatment of neurological conditions. Here, we briefly discuss the implications of WA as a neuroprotectant in several neurological disorders.

PD: Common characteristics of PD include loss of dopaminergic neurons in the substantia nigra, rigid muscles, unstable posture, slowed movement, and frequent tremors throughout the body. Abnormalities in leucine-rich repeat kinase 2 (LRRK2) activity and aggregation of α -synuclein (α -syn) have been reported as two major molecular hallmarks of PD. Heat shock protein 90 (Hsp90) and co-chaperone cell division cycle 37 form a complex to stabilize and interact with multi-domain protein LRRK2. In endogenously expressing LRRK2 N9 microglial cells, treatment with 2 μ M WA led to an 80% reduction of LRRK2 with only 16% cytotoxicity. Clearance and destabilization of LRRK2 by WA is suggested to occur by disruption of the Hsp90-cell division cycle 37 complex (Narayan et al., 2015b). Furthermore, a study presented at University of South Florida Health Research Day 2017 from our lab (published online at eposter.net: <https://www.eposters.net/poster/characterizing-the-effect-of-withaferin-a-on-alpha-synuclein>) showed that 2 μ M WA treatment not only led to clearance of wild-type α -syn, but also to A30P, E46K and A53T point mutants of α -syn in stably transfected M17 neuroblastoma cell models. In another study, a stable isotope labelling by amino acids in cell culture-based proteomics analysis of WA-treated mouse microglial cells showed an increase in the sequestosome-1/p62 complex, a key regulator of the autophagy pathway, upon 2 μ M WA treatment (Narayan et al., 2015a). Interestingly, our presented work that showed clearance of α -syn also showed an increase in p62 levels. Overall, these results indicate that WA-mediated clearance of α -syn could be facilitated by p62. More studies are necessary to effectively understand this relationship.

Additionally, WA treatment can potentially improve the integrity of motor neurons, which are known to functionally decline with the progression

of PD due to oxidative damage. A study using a rat model assessed the effects of WA on age-mediated locomotor and neurotransmitter alterations (Banu et al., 2019). Motor impairments in aged rats are correlated with low levels of dopamine and homovanillic acid in the midbrain and striatum. Age-mediated decline of dopamine and locomotion in aged rats could be due to the accumulation of reactive oxygen species. When aged rats were administered 50 mg/kg of WA by oral gavage, levels of both homovanillic acid and dopamine were markedly increased. This suggests that WA possesses anti-oxidant properties or free radical scavenging potential to reverse oxidative damage and improve motor function. Moreover, coordination, balance, and motor impairments in aged rats treated with WA markedly improved (Banu et al., 2019). These findings highlight the neuroprotective function of WA in ameliorating pathological hallmarks of PD through reduction in both disease-associated protein aggregates and motor impairment.

CI: CI is a condition characterized by neuronal cell death in the brain and is well-documented in carotid and vertebralbasilar ischemic stroke. WA has been suggested to protect against cellular damage associated with CI (Zhang et al., 2017). In male rats, treatment of induced cerebral infarction with orally administered WA led to significant reduction in the infarct area in a dose-dependent manner (25, 50, 100 mg/kg). Additionally, intimal hyperplasia, which is directly linked to cerebral tissue damage and ischemia, is a result of abnormal vascular smooth muscle cell migration and proliferation. Moreover, the process of cell migration is critically affected by matrix metalloproteinases. A platelet-derived growth factor-stimulated A7r5 (vascular smooth muscle cell) cell model-based study suggests that WA treatment not only reduces matrix metalloproteinase expression, but also cell migration and viability in a dose-dependent manner when exposed to 75, 150, and 300 μ g/mL WA.

The phosphoinositide 3-kinase/Akt (PI3K/Akt) pathway is hypothesized to play an important role in neuroprotection against damage caused by ischemia-induced apoptosis. Assessment of PI3K/Akt pathway proteins in a carotid ligation animal model upon 25, 50, or 100 mg/kg b.w. WA treatment showed suppression of pro-apoptotic phosphatase and tensin homolog and increased phosphorylation of anti-apoptotic Akt, glycogen synthase kinase-3 beta, cyclinD1, p65 and mTORC1. These findings indicate that WA may provide neuroprotective effects via activation of the PI3K/Akt pathway. Additionally, WA treatment following induced injury led to a decrease in the expression of activated caspase-3 and an increase in the expression of anti-apoptotic proteins Bcl-xL and Bcl-2. Therefore, WA has the potential to serve as a neuroprotectant in ischemic injuries via reducing apoptosis, vascular smooth muscle cell migration and proliferation, and cerebral infarct area (Zhang et al., 2017).

Reactive gliosis: It is also known as astrocyte reactivity, is a common indicator of many early-stage neurodegenerative diseases, including Alzheimer's disease (AD), PD, and ALS. Stress or injury induces characteristic changes in astrocytes, such as release of cytokines and higher expression and polymerization of both vimentin and glial fibrillary acidic protein, type III intermediate

filaments. To study the therapeutic effect of WA on reactive gliosis, Livne-Bar et al. (2016) used a combination of two different models, a kainic acid-induced retinal injury mouse and a debridement-induced astrocyte reactivity mouse. They found that intravitreal injections of 2 mg/kg WA not only led to the clearance of glial fibrillary acidic protein and vimentin, but also a reduction in neuronal apoptosis. Additionally, in retinal astrocyte cultures, both vimentin and glial fibrillary acidic protein displayed shorter aggregate formation and reduced filament signal intensity upon 2 μ M WA treatment (Livne-Bar et al., 2016).

Furthermore, cytokines released from astrocytes following excitatory injury or disease have been linked to neuronal apoptosis. A proinflammatory cytokine and extrinsic apoptotic cascade inducer, tumor necrosis factor- α , was found to be significantly reduced upon treatment with 2 μ M WA in cultured astrocytes. This reduction in tumor necrosis factor- α was shown to be dependent on p38 mitogen-activated protein kinase signaling. These findings imply that WA serves as a neuroprotectant for retinal neurons that are susceptible to excitotoxic injury as a result of reactive gliosis by reducing neuronal cell death and inflammatory responses (Livne-Bar et al., 2016).

ALS: ALS is characterized by the fatal degeneration of muscles, which can lead to death within 2–5 years of disease onset. Several cases of familial and sporadic amyotrophic lateral sclerosis are linked to abnormalities in Cu/Zn superoxide dismutase (SOD1). Upon intraperitoneal administration of 4 mg/kg WA, mouse models harboring SOD1^{G93A} and SOD1^{G37R} mutants showed a reduction in neuroinflammation, a decrease in misfolded SOD1 species, and an extended lifespan. The anti-inflammatory effects of WA are supported by an increase in anti-inflammatory cytokine levels, such as interleukin-10, a decrease in expression of pro-inflammatory cytokine granulocyte macrophage colony-stimulating factor, and a decrease in astrogliosis and microgliosis. Furthermore, WA treatment in mice harboring SOD1^{G93A} mutation resulted in an increase in Hsp25 and Hsp70 levels. Hsp25 upregulation, possibly leading to the reduction of misfolded SOD1, may lead to the observed increase in motor neuron survival. Additionally, assessment of growth-associated protein 43, an early neuronal injury biomarker, using a double transgenic growth-associated protein 43-luc/gfp/SOD1^{G93A} mouse model showed a decrease in growth-associated protein 43 levels upon 4 mg/kg WA treatment. This decrease in neuronal injury response also supports the evidence that WA increases the survival of motor neurons (Patel et al., 2015).

Aggregation of transactive response DNA-binding protein 43 (TDP-43) in the brain and spinal cord is considered a feature of some familial and sporadic ALS cases. Co-immunoprecipitation data suggests that TDP-43 interacts with nuclear factor-kB p65, a modulator of p38 mitogen-activated protein kinase-dependent apoptosis, in sporadic ALS cases. This interaction has also been established in TDP-43 transgenic mouse models, as well as mouse neuroblastoma Neuro2a cells overexpressing p65 and TDP-43^{WT}. Particularly, TDP-43 was found to be a co-activator of p65. Treatment with 1 μ M WA, a known inhibitor of nuclear factor-kB, led to neuronal protection from TDP-43-mediated cell death in primary cortical neurons isolated from wild-type or mutant TDP-43 expressing transgenic mice. Additionally, WA-treated TDP-43^{WT} mice exhibited improved mobility and reduced neuroinflammation. This was supplemented with reduction in both peripherin aggregation and partially denervated neuromuscular junctions, two phenomena common in ALS (Swarup et al., 2011). This evidence indicates that WA serves a significant neuroprotective function in the pathogenic progression of ALS by alleviating neuroinflammation, increasing neuronal survival,

Received: February 1, 2020
 Peer review started: February 22, 2020
 Accepted: April 26, 2020
 Published online: August 24, 2020

<https://doi.org/10.4103/1673-5374.290894>
 How to cite this article: Ram N, Peak SL, Perez AR, Jinwal UK (2021) Implications of Withaferin A in neurological disorders. *Neural Regen Res* 16(2):304-305.

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Open peer reviewer: John Mitrofanis, University of Sydney, Australia.

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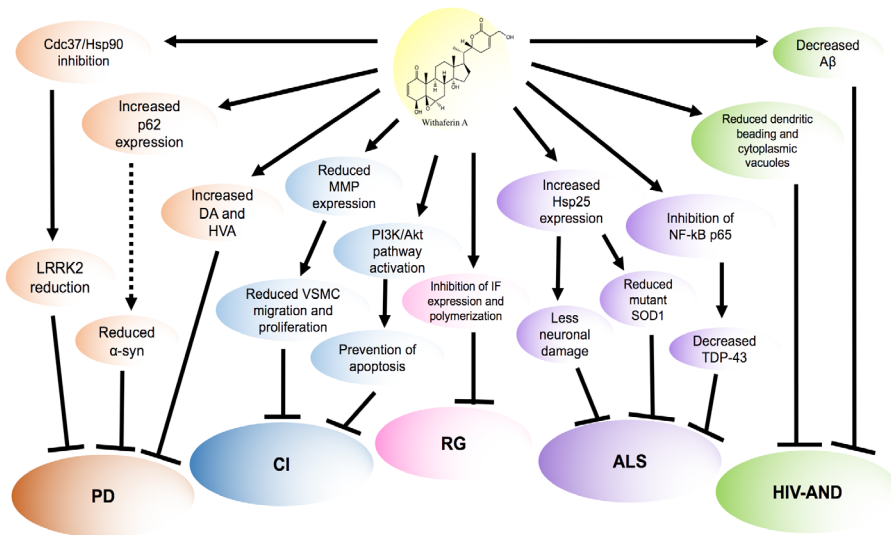


Figure 1 | Proposed neuroprotective role of Withaferin A (WA) in the modulation of pathways implicated in neurological abnormalities and dysfunctions.

Parkinson's disease (PD): WA is a known inhibitor of the Cdc37/Hsp90 complex. Inhibition of Cdc37/Hsp90 has resulted in a decrease of pathological protein LRRK2 in cellular models. WA exposure also increased the expression of p62 intracellularly, which could potentially be responsible for decreased levels of α -synuclein. WA also rescued low levels of dopamine (DA) and homovanillic acid (HVA) to improve motor deficits. Cerebral infarction (CI): *In vitro* WA treatment reduced expression of matrix metalloproteinases (MMPs), which mitigate VSMC migration and proliferation. *In vivo* the PI3K/Akt pathway was activated through induced CI, leading to a prevention of apoptosis. Reactive gliosis (RG): inhibition of both expression and polymerization of intermediate filaments (IFs) by WA led to a decrease in astrocyte reactivity and neuronal apoptosis. Amyotrophic lateral sclerosis (ALS): WA induced expression of Hsp25, which led to a decrease in mutant superoxide dismutase 1 (SOD1) and a decrease in neuronal apoptosis. Suppression of nuclear factor kappa B (NF- κ B) by WA is linked to clearance of transactive response DNA-binding protein 43 (TDP-43). Human immunodeficiency virus-associated neurological dysfunctions (HIV-AND): WA treatment lowered amyloid beta ($A\beta$) aggregation in SH-APP cells. Reduced dendritic beading and cytoplasmic vacuoles were also shown in cells treated with WA. Uncertain until further study ($\cdots\rightarrow$), leads to (\rightarrow), prevents (\dashv).

decreasing levels of misfolded proteins, and reducing neuronal injury responses.

HIV-AND: Oftentimes, older individuals with HIV develop AD or pathologies resembling AD. With a rising elderly HIV-positive population, there is a greater prevalence of neurocognitive dysfunction. One of the major causes of neuronal toxicity seen in AD and AD-like pathologies is the aggregation of extracellular amyloid beta ($A\beta$). Increased $A\beta$ accumulation is reported in HIV-positive patients. Additionally, cocaine use has been shown to augment $A\beta$ secretion and neurocognitive abnormalities in HIV cases.

Treatment with 2 μ M WA led to reduction of both neurotoxin protein HIV-1 Tat-induced and cocaine-induced $A\beta$ aggregates in SH-APP cells, a neuroblastoma cell line stably expressing amyloid precursor protein. Notably, 2 μ M WA treatment resulted in a significant decrease in $A\beta$ levels without causing cytotoxic effects. It is also suggested that WA decreases cocaine-induced dendritic beading and cytoplasmic vacuoles, two signs of neurotoxicity. This demonstrates that WA potentially reverses the neuronal damage caused by cocaine abuse in HIV cases through the clearance of protein accumulation. However, this neuroprotective implication requires further investigation to confirm WA's efficacy *in vivo* (Tiwari et al., 2018).

BBB permeability: The ability of a chemical to pass through the BBB is an important factor for its effectiveness in the treatment of neurological disorders. Lipinski's Rule of Five is effective at assessing the BBB permeability of orally administered drugs. WA obeys the criteria of Lipinski's Rules based on its below-500 Da molecular weight and other chemical properties, such as lipophilic structure. The ability to penetrate the BBB is also supported by WA's favorable blood/brain partition coefficient (Marlow et al., 2017). An *in silico* study further provided evidence for this permeability; a BBB predictor identified that WA has the ability to diffuse across the BBB from

a group of WS phytochemicals known to satisfy Lipinski's Rules (Kumar and Patnaik, 2016). In an *in vivo* study using a human glioma xenograft mouse model, Santagata et al. (2012) demonstrated WA's potential to enter the central nervous system and reach the diseased area with administration of 12 mg/kg WA intraperitoneally. Furthermore, WA's ability to penetrate the BBB is also evident from a study mentioned above in the PD section, where an increase in levels of dopamine and homovanillic acid in the midbrain of aged rats was observed upon oral administration of WA (Banu et al., 2019).

Collectively, the various findings discussed here demonstrate that WA works as a neuroprotective agent in multiple neurological conditions, affecting processes such as cell migration, protein aggregation, apoptosis, and inflammation (Figure 1). Additionally, WA's ability to overcome the BBB signifies its importance as a potential molecule for the treatment of neurological disorders. Notably, the phase I clinical trial conducted to evaluate the pharmacokinetics and safety of orally administered WA in late stage osteosarcomas reported a good safety profile due to the observation of Grade 1 and 2 adverse effects with tolerable doses (Pires et al., 2020). This study reveals WA's potential for human use. Overall, it would be worthwhile to pursue WA for further studies in neurological diseases and ultimately in human trials.

The authors thank Taneja College of Pharmacy, College of Arts and Sciences, and Office of Undergraduate Research, USF for providing supports.

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