COVER SHEET

TITLE: The Effects of Withaferin A on Mouse Models of Alexander Disease

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ABSTRACT

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Alexander disease is a rare neurodegenerative disorder which is caused by the accumulation of glial fibrillary acidic protein (GFAP) resulting in aggregates termed Rosenthal Fibers (RFs) in the central nervous system. TAR DNA-binding protein 43 (TDP-43) is a DNA/RNA binding protein that is involved in the pathology of disorders such as amyotrophic lateral sclerosis (ALS) as well as Alexander disease. Withaferin A (WA) is a plant derivative and known inhibitor of NF-κB, and activated NF-κB targets the GFAP promoter. We proposed that treatment of the mice with WA should down-regulate the GFAP promoter. We treated 10 week old Alexander disease mutation GFAP knock-in mice with WA twice a week for 4 weeks. After treatment we analyzed the amount of GFAP protein concentration with an ELISA. Results showed an initial significant decrease in GFAP, but after repeating the experiment and analyzing specific brain regions, treatment was determined to be slightly toxic.

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Abstract

Alexander disease is a rare neurodegenerative disorder caused by the accumulation of glial fibrillary acidic protein (GFAP) which results in aggregates termed Rosenthal Fibers in the central nervous system. TAR DNA-binding protein 43 (TDP-43) is a DNA/RNA binding protein that is involved in the pathology of amyotrophic lateral sclerosis as well as Alexander disease. It is hypothesized that deregulated TDP-43 co-activates NF-κB, which then targets the GFAP promoter. We proposed that treatment of mice with Withaferin A, a known inhibitor of NF-κB, should therefore inhibit activation of the GFAP promoter. We treated 10 week old Alexander disease mutation GFAP knock-in mice with WA twice a week for 4 weeks. After treatment, the concentration of GFAP was analyzed with an ELISA. Results showed an initial significant decrease in GFAP in mutant mice, but after repeating the experiment and analyzing specific brain regions, treatment was determined to be slightly toxic.

Intro:

Alexander disease is caused by a dominant gain of function mutation in *gfap,* the accumulation of the resultant protein creates characteristic aggregates of the disease termed Rosenthal Fibers (Quinlin, *et al.* 2007; Messing, *et al.* 2012). Rosenthal fibers are made up of multiple components including heat shock proteins, α-B-crystallin and many other proteins (Quinlin, *et al.* 2007; Messing, *et al.* 2012). While rare, this disorder is fatal, the age of onset is variable, with the most common onset recorded in the literature occurring in the infantile years (0-2 years) (Quinlin, *et al.* 2007). Common clinical manifestations during this time period of the disease include frontal leukodystropy, macrocephaly, seizures and developmental delays (Quinlin, *et al.* 2007). There are some reported cases of Alexander disease onset in the adult years (>12yrs) but the amount of cases is generally considered under-reported due to the high likelihood that individuals are misdiagnosed with other neurodegenerative disorders (Quinlin, *et al.* 2007). While Alexander disease is rare, the insights into how inhibitory compounds such as

WA can address neurological effects can be applied to more common neurological disorders (Messing, *et al.* 2012).

In astrocytes with high levels of GFAP, there were correspondingly high levels of TDP-43 at a similar level of severity (Walker, *et al.* 2014). While TDP-43 is normally found in the nucleus of cells, it is detected in the cytoplasm of diseased neurons and glial cells in ALS and Alexander disease (Swarup, *et al.* 2011, Walker, *et al.* 2014). While the basis for the WA study was done on mutant TDP-43 mice to study ALS, TDP-43 pathology is relevant to Alexander disease as well. TDP-43 was phosphorylated and mis-localized in the astrocytes of Alexander disease mice and increased TDP-43 pathology corresponded to abundant Rosenthal fibers as well as co-localization with GFAP (Swarup, *et al.* 2011; Walker, *et al.* 2014). While the role of TDP-43 and how it contributes to both ALS and Alexander disease is not known, it is reasoned that TDP-43 accumulation has the same effect as GFAP accumulation, in that the pathology creates positive feedback loops that hinder protein degradation and disrupt protein synthesis (Swarup, *et al.* 2011; Messing, *et al.* 2012).

It is also hypothesized that TDP-43 is a co-activator of NF-κB, and with deregulation of TDP-43 as seen in these neurodegenerative disorders, there is more binding opportunity for TDP-43 to interact with p65, a protein subunit of NF-κB (Swarup, *et al.* 2011). TDP-43 and p65 co-localize in astroglial cells as well; and activated NF-κB in turn targets GFAP promoter activity (Swarup, *et al.* 2011). WA, a plant derivative with anti-tumor and anti-inflammatory properties is a known inhibitor of NF-κB (Oh, *et al.* 2008). The activation of NF-κB requires dissociation from IκB, an inhibitory molecule, it is proposed that WA stabilizes and prevents degradation of IκB, which is required in order to 'free' NF-κB and allow it to travel to the nucleus and target the GFAP promoter (Oh, *et al.* 2008; Swarup, *et al.* 2011). In the mutant TDP-

43 mice, WA caused a reduction in GFAP expression as well as down-regulation of GFAP promoter activity as determined with a luciferase assay (Swarup, *et al.* 2011). Because less NF-κB was entering the nucleus with the WA stabilized inhibitor, there was less active p65 and therefore less of an interaction between p65 and TDP-43 (Swarup, *et al.* 2011). It was also found that WA inhibition of NF-κB reduced microglia toxicity and cell death in neurons over expressing TDP-43 (Swarup, *et al.* 2011).

Since GFAP expression is an indicator of Alexander disease severity, we propose that by treating mouse models with the GFAP Alexander disease point mutation (GFAP-R236H) with WA, an inhibitor of NF-κB, that there will be less GFAP accumulation and therefore less aggregates and a reduction of the positive feedback loop impacting degradation and synthesis of necessary proteins (Quinlin, *et al.* 2007; Messing, *et al.* 2012; Walker, *et al.* 2014). Reduced toxicity of TDP-43 as well as GFAP is a therapeutic option for treatment of Alexander disease.

Methods:

To test whether GFAP was reduced with WA treatment in an inhibitory manner, we treated mice with the Alexander disease GFAP mutation (GFAP-R236H) twice a week with either the drug or a negative control of water and saline (0.9%) (Swarup, *et al.* 2011).

The treatments were started in the mice between 6 and 7 weeks of age, and the mice were treated for 4 weeks. The late treatment start was to determine if GFAP accumulation can be prevented and/or reversed. Each individual mouse received either the drug or control, and treatment and analysis was administered blind of the genotype. This was a 10ul/g of body weight intraperitoneal (IP), into the body cavity, injection. The mice were sacrificed by CO_2 inhalation 12-24 hours after the final injection.

Two rounds of this experiment were conducted, with the first trial of mice used for half brain analysis, and the second trial analyzed by brain region. The regions collected were the cervical spinal cord, olfactory bulb, hippocampus and cerebral cortex. These regions were chosen based on the location of common manifestations of the disease. For both treatment groups, the brains were collected, with one half being used for protein analysis and the other for mRNA. To determine the amount of mRNA, quantitative PCR (qPCR) was used after Trizol RNA extraction, with a SYBR green indicator and normalized to ribosomal 18S as well as mGFAP. For the ELISA protein analysis, we used an anti-rabbit antibody and horseradish peroxidase (HRP) to determine concentration via a color change. The data was analyzed using GraphPad Prism 5.01 (GraphPad Software). Comparisons among the four experimental groups were done using one-way ANOVA with post Bonferroni t-tests for 4 selected comparisons.

Results

For the first trial of the experiment, the half-brains of the GFAP mutant mice ($GFAP^{+/R236H}$) was found to contain less GFAP per amount of protein in the group receiving treatment than the group receiving vehicle (Figure 1). This decrease in protein was found to be significant ($P \leq 0.01$). In the wild-type mice, the same trend is evident, with a smaller amount of GFAP protein in the group receiving treatment, but this difference is insignificant (Figure 1).

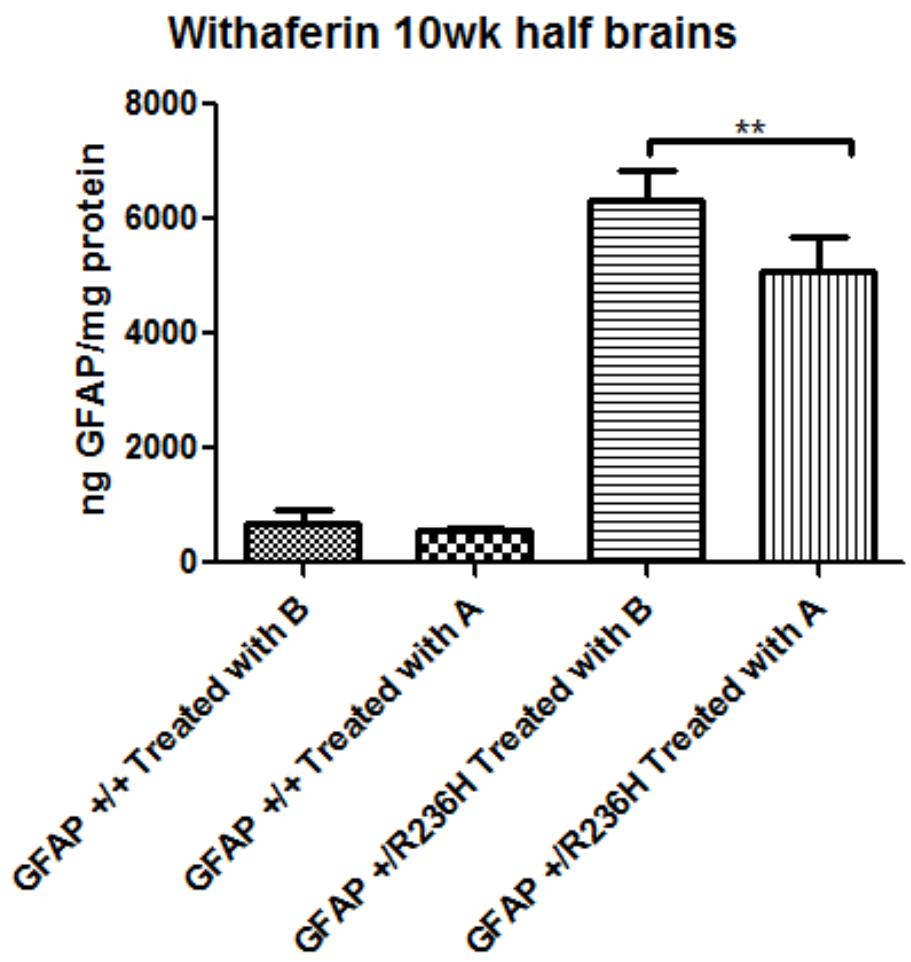

Figure 1: Half Brain Analysis for GFAP in 10 Week Old Wild-type and GFAP+/R236H Mutant Mice The wild-type (*GFAP* $^{+/+}$) mice are listed on the left, and the mutant (*GFAP*$^{+/R236H}$) mice are listed on the right. The saline vehicle was B, and the Withaferin A treatment was A. Bars represent averages across genotype and treatment, with error bars +/- SD of the mean. N=18.

For the second trial of the experiment, all brain regions were found to have differing levels of GFAP. The hippocampus (Hipp) showed more GFAP in the mutant mice compared to wild-type. There was an insignificant difference in the amount of GFAP between the treatment and vehicle for the mutant mice (Figure 2a). The difference in the amount of GFAP in the wild-type mice for both treatment and vehicle was insignificant. The cerebral cortex (cc) showed

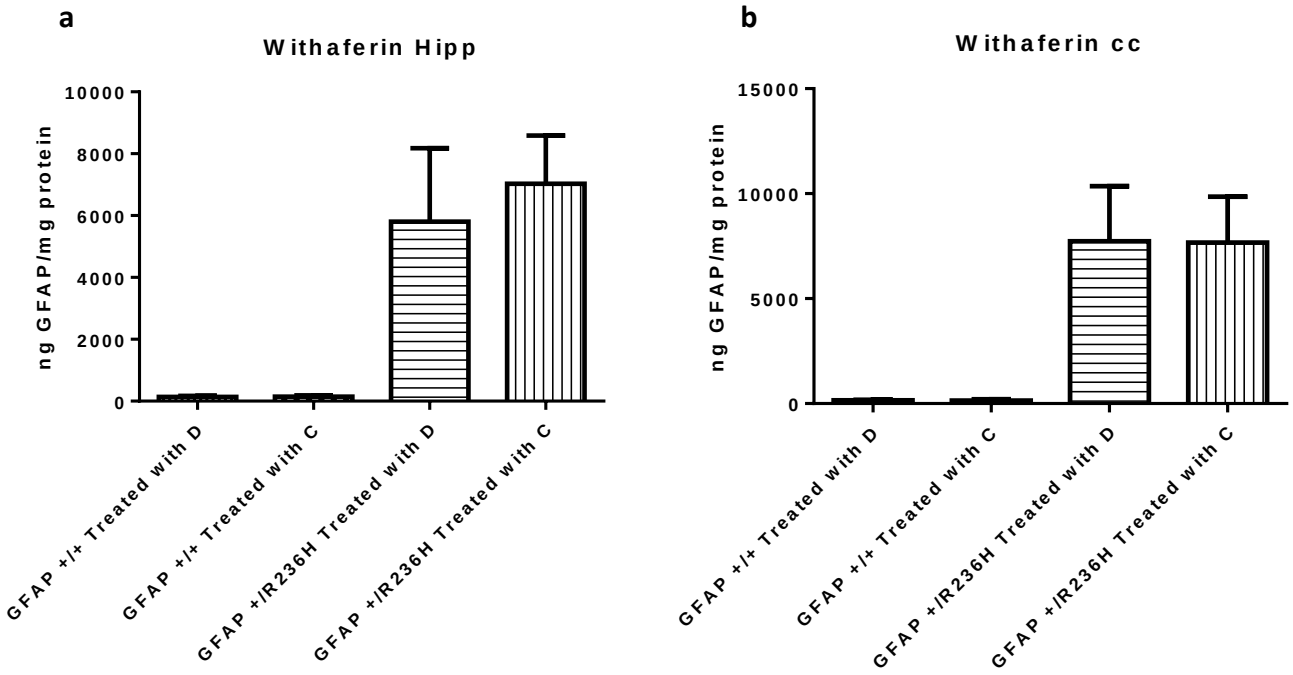

Figure 2(a and b): Brain Region Analysis for the Hippocampus (Hipp) and Cerebral Cortex (cc) of wild-type and GFAP+/R236H Mutant Mice. The wild-type (*GFAP* $^{+/+}$) mice are listed on the left, and the mutant (*GFAP*$^{+/R236H}$) mice are listed on the right. The saline vehicle was D, and the Withaferin A treatment was C for both graphs. Bars represent averages across genotype and treatment, with error bars +/- SD of the mean. N=34

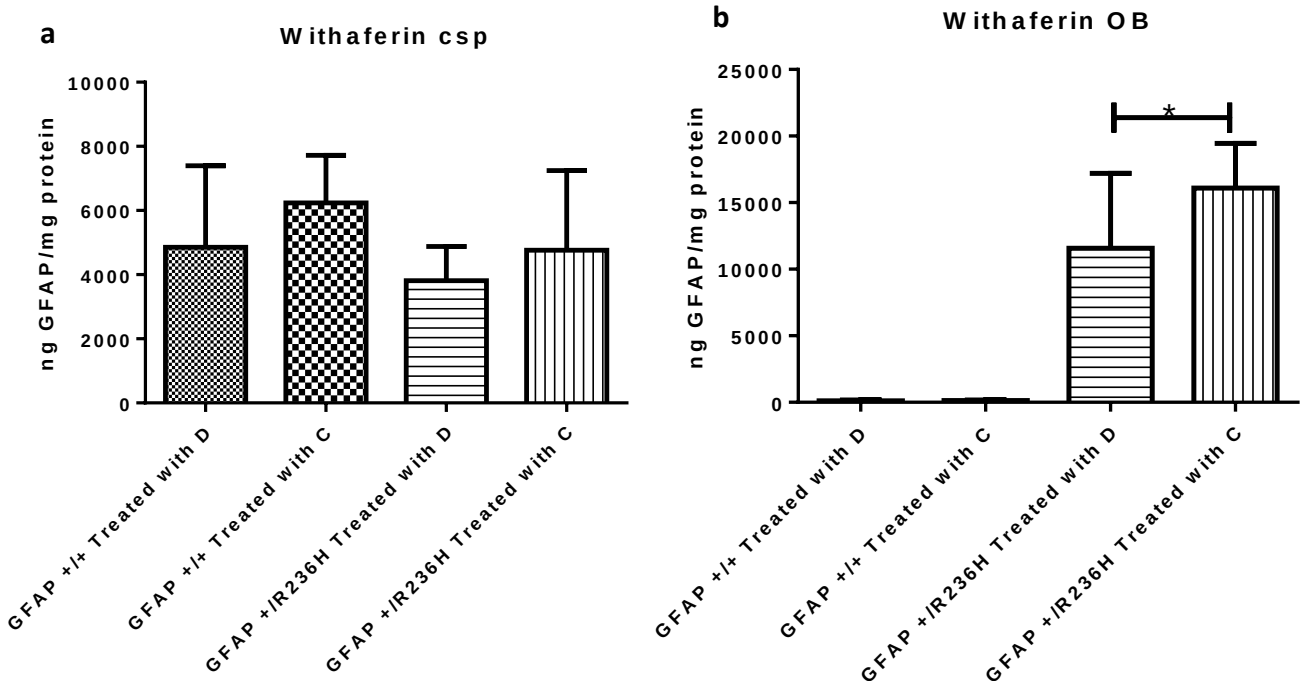

Figure 3(a and b): Brain Region Analysis for the cervical spinal cord (csp) and olfactory bulb (OB) of wild-type and GFAP+/R236H Mutant Mice. The wild-type (*GFAP* $^{+/+}$) mice are listed on the left, and the mutant (*GFAP*$^{+/R236H}$) mice are listed on the right. The saline vehicle was D, and the Withaferin treatment was C for both graphs. Bars represent averages across genotype and treatment, with error bars +/- SD of the mean. N=34

similar results to the hippocampus, but the amount of GFAP was approximately equal for both treatment and vehicle (Figure 2b). The cervical spinal cord showed higher overall levels of GFAP in the wild-type mice compared to the mutant. For both genotypes there were higher levels of GFAP in the mice treated with WA compared to vehicle. The differences for both were insignificant (Figure 3a). For the olfactory bulb, there was significantly more GFAP in the mutant mice with WA treatment compared to vehicle. The difference in the amount of GFAP in the wild-type mice between treatment and vehicle was insignificant (Figure 3b).

Conclusions

While the results were initially promising, further investigation proved that WA treatment was not decreasing the amount of GFAP in the brain of Alexander Disease mice. One of the possible reasons for the discrepancy could be the smaller sample size with the half-brain analysis; the total sample size was 18 for the half-brain analysis compared to a total of 34 for the brain regions. The smaller sample size could have provided inaccurate results. Another potential source of error is the large variations of GFAP concentration in different areas of the brain. Collecting brain regions for analysis allowed for specific evaluation of GFAP concentrations in relevant treatment areas. While the difference in collection likely had a small effect, it may have played a role in the discrepancy. These results show the importance of multiple trials and using a protocol that is as specific as possible.

While we have some insight into the pathways proposed earlier, there is still much that is unknown about Withaferin A and how it inhibits NF-κB. While it likely stabilizes IκB, there may be other pathways involved in inhibition that have unknown effects on GFAP, or the GFAP promoter. Additionally, discoveries are just being made about the relationship between TDP-43

and Alexander disease. While deregulated and insoluble TDP-43 has been found in Rosenthal fibers, the pathway and pathology of TDP-43 in Alexander disease remains largely unknown. (Walker, *et al.* 2014). Because of this, it is possible that an element of the pathological TDP-43 pathway is different than how we predicted or interacts with other promoters and binding elements related to GFAP, that WA cannot inhibit.

While the data showed a potentially toxic effect of WA on the mice, investigations into the treatment and regulation of Alexander disease can provide a better understanding of TDP-43 as well as GFAP accumulation in astrocytes and how it leads to the pathology of other neurological disorders as well as potential therapeutic treatment options.

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