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RETINOIC ACID HYDROXYLASE INHIBITORS AS A NOVEL THERAPY FOR
ALZHEIMER'S DISEASE

By
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Abstract

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Russian

Retinoic Acid Hydroxylase Inhibitors as a Novel Therapy for Alzheimer's Disease

Faculty Mentor: Philippe Diaz

Alzheimer's Disease (AD) is one of the most prevalent neurodegenerative diseases afflicting the modern world. As no cure for AD has yet been discovered we sought to explore a potential treatment option based on the inhibition of retinoic acid (RA) metabolism, the active metabolite of vitamin A. Beta-amyloid plaques form in the brain and decrease cognitive function in AD patients. In a recent preclinical study RA was shown to decrease these plaques and rescue memory deficits in an Alzheimer's mouse modelⁱ. One key limitation is that when RA is administered to humans it induces its own breakdown, making it less effective for long-term treatment. The proteins mediating RA breakdown are named CYP26. Of the CYP26 enzymes identified as clearing RA, CYP26B1 appears to be the predominant brain isoform. We hypothesize that the inhibition of CYP26 would cause an increase in the amount of RA concentration in the brain, thereby improving AD patient outcomes. Using two behavioral maze tests, the Morris Water Maze (MWM) and Y maze, we assessed cognitive function based on how quickly the mice maneuvered the mazes. We have begun preclinical testing of the prototypical CYP26 inhibitor in Alzheimer's mice relative to wild type (WT) litter-mates. A MWM measure of latency to the end of the maze showed a discrepancy between the performances of AD and WT mice, with the AD mice exhibiting a considerable cognitive deficit. The mice then received 8 weeks of treatment with the first selected CYP26 inhibitor NMP300 (3 times per week at 10 mg/kg i.p.). A follow-up MWM was performed and no significant reversal of cognitive deficits in AD mice was detected. A more recent MWM study of 31 treated AD mice is currently being analyzed and shows promising similarities

between AD and WT latencies. Furthermore, the stained brain tissues of these mice show a decrease in Beta-Amyloid plaques in AD mice that have received treatment. These results provide a strong foundation from which to further explore the use of these CYP26 inhibitors in treating AD.

Introduction

The Role of Retinoic Acid in the Body

Retinoic acid (RA) isomersⁱⁱ, the metabolites of vitamin A, are important endogenous signaling molecules critical for a number of biological processesⁱⁱⁱ including cell differentiation^{iv} and neural plasticity^v. RA isomers are involved in maintaining homeostasis of various tissues, including the brain^{vi}. The biological activity of retinoids is largely mediated by their binding to the retinoic acid receptors (RAR)^{vii} and the retinoid X receptors (RXR)^{viii} located in the cell nucleus.

RA and synthetic retinoids have been suggested to have therapeutic uses in neurodegenerative disorders^{ix}. However, the therapeutic chronic uses of currently available synthetic retinoids are limited due to potential side effects, such as retinoic acid syndrome^x.

Examining CYP26 Inhibitors as Treatment for Alzheimer's Disease

The clearance of RA is predominantly mediated by cytochrome P450 family 26 enzymes (CYP26)^{xi}. The CYP26 family has three isoforms: CYP26A1, CYP26B1 and CYP26C1^{xii}. While CYP26A1 is the human liver *atRA* hydroxylase, CYP26B1 is predicted to be responsible for *atRA* metabolism in extrahepatic tissues and CYP26C1 is responsible for *9-cis* metabolism^{xiii}. Of the RA clearing enzymes, CYP26B1 appears to be the predominant brain isoform, whereas CYP26A1 seems to function in tissues with high metabolic capacity such as the liver^{xiv}. When *atRA* is administered to humans, it induces its own metabolism likely *via* CYP26, which leads to resistance to RA treatment. **Hence, we hypothesize that selective inhibition of CYP26B1 in the brain will increase concentrations of neuronal RA and provide a therapeutic advantage in AD.** Inhibition of CYP26A1 would

increase circulating RA concentrations but will have a smaller effect on brain RA concentrations than a CYP26B1 inhibitor and will result in systemic side effects.

Methods

Transgenic mice and CYP26 Inhibitor Treatment

All procedures were performed in accordance with the University of Montana Institutional Animal Care and Use Committee. A transgenic (Tg) 5-Familial Alzheimer's disease mouse line was acquired from Jackson Laboratories in Bar Harbor, Maine. The original mice were crossed and F2 and F3 generations were genotyped using polymerase chain reaction (PCR) to establish the presence of the AD genes. This was done using primers #123513562, #123513561, #123513564 and #123513563. Transgenic mice were further crossed to establish a colony and each generation was subsequently genotyped. Transgenic and wild type (WT) mice were divided into groups for experimental use and were treated with different dosages of the CYP26 inhibitor, NMP308. The treatment was continued for the duration of 13 weeks, with three treatments per week. This exceeded the 8-week treatment period but an exception was made due to time constraints. The drug was introduced through intraperitoneal injections. The mice were weighed once a week during the first eight-weeks of treatment to ensure that no dramatic weight fluctuations occurred due to drug toxicity.

Morris Water Maze Protocol

After treatment the mice performed a Morris Water Maze (MWM) in order to test cognitive function. This test examines spatial learning and memory in mice^{xv}. The MWM consisted of a 1.76 ft tall and 4 ft across round galvanized steel tank with the edges and walls painted matte white to reduce glare. A platform made of a section of 10 cm wide PVC pipe, mounted on a stabilizing block with weights was covered with mesh and placed in the tank to act as a landing site for the animals. Non-toxic white tempera paint was added to the water to make it opaque and distal cues were placed 3 feet from the edge of the tank. The tank was divided into four quadrants with AnyMaze tracking software and the platform was left in the same quadrant

throughout the entire test. On the first day of the MWM a colleague determined treatment groups and the mice were placed alone in empty cages before being observed and weighed. A probe trial was then performed on Day 0 of the experiment. First a small flag was attached to the platform and the tank was filled with 22.5 cm of water at 20-22°C. The first mouse was placed in the tank at a predetermined location and the tracking software on the computer was started. The mouse was allowed to swim for 60 seconds and if it did not locate the platform it was placed there and allowed to rest for 30 seconds. Then, the mouse was placed in its empty cage underneath the heat lamp and allowed it to remain for the trials of two more mice to prevent hypothermia. During days one through three of the MWM the platform was placed in the same location and the mice performed four 60-second trials, being placed in the tank at four different starting locations, facing the wall each time. If the mouse did not find the platform, it was guided there and allowed it to rest for 30 seconds. As with the probe trial, the mouse was allowed to sit under the heat lamp for two more trials. Day five was a rest day and a final probe was performed on day six to assess memory function in the mice. During this final probe trial the platform was removed and all other conditions were kept constant. The mouse was placed in the tank facing the wall and allowed to explore for 60 seconds. As in the first probe trial, each mouse was allowed one session in the tank before being removed to its cage.

This protocol was modified from (Vorhees and Williams, 2006).

Y Maze Test

A prescreen was performed the day before the Y-maze. This included observing the mouse in the home cage, assessing eye, ear and whisker reflexes and weighing the mouse. The mice were individually placed in empty cages and randomized by a colleague. The test was performed the following day. A mouse was placed in the start arm of the maze and the computer software was started. The mouse was allowed to roam for five minute, exploring the maze at will. The number of alternations to various arms was recorded during the five-minute trial, and

provided the basis of the behavioral analysis. The mouse was then removed, the maze was cleaned with 10% ethanol, and the next mouse was placed in the maze. The results of this test were determined by the percentage of alternations made by the mouse. A normal WT mouse will theoretically travel to each arm of the maze in turn, alternating evenly between all of them. A mouse with a functioning memory is unlikely to travel to the same arm twice in a row. Consequently, the AD mice are expected to return to the same arm multiple times in a row because they lack the memory of having recently visited said arm.

Perfusion and Histology

After the Y-maze and MWM behavior tests were performed the mice were anesthetized with Isoflurane in an anesthetizing box and then perfused and with PBS and 4% paraformaldehyde. The brains were soaked for 24 hours in PBS, then 24 hours in 4% paraformaldehyde and then were placed in 70% ethanol until they could be embedded in paraffin. Once the tissue was embedded the paraffin blocks were vertically sectioned with a microtome setting of 7 microns. The sections were floated on a warm water bath and mounted on SuperFrost glass slides. The sections were collected at intervals of 8. They were then deparaffinized and hydrated in distilled water. The slides were stained for 12 minutes in Congo Red solution, rinsed and differentiated in an alkaline alcohol solution. The slides were rinsed in tap water for 5 minutes, dehydrated with 95% and 100% alcohol, cleared in xylene and mounted with a resinous mounting medium. Following this procedure the brain sections were imaged on a confocal microscope with the 10x objective.

Statistical Analysis

Analyses were performed using Microsoft Excel. Data is shown with a mean +/- SEM.

Results

Behavioral Tests

To examine the effects of the CYP26 inhibitor NMP308 in the brains of AD mice we performed Y-maze and Morris Water Maze (MWM) behavioral tests. Four groups of mice, age 10 months, were injected with the drug for 13 weeks. A group of Transgenic (Tg) mice (n=7) and a group of WT mice (n=9) were injected with 10 mg/kg of the drug. Another group of Tg (n=6) and a group of WT (n=9) mice were injected with 20 mg/kg doses. The MWM was executed after treatment and allowed us to determine whether the CYP26 inhibitor was affecting cognitive function in the mice. This was accomplished by comparing the groups to one another and to a previously tested untreated group of mice. The most indicative measure of cognitive function in the MWM is latency to platform (see methods). The latencies of mice (Fig 1.) to the MWM platform were within a range of statistical insignificance (t-test > .05) between the four groups of mice.

We then perfused the mice and prepared stains for imaging (see methods). The Congo Red stain highlights the Beta-amyloid plaques within the brain tissue of the mice. It can be seen in Figure 2 that WT mice have no plaques whatsoever. The Tg mice that were treated with the 20 mg/kg dosage of the drug have fewer plaques than those treated with the 10 mg/kg dosage. A method to quantify the number of plaques in each tissue sample has not yet been determined

Discussion

Through the course of this study we have gained insight into the accuracy and effectiveness of the MWM behavioral test as well as into the effects of the CYP26 inhibitor on Beta-amyloid plaques in the brain tissue.

Latency to Platform as Measure of Cognitive Function in MWM

Figure 1 and the accompanying statistical tests indicate that there is no significant difference in the performances of WT and Tg mice in the MWM. Nor is there a discrepancy in the performance of the two different treatment groups (20 mg/kg versus 10 mg/kg) Tg mice. This could indicate that the cognitive function of the Tg

mice is improving and causing them to perform more like their WT litter-mates. However, MWM tests performed on previous groups of WT and Tg mice have shown a similar lack of statistical significance ($t\text{-test} > .05$) between WT and Tg latencies in untreated mice. This is unexpected, as WT mice should show significantly better cognitive function when compared to mice with advanced Alzheimer's disease. Because these initial MWM tests did not provide the expected results it is difficult to trust the most recent test displayed in Figure 1. Therefore, we have determined to place little faith in the MWM behavioral test as an indicator of the effectiveness of our drug and instead turn to histology for answers.

Beta-Amyloid Plaques in Congo Red Stained Brain Tissue

The Congo Red stain used in the histology process is one that indicates Beta-Amyloid plaques in the brain. We stained Tg tissue that was vehicle treated (Figure 3), tissue from mice that were treated with 10 mg/kg doses (Figure 4) and 20 mg/kg doses of NMP308 (Figure 5). Although no method of quantifying the plaques has yet been determined, we used visual analysis to conclude that mice treated with 20 mg/kg doses for 13 weeks showed fewer plaques in their brain tissue than mice that were untreated or treated with the lower dosage. There is not a noticeable difference between the vehicle treated and 10 mg/kg treated Tg mice, possibly because the 10 mg/kg dose was not concentrated enough to affect the plaques. The decrease of plaques among 20 mg/kg treated mice seems to be a trend consistent between all samples. To further determine the implications of the histology slides a quantification method will need to be determined to accurately gauge the number of plaques in each tissue sample and draw further conclusions.

Implications for Alzheimer's Disease Treatment

Through this study we have gained valuable information regarding Alzheimer's disease mouse models as well as the effects of our CYP26 inhibition drug on said mice. Beta-amyloid plaques are strongly associated with the development of Alzheimer's disease. Therefore, the measurement of these plaques is an indicator of

whether or not a mouse has AD and whether the drug therapy is decreasing the effects of the disease. Future steps for this study include developing a Beta-amyloid plaque measurement technique and performing tests with varying concentrations of our drug to ascertain effective dosing for future studies.

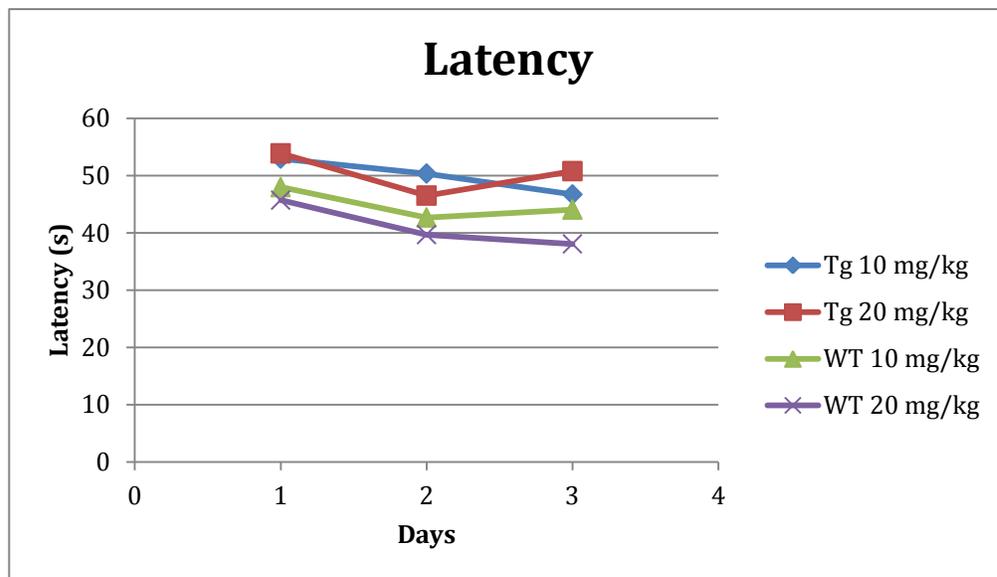


Figure 1. Latency to Platform of WT and Tg mice from 20 mg/kg and 10 mg/kg NMP308 Treatment Groups. Mice performed four 60 second trials over the course of three days, during which they are expected to learn the location of the underwater platform aka their “escape route”. Theoretically the mice should reach the platform with increasing speed each day as their knowledge of the maze increases. It is expected that WT mice will reach the platform more quickly than Tg mice, which is not the case here; all four groups of mice in this test performed similarly enough that their results showed no statistically significant difference (t-test > 0.05).

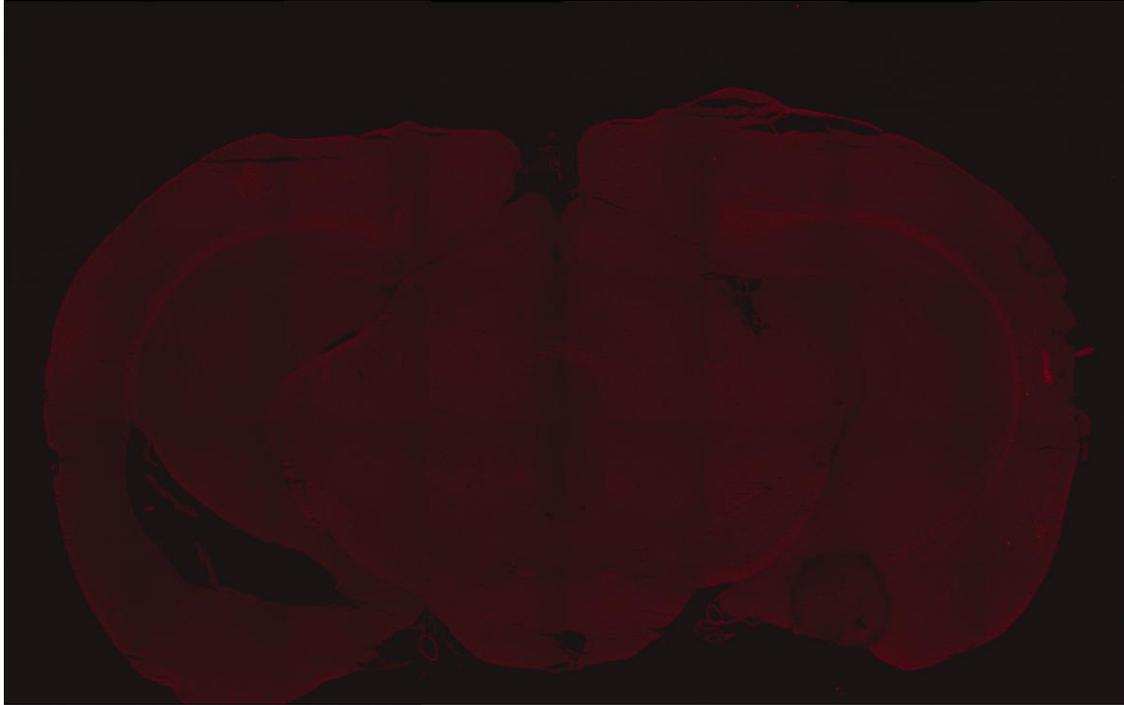


Figure 2. NMP308 10 mg/kg Treated WT Mouse. This mouse was treated with a 10 mg/kg dosage of the CYP26 inhibitor over the course of 13 weeks. No bright red dots (representing Beta-amyloid plaques) are present in this tissue, which is to be expected in any WT mouse. The treatment was not expected to have an effect on the WT tissue but was administered as a control.

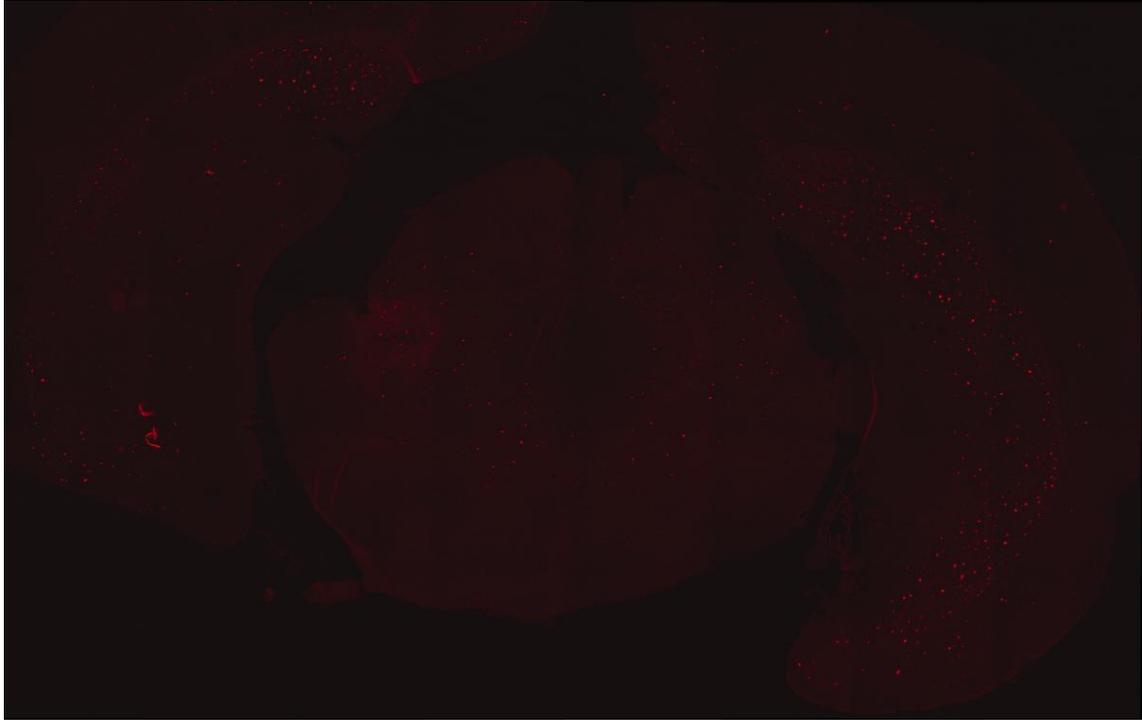


Figure 3. Vehicle Treated Tg Mouse. This mouse was treated for 13 weeks with the vehicle that is used to administer the CYP26 inhibitor. In theory this mouse should have felt no effects since the vehicle is intended to be merely a means of transportation and not to have a tangible effect. Therefore, the plaques in this mouse should be the same as those in an untreated AD mouse.

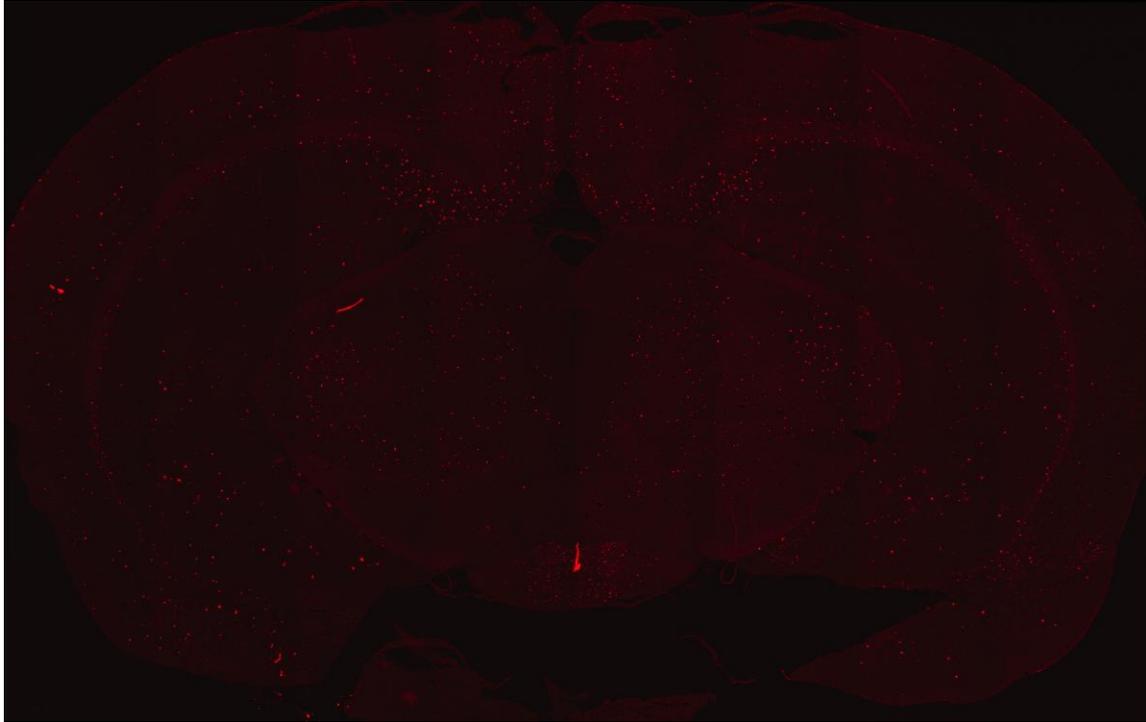


Figure 4. NMP308 10 mg/kg Tg Mouse. This animal was treated with a dosage of 10 mg/kg for the course of 13 weeks. Though we have not been able to quantify the plaques present, we can see by comparison that this tissue seems to have slightly fewer plaques than the vehicle treated Tg mouse, although not substantially so.

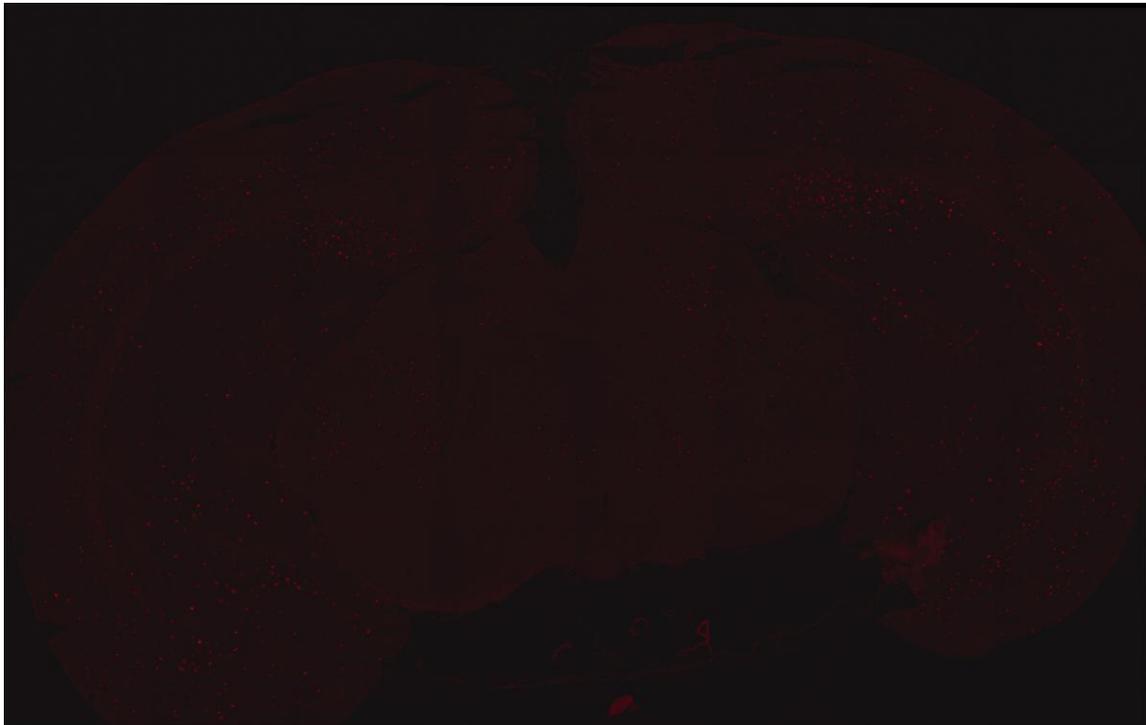


Figure 5. NMP308 20 mg/kg Treated Tg Mouse. This Tg mouse was treated with 20 mg/kg doses of CYP26 inhibitor for 13 weeks. Again, there is no quantification

method for comparing the plaques but this mouse appears to have significantly fewer plaques than the vehicle treated Tg shown in Figure 3. This tissue also has fewer plaques than are present in the 10 mg/kg treated mouse shown in Figure 4. This suggests that the higher dosage has a stronger effect in clearing Beta-Amyloid plaques from the brain.

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