Progress Report

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GTA Treatment in Tremor Rat Model of Canavan Disease: Effectiveness and Lack of Toxicity

In our ongoing efforts, we have performed preclinical studies on acetate supplementation using glyceryltriacetate (GTA) as a treatment for Canavan disease (CD) using the tremor rat model. These studies were based on our finding that in CD-mice there was deficiency in the synthesis of myelin lipids due to limited supply of N-acetylaspartate (NAA)-derived acetate resulting from the loss of aspartoacylase (ASPA) activity due to mutations in the gene. The data provide convincing evidence that this therapeutic approach has a very high chance for success and that the treatment has no detectable toxicity on chronic use up to 4-6 months, the duration of the present study.

Two animal models of CD exist; the *tremor rat* model and the aspartoacylase (ASPA) gene knockout mouse model. The tremor rat model is a stable line of a naturally occurring mutant with a genomic deletion on chromosome 11 spanning 4 genes, including the aspartoacylase gene, olfactory receptor gene, vanilloid receptor subtype I gene, and the calcium/calmodulin-dependent protein kinase IV gene. The tremor rat shows no ASPA activity in brain, and greatly reduced activity in kidney, and also exhibits increased brain NAA levels similar to the CD-mouse. Tremor rats exhibit muscular tremors starting at about 2 weeks of age, which give way to absence-like seizure activity in later development stages. Pathology in the CNS involves white matter spongiform degeneration and hypomyelination similar to the ASPA gene knockout mouse. In both models, homozygous mutants alone show Canavan disease symptoms. A tremor rat colony has been established in our laboratory at the USUHS, Bethesda, MD, and the homozygous mutant rats were orally treated with GTA as a treatment for CD. Here we present the unpublished observations of at least 6 months of GTA treatment on the tremor rats and 3-4 months of treatment on the normal wild type (WT) rats of the tremor rat strain (Kyoto Wistar albino).

Generation of mutant pups and GTA treatment

Tremor rats were bred using heterozygous pairs. The tremor-rat pups were identified by curled whiskers as early as 2 days after birth. GTA was orally given to the mutant pups once in the morning (9:00-10:00 am) and once in the evening (4:30-5:30pm) starting from day 7 after birth at a dose of 4.2 g/kg until 14th day. From 15th day, the dose of GTA was increased to 5.8 g/kg until the pups were weaned (22/23 days after birth). After weaning, the pups received GTA in the rat feed (7.5% of GTA by weight) and water (5% of GTA by weight), until they are sacrificed for histopathology and biochemical analyses. The untreated tremor-rat pups (control) were given the normal rat feed and water after weaning. Effectiveness of GTA treatment was evaluated by behavioral studies. A comparative study on the toxic effect of GTA was conducted on wild type (WT) rats of tremor rat strain (Kyoto Wistar albino). A litter of 10 divided randomly into two groups containing both male and female in each group of 5 animals and one group received GTA exactly as the mutants were given and the other group received normal feed and tap water. At the end of the study (110 days) blood was collected for biochemical analyses and these rats were sacrificed for histopathology analysis.

GLP Status of our Laboratories

The laboratory of animal medicine (LAM) at USUHS, Bethesda, MD, has a AALAC approved animal facility and College of American Pathologists-approved laboratories for biochemical analyses of serum and histopathological analyses of different organs. All the toxicity work described here was performed in these facilities.

Blood serum analyses to determine toxicity of GTA treatment

Approximately 1 ml blood collected from the tail vein was centrifuged (10,000 g for 15 min), the serum separated and stored at -20 °C until analyzed. Analyses of the rat sera were done at the USUHS clinical pathology facility. All the organic and inorganic biochemicals were analyzed from the rat sera using an automated System called Vitros 250 by the Ortho-Clinical Diagnostics, Rochester, NY 14626, of the Johnson & Johnson Company.

Histopathology

For histological studies, brain tissues were fixed with 10% formalin in phosphate buffered saline by transcardial perfusion of the rat under anesthesia and the tissues were rapidly removed and post fixed in the same fixative for 24h. Subsequently, the tissues were transferred to 80% ethanol and processed for histochemical analysis. The tissues were sliced to produce standard blocks embedded in paraffin and sectioned at 7 μm. The slices on the glass-slides were then submitted for standard paraffin processing (dehydration through graded alcohols, clearing with xylene and paraffin infiltration). Adjacent sections from each block were stained using standard histological procedures for hematoxylin and eosin (H&E).

Behavioral Studies

Two types of behavioral parameters were analyzed to determine if GTA treatment affects the brain in an adverse way and/or improves the behavioral parameters. The two parameters were: 1) different types of locomotor activity and 2) the ability of the animals to balance on rotating rotarod.

Locomotion: Locomotor activity was measured using an Omnitech Electronics Digiscan infrared photocell system [Test box model RXYZCM (16 TAO); Omnitech Electronics, Columbus, OH], located in a dedicated room within the animal facility as previously described. Briefly, one hour duration activity was measured. Animals were placed singly in a 40 X 40 X 30 (L X W X H) cm clear Plexiglas arena and a Plexiglas lid with multiple 3.5 cm diameter holes placed on top of the arena. Data were automatically gathered and transmitted to a computer via an Omnitech Model DCM-I-BBU analyzer. Our collaborator, Dr. Neil Grunberg, has extensive experience with this measure and his collaborative support was central to the success of our efforts.

Rotarod balance test: The Rotarod test measures an animal's fatigue and ability to maintain balance by coordinating the movement of all four feet and making the necessary postural adjustments. It also measures the animal's ability to improve on these skills with practice. Each Rota-Rod treadmill consisted of a motor-driven drum with constant speed or accelerating speed modes of operation. The drum allows each animal to maintain a suitable grip. It was divided into four test zones so that up to four animals could be tested at the same time. The device consisted of a smooth hard plastic cylinder with concentric circular plastic sides attached to prevent the rat from climbing off the cylinder laterally. The cylinder was connected to a variable speed reversible motor, allowing the speed and direction of rotation of the cylinder to be changed. An accelerated Rotarod task was used with a Med Associates Inc, Georgia, VT, deviced rotarod instrument. The equipment consisted of a metal frame of rotating rods (25.5 cm high from the bench, 6.0 cm diameter). Rats were placed on the device such that they had to use their paws to pace backwards to avoid falling off the rod, and were tested several times on this device. In each trial, the speed of rotation was slowly increased from 0 revolutions per minute (rpm) to 30 rpm for a maximum of 3 min and the duration (in seconds) for which the rats managed to stay was recorded. The mean of 5 trials per experiment was expressed in seconds for scoring each rat.

Results and Discussion

In the effectiveness studies, we found that performance of the treated Tremor rats improved significantly in rotarod balancing as well as in locomotion tests by about 8 weeks of treatment that was described above. The female rats seemed to perform better than the males in the rotarod balancing test and the performance of females in the present study formed about 50% of that of the wildtype. While the reason for the performance difference between females and males remains unclear, studies are in progress to improve the performance using higher doses and longer duration. Locomotor activity also showed significant improvement on GTA treatment although male-female difference was not detectable in the various locomotion parameters. The values were analyzed for the time period of 70 to 120 day and this time period showed the maximum differences between the treated and untreated groups. The ANOVA was significant ($p<0.05$) for this time period and independent comparison between the treated and untreated groups showed statistically significant differences by two-tailed t-test ($p<0.05$). The pattern of the values remained similar at 100 day and 115 day data. The openfield parameters thus corroborated the rotarod performance of the GTA

treated Tremor rats in that GTA treatment improved the locomotor parameters statistically significantly over the untreated Tremor rats.

In the chronic toxicity studies, we found that the weight gain of the Wild Type treated and untreated rats remained the same, showing a general lack of any overt toxicity. The biochemical analyses of the rat sera included estimation of the commonly used enzymes in toxicity studies: lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyltranspeptidase (GGT) in addition to alkaline phosphatase (ALKP), creatine kinase (CK) and amylase. Serum lipids (cholesterol, high density lipid cholesterol (HDLC), triglycerides and very low density lipoproteins (VLDL)), glucose and serum osmolality were also analyzed. Statistically significant differences between the treated and untreated groups were not found for either females or males. When the F-statistic was significant the Tukey's HSD showed difference between the male and female values but not between treated and untreated groups suggesting that there may be sex dependent differences for the analyte as in case of alkaline phosphatase (ALKP) and very low density lipoproteins (VLDL). All test analytes in most of the treated and untreated rats in both category (wild type and Tremor) were found to be within the normal range. When the values were outside the normal range they were so in all three categories: Tremor females (treated and untreated), Tremor males (treated and untreated) and wild type normal, suggesting a characteristic of the Kyoto Wistar Strain. These data suggest that there was no detectable GTA induced toxicity at the GTA dose and frequency used to orally supplement the Tremor rats.

For histopathology analyses, slides from 2-4 rats per group (four groups: background wild-type strain untreated, wild-type strain treated, tremor rat untreated, and tremor rat treated) were examined by a board-certified veterinary pathologist at USUHS. Scoring of the slides for acute, subacute or/subchronic, and chronic lesions was: 0 = normal or no significant lesions; 1 = minimal; 2 = mild; 3 = moderate; 4 = marked; 5 = severe. The following tissues were included: stomach, liver, kidney, spleen, heart, lungs, small intestine, large intestine, cerebrum, cerebellum and spinal cord. The majority of organs examined had no significant findings noted. No grade 3 or higher lesions were noted in any group for any of the organs tested (data not shown due to huge size of the micrographs. Each micrograph is about 40 megabytes). Therefore, we believe that GTA treatment is likely to be safe for the treatment of Canavan disease in children.

Acetate Supplementation using Glyceryltriacetate as a treatment for Canavan Disease.

In our ongoing efforts, we have performed preclinical studies on acetate supplementation using glyceryltriacetate (GTA) as a treatment for Canavan disease (CD) using rat and mouse models. These studies were based on our findings that in CD-mice there was deficiency in the synthesis of myelin lipids due to limited supply of N-acetylaspartate (NAA)-derived acetate resulting from the loss of aspartoacylase (ASPA) activity due to mutations in the gene. During the current year, we have established effectiveness of acetate supplementation using the mouse as well as rat models of Canavan disease and the data are being prepared for publication. Additionally, no toxicity was detected in a chronic study in the rat for four months. Based on these encouraging results, we have got Dr. Bill Gahl, Clinical Director, NHGRI, interested in starting clinical trials. In our subsequent efforts, FDA has given approval for single patient INDs, starting at a low dose and increasing to a very high dose, based on the effectiveness data and lack of toxicity data. Currently, Dr. Yair Anikster, our collaborator in Israel, is involved in treating a Canavan disease patient with high dose of GTA and the results are awaited.