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Michael Zorniak
Lake Forest College

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Effects of Cystamine, a Transglutaminase Inhibitor, in a Mouse Model of Progressive Supranuclear Palsy

Michael Zorniak*
Department of Biology
Lake Forest College
Lake Forest, Illinois 60045
Department of Pharmacology
Loyola University Stritch School of Medicine  Maywood, Illinois 60153

Summary

In Alzheimer’s disease (AD) and Progressive Supranuclear Palsy (PSP), the cross-linking of phosphorylated tau into stable insoluble aggregates may lead to formation of paired helical filaments (PHFs) and neurofibrillary tangles (NFTs). NFT pathology is accompanied by oxidative stress in AD and PSP. Previous investigations have uncovered that phosphorylated tau is cross-linked by transglutaminase in the hindbrain of P301L tau transgenic mice. We wanted to determine whether or not P301L mice would exhibit oxidative pathology. To analyze this, we performed immunoblots for the detection of modified carbonyl groups on proteins in spinal cord tissue of P301L tau transgenic, four-repeat wild-type (4RWT) tau, and nontransgenic mice. Carbonyl levels were used as a measure of oxidative stress. We found that P301L tau transgenic mice exhibit more oxidative stress than 4RWT tau and nontransgenic animals. We are currently orally administering cystamine, a transglutaminase inhibitor and antioxidant, to P301L mice. Future investigations will use this immunoblot technique to evaluate the pleiotropic effects of cystamine on P301L mice.

Introduction

Alzheimer’s disease (AD), Pick’s disease, Progressive Supranuclear Palsy (PSP), and corticobasal degeneration are characterized by neuropathological changes including the aggregation of microtubule-associated protein tau (Lewis et al., 2000). In the above tauopathies, abnormally hyperphosphorylated tau polymerizes into straight and paired helical filaments (PHF), which comprise neurofibrillary tangles (NFT) (Halverson et al., 2005). The exact mechanism that causes the formation of NFTs is unknown. The accumulation of tau in neurodegenerative disorders (NDS) is linked to a class of calcium dependent enzymes known as transglutaminases (TGases) (Selkoe et al., 1982a). In vitro studies demonstrate that tau is a substrate of transglutaminase (Nortlund et al., 1999), where tau is modified at two glutamine and ten lysine residues to form a cross-linked insoluble protein complex (Murthy et al., 1998). In brain regions with extensive neurofibrillary pathology in both AD and PSP, transglutaminase activity (Johnson et al., 1997; Zemaitaitis et al., 2003) and transglutaminase catalyzed bonds found in PHFs and NFTs are significantly elevated (Balin et al., 1998; Nortlund et al., 1999; Zemaitaitis et al., 2000; Singer et al., 2002). Despite recent findings, it remains unclear whether PHF formation and tau cross-linking are involved in neurodegenerative pathogenesis or whether they may have a neuroprotective function (Karpuj et al., 2004).

TGase may play a role in the pathogenesis of CAG trinucleotide-repeat disorders, which are characterized by proteins with expanded polyglutamine domains (Karpuj et al., 2002). Some examples of these proteins are tau and huntingtin. TGase catalyzes the formation of ___-glutamyl isopeptide bonds between polyglutamine tracts and lysine substrates (Folk et al., 1983; Green et al., 1993). The resulting cross-linked protein complex is insoluble and resistant to both proteolysis and denaturation (Selkoe et al., 1982b). The understanding of the behavior of TGases in vivo has led to investigations into the development of novel therapies involving the use of TGase inhibitors such as cystamine.

Recent studies have shown that cystamine is an in vitro TGase inhibitor (Gentile et al., 2004). Cystamine is known to inhibit TGase through a disulfide exchange reaction (Folk, 1980; Lorand et al., 1984; Lorand, 1998). As a primary amine, cystamine has a high affinity for TGase and acts as a competitive inhibitor by blocking the active site of the enzyme for glutamine residues (Lorand et al., 1979). As a result, cystamine may regulate the redox status of neurons, playing a possible neuroprotective role. Cystamine has been found to be cytoprotective by increasing glutathione levels in vitro. Glutathione is an antioxidant that is a substrate for peroxides (Dedeoglu et al., 2002). The understanding of oxidative stress in NDS may provide a comprehensive paradigm for the behavior of tau in aggregation pathology.

In our previous investigation, we used P301L transgenic mice which over-express a human isoform of tau associated with frontotemporal dementia (FTDP-17) (Lewis et al., 2000). We also used four-repeat wild-type (4RWT) tau transgenic and nontransgenic mice. The latter two model organisms display no NFT pathology or symptoms (Lewis et al., 2000). Our results have illustrated that tau protein from the brain and spinal cord tissues of P301L tau transgenic mice contain significantly more transglutaminase-catalyzed cross-links than 4RWT tau and nontransgenic mice (Halverson et al., 2005). Therefore, we hypothesized that P301L mice would exhibit more oxidative stress in the spinal cord than 4RWT tau and nontransgenic mice. This hypothesis also followed the paradigm that neurodegeneration is accompanied by aggregation pathology and oxidative stress (Park et al., 2001). We also sought to determine whether or not P301L mice were a good model of tauopathies by determining the level of oxidative pathology found in their spinal cords. Results from these experiments will aid in understanding of current PSP investigations.

Our current research focuses on the investigation of a potential drug therapy for PSP, using P301L mice treated orally with cystamine. Other researchers have used oral treatments of cystamine in R6/2 mice. A mutant form of huntingtin protein is expressed in R6/2 mice and may be cross-linked by transglutaminase to form intranuclear aggregates. This event is followed by neuronal loss in the striatum and cortex. Cystamine treatment was found to increase survival by 20% in comparison to saline and untreated littersmates. Manifestations of aggregated huntingtin protein were reduced by 70% in the striatum and by 50% in the cortex (Dedeoglu et al., 2002). These findings suggest a possible usefulness of cystamine in P301L mice. We investigated whether or not cystamine would prolong...
Figure 1A. OxyBlot Control Experiment
Gel displays control experiments run to determine efficacy of reagents. DNPH indicates that a derivatization reaction was performed on the tissues specified. Oxidized proteins were detected in relatively equal concentrations within AD cortex, nontransgenic (NTG), and 4RWT spinal cord tissues. P301L tau transgenic tissue showed very little oxidation in comparison to other derivatized samples. The immunoblots were reprobed with an antibody against actin to demonstrate relatively equal protein loading among lanes. Molecular mass in kilodaltons (kDa) is indicated on the left. The film was exposed for 3 minutes.

Figure 1B. OxyBlot DNPH Experiment
AD tissue loaded into the gel was not derivatized. Nontransgenic (NTG), 4RWT, and P301L spinal cord tissues were derivatized. Nontransgenic and 4RWT tissues exhibited uniform oxidation. One P301L sample appeared to display significantly (statistics were not performed) greater intensity than any other sample. The immunoblots were reprobed with an antibody against actin to demonstrate relatively equal protein loading among lanes. Molecular mass in kilodaltons (kDa) is indicated on the right. The film was exposed for 3 minutes.
one symptomatic transgenic animal (lane 12, 13 months of electrophoresis. Lack and band expected. 1A).

To measure spinal oxidative pathology found in P301L transgenic mice. We wanted to determine if oxidative stress is elevated in the spinal cords of symptomatic P301L tau transgenic mice. To measure oxidative stress, we utilized the OxyBlot protein oxidation detection kit from Chemicon. A control blot was run to determine efficacy of reagents in the OxyBlot kit (Figure 1A). Alzheimer’s disease parietal cortex tissue was used as a positive control where oxidative stress pathology is expected. There seems to be no significant difference in band intensity among the three positive control lanes (3, 5, and 7). Tissue used in lane 9 was obtained from a symptomatic animal of unknown age. This may explain the lack of intensity found in this lane. A high range rainbow molecular weight marker (BioRad) with 2-mercaptoethanol (reducing agent) served as an internal control for electrophoresis.

Oxidized proteins were significantly increased in one symptomatic transgenic animal (lane 12, 13 months of age, Figure 1B) compared to age-matched non-transgenic controls. Symptomatic P301L transgenic animal tissue used in lanes 10 (4.3 months), 11 (4.3 months), and 13 (unknown age) were not age-matched. These symptomatic mice did not show significant differences in oxidized proteins when compared to asymptomatic animals.

Table 1. Behavioral Scoring Rubric
Scores used to assess criteria for sacrificing mice. Behavioral tests adapted from Karl and colleagues (2003).

<table>
<thead>
<tr>
<th>S1</th>
<th>Righting Reflex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tail Hang</td>
<td>0</td>
</tr>
<tr>
<td>0- Mouse when lifted by tail displays standard escape response, legs in “V” position</td>
<td></td>
</tr>
<tr>
<td>1- Mouse does not exhibit full escape response (poor escape), inward trend with legs</td>
<td></td>
</tr>
<tr>
<td>2- Mouse maintains legs in a scissored, clasped (clenched), or dystonic position</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>S2</th>
<th>Wire Hang Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wire Hang Behavior</td>
<td>0 (120s)</td>
</tr>
<tr>
<td>0- Can complete test with at least three paws and tail within two minutes, climber</td>
<td></td>
</tr>
<tr>
<td>1- Can hold on to rope without falling until end, does not complete</td>
<td></td>
</tr>
<tr>
<td>2- Falls before two minutes without completion</td>
<td></td>
</tr>
<tr>
<td>3- Unable to perform test, slips off the wire</td>
<td></td>
</tr>
</tbody>
</table>

Wire Hang Behavior (Scoring 0-3)
0- Can complete test with at least three paws and tail within two minutes, climber
1- Can hold on to rope without falling until end, does not complete
2- Falls before two minutes without completion
3- Unable to perform test, slips off the wire

Wire Hang Time (Scoring 0-3)
0- Hangs on for full 120 seconds
1- Hangs on between 60 seconds and 120 seconds
2- Hangs on between 5 seconds and 60 seconds
3- Hangs on for less than 5 seconds

Total Score = S1 + S2

<table>
<thead>
<tr>
<th>Normal</th>
<th>Mild/Early</th>
<th>Moderate/Mid</th>
<th>Severe/End</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-12</td>
<td>13-20</td>
<td>21-23</td>
<td>24-30</td>
</tr>
</tbody>
</table>

Survival and decrease motor impairments in P301L mice when compared to untreated P301L mice.

Results

Oxidative Pathology Found in P301L Transgenic Mice
We wanted to determine if oxidative stress is elevated in the spinal cords of symptomatic P301L tau transgenic mice. To measure oxidative stress, we utilized the OxyBlot protein oxidation detection kit from Chemicon. A control blot was run to determine efficacy of reagents in the OxyBlot kit (Figure 1A). Alzheimer’s disease parietal cortex tissue was used as a positive control where oxidative stress pathology is expected. There seems to be no significant difference in band intensity among the three positive control lanes (3, 5, and 7). Tissue used in lane 9 was obtained from a symptomatic animal of unknown age. This may explain the lack of intensity found in this lane. A high range rainbow molecular weight marker (BioRad) with 2-mercaptoethanol (reducing agent) served as an internal control for electrophoresis.

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Treated Mice Consume More Water
Current research in our laboratory involves the use of cystamine as a therapeutic agent to treat symptomatic P301L tau transgenic mice. Cystamine dihydrochloride was orally administered (157mg/kg/day) via water bottles. Control group received regular tap water. Consumption was checked everyday in the mid-morning. Cystamine treated mice consumed about 14.9%, on average, more than non-treated mice over the past year (July 2004 to July 2005) (Figure 2).

Cystamine does not Ameliorate Weight Loss
Mice were weighed twice a week during behavioral testing to normalize the concentration of cystamine administered(157mg/kg/day). A hallmark of disease progression in P301L mice is weight loss. Dedeoglu and colleagues (2002) reported improved body weight by 12.6%
For R6/2 mice supplemented with cystamine. Based on this study we expected cystamine to ameliorate weight loss in P301L transgenic animals. Preliminary data shows no significant difference in weight among treated and non-treated groups (Figure 3).

Cystamine Treated Mice do not Live Longer
We hypothesized that cystamine treatment would extend the survival of P301L mice based on similar work done by Dedeoglu and colleagues (2002). Our preliminary data suggest that cystamine does not extend survival in P301L transgenic mice. Data does, however, show that after onset of first symptoms cystamine treated mice develop moderate symptoms (Table 1) about 33 days after non-treated mice. Figure 4 in the appendix shows the behavioral progress of treated and non-treated P301L mice. Results of preliminary data on motor performance suggest no apparent differences in phenotypic onset of symptoms between treated and non-treated mice.

Discussion
The central goal of taupathy research is to understand how tau accumulates and determine whether or not it leads to neurotoxicity in Alzheimer’s disease, Progressive Supranuclear Palsy, and Frontal Temporal Dementia. This research may then provide scientists with therapeutic targets to prevent the processes that lead to neuronal death. Yet, in order to understand the mechanisms underlying these diseases, a holistic picture including oxidative stress must be rendered.

P301L Transgenic Mice Exhibit Oxidative Pathology
OxyBlot experiments performed with P301L spinal cord tissue gave insight into the progression of oxidative pathology in P301L tau transgenic mice. Animals used for lanes 10 (5.5 months) and 11 (5.5 months) (Figure 1B) displayed onset of disease related behavioral phenotype, yet did not show any significant differences in oxidative pathology when compared to asymptomatic mice. We can speculate that levels of oxidative stress in P301L spinal cord tissue cannot be used as an early marker for disease progression. Tissue of a much older specimen (13 months, lane 12, Figure 1B) showed significantly more oxidation than any other sample. This result shows the potential of oxidative damage that can be done in this animal model. These experiments advocate the use of P301L mice supplemented with cystamine in OxyBlot assays. The animal model manifests extreme oxidative pathology in end-stage (Table 1) behavioral phenotypes which provide cystamine an opportunity to demonstrate the extent of its suggested antioxidant effects.

Future OxyBlot Assays with Cystamine Treated Mice
Oxyblot experiments were performed for two reasons. First, we wanted to determine if there are higher levels of oxidatively modified proteins in the spinal cord and hindbrain of P301L tau transgenic mice when compared to asymptomatic 4RWT tau and nontransgenic mice. Second, we wanted to optimize the efficacy of the OxyBlot protocol for testing cystamine treated and non-treated P301L tau transgenic mice.

Figure 2. Average Monthly Consumption of P301L Tau Transgenic Mice
An experimental group of P301L tau transgenic mice were supplemented with cystamine (157mg/kg/day) at 2 months of age. A control group was treated with water. Consumption was recorded daily. Graph displays average consumption for every month. Preliminary data shows that cystamine supplemented animals consume more than non-supplemented animals.
Figure 3. Average Monthly Weight Gain for P301L Tau Transgenic Mice
Weights were recorded twice every week during behavioral testing. Graph shows that there is no difference in weight gain for treated and non-treated animals.

Figure 4. Behavior and Symptom Summary
The graph shows a record of the number of P301L mice in a particular symptom category as of July 2005. Tail hang, righting, and wire hang behavioral tests were performed twice a week beginning in July 2004. The criteria for each symptom group (normal, mild, moderate, severe, dead) was determined by a scoring rubric (Table 1). We began with a total of 32 mice. Four water treated mice died at an age of 5.5 months from hypothermia due to a leaky water bottle.
transgenic mice in the future. We expect to see decreased levels of oxidative stress in cystamine treated animals because of the drugs recognized anti-oxidant effects. The drug’s ability to inhibit transglutaminase may decrease NFT formation which may directly impact the redox status of the cell.

**Cystamine may have an Effect on Energy Metabolism**

Knowledge of cystamine’s antioxidant effects may give insight into abnormalities in glucose metabolism found in PSP (Park et al., 2001). Current research has shown P301L mice supplemented with cystamine consume more than non-treated animals (Figure 2). This begs the question that cystamine’s antioxidant effects may have an effect on energy metabolism in symptomatic P301L tau transgenic mice. Supplemented animals seem to be more active than non-treated mice, especially from October 2004 to March 2005. However, behavioral and weight assessments show no significant differences (Figure 3) in treated mice. In a different tauopathy, Dedeoglu and colleagues (2002) found that cystamine significantly improved motor performance and delayed pathology in a murine model of Huntington’s disease. Our evaluations do not support their findings.

**Mitochondrial Impairment Increases Oxidative Stress in PSP**

It has been shown that oxidative stress is present in PSP brains with decreased activity of a mitochondrial enzyme, alpha-ketoglutarate dehydrogenase (KGDHC) (Park et al., 2001). KGDHC is a rate-limiting enzyme in the Krebs cycle. Irregularities in energy metabolism by mitochondrial dysfunction and oxidative stress are associated with neuronal loss in AD and PSP. This has been confirmed by studies of glucose metabolism in PSP patients using positron emission tomography (PET). PSP patients are seen to have reduced glucose utilization in the frontal cortex and basal ganglia structures (Chirichigno et al., 2002). The cause and consequences of such mitochondrial dysfunction remain unknown. However, Albers and colleagues (2001) have proposed a mechanism involving oxidative pathology found in PSP.

**ROS Increase Cytoplasmic Calcium Levels which Activate T-Gase**

A compensatory cellular response to increased free radical production is upregulation of antioxidant defense systems, such as superoxide dismutase (SOD) and glutathione (Albers et al., 2001). Yet, these defense mechanisms do not have the capacity to scavenge ROS found in PSP pathology. Oxidative pathology has also been shown to increase intracellular calcium levels in two ways. First, ROS decreases activity of plasma membrane calcium ATPase activity (Powers et al., 2005). The inactivity of this enzyme hinders the movement of calcium in and out of the cell. Second, the endoplasmic reticulum serves as an intracellular calcium store. In the presence of ROS, these calcium stores are lysed open by lipid peroxidation (Mattson et al., 1997). Calsequestrin, which binds calcium in the cytoplasm, is also upregulated in the presence of ROS (Hunter et al., 2001). Oxidative stress is also associated with abnormalities in energy metabolism, as mentioned above. Hypoglycemia has been shown to increase intracellular calcium levels (Cheng et al., 1992). All of these conditions make a suitable environment for the activity of transglutaminase. The concentration of calcium required for activation of T-Gase is higher than the physiological range associated with most intracellular processes (Karpju et al., 2004). High intracellular calcium levels also upregulate caspases, which are a group of cysteine proteases. Tau is a substrate for the apoptotic protease caspase-3. After cleavage, tau is turned into an effector of apoptosis, generating a self-propagating, positive-feedback loop (Fasulo et al., 2005). These caspase cleaved fragments of tau may serve as a better substrate for T-Gase (Karpju et al., 2004). This evaluation gives us clues into how cystamine will perform in P301L mice.

**P301L Transgenic Mice are not Effective Tauopathy Models**

Cystamine may provide effective therapies to the aforementioned conditions in PSP neuropathology. Cystamine has been mentioned to increase antioxidant levels within the cell and inhibit transglutaminase. Cystamine also serves as a caspase-3 inhibitor, which would possibly decrease the probability of transglutaminase catalyzed cross-linking of tau (Fentile et al., 2003). Future directions may involve the creation of better transgenic mice to model PSP. P301L tau transgenic mice used are an extreme model of PSP, which may set the bar higher for any successful therapeutic approach with cystamine.

**Experimental Procedures**

**Animals**

Female P301L tau transgenic mice were obtained from Taconic™ (Germantown, NY) at two months of age and were assessed for motor disturbances using behavioral tests as an indication for disease progression twice a week. Body weights were recorded during this time. A behavioral scoring rubric was constructed to chart this progress (Table 1). Tail hang, righting reflex, and wire hang tests were performed according to Karl and colleagues (2002). Once mice arrived to an end-stage behavioral phenotype consisting of a score between 24 and 30 (Table 1), they were sacrificed. Mice were also observed for poor grooming and gait, deficient body posture, reduced weight loss, and development of eye irritations. These criteria were evaluated before euthanasia by cervical dislocation/RWT tau and nontransgenic mice were also obtained from Taconic. The age of the animals used in this experiment ranged from 10 to 16 months, where 4RWT tau (n = 4) and nontransgenic mice (n = 4) were age matched to P301L tau transgenic mice (n = 1). Due to lack of aged mice tissue, spinal cord OxyBlot experiments were performed with two P301L tau transgenic mice that were sacrificed at 4.3 months.

**Drug Treatment**

Cystamine dihydrochloride (MW 225.2), purchased from ICN Biomedicals Inc. (Irvine, CA), was orally administered (15mg/kg/day) to P301L tau transgenic mice via water bottles. Control group was given regular tap water. Consumption was recorded daily in the mid-morning.

**Human Tissue**

Human AD cortex tissue from pathologically confirmed cases was obtained from the Loyola University Brain Bank (Maywood, IL).

**Tissue Harvest**

Brain and spinal cord tissue were extracted from sacrificed mice and then cooled rapidly to minus 80°C. This procedure used a flash freezing technique. After the brain was extracted, it was rinsed in 0.9 % saline solution and was cut into two hemispheres. A prepared methanol bath was cooled with dry ice. A 10 ml beaker was filled with isopentane and placed in the ethanol bath to cool. Each hemisphere was placed in the isopentane for approximately 5 minutes or until air bubbles finished emerging from the tissue and changed to a whitish hue. The spinal cord was extracted and cooled by placing it in paraffin on dry ice. Spinal cord could not be flash frozen because of its delicate make-up. The tissues were massed before storage at minus 80°C.

**Tissue Preparation**

Spinal cord tissue from P301L tau, 4RWT tau, and nontransgenic mice was processed using Dounce homogenizing tubes with glass pestles in three volumes of TBS (10mM Tris-HCl, pH 7.5, 0.14 M NaCl), 1 mM EDTA, and 1:1000 protease inhibitor cocktail from Sigma (St. Louis, MO) (Halverson et al., 2005).

**Determining Protein Concentration**

BCA Protein Assay Kit from Pierce (Rockford, IL) with a detergent-compatible formulation based on bicinchoninic acid (BCA) was used
for the colorimetric detection and quantification of total protein in homogenate. Unknown protein concentrations were determined by comparison to serial dilutions (0, 1, 2, 4, 7, 15, 20 μg/ml) of known standard protein concentrations from bovine serum albumin (BSA). Each prepared standard and sample were loaded into a 96 microwell plate in triplicates. The plate was then gently shaken and allowed to incubate at 37°C with a cover plate for 30 minutes. Samples were read on a BioRad Microplate Spectrophotometer (Model 680) at 540 nm. Final unknown protein concentrations were determined by interpolating values made from a curve of standard values from BSA.

Derivatization of Carboxyl Groups
OxyBlot Protein Oxidation Detection Kit was obtained from Chemicon International (Temecula, CA) and was used to perform immunoblot detection of modified proteins by carbonyl groups. Samples were prepared with a final protein concentration of 20 μg. Carbonyl groups in samples were derivatized to 2,4-dinitrophenylhydrazone (DNPH) by reaction with 2,4-dinitrophenylhydrazine (DNPH). Samples were stored at 4°C until ready to load on polyacrylamide gel.

Western Blotting
The derivatized samples were separated by a polyacrylamide gel (12%) electrophoresis at 200 volts and transferred onto a nitrocellulose membrane at 100 volts for 2 hours at 16°C. The membranes were then blocked with 1% BSA-PBS-Tween 20 for 1 hour at room temperature. A rabbit anti-DNP (1:150 diluted in 1% BSA-PBS-Tween 20) primary antibody was used to probe the membranes overnight at 4°C. Membranes were rinsed twice with 1X PBS-Tween 20 and then washed for 15 minutes, followed by 2 washes for 5 minutes. A goat anti-rabbit IgG (1:300 diluted in 1% BSA-PBS-Tween 20), Horse radish peroxidase (HRP) conjugated, secondary antibody was used to probe the membranes for 1 hour at room temperature. The membranes were rinsed and washed as they were after probing with the primary antibody. Chemiluminescent reagents (1:1 luminal and enhancer) from Amersham Biosciences (Buckinghamshire, England) were used to treat the membranes for development by short exposure to blue-light sensitive films from Denville Scientific Inc. (Metuchen, NJ). Antibodies for immunodetection were supplied by the OxyBlot kit.

Actin Probing
Membranes were washed twice with 1% BSA-PBS-Tween 20 for 5 minutes and then stripped with 1M NaOH for 10 minutes at room temperature and washed again. The membranes were then reblocked with 5% BSA-PBS-Tween 20 for 1 hour at room temperature and subsequently probed with monoclonal actin (clone 4, which is a mouse IgG (gamma 1 heavy/kappa light chain) antibody (1:15,000 diluted in 1% BSA-PBS, pH 7.6, with 0.1% sodium azide) from MP Biomedicals, Inc. (Aurora, OH) for 1 hour at room temperature. Washing was then performed three times for 5 minutes. A rabbit anti-mouse IgG, HRP-conjugated, secondary antibody (1:10,000 diluted in 1% BSA-PBS-Tween 20) from Jackson Immuno Research Laboratories, Inc. (West Grove, PA) was used for 1 hour. Membranes were developed as done in the OxyBlot protocol.

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