

Stimulator of neurotropic effects determining the mechanism of action of the MS-818 compound through protein identification by affinity chromatography and SDS-PAGE

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STIMULATOR OF NEUROTROPIC EFFECTS: DETERMINING THE
MECHANISM OF ACTION OF THE MS-818 COMPOUND THROUGH
PROTEIN IDENTIFICATION BY AFFINITY CHROMATOGRAPHY AND
SDS-PAGE

by

CHARLENE SERAPHINA DASS

A thesis submitted in partial fulfillment of the requirements
for the Honors in the Major Program in Molecular Biology and Microbiology
in the College of Medicine
and in the Burnett Honors College
at the University of Central Florida
Orlando, Florida

Summer Term 2011

Thesis Chair: Dr. Kiminobu Sugaya

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DEDICATION

To my wonderful parents, who have been there for me every step of the way. I am forever grateful for everything you have done for me. Your endless love and support have made me the person I am today. I only want to make you proud.

I love you Mom and Dad, more than I can ever say.

To Shannon and Dane, thank you for always being there for me. I am very lucky to have you as a sister and brother. I love you very much.

To Stephen, my everything.

Thank you for all of your love, support, and guidance.

You are the reason for everything I do.

I love you with all of my heart now and forever.

ACKNOWLEDGEMENTS

Dr. Kiminobu Sugaya, thank you for inviting me into your lab and trusting me to take on this project. I really appreciate all of the patience and advice you have given me. Your knowledge and enthusiasm for science inspires me to continue doing research throughout my future career.

Stephanie Merchant, thank you for everything you have done in the past two years. Your kindness and warm heart made me feel at home the day I started volunteering in the lab. I am very grateful for all of your support and advice.

Dr. Borgon, thank you for inspiring me to get involved in research. I am very appreciative of all of your help. You welcomed any questions I had and provided me with great guidance. Without you, I would not have been able to complete my research.

Dr. Kevin Belfield, thank you for being a part of my committee.

Thank you to all of my family and friends who have helped me on this journey.

ABSTRACT

The MS-818 compound is used in the proliferation process of neuronal cells and many biological activities that accompany this process such as astrocyte differentiation, inhibition of neuronal apoptosis, and fraction repairs. We do know the effects of this compound, but the mechanism of action remained uncertain until now. To determine the pathway of this compound, NT2 cells were cultured and lysed to isolate the proteins. Affinity Chromatography was performed in order to immobilize the MS-818 compound to a Hi-Trap NHS column. The NT2 protein sample was injected through the column and eluted with a MS-818 concentrated, high salt content elution buffer. SDS-PAGE was then performed to isolate the proteins that bound to MS-818. The gel was visualized using Coomassie Blue.

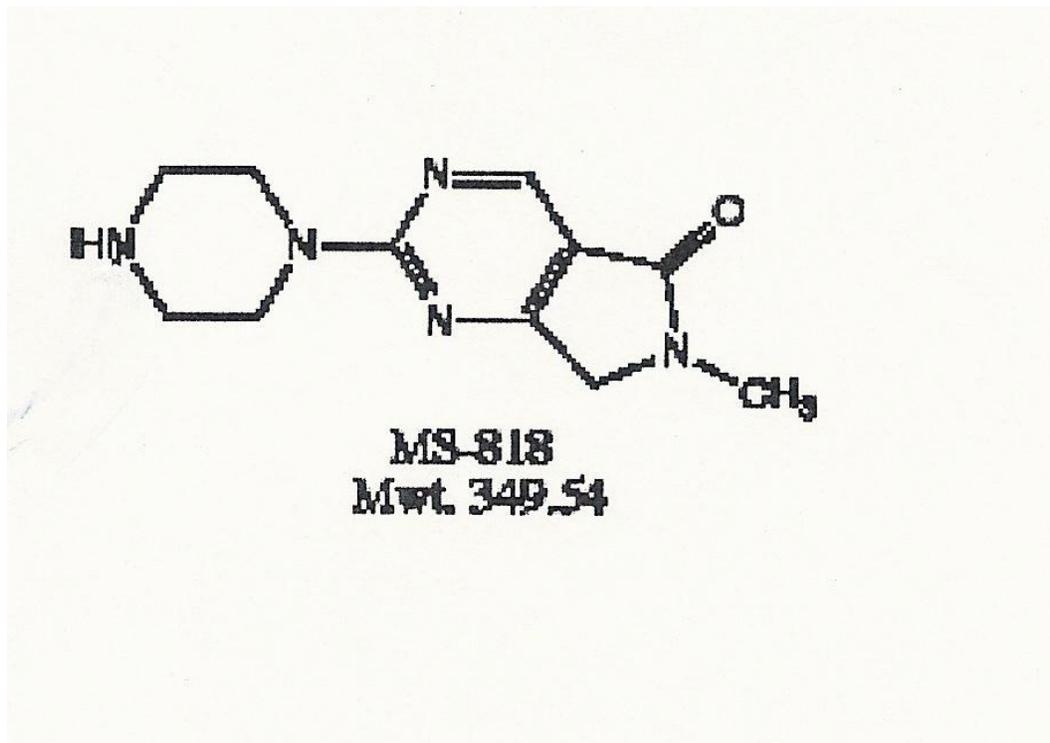
The results indicate that there are two proteins associated in the mechanism of this compound. A standard protein marker ranging from 10 kDa to 250 kDa was used to compare the bands. The findings indicate that one of the protein bands is slightly less than 250kDa and the other is between 50-75 kDa. When the proteins are confirmed by mass spectrometry sequencing, this will help to promote this compound as a drug candidate.

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INTRODUCTION

The nervous system is divided into two parts: the peripheral nervous system and the central nervous system. While the peripheral nervous system is able to regenerate and repair itself, the central nervous system is incapable of doing so without the assistance of regenerative compounds such as MS-818 (Kamm, and Zettl, 2011). The MS-818 compound (2-piperadino-6-methyl-5-oxo-5, 6-dihydro (7H) pyrrolo [2, 3-d] pyrimidine maleate) is a heterocyclic pyrimidine derivative (Jiang, Mei, Ohnishi, Yamamoto, Murai, Awaya, and Ikeda, 1995).



Although there have not been many studies that have identified the mechanism of this compound, there have been studies that test the effectiveness of this endogenous cell stimulator. Since MS-818 has been shown to improve neuronal growth, Human Tetracarcinoma cells (NT2)

were used as a model system to isolate proteins. The NT2 cell line is derived from human testicular cancer and is induced to differentiate into neurons (Podrygajlo, 2009).

According to Sanjo, Owada, Kobayashi, Mizusawa, Awaya and Michikawa (1998) it was observed that MS-818 promotes neurite outgrowth in neuronal cell lines, elevates mitogen-activated protein kinase levels in neurons, and increases the neurotrophic levels in the basic fibroblast growth factor in mouse cortices that were treated with specific doses of the compound. While the basic fibroblast growth factor is known to stimulate the proliferation of mesodermal tissues such as fibroblasts and endothelial cells, it also stimulates neuroectodermal cells (Kuhn, Page, Nguyen, Ko, Wang, Wachtel, and Robson, 2001). MS-818 acts as an agonist in the regeneration of neuronal growth in conjunction with the basic fibroblast growth factor. This is just one of many studies that have shown MS-818 to promote neuronal growth among mammalian cell lines.

In the study performed by Mitsumaya, Kawamata, Yamane, Awaya and Hori (2002) rats were given permanent right middle cerebral artery occlusion. The rats were dispersed into four groups, each given a different treatment dose of MS-818 (1, 5, or 10 mg/kg) and a control group that was administered phosphate-buffer saline. The results showed a number of activates had occurred in the three groups that were administered MS-818. There was evidence of neurite outgrowth, astrocyte differentiation, inhibition of neuronal apoptosis, fracture repairs, angiogenesis, regeneration of injured peripheral nerves and super-ovulation.

Since there is evidence that this might be a membrane bound protein, the goal of my research was to isolate and identify the proteins that interact and bind to this compound. The effects of this non-invasive enhancer are substantial with no known adverse side effects as studied thus far. If we can potentially target the proteins and find the pathway associated with MS-818 this novel compound could be the key to curing neurodegenerative diseases such as Multiple Sclerosis, Parkinson's, Alzheimer's and Huntington's disease (Plunet, Ward, Kwon, and Tetzlaff, 2002).

METHODS

NT2 Cell Lysis and Protein Isolation

NT2 cells were cultured in a 75cm² flask and incubated for several days at 37°C. Once the cells were 100% confluent, the culture was washed twice with cold Phosphate Buffered Solution. 10 µl of Protease Inhibitor Cocktail was added immediately before being treated with 1 ml of lysis buffer (25mM Tris-pH 7.4, 20mM NaCl, 1% deoxycholic acid, 1% Triton X-100, 1 mM Phenylmethylsulfonyl Fluoride, 1 µg/ml Pepstatin, 1 µg/ml Leupeptin). The flask was kept on ice for 5 minutes, on an orbital shaker. A cell scraper was used to gather the lysate to one corner of the flask and then transferred to a microcentrifuge tube. The sample was centrifuged at 14,000 * g for 15 minutes. The supernatant was transferred to a new microcentrifuge tube. The sample was stored at -20°C.

Affinity Column Preparation and Coupling

A 1 ml Hi Trap NHS-activated HP column was used to purify the protein sample using a syringe. The MS-818 Compound was dissolved in a coupling buffer (0.2 M NaHCO₃, 0.5 M NaCl, pH 8.3). Before beginning the ligand coupling, a drop of ice cold 1 mM HCl was applied to the top of the column in order to avoid air bubbles. The column was stored in 100% isopropanol to prevent deactivation of the NHS groups; 6 ml of ice cold 1 mM HCl was used to wash out the isopropanol at a rate of 1 ml/min. Then, 1 ml of the ligand coupling buffer was immediately injected into the column. The column was then sealed and stored at 25°C for 30 minutes.

The column was then subjected to a series of washing and deactivation to inactivate any excess active groups that did not couple to the ligand. Buffer A (0.5 M ethanolamine, 0.5 M NaCl, pH 8.3) and Buffer B (0.1 M acetate, 0.5 M NaCl, pH 4) were prepared. 6 ml of Buffer A was injected first into the column, then 6 ml of Buffer B and again 6 ml of Buffer A. The column was left at room for 30 minutes and the procedure was repeated once again. Lastly, 2 ml of a neutral pH buffer was injected into the column. The coupling efficiency was measured using a low pH solution. Once the coupling procedure was finished, the solution was washed out with the standard coupling buffer (0.2 M NaHCO₃, 0.5 M NaCl, pH 8.3). 1 ml of the coupling buffer was mixed with 1 ml of 2 M glycine-HCl, pH 2.0. The absorbance was measured at 280 nm. The following formula was used to calculate the coupling yield:

Calculation

Loaded coupling solution, A_{280} * ml:

$$A = A_{280} * V$$

where

A_{280} = A_{280} of coupling solution after the ligand was dissolved.

V = loaded volume of coupling solution

Amount not coupled, A_{280} * ml:

$$B = A_{280} * V * 2$$

where

A_{280} = A_{280} of coupling solution after the ligand was dissolved

V = loaded volume of coupling solution

2 = dilution when acidified

Coupling yield, %: (A-B) A * 100

Affinity Column Purification

The column was prepared by first washing with 3 ml of binding buffer (25 mM Tris-HCl, pH 8) and 3 ml of elution buffer (25 mM Tris-HCl, 115 μ M MS-818, 1.37 M NaCl). The column was equilibrated with 10 ml of binding buffer. The NT2 protein sample was injected into the column at a rate of 0.5 ml/min. The column was again washed with 5 ml of binding buffer and then eluted with 3 ml of elution buffer. The sample was collected into microcentrifuge tubes and stored at -20°C.

Protein Separation by SDS- PAGE

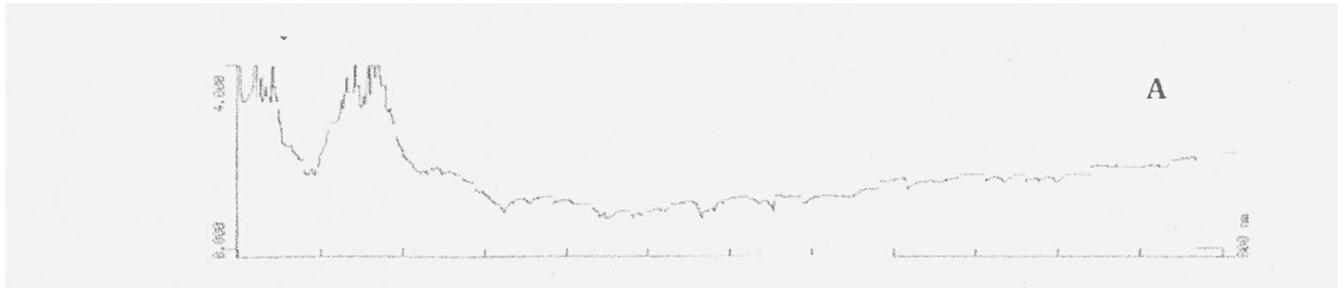
The samples collected from the affinity chromatography were mixed with 2X sample loading buffer (0.5 M Tris-HCl, pH 6.8, glycerol, 10% (w/v) SDS, 0.1% (w/v) Bromophenol Blue, deionized water) and heated for 10 minutes. 15 μ l of the sample was loaded into each well along with the Precision Plus Protein Unstained Standard marker in Lanes 1 and 11. The gel was run at 200V for 60 minutes using the Biorad Mini-Protean Tetra System and the PowerPac HC Power Supply.

Protein Visualization using Coomassie Blue

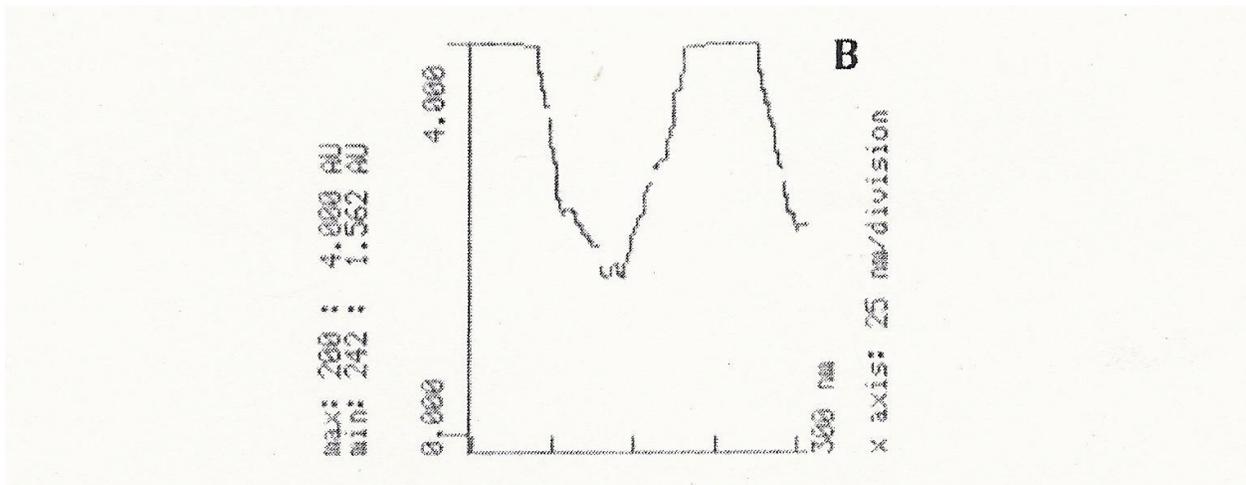
The gel was placed in staining solution (Methanol, Ultrapure water, Glacial Acetic Acid, Coomassie Brilliant Blue R-250) and microwaved for 30 seconds in order to facilitate the uptake of the dye into the gel. It was then placed on an orbital shaker for 60 minutes. The gel was removed and washed off with deionized water. The gel was then placed in destaining solution (Ethanol, Glacial Acetic Acid, Ultrapure water) and microwaved again for 30 seconds. Kimwipes were added to the container and stored at 4°C overnight.

RESULTS

UV- Vis Absorbance of MS-818 at 275 nm

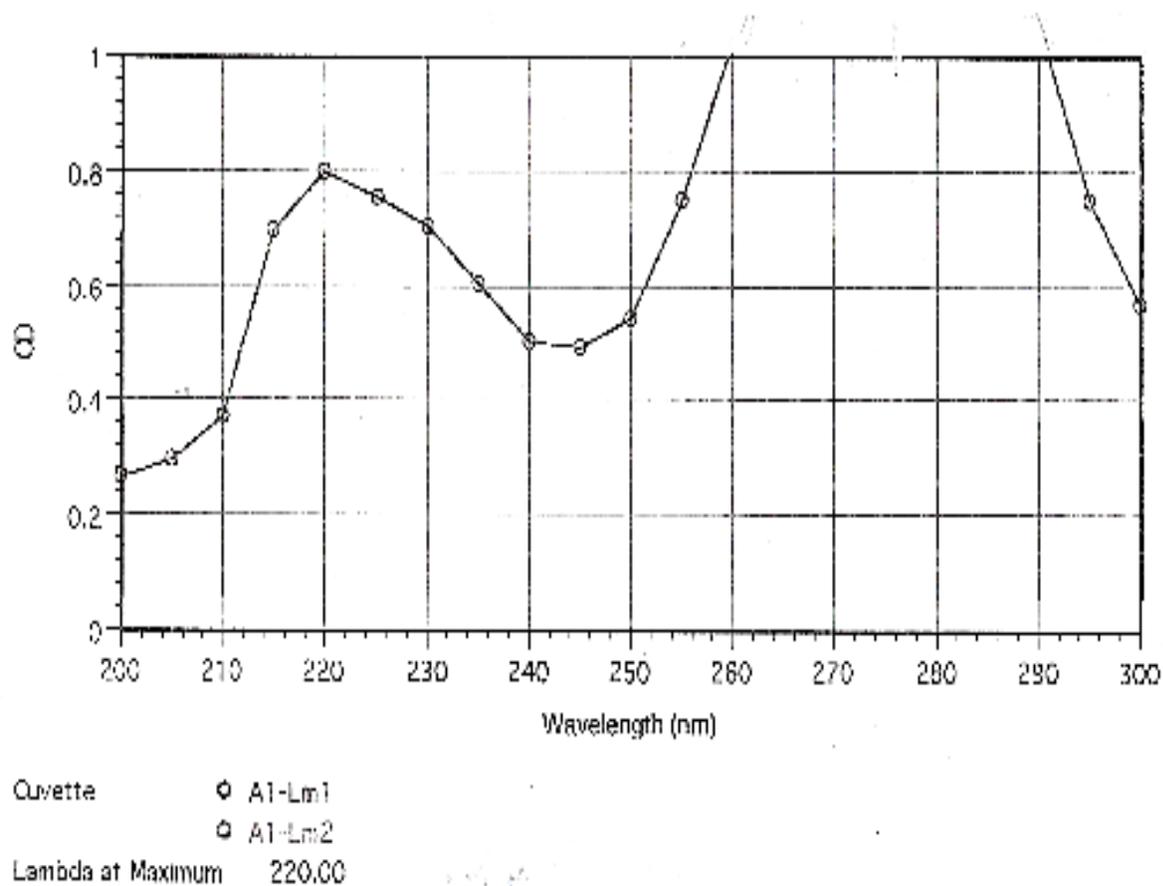


In Figure A, MS-818 was measured in a concentration dependent path. The range is from 200-800 nm.



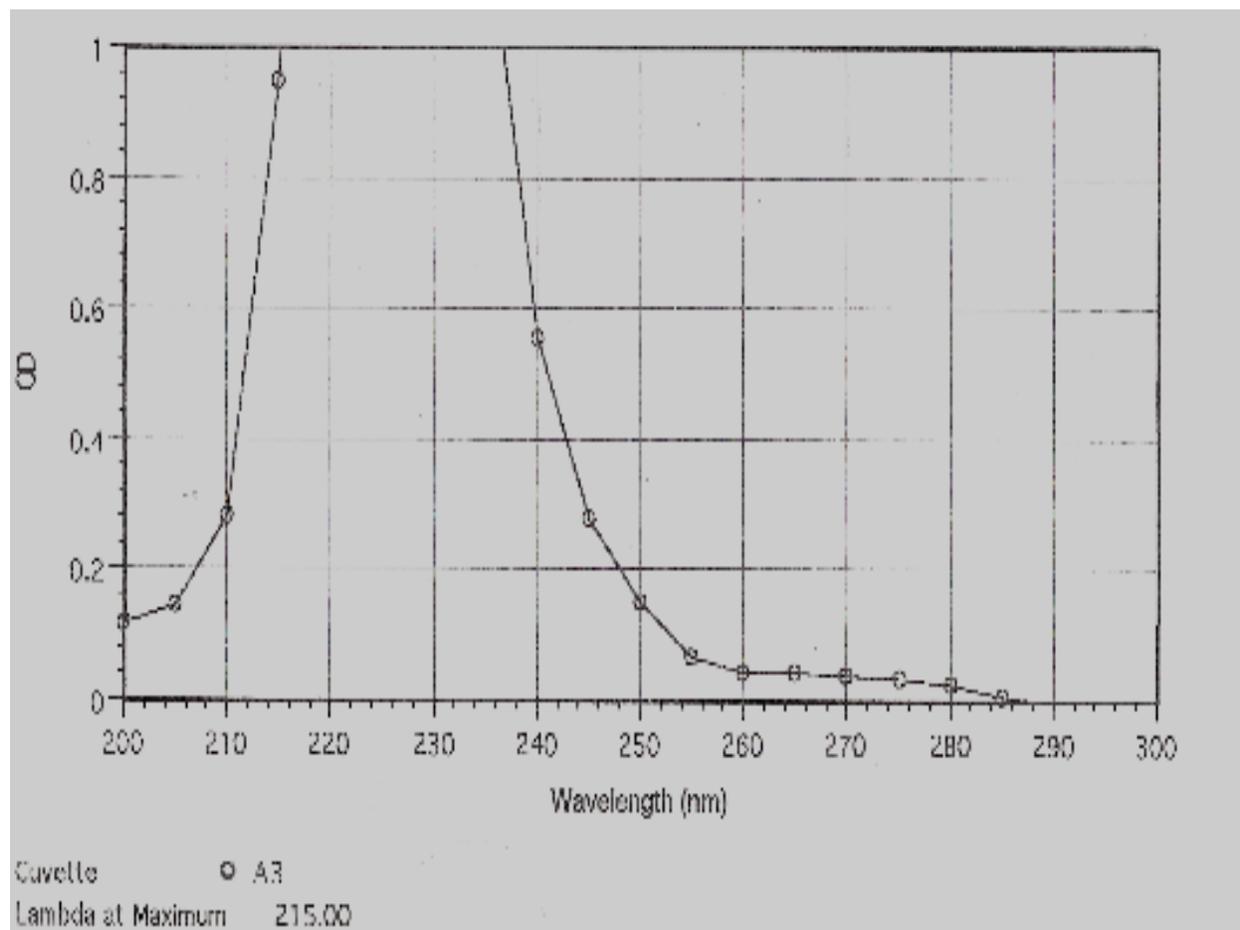
In Figure B, MS-818 can be seen to absorb at 275 nm. The range is from 200-300 nm.

UV-Vis Absorbance of MS-818 before Affinity Column Coupling



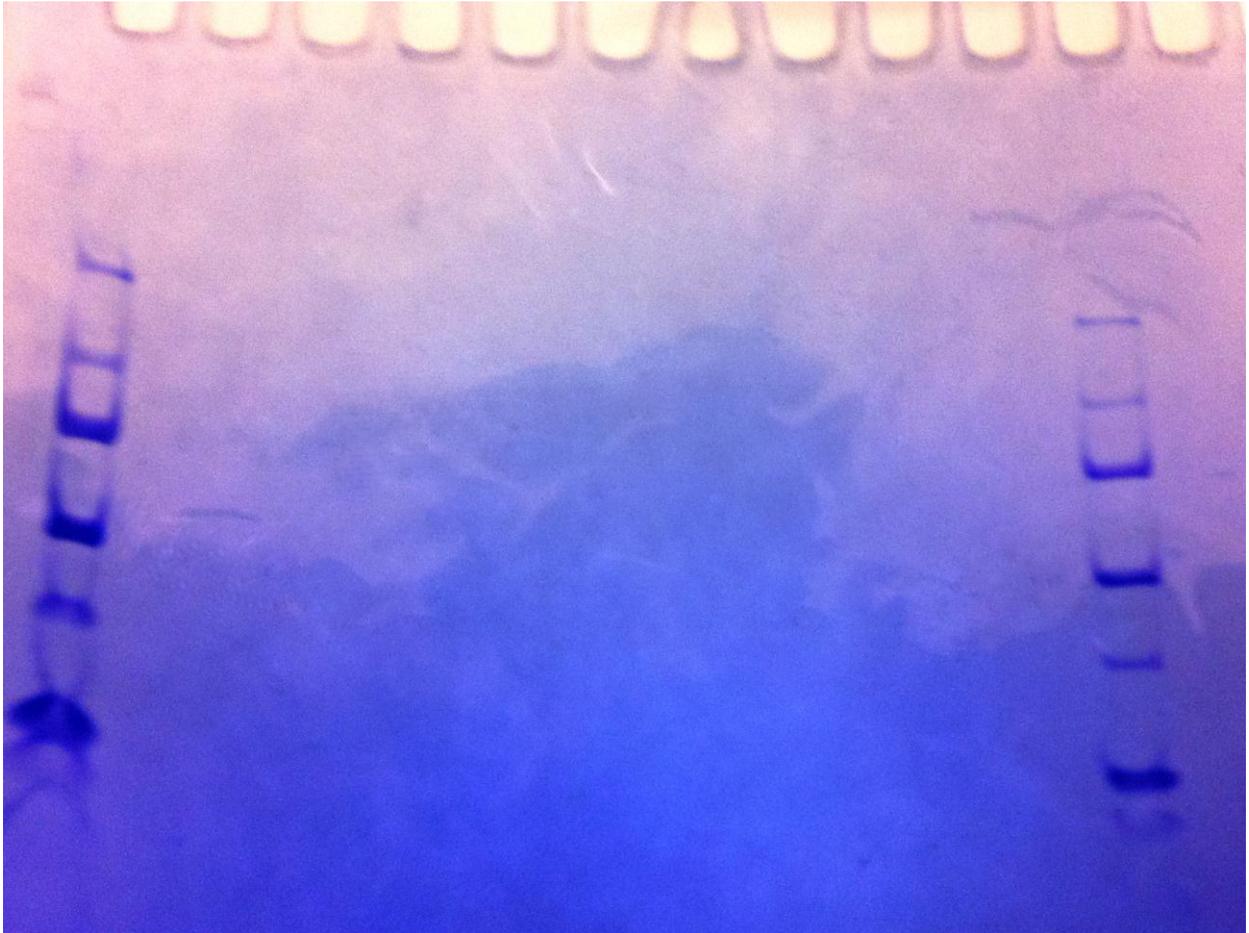
0.4 mg of MS-818 was dissolved in 10 ml of coupling buffer (0.2 M NaHCO₃, 0.5 M NaCl, pH 8.3). The absorbance was measured before loading onto the column. From this graph, there would be a peak at 275 nm where MS-818 absorbs.

UV-Vis Absorbance of MS-818 after Affinity Column Coupling



After the MS-818 was dissolved in the coupling buffer and injected through the column, it was mixed with a low pH, glycine buffer. The effluent was collected and the absorbance was then measured to assess the coupling efficiency of MS-818 to the column. From this graph, there is no peak in the 275 nm region where the ligand absorbs, indicating that MS-818 did in fact stick to the column.

SDS-PAGE using Coomassie Blue Staining



Lanes 1 (left) and 11 (right) contain 15 μ l of Precision Plus Protein Unstained Standard marker ranging from 10 to 250kDa. In Lane 2 there is a band between the 50 to 75 kDa scale. In Lane 10 there is a clear band right below the 250 kDa marker.

DISCUSSION

From the results of the SDS-PAGE, there are clearly two bands of proteins that can be seen. This indicates that MS-818 does interact with proteins from mammalian cell lines, in this case NT2 cells. Currently, I am working on repeating the experiment and performing mass spectroscopy after SDS-PAGE. The bands will be excised and sent to the New York University Medical School to undergo a series of steps to identify the proteins. After, I will perform a Western Blot to confirm the presence of those specific proteins.

There are a couple of improvements that are going to be made to ensure the maximal amount of protein interaction in the next experiment. One of the main reasons I believe there was not a lot of protein in the gel was because of the lack of starting protein isolated from the NT2 cells. This time instead of one 75cm² flask, I will be culturing and using five times more cells. This way there will be more proteins for the MS-818 to interact with during the purification step of affinity chromatography. Also, when purifying the column I will use 2 ml less binding buffer to run through the column after the protein sample is injected; this will maximize the protein yield during SDS-PAGE.

Once the proteins are identified and confirmed by Mass Spectroscopy and Western Blot, we can begin the process of promoting the drug to be a candidate by the U.S. Food and Drug Administration. This novel compound can be the key to helping over five million Americans

suffering from Parkinson's ("What Is PD? - Parkinson Foundation" 2011) and Alzheimer's disease ("Alzheimer's Disease" 2011).

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