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Vilma S. Carson  
*University of North Florida*

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## Characterization of the Basigin-MCT1 complex in vertebrate retina

Vilma S. Carson

Faculty Sponsor: Dr. Judith D. Ochrietor

### Abstract

**It has been hypothesized that the cell adhesion molecule Basigin and the lactate transporter monocarboxylate transporter-1 (MCT1) interact to form a lactate shuttle necessary to fuel photoreceptor neurons in the retina. The purpose of this study was to characterize the interaction between the two proteins. Specifically, the amino acids within MCT1 that interact with Basigin were investigated. MCT1 is a large protein. Therefore, the complementary DNA (cDNA) for MCT1 was divided in half via the polymerase chain reaction (PCR) and each half was cloned into a bacterial expression vector to generate protein probes. The probes were then used to test for binding to Basigin isolated from mouse retina via enzyme-linked immunosorbant assay (ELISA). The results of the ELISA studies indicate that amino acids within the second half, or carboxy (C)-half, of MCT1 are used in the interaction with Basigin. Future studies will be aimed at determining which amino acids within the C-half of MCT1 are specifically involved.**

### Introduction

The retina is a highly organized tissue that converts light signals captured by photoreceptor neurons to electrical signals that are interpreted by the brain as vision. Light enters through the front of the eye and is refracted on the back of the eye, where the retina resides (reviewed in Wade, 2007). The light then travels through several layers of neuronal cells within the retina until it reaches the photoreceptors, also known as rods and cones (reviewed in Wade, 2007). Photoreceptor cells contain a photopigment known as rhodopsin, which is made of the protein opsin and the chromophore retinal (reviewed in Ridge and Palczewski, 2007). Upon light stimulation, the chromophore changes its conformation and initiates a cascade of events that leads to neurotransmitter release from the photoreceptor and subsequent electrical signaling through the other neurons of the retina tissue (reviewed in Ridge and Palczewski, 2007). This process of light detection and visual signaling requires a great amount of energy (Winkler, 1981). It is therefore not surprising that the retina is a highly metabolic tissue (Winkler, 1981).

Like most tissues in the body, the proper interactions between cells must occur for proper development and function of the retina. It has been demonstrated that photoreceptor neurons depend on Müller glial cells for proper function within the retina (Winkler, 1981; Poitry-Yamate et al., 1995). Previous studies by this laboratory, using mouse retina, suggest that a specific interaction between photoreceptors and Müller glial cells, involving the proteins Basigin and monocarboxylate transporter 1 (MCT1), is essential for photoreceptor cell function (Philp *et al.*, 2003). Without these two proteins in the proper location in the retina, the photoreceptors do not convert the light signal to an electrical signal and the mice are blind (Ochrietor *et al.*, 2002).

Basigin, also called CD147, EMMPRIN, HT7, and RPE7, is a membrane glycoprotein that contains extracellular immunoglobulin-like domains and a separate domain that spans the plasma membrane (reviewed in Ochrietor and Linser, 2004). Basigin is expressed in a wide variety of tissues, including the brain, thymus, testes, and retina

(Ochrietor *et al.*, 2003). In the mouse retina, Basigin is expressed on Müller cells and photoreceptor cells (Ochrietor *et al.*, 2001). The biological function of Basigin is unknown, but it is thought to participate in cell to cell interactions (reviewed in Ochrietor and Linser, 2004). Previous studies by this laboratory and others also indicate that Basigin interacts with MCT1, another membrane protein within the same cell (Kirk *et al.*, 2000; Wilson *et al.*, 2002; Philp *et al.*, 2003).

MCT1 is a glycoprotein consisting of twelve membrane-spanning domains (Halestrap and Price, 1999). MCT1 is expressed in a wide variety of tissues and is usually co-expressed with Basigin (Clamp *et al.*, 2004). In the mouse retina, MCT1 is expressed on Müller cells and photoreceptor cells (Philp *et al.*, 2003). As the name suggests, MCT1 is a transporter of monocarboxylates such as pyruvate and lactate (Halestrap and Price, 1999). Although lactate is typically produced as a result of anaerobic conditions, the retina and brain produce lactate even in the presence of oxygen (Poitry-Yamate *et al.*, 1995). In the retina, Müller glial cells produce and release lactate via facilitated diffusion through MCT1 (Poitry-Yamate *et al.*, 1995; Halestrap and Price, 1999). The lactate is then taken up by photoreceptor neurons also expressing MCT1, converted to pyruvate, and used to generate the energy needed for the rods and cones to function (Poitry-Yamate *et al.*, 1995; Philp *et al.*, 2003).

Studies using a strain of mice in which the Basigin gene is deleted (Basigin null mice; Igakura *et al.*, 1998) suggest that Basigin and MCT1 are needed for proper photoreceptor cell function (Philp *et al.*, 2003). When Basigin is not expressed by photoreceptors and Müller glial cells, MCT1 does not translocate to the plasma membrane, but rather remains in intracellular vesicles within those cells (Philp *et al.*, 2003). It is likely that the two proteins must associate after protein synthesis and translocate to the plasma membrane together (Kirk *et al.*, 2000; Wilson *et al.*, 2002; Philp *et al.*, 2003). It was hypothesized that the two membrane proteins function together to comprise a lactate shuttle between Müller cells and photoreceptors. Without the lactate shuttle, photoreceptors do not have the energy necessary to function properly, as seen in the Basigin null mice (Philp *et al.*, 2003).

Previous studies using immunoprecipitation and fluorescence resonance energy transfer (FRET) indicate that Basigin and MCT1 do physically interact (Kirk *et al.*, 2000; Wilson *et al.*, 2002; Philp *et al.*, 2003). However, the details of the interaction remain unknown. This present study is aimed at determining which amino acids within MCT1 interact with Basigin. MCT1 is a large protein and was therefore cut in half via molecular biology techniques to make the search for critical amino acids more approachable. Each half of MCT1 (the amino [N]-half and the carboxy [C]-half) was then used as a probe to determine if it binds to Basigin. The data suggest that amino acids within the C-terminal half of MCT1 are used in the interaction with Basigin.

## **Materials and Methods**

### Plasmid Construction

The plasmid pTopo-MCT1, which contains the full-length MCT1 cDNA sequence (accession number NM 009196), was used as template for PCR amplification of the N-terminal and C-terminal halves of the protein. Primers for the N-terminal half were: pET102-MCT1-start, 5' CACCATGCCACC-TGCGATTGGAG and pET102-MCT-N-RV, 5' CCCCTATGGGTAAACAGCGACA. Primers for the C-terminal half were: pET102-

MCT1-C-Fwd, 5'-CACCTTGTCGCTGT-TTACCCATAGG and pET102-MCT1-end RV, 5'GACAGGGCTCTCCTCCTCTGTGG. The polymerase chain reaction (PCR) cycling protocol used was as follows: 95°C for 2 minutes followed by 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for one minute. The DNA fragments were each cloned into the bacterial expression vector pET102/D (Invitrogen Corporation, Carlsbad, CA), which adds a six-histidine tag (6X-His) to the carboxy-terminus of recombinant proteins. The plasmids pET102-MCT1-N (N-terminus) and pET102-MCT1-C (C-terminus) were transformed into Top10 *E. coli* cells (Invitrogen Corporation). The resultant colonies were analyzed via a PCR screen and the plasmids from the positive colonies were sequenced for accuracy using the Beckman-Coulter Quickstart protocol on a Beckman-Coulter CEQ automated DNA sequencer (Fullerton, CA).

#### Recombinant protein expression

The pET102-MCT1-N and pET102-MCT1-C plasmids were used as templates for *in vitro* transcription and translation using PROTEINscript II (Ambion, Austin, TX). Plasmid (0.5 µg) was combined with transcription mix and T7 enzyme mix and incubated at 30°C for 1 hour to generate MCT1-N and MCT1-C transcripts. The transcripts were then combined with translation mix, methionine, and retic lysate and incubated at 30°C for 1 hour to generate the recombinant proteins MCT1-N-6XHis and MCT1-C-6XHis.

#### Mouse retina proteins

Mouse retina lysates were obtained from Dr. Paul Linser at the University of Florida Whitney Laboratory for Marine Bioscience. Two mice were sacrificed according to accepted protocols and the eyes were immediately removed. The retinas were isolated from the four eye cups and purified water (400 µL) was added to the tissue. The tissue was disrupted by vortexing and stored at -80°C.

#### ELISA analyses

The mouse retina lysates were tested for the presence of Basigin protein via enzyme-linked immunosorbent assay (ELISA). Wells of a 96-well plate were coated with antibodies specific to Basigin (500 ng/mL; Fan *et al.*, 1998) in phosphate buffered saline (PBS; 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 2.7 mM KCl; pH 7.4) overnight at 4°C. Wells coated with PBS alone served as the control. Unbound antibody was removed with three washes of PBS-T (PBS plus 0.5% Tween 20). Mouse retina lysates (1µg/mL) were added to the wells and incubated at room temperature for 30 minutes. Unbound proteins were removed by three washes with PBS-T. The antibody specific to Basigin (Fan *et al.*, 1998) was added to the wells and incubated at room temperature for 30 minutes. Unbound antibody was removed by three washes with PBS-T. Alkaline phosphatase-conjugated goat-anti-rabbit was added to the wells and incubated at room temperature for 30 minutes. Unbound antibody was removed by three washes with PBS-T. Alkaline phosphatase substrate (PNPP, Pierce Biotechnologies, Rockford, IL) was added to the wells and incubated at room temperature for 2 minutes. The control wells received PBS throughout. PNPP substrate was added in the final step and incubated at room temperature for 2 minutes. The reaction was stopped by the addition of 2N NaOH and the absorbance at 405 nm was read and recorded on a Biotek plate reader. Microsoft Excel software was used to graph and analyze the data.

ELISA was then performed to test for recombinant MCT1 binding to endogenous Basigin from mouse retina lysates. Wells of a 96-well plate were coated with antibodies specific to Basigin (500 ng/mL; Fan *et al.*, 1998) in PBS overnight at 4°C. Wells coated

with PBS alone served as the control. Mouse retina lysates (1µg/mL) were added to the wells and incubated at room temperature for 30 minutes. Unbound proteins were removed by three washes with PBS-T. Recombinant protein (either MCT1-N-6XHis or MCT1-C-6XHis) was added to the wells and incubated at room temperature for 30 minutes. Unbound proteins were removed by three washes of PBS-T. Antibody specific for the 6X-His (anti-6XHis C-term; Invitrogen Corporation) was added to the wells at a 1:1000 dilution in PBS and incubated at room temperature for 30 minutes. Unbound antibody was removed by washing the wells three times with PBS-T. Alkaline phosphatase-conjugated goat-anti-mouse secondary antibody was added to the wells at a 1:1000 dilution in PBS and incubated at room temperature for 30 minutes. Unbound antibody was removed by washing the wells three times with PBS-T. Alkaline phosphatase substrate (PNPP, Pierce Biotechnologies) was added to the wells and incubated at room temperature for 2 hours. The control wells received PBS throughout. PNPP substrate was added in the final step and incubated at room temperature for 2 hours. The reaction was stopped by the addition of 2N NaOH and the absorbance at 405nm was recorded on a BIORad plate reader. Excel software was used to construct a graph and for statistical analysis.

## **Results**

### **Recombinant protein expression**

MCT1 is a membrane protein containing twelve membrane-spanning domains (Halestrap and Price, 1999). The protein was divided in half via molecular biology methods to generate probes to determine which region of the protein interacts with Basigin. Primers were designed based on the sequence for MCT1 cDNA found in the Genbank database (accession number NM 009196) to amplify the N-terminal and C-terminal halves of the resultant protein. The DNA fragments were then cloned into the pET102/D vector (Invitrogen Corporation) for recombinant protein expression. The pET102/D vector adds a six histidine (6XHis) tag to the carboxy terminus of recombinant proteins, which can be used for purification and visualization of the resultant protein. The plasmids were sequenced and translation software (<http://arbl.cvmbs.colostate.edu/molkit/translate/index.html>) was used to determine the corresponding amino acid sequences from the nucleotide sequences obtained. Figure 1 shows the amino acid sequences for MCT1-N-6XHis and MCT1-C-6XHis.

### **Analysis of binding**

Once the MCT1 probes were generated, it was necessary to determine that the mouse retina lysates contain Basigin protein. A sandwich ELISA was performed to verify the presence of Basigin protein in the lysates. Basigin was captured from the lysates using a Basigin-specific antibody (Fan *et al.*, 1998) and then probed with that same antibody to verify its presence. Figure 2 shows the results of these analyses. Although this method does not allow the concentration of Basigin protein in the lysate to be determined, it is evident that Basigin is present in the mouse retina lysate.

The ability of the MCT1-N-6XHis and MCT1-C-6XHis probes to bind Basigin isolated from the mouse retina lysate was then performed. Endogenous mouse Basigin was captured as described and probed with either MCT1-N-6XHis or MCT1-C-6XHis. An antibody specific for the 6XHis was then used to determine whether the probes were retained in the wells containing Basigin or removed upon washing. Figure 3 shows the results of these analyses. Both probes generated a positive signal when incubated with the

captured Basigin protein; however, only MCT1-C-6XHis probe binding was statistically significant when compared to the control (Figure 3).

## **Discussion**

Previous studies by this laboratory and others, using various biochemical techniques, indicate that Basigin and MCT1 interact with one another within the plasma membrane of cells expressing the two membrane proteins (Kirk *et al.*, 2000; Wilson *et al.*, 2002; Philp *et al.*, 2003). Studies of the Basigin null strain of mice suggest that these proteins comprise a lactate shuttle that is necessary for proper photoreceptor cell function within the retina (Philp *et al.*, 2003). Mice lacking Basigin do not express MCT1 at the plasma membrane of Müller glial cells or photoreceptor cells and are considered blind via electrophysiological analyses (Ochriotor *et al.*, 2002; Philp *et al.*, 2003). It is thought that the two proteins interact via their transmembrane domains (Wilson *et al.*, 2002). Basigin has only one membrane-spanning domain, whereas MCT1 possesses twelve such domains. A related study by this laboratory is aimed at determining the amino acids within the Basigin transmembrane domain that interact with MCT1. The purpose of this study was to examine the MCT1 protein to identify amino acids that interact with Basigin. The data obtained indicate that amino acids within the C-half of MCT1 are involved in the interaction.

This study used molecular biology and biochemical techniques to investigate the Basigin-MCT1 interaction. Since MCT1 is a large protein, consisting of twelve membrane-spanning domains, the protein was cut in half and each half was assessed for binding to Basigin. PCR was used to divide the cDNA for MCT1 into the N- and C-halves and the resulting cDNAs were then cloned into a bacterial expression vector to generate what are known as recombinant protein probes. The bacterial expression vector used (pET102/D, Invitrogen Corporation) adds a tag of six histidine amino acids to the C-termini of the recombinant proteins, which aids in purification and identification of those recombinant proteins. Ideally, the expression vector is then introduced to bacterial cells for protein production. However, it was determined that the MCT1 proteins were too complex for the bacteria to synthesize. Therefore, *in vitro* transcription and translation were performed to generate the MCT1 probes for the study. The MCT1 probes were not purified, but rather the entire translation mixture was used when probing via ELISA.

ELISA proved to be an appropriate methodology for studying the interaction, as Basigin protein was efficiently captured from mouse retina lysates. The ELISA protocol involved first capturing Basigin from total mouse retina proteins using an antibody specific for Basigin. After the capture step, the presence of Basigin in the wells was verified using the same Basigin-specific antibody in what is known as a “sandwich” ELISA. A secondary antibody conjugated to the enzyme alkaline phosphatase and substrate for the enzyme was then added to the wells to visualize the presence of Basigin. The alkaline phosphatase substrate produces a color change in the presence of the enzyme, which can be quantified by spectrophotometric means. The wells containing trapped Basigin generated a statistically greater absorbance at 405 nm than the control wells containing only PBS, indicating that Basigin was indeed present in those wells (Figure 2).

The ELISA analyses suggest that amino acids within the C-terminal half of MCT1 interact with Basigin. In these analyses, captured Basigin was probed with MCT1-N-6XHis or MCT1-C-6XHis. Then an antibody specific for the 6XHis, a secondary antibody conjugated to alkaline phosphatase, and alkaline phosphatase substrate were used to detect probe binding to the captured Basigin. Again, spectrophotometric analyses were used to

quantify the color development of the substrate. Wells in which MCT1-C-6XHis was used as probe generated a color reaction that was statistically greater than that of the control containing only PBS via a Student's T-test (Figure 3). The data suggest that amino acids within the MCT1-C-6XHis probe interact with Basigin. This is consistent with recent studies by another laboratory presented at the annual meeting of the Association for Research in Vision and Ophthalmology (Deora *et al.*, 2007). Wells in which MCT1-N-6XHis was used as probe also generated a color reaction; however, the change was not statistically greater than that of the control wells via Student's T-test (Figure 3). Deora *et al.* (2007) did not observe any binding of the N-terminal half of MCT1 to Basigin. The difference in these results may be caused by differences in the exact location at which MCT1 was divided. There is a rather large intracellular loop region between transmembrane domains 6 and 7 of MCT1 (see Figure 1). In this study, MCT1 was cut immediately before transmembrane domain 7 such that the loop region is contained within MCT1-N-6XHis. It was unclear exactly where MCT1 was divided in the Deora *et al.* (2007) study. However, it is possible that the MCT1-C probe, rather than the MCT1-N probe used by Deora *et al.*, (2007) contains this loop region. Such speculation suggest that the loop between transmembrane domains 6 and 7 accounts for the moderate binding of MCT1-N-6XHis to Basigin in the present study. Additional experiments are necessary to determine whether that loop region is involved in the MCT1-Basigin interaction.

The results of this study provide a foundation for future investigations. An important first step has been taken to determine which amino acids within MCT1 interact with Basigin. Future studies will involve a closer examination of the C-terminal half of MCT1 to identify amino acids within that region that may bind to Basigin during lactate shuttle formation in the retina.

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## Figure legends

### Figure 1. Amino acid sequences for the MCT1-N-6XHis and MCT1-C-6XHis probes.

The single letter code for amino acids is shown for each probe. The sequences match that of MCT1 (accession number NM 009196). Each domain of MCT1 contains six transmembrane domains, which are indicated by underlining. The 6XHis tag is indicated in bold.

**Figure 2. Presence of Basigin protein in mouse retina lysate.** Sandwich ELISA was performed to verify the presence of Basigin protein in the retina lysate. Basigin protein was captured and then probed with an antibody specific for Basigin (Fan *et al.*, 1998). The experiment was performed in duplicate and the error bars represent standard deviations. \* =  $p < 0.005$  via Student's T-test.

**Figure 3. Binding of the N-terminal and C-terminal MCT1 probes to Basigin protein.** ELISA was performed to test for binding of the MCT1 probes to mouse Basigin. Basigin protein was captured and then probed with either MCT1-N-6XHis or MCT1-C-6XHis. An antibody specific to the 6XHis (Invitrogen Corporation) was added to visualize the interaction between Basigin and the probe. The experiment was performed in duplicate and the error bars represent standard deviations. \* =  $p < 0.05$  via Student's T-test.

#### N-terminus of MCT1:

MPPAIGGPVGYTPPDGGWGWAVLVGAFISIGFSYAFP  
KSITVFFKEIEVIFSATTSEVSWISSIMLAVMYAGGPISS  
ILVNKYGSRPVMIAGGCLSGCGLIAASFCNTVQELYLC  
IGVIGGLGLAFNLNPALTMIGKYFYKKRPLANGLAMAG  
SPVFLSTLAPLNQAFFDIFDWRGSFLILGGLLLNCCVA  
GSLMRPIGPEQVKLEKLSKESLQEAGKSDANTDLIGG  
SPKGEKLSVFQTINKFLDLSLFTHR**HHHHHH**

C-terminus of MCT1:

LSLFTHRGFLLYLSGNVVMFFGLFTPLVFLSSYGKSKDF  
SSEKSAFLLSILAFVDMVARPSMGLAANTKWIRPRIQYF  
FAASVVANGVCHLLAPLSTTYVGFCVYAGVFGFAFGW  
LSSVLFETLMDLIGPQRFSSAVGLVTIVECCPVLLGPPL  
LGRLNDMYGDYKYTYWACGVILIIAGIYLFIGMGINYRLL  
AKEQKAEEKQKREGKEDEASTDVDEKPKETMCAAQSP  
QQHSSGDPTEEEESP**HHHHHH**

Figure 1.

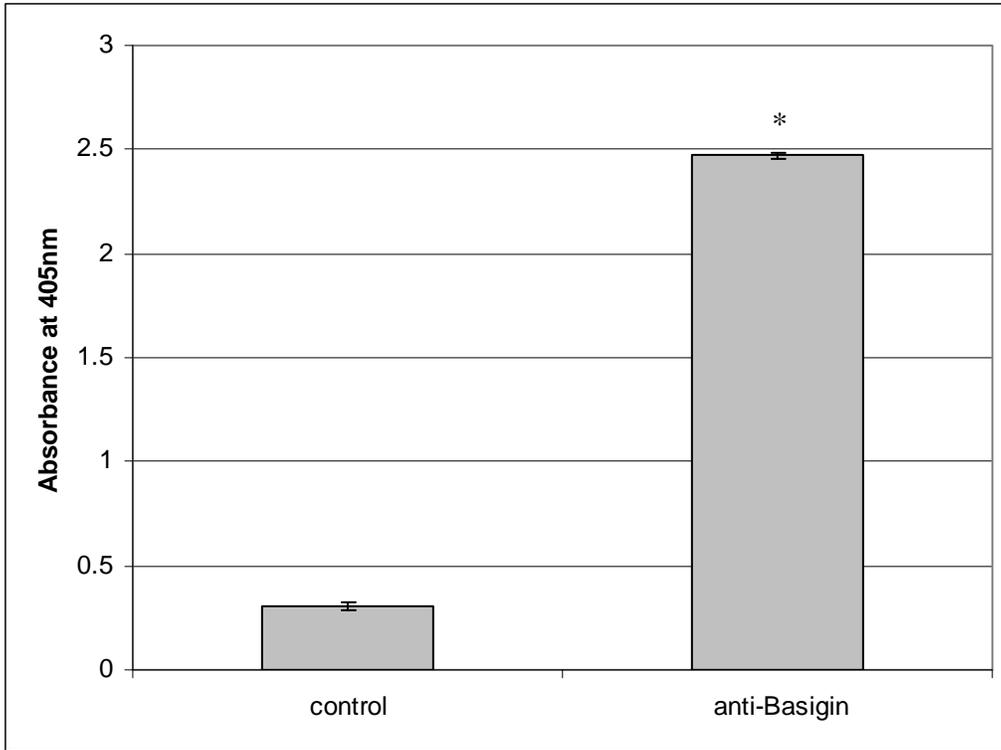


Figure 2.

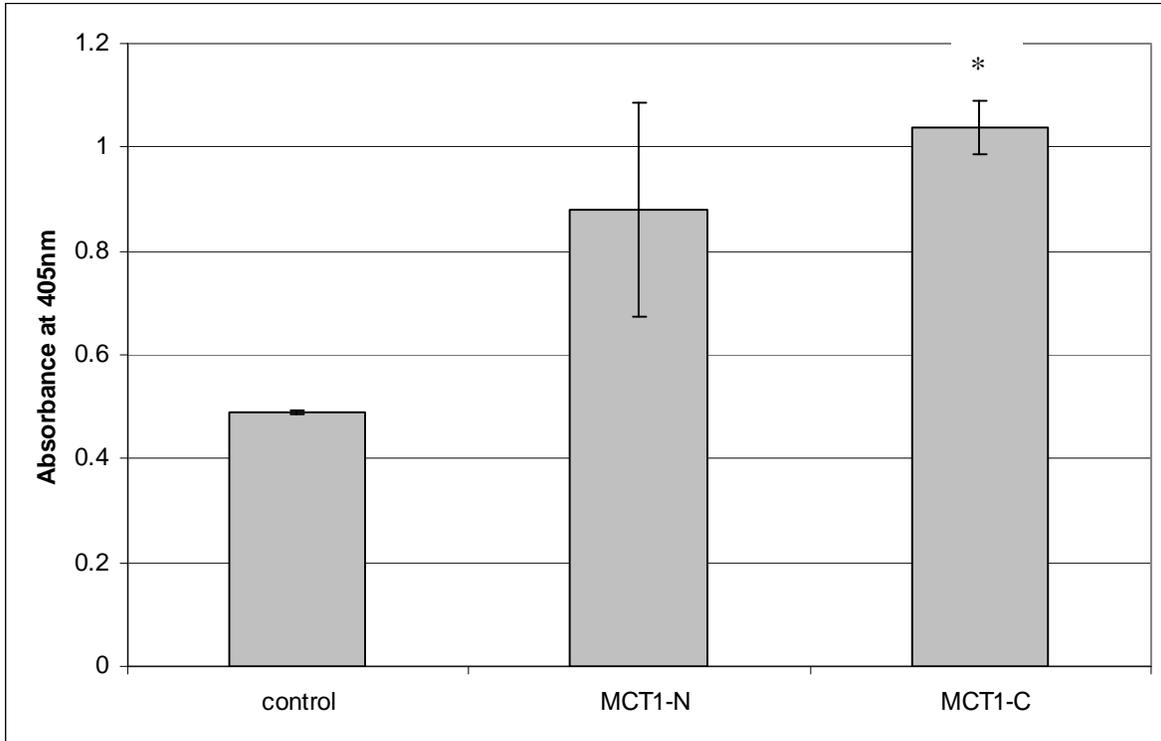


Figure 3.