DETERMINING THE MECHANISMS BY WHICH MEMBRANE TARGETED DIETARY BIOACTIVES REDUCE ONCOGENIC KRAS DRIVEN NUTRIENT SCAVENGING

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Determining the mechanisms by which membrane targeted dietary bioactives reduce oncogenic KRas driven nutrient scavenging

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Approximately 30 to 50% of colorectal cancers contain KRas mutations, which confer resistance to standard therapy and have therefore been termed "undruggable." Since no curative treatments for KRas driven colon cancer are available, there is a critical need to develop toxicologically innocuous KRas therapeutic approaches that are free of safety problems intrinsic to drugs administered over long periods of time. The present study will investigate the mechanisms underlying previous observations that membrane targeted dietary bioactives (MTDBs) attenuate oncogenic Ras driven nutrient scavenging. The overall goal of the study is to link perturbations in plasma membrane organization and cytoskeletal remodeling to reductions in macropinocytosis and ultimately cell proliferation. For this purpose, we have utilized a human colon adenocarcinoma cell line as an *in vitro* model. These experiments are relevant to the chemoprevention field, i.e., colorectal cancer prevention**.**

DEDICATION

To my family and to all of the people that have believed in me. Thank you for always encouraging me to do my best.

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I would like to start by thanking my research advisor, Dr. Robert S. Chapkin. He has encouraged me to conduct undergraduate research and has supported me all the way through. I will always be grateful for the opportunities he has given me. He has been such a great source of encouragement and knowledge.

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Lastly, I would like to thank the Chapkin Lab for accepting me into such a supportive lab, and allowing me to perform research by their side.

NOMENCLATURE

DHA	Docosahexaenoic Acid
DPBS	Dulbecco's Phosphate Buffered Saline
EIPA	5-(N-ethyl-N-isopropyl)amiloride
EPA	Eicosapentaenoic Acid
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
FOV	Field of View
GP	Generalized Polarization
LSD	Least Significant Difference
MTDB	Membrane Targeted Dietary Bioactives
MW	Molecular Weight
n-3 PUFA	Omega-3 Polyunsaturated Fatty Acids
PBS	Phosphate Buffered Saline
TMR	Tetramethlyrhodamine
UV	Ultraviolet

CHAPTER I

INTRODUCTION

Colorectal cancer is one of the major causes of death from cancer in the United States and around the globe (Jemal *et al*., 2011). Ras mutations are found in three out of the four most lethal types of cancer which are the colon, lung, and pancreatic cancer (Cox *et al*, 2014). There are three Ras isoforms H-, N-, and KRas, with the latter being the most common in colon cancer. Recently, Ras had been termed "undruggable" due to no effective pharmacological inhibitors reaching clinical studies (Cox *et al*., 2014). Although direct pharmacological drugs had been shown to be ineffective, targeting the Ras driven dependencies may be a viable strategy (Commisso *et al.*, 2013).

Substantial evidence exists that long chain n-3 PUFA, e.g. docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are chemoprotective agents which protect against colon tumorigenesis (Reddy *et al*., 2005; Chapkin *et al*., 2014). Furthermore, other dietary compounds such as curcumin, and walnut-derived procyanidins appear to exhibit chemoprotective properties (Park *et al*., 2013; Falasca *et al*., 2014). Interestingly these dietary compounds are known to influence the biophysical properties of plasma membranes (Erlejman *et al*., 2004; Kim *et al*., 2008; Tsukamoto *et al*., 2014). This is noteworthy because the biophysical properties of the membrane lipid bilayer are known to influence many cell signaling events (Nicolson, 2014). Therefore, we propose that these dietary compounds fall into a unique class of membrane targeted dietary bioactives (MTDBs) that influence aspects of cellular signaling through direct perturbation of plasma membrane organization.

KRas driven oncogenic proliferation is dependent on the uptake of extracellular lipids and proteins to be used as fuel in the process termed macropinocytosis (Commisso *et al*., 2013; Salloum *et al*., 2014). Since the biophysical properties of the plasma membrane can influence uptake of extracellular lipids and proteins (Magenau *et al*., 2011), we have hypothesized that MTDB modulation of the plasma membrane organization may underlie their ability to reduce macropinocytosis and therefore cancer cell related growth (Commisso *et al*., 2013).

Objectives

The purpose of the conducted experiments was to determine how membrane active dietary compounds influence the proliferation of KRas driven colon cancer. Using plate reader assays, we have validated that n-3 PUFAs, curcumin, and procyanidins actively influence plasma membrane organization. We initially determined the minimal effective dose of select MTDB compounds with respect to SW48 plasma membrane organization. This is important because membrane organization is known to influence cellular processes (Nicolson, 2014). We subsequently determined how these dietary compounds alter macropinocytosis, since membrane organization and cytoskeletal remodeling are important steps regulating this process (Butler *et al*., 2013; Fujii *et al*., 2013). Since KRas driven cancer cells are dependent on macropinocytosis for proliferation, we hypothesize that MTDB inhibition of KRas driven macropinocytosis is mediated by disruption of Rac1 activation and alterations in plasma membrane organization.

CHAPTER II

METHODS

Cell culture and treatments

SW48 human colorectal adenocarcinoma cells (SW48-KRas-wild type (control) and SW48-KRas-G12D engineered to express oncogenic KRas) were provided by Horizon Discovery, Cambridge, United Kingdom were utilized. The two cell lines were cultured at 37°C and 5% CO_2 in the presence of 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and 1% Penicillin/Streptomycin. Select cultures were treated with physiologically relevant doses of MTDBs added to standard culture media for specific time intervals (Conquer *et al*., 1998; Kim *et al*., 2008; Irving *et al*., 2013; Verstraeten *et al*., 2013).

Quantification of macropinocytosis

Cells were seeded onto poly-L-lysine treated chamber slides. Twenty-four to 48 hours after cells were seeded, cells were serum starved for 16-24 hours. During the final hours of starvation, cells were treated with varying doses and combinations of MTDBs. Cells were also treated with 5-(N-ethyl-N-isopropyl)amiloride (EIPA), a macropinocytosis inhibitor specifically a Na^+/H^+ exchange inhibitor, utilized as our positive control of macropinocytosis (Commisso et al., 2013). After treatment, fluorescein isothiocyanate (FITC)-dextran 70,000 MW (1 mg/mL), CellMask Deep Red Plasma Membrane dye (1 µL/mL), and Hoechst 33342 (1 µg/mL) were added to the cell media. Cells were incubated with the dyes for 30 minutes at 37°C. At the end of the incubation and starvation period, cells were placed on ice, rinsed five times with ice cold PBS and fixed in 4% paraformaldehyde for 10-30 minutes. To complete the macropinocytosis assay,

cells were imaged using fluorescent microscopy. Cell images were then analyzed and quantified using ImageJ software (Commisso *et al*., 2013; Commisso *et al*., 2014). Adapted from a *Nature* protocol, a macro was created to determine the macropinocytic index (Commisso *et al.*, 2014). The total cell area is determine by a threshold set according to the plasma membrane dye channel. The total macropinosome area was identified by subtracting the background from the FITC-dextran dye channel, and setting a threshold for the relative size of the puncta. The macropinocytic index was calculated by the division of the total macropinosome area by the total cell area then multiplied by 100.

Quantification of cell proliferation

Cell proliferation was monitored by CyQUANT Direct Cell Proliferation Assay (ThermoFisher, Waltham, MA). Cells were seeded in 96-well plates, and treated with curcumin (1 µM, 10 µM, and 50 µM), (+)-catechin (25 µM, and 50 µM), and procyanidin B2 (25 µM, and 50 µM). The proliferation assay was performed according to the manufacturer's instructions. The fluorescence signal was monitored and recorded every other day for seven days. Cell media was replaced with new treated media on the third day and every other day after.

Quantification of cell viability

Cell viability was assessed using LIVE/DEAD Cell Viability Assay Kit (ThermoFisher, Waltham, MA). Cells were seeded in 96-well plates and treated with MTDBs. Cells were treated for seven days, and new treated media was added every other day. C_{12} resazurin and SYTOX Green from the LIVE/DEAD Cell Viability Assay Kit (ThermoFisher, Waltham, MA)

were added to each well according to the manufacturer's instructions. After 15 minutes of incubation, the cells viability was measured by fluorescent microscopy.

CHAPTER III

RESULTS

Macropinocytosis

Because select MTDBs modify membrane organization (Holy, 2004; Magenau *et al*., 2011), we expected to see an inhibition of macropinocytosis upon exposure to MTDBs. Certain SW48 cells treated with curcumin (1 µM, 10 µM, or 50 µM), (+)-catechin (25 µM, or 50 µM), or procyanidin B2 (25 µM, or 50 µM) for one hour exhibited a reduction in FITC-dextran uptake illustrating a reduction in macropinocytosis.

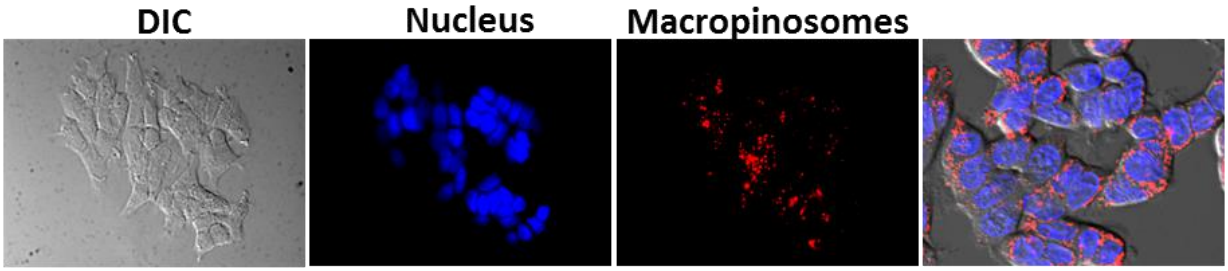

Figure 1. Macropinocytosis and macropinosomes. SW48 cell's nuclei were stained with Hoechst and can be seen in blue. TMR-dextran was taken up by the SW48 cells through macropinocytosis and are in macropinosome vesicles seen in red. Macropinosomes are then set to appropriate thresholds and surface area.

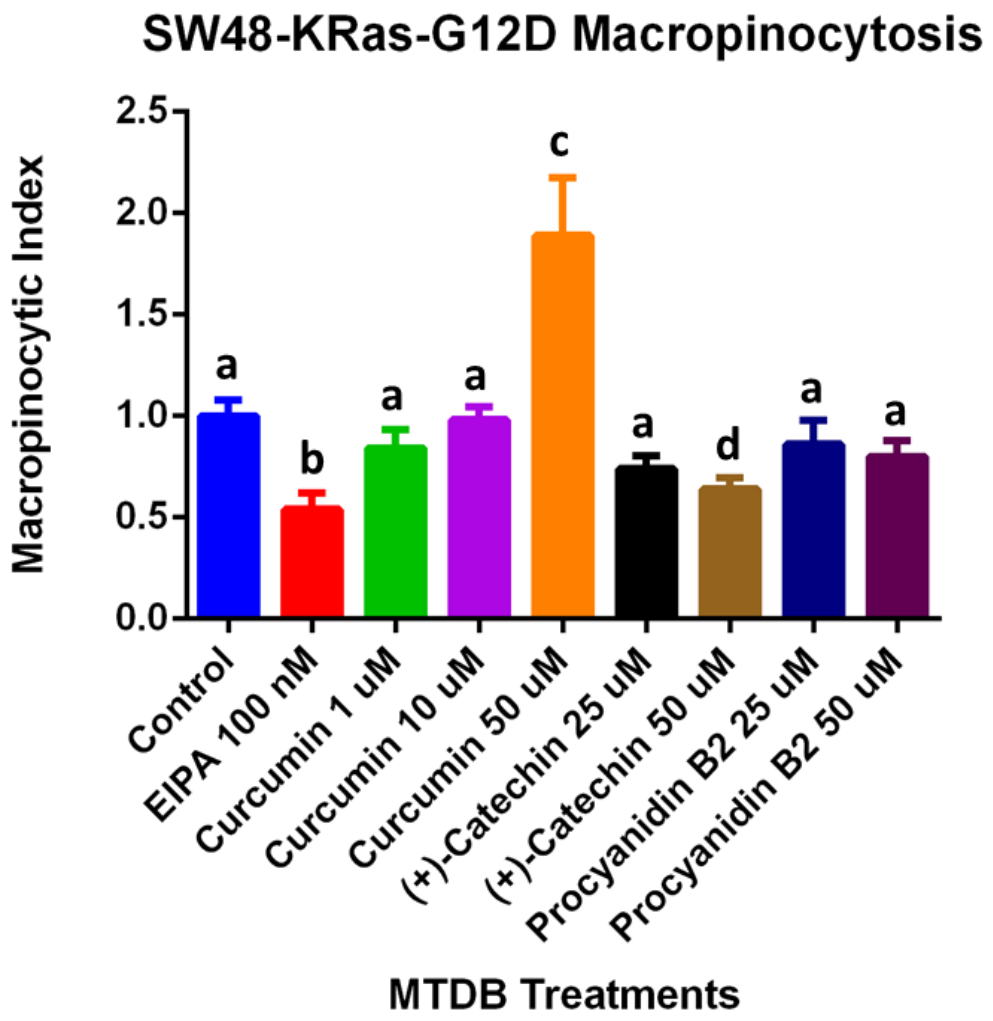

Figure 2. MTDBs inhibit macropinocytosis. SW48 cells were treated with 100 nM EIPA, curcumin (1 µM, 10 µM, or 50 µM), (+)-catechin (25 µM, or 50 µM), or procyanidin B2 (25 µM, or 50 µM) for one hour. FITC-dextran absorption was utilized to determine macropinocytosis uptake. Cells were imaged using a 40X objective on a TE 300 Nikon Eclipse fluorescence microscope. n=15 separate FOV (~300 cells) for each group from 1 experiment. Statistical significance was measured using Fisher's Protected Least Significant Difference (LSD) test.

Curcumin has been known to autofluoresce when excited with UV-light and can fluoresce or crosstalk or bleedthrough to the FITC-channel (Chignell et al., 1994). This crosstalk is what lead to the apparent increase of "macropinosome" signal.

Cell proliferation

Multiple concentrations of MTDBs reduced G12D mutated SW48 human colorectal adenocarcinoma cell proliferation (**Figure 3**). The concentrations of MTDBs were selected based on human blood levels and levels of MTDBs in the human colonic mucosa (Conquer *et al*., 1998; Kim *et al*., 2008; Irving *et al*., 2013; Verstraeten *et al*., 2013).

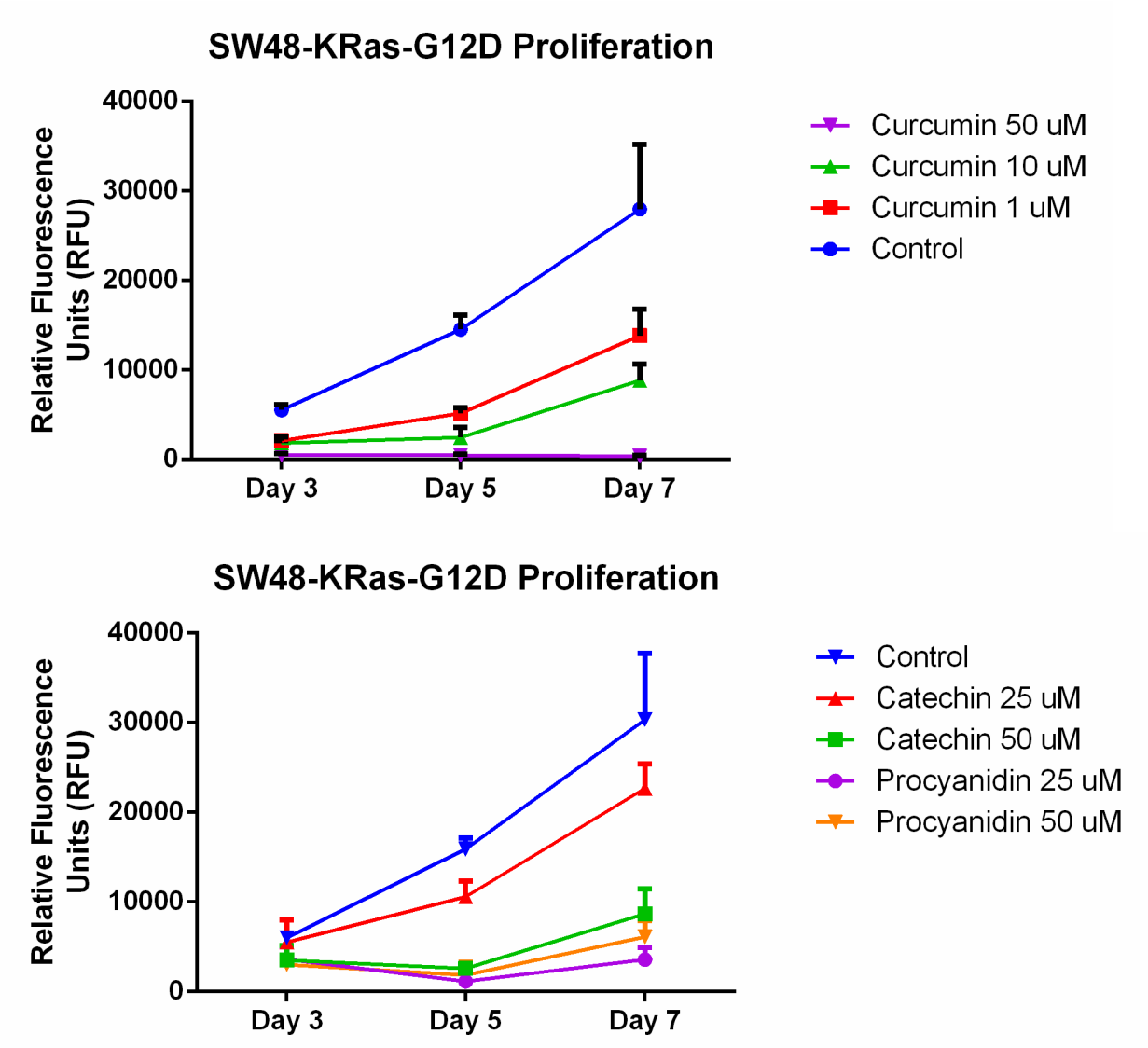

Figure 3. MTDBs reduce proliferation of SW48-Kras-G12D cells. SW48 cells were treated with curcumin (1 µM, 10 µM, and 50 µM), (+)-catechin (25 µM, and 50 µM), or procyanidin B2 (25 µM, and 50 µM) for 7 days. Media was changed on days 3, 5 and 7 with fresh treated McCoy's media. CyQUANT Direct Cell Proliferation Assay was utilized to quantify cell counts using a fluorescence microplate reader. n=12 separate wells for each group from 1 experiment.

Cell Viability

Curcumin at 1 µM, 10 µM and 50 µM had no effect on cell viability after one hour of treatment (**Figure 4**). Similarly, curcumin (1 µM, and 10 µM), (+)-catechin (25 µM, and 50 µM), and procyanidin B2 (25 µM, and 50 µM) had no effect on cell viability after seven days of treatment. Curcumin at 50 µM reduced SW48-KRas-G12D cell viability after seven days.

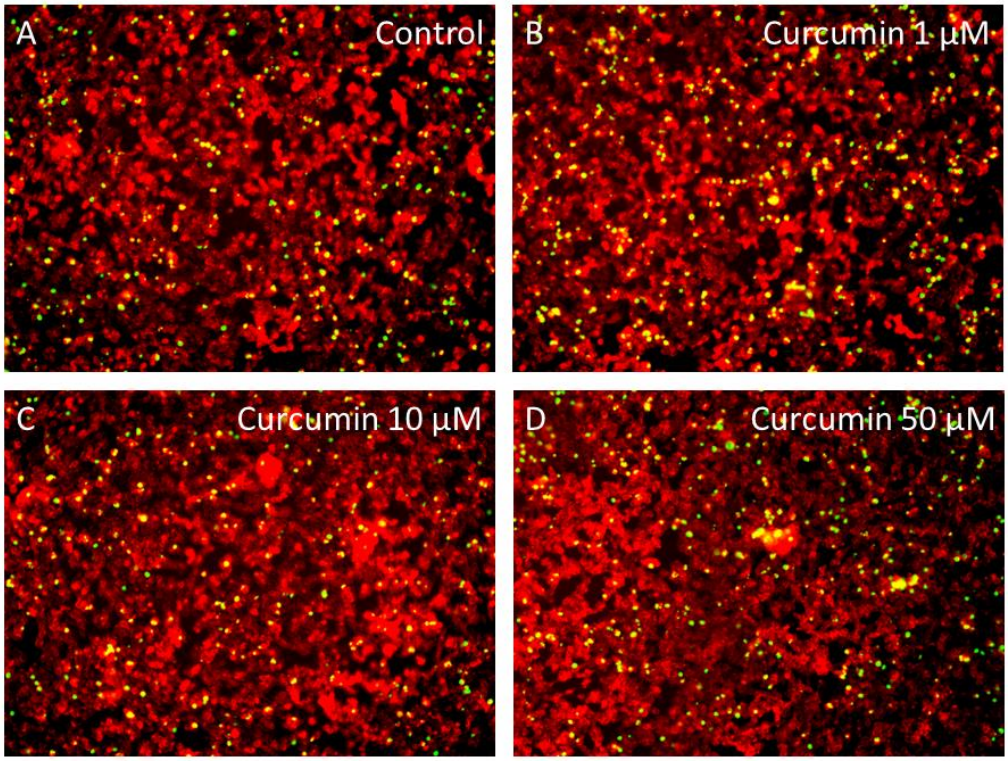

Figure 4. Curcumin exhibited no effect on cell viability. SW48 cells were treated with curcumin (1 µM, 10 µM, and 50 µM) for one hour. LIVE/DEAD Cell Viability Assay Kit was utilized to determine cell viability. Cells were imaged using a 10X objective on a TE 300 Nikon Eclipse fluorescence microscope. n=3 separate FOV (~1,200 cells) for each group from 1 experiment.

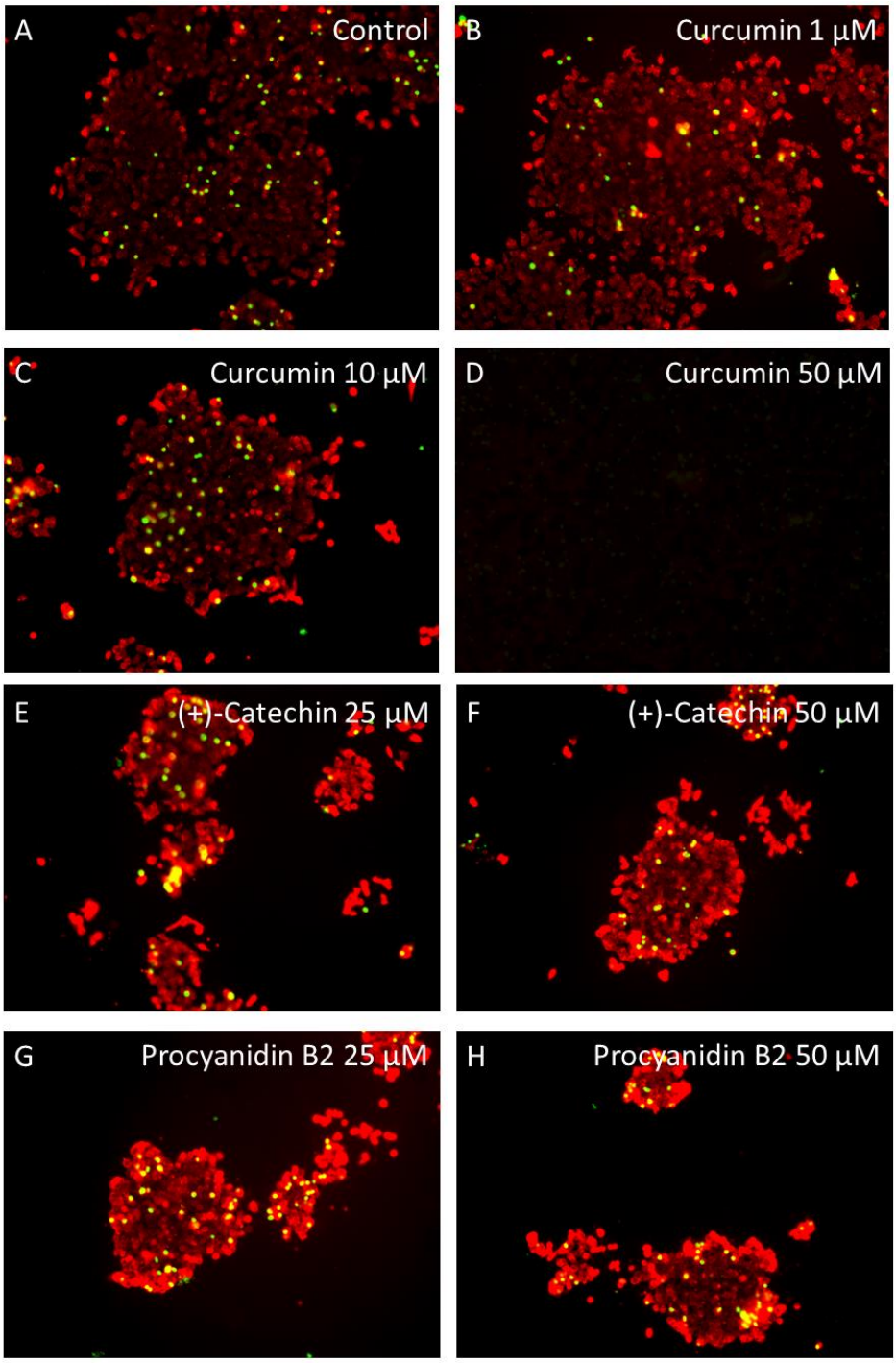

Figure 5. MTDBs exhibited no effect on cell viability. SW48 cells were treated with curcumin (1 µM, 10 µM, and 50 µM), (+)-catechin (25 µM, and 50 µM), or procyanidin B2 (25 µM, and 50 µM) for seven days. LIVE/DEAD Cell Viability Assay Kit was utilized to determine cell viability. Cells were imaged using a 10X objective on a TE 300 Nikon Eclipse fluorescence microscope. n=3 separate FOV (~1,000 cells) for each group from 1 experiment.

CHAPTER IV

CONCLUSION

Curcumin, (+)-catechin, and procyanidin B2 have previously been shown to modify plasma membrane signaling and fluidity (Erlejman *et al*., 2004; Park *et al*., 2013; Falasca *et al*., 2014; Tsukamoto *et al*., 2014). Upon incubation of SW48 human colorectal adenocarcinoma cells with MTDBs, macropinocytosis was inhibited and proliferation was reduced. Our data demonstrate that amphiphilic molecules such as (+)-catechin are capable of modifying KRas-driven macropinocytosis (**Figure 2**). Since macropinocytosis plays a vital role in the uptake of amino acids and lipids for KRas-dependent cancers, inhibition of macropinocytosis leads to a reduction in cellular growth and proliferation (Commisso et al., 2013; White, 2013). Our findings suggest the ability of (+)-catechin to suppress Ras membrane signaling may be linked to the reduction of macropinocytosis. It is important to further determine the mechanism by which these MTDBs modulate plasma membrane signaling, so that the role of MTDBs can be assessed with regard to mutant KRas inhibition.

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APPENDIX A

FUTURE EXPERIMENTS

Quantification of plasma membrane order

The organization of the plasma membrane was monitored by fluorescence spectroscopy of the polarity sensitive dye Di-4-ANEPPDHQ as previously described (Owen *et al*., 2012). Briefly, 5 μM of Di-4-ANEPPDHQ will be added to SW48 cells in 96-well plates, and immediately analyzed. Generalized polarization (GP) was calculated by obtaining the emission intensity (I) from the ordered (510-550 nm) and disordered (650-700 nm) when excited at 488 nm and applying the following equation:

$$GP = (I_{(510\text{-}550)} - I_{(650\text{-}700)})/(I_{(510\text{-}550)} + I_{(650\text{-}700)})$$

Cells were imaged using a fluorescence microplate reader (CLARIOstar, BMG LABTECH, Cary, NC).

Quantification of active Rac1

Activation of Rac1 will be determined using a kit from Cytoskeleton (Denver, CO) as we have described previously (Turk *et al*., 2013). Briefly, samples for these assays will be harvested as described above using the lysis buffer provided and supplemented with protease and phosphatase inhibitors (Sigma). Activation of Cdc42 and Rac1 will be analyzed using G-LISA Cdc42 and Rac1 Activation Assay Biochem kits, respectively, in the colorimetric format. The assays will be performed using 25 μg of cell protein according to the manufacturer's instructions. Absorbance will be measured using a microplate reader (SpectraMax 190, Molecular Devices, Sunnyvale, CA).

Quantification of macropinocytosis using MTDB combinations

A combination of MTDBs at effectively low doses will be utilized to assess the synergistic effects on macropinocytosis inhibition. The methods utilized will be the same as the prior macropinocytosis experiments that we conducted (described above). Previous data from our lab have shown synergistic effects on outcomes when treated with a combination of extrinsic factors such as MTDBs (Kolar et al., 2007; Turk et al., 2011; Chapkin et al., 2014; Triff et al., 2015).

APPENDIX B

PROTOCOL: CELL SEEDING AND FEEDING

Purpose: To seed SW48 cells for macropinocytosis, proliferation, and viability assays.

Materials:

- Complete McCoy's 5A Media
- Trypsin
- 1X DPBS
- T-75 flask
- 50 mL conical tube
- (2) 15 mL conical tube
- Plastic pipettes
- Glass pipettes
- Vacuum aspirator
- 37°C/5% CO_2 cell culture incubator

Procedure:

1. Sterilize the hood.
2. Aliquot 50 mL of McCoy's media into a 50 mL conical tube. Warm media to 37°C.
3. Aliquot 6 mL of trypsin into a 15 mL conical tube. Warm trypsin to 37°C.
4. Open the T-75 flask under the hood using sterile techniques.
5. Aspirate media from flask. Wash with 10 mL of sterile 1X DPBS. Aspirate DPBS.
6. Add 5 mL trypsin and incubate at 37°C for 5 minutes.
7. Add 10 mL of warm media into the flask to stop trypsinization. Transfer trypsinized cells into a sterile 15mL conical tube to spin down cells.
8. Centrifuge the cells in the tube at 200 x g in the tabletop centrifuge for 5 minutes.
9. After completion of the spin, aspirate supernatant to remove the media and trypsin from the cells and leave cell pellet.
10. Resuspend the pellet in 5 mL of warm media.
11. Open a new sterile T-75 flask under the hood using sterile techniques.
12. Add 10 mL of warm media into the T-75 flask.
13. Count the cells and seed approximately 2 x 10^6 cells into the T-75 flask. Cell number needed varies per assay. Need approximately 1 x 10^6 cells per chamber for macropinocytosis assay, approximately 1 x 10^5 cells per well for viability assay, and approximately 1 x 10^4 cells per well for proliferation assay. Adjust for cell growth rate and size.
14. Add the resuspended cells to the T-75 flask and rotate it gently to distribute the cells evenly.
15. Label the flask. Specify the cell type, passage number, date, and initials.
16. Incubate the flask at 37°C under 5% CO_2 atmospheric pressure.

APPENDIX C

PROTOCOL: MACROPINOCYTOSIS ASSAY

Purpose: To determine macropinocytosis in SW48 cells

Materials:

- Complete McCoy's 5A Media
- McCoy's 5A Media (0% FBS)
- 70 kDa FITC-Dextran
- 5-(N-ethyl-N-isopropyl) amiloride (EIPA)
- CellMask Deep Red Plasma Membrane dye
- Hoechst 33342 dye
- Poly-L-lysine
- Paraformaldehyde (PFA)
- Glycine
- Curcumin
- (+)-Catechin
- Procyanidin B2
- PBS
- 1X DPBS
- Chambered slides
- Plastic pipettes
- Glass pipettes
- Vacuum aspirator
- 37°C/5% CO_2 cell culture incubator
- 40X objective
- fluorescence microscope (TE 300 Nikon Eclipse)

Procedure (Prior to assay):

1. Sterilize the hood.
2. Aseptically coat chamber slides or other cell culture surface with 1 mL/25cm^2. Rotate it gently to ensure even coating of the surface.
3. After 5 minutes, remove the solution by aspiration and thoroughly rinse the surface with sterile water.
4. Allow to dry for at least 2 hours before introducing cells or media.
5. Seed appropriate number cells in the necessary number of chamber slides using sterile cell culture techniques.
6. Incubate slides at 37°C under 5% CO_2 atmospheric pressure.
7. Serum starve cells 16 to 24 hours before continuing with assay.

Procedure:

1. Aspirate serum free media from chamber slides.
2. Add 500 µL of serum free media (negative control), 100 nM EIPA (positive control), curcumin (1 µL, 10 µL, or 50 µL), (+)-catechin (25 µL, or 50 µL), or procyanidin B2 (25 µL, or 50 µL) treated media to respective cells in the chambers.
3. Incubate at 37°C under 5% CO_2 atmospheric pressure for 1 hour.
4. After 1 hour of incubation, aspirate media and replace with 500 µL of media containing dyes. One mg/mL FITC-dextran, 1 µL/mL CellMask Deep Red Plasma Membrane dye, and 1 µg/mL Hoechst 33342. For the EIPA treated cells, EIPA needs to be present in the dye media at a concentration of 100nM.
5. Incubate at 37°C under 5% CO_2 atmospheric pressure for 30 minutes.
6. After 30 minutes of incubation, place cells on ice.
7. Aspirate media and wash with ice cold PBS 5 times.
8. Add 500 µL of 4% PFA to each chamber for 10 minutes.
9. Wash with PBS 2 times. Add 500 µL of 100 mM glycine for 10 minutes.
10. Wash with PBS 2 times. Add 1 mL of DPBS to each chamber.
11. Cells can be imaged or stored at 4°C. Images were taken with a 40X objective on a TE 300 Nikon Eclipse fluorescent microscope.

APPENDIX D

PROTOCOL: CELL PROLIFERATION ASSAY

Purpose: To determine SW48 cell proliferation.

Materials:

- Complete McCoy's 5A Media
- McCoy's 5A Media (0% FBS)
- 5-(N-ethyl-N-isopropyl) amiloride (EIPA)
- Curcumin
- (+)-Catechin
- Procyanidin B2
- CyQUANT Direct Cell Proliferation Assay Kit
    - CyQUANT Direct nucleic acid stain
    - CyQUANT Direct background suppressor I
- 1X PBS
- 15 mL conical tube
- 96-well microplate(s)
- Plastic pipettes
- 37°C/5% CO_2 cell culture incubator
- Microplate reader (CLARIOstar microplate reader)

Procedure:

1. Prepare 12 mL of 2X detection reagent, which is sufficient for 100 µL/well in a 96-well plate.
2. Combine the following in 15 mL conical tube and mix well: 11.7 mL of 1X PBS, or cell culture media, 48 µL of CyQUANT Direct nucleic acid stain, and 240 µL of CyQUANT Direct background suppressor I.
3. Add an equal volume of 2X detection reagent to cells in culture. For example, add 100 µL of 2X detection reagent to 100 µL of cells in cell culture media.
4. Incubate cells with the detection reagent for 60 minutes at 37°C.
5. Read fluorescence of samples using standard "green" or FITC filter sets or appropriate wavelengths (480/535 nm) if using a monochromatic instrument such as CLARIOstar microplate reader.

APPENDIX E

PROTOCOL: CELL VIABILITY ASSAY

Purpose: To determine SW48 cell viability.

Materials:

- Complete McCoy's 5A Media
- McCoy's 5A Media (0% FBS)
- 5-(N-ethyl-N-isopropyl) amiloride (EIPA)
- Curcumin
- (+)-Catechin
- Procyanidin B2
- LIVE/DEAD Cell Viability Assay Kit
    - C_{12}-Resazurin
    - SYTOX Green stain
    - Dimethylsulfoxide (DMSO), anhydrous
    - 10X Phosphate-buffered saline
- 96-well microplate(s)
- Plastic pipettes
- Glass pipettes
- Vacuum aspirator
- 37°C/5% CO_2 cell culture incubator
- Microplate reader (CLARIOstar microplate reader)

Procedure:

1. Prepare a 1 mM stock solution of C_{12}-resazurin. Dissolve the contents of the vial of C_{12}-resazurin in 100 µL of DMSO. Prepare a fresh 50 µM working solution of C_{12}-resazurin by diluting 1 µL of the 1 mM C_{12}-resazurin stock solution in 19 µL of DMSO.
2. Prepare a 1 µM working solution of SYTOX Green stain. Dilute 5 µL of the 10 µM SYTOX Green stain stock solution in 45 µL of DMSO.
3. Prepare a 1X phosphate-buffered saline (PBS) solution. Dilute 2 mL of the 10X PBS in 18 mL of deionized water.
4. Prepare a dye solution with a final concentration of 10 nM C_{12}-resazurin and 5 µM SYTOX Green dye when added to cell media. For example, dilute 1 µL of the 50 µM C_{12}-resazurin working solution and 2 µL of the 1 µM SYTOX Green stain working solution to 200 µL of 1X PBS.
5. Aspirate cell media and add 50 µL of the newly prepared dye solution to the wells.
6. Incubate at 37°C under 5% CO_2 atmospheric pressure for 15 minutes.
7. Analyze the cell sample. As soon as possible, analyze the stained cells by microplate reader, exciting at 488 nm and measuring the fluorescence emission at 530 nm and 575 nm.