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RECOMBINANT MOJASTINS AND THEIR AFFECT ON THE INDUCTION OF APOPTOSIS IN HUVEC

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San José State University

In Partial Fulfillment

of the Requirements for the Degree

Masters of Science

by

Victoria Lynn Tran

May 2008

UMI Number: 1458151

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ABSTRACT

RECOMBINANT MOJASTINS AND THEIR AFFECT ON THE INDUCTION OF APOPTOSIS IN HUVEC

by Victoria Lynn Tran

The mojastin disintegrin, isolated from *Crotalus scutulatus scutulatus* snake venom, has been shown to inhibit platelet aggregation. However, the use of disintegrins has also been suggested for the treatment of metastasis. Disintegrins bind to specific integrin receptors, via the Arg-Gly-Asp motif (RGD-loop), to induce cell migration, proliferation, and apoptosis. The specificity of the disintegrin to a particular integrin has been attributed to C-terminal amino acids immediately following the RGD tripeptide. Thus mutating one or two amino acids in this region of mojastin (RGDWN) may cause mojastin to bind to apoptosis affecting integrins. In this study four recombinant proteins (RGDDM, RGDMP, RGDWP, and RGDNN) were produced and tested for their ability to induce apoptosis. The results showed that the mojastin wild-type and four mutant fusion proteins did not induce apoptosis in HUVEC cells. This data suggests that other portions of the disintegrin molecule are important for integrin recognition.

ACKNOWLEDGEMENT

Thank you to Dr. Julio Soto for your guidance and support throughout this project. Thank you to my labmates and fellow graduate students who persevered side by side with me and whose kind words and gentle support helped me to push forward through the long hours and late nights. Thank you to Dr. Robert Fowler, Dr. Brandon White, Dr. Ruth Kibler, Tim Andriese, and the technical staff for your input and assistance.

Thank you to my late husband, Joseph Kavanagh, for your encouragement and support of my goals. Thank you to my brother, Liem Tran, for showing me, that in the end, your unconditional love was indeed enough and for opening up your home to me. Thank you to my sister-in-law, Kim-Uyen Le, for your generosity and kindness, without you this journey would have taken much longer to complete. Thank you to my mom, dad, mother, and father in-law for their continued support. Finally, thank you to all the Trans, Kavanaghs, Biscans, soon to be Yerkes, and my dear friend Janelle Clark for your love. To all of you I dedicate this thesis, with my love in return and my deepest gratitude.

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1. Introduction

1.1 *The Emerging Role of Apoptosis in Treating Metastatic Cells*

One of the characteristics of cancer is metastasis, the ability of a cell to break free from the surrounding extracellular matrix, migrate, and invade other organs. The most effective cancer therapeutic is targeted at killing these invasive cells rather than inducing cell senescence (Meng et al., 2006). Successful cancer therapeutics cause neoplastic cells to undergo apoptosis, ultimately preventing both proliferation and migration of cancer cells. Apoptosis is an essential part of cell development. In order for humans to develop fingers and toes or to form new synapses in the brain, requires the removal of extraneous cells. Apoptosis is also a way to remove cells that threaten the structural organization which allows multicellular organisms to thrive (Kerr et al., 1972).

1.2 *The Interaction of Cell Surface Receptors and Signaling Systems They Affect*

In a homeostatic environment cells, within a multicellular organism have extracellular and intracellular signals that convey cell boundaries and are the basis for the formation of tissues. Cells interact with the extracellular matrix (ECM) through the binding of ECM adhesion molecules and cell surface receptors, one of which is a family of receptors known as integrins. Integrins are transmembrane receptors composed of a combination of different alpha and beta subunits. Depending upon the combination of the alpha and beta subunit, an integrin bound to insoluble proteins (fribonectin and vitronectin), soluble proteins (disintegrins), or neighboring cells, transfers a signal that

induces the cell to migrate, proliferate, or undergo apoptosis. Recent cancer research has capitalized on the role of integrins in the signaling of apoptosis and focused on finding antagonist molecules that will induce this signal (Aguzzi et al., 2004).

Among these molecules are disintegrins, a low molecular weight, snake venom proteins that are released as part of a larger protein, matrix metolloprotease, known as snake venom metalloprotease (SVMP), or after proteolytic cleavage as a monomer or dimer (Ramos et al., 2008). Disintegrins are non-enzymatic molecules, with a cysteine rich portion, and a tripeptide (arginine/glycine/aspartic acid) called the RGD-loop. In its tertiary arrangement the cysteine rich portion forms disulfide bonds creating a hairpin loop, exposing the RGD tripeptide in the loop region (McLane et al., 2004). The RGD-loop when bound to an integrin provides potency of signal and the amino acids immediately flanking the RGD tripeptide, in the N-terminal and C-terminal regions, provide specificity and affinity for a particular integrin (Kini, 1998; Rahman et al., 1998). Other tripeptide binding motifs include KGD, MVD, MLD, VGD, ECD, and MDG (Lu et al., 2006).

1.3 *RGD-Loop and Disintegrin Specificity*

The binding specificity and affinity of integrins has been attributed to the amino acids flanking the RGD-loop of disintegrins (Kini and Evans, 1995a; Kini and Evans, 1995b; Rahman et al., 1998). In experiments where Kini and Evans (1995) replaced two amino acids, one in the N-terminal and one in the C-terminal region adjacent to the RGD-loop of the peptide **I**ARGDM**N**A, with a Pro residue, converting it to **I**PRGDM**P**, they

found that the antiplatelet activity increased by up to 13-fold. Rahman et al. (1998) found that by replacing the N-terminal and C-terminal amino acids flanking the RGD-loop of elagantin, changing the 50th amino acid from Ala to Pro, and the 54th amino acid from Met to Asn, they were able to affect inhibitory potency of elagantin (Figure 1). To this end, the mojastin disintegrin was mutated at the 54th and 55th amino acids (the two amino acids immediately C-terminal to the RGD tripeptide) to mimic those of echistatin (RGDDM) and rhodostomin (RGDMP). *In vivo* studies have shown the induction of apoptotic activity of endothelial cells after exposure to accutin, agkisrin-s, echistatin, and rhodostomin (Brassard et al., 1999; Ren et al., 2006; Wu et al., 2003; Yeh et al., 1998). These disintegrins bind to $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin receptors phosphorylating FAK and inducing caspase-3 activation of apoptosis (Alimenti et al., 2004; Wierzbicka-Patynowski et al., 1999). Studies using recombinant disintegrins are aimed at increasing their binding specificity to integrins and also to improve their bioavailability (Eble and Haier, 2006).

Mojastin, a disintegrin found in the *Crotalus scutulatus scutulatus* venom, has 70 amino acids with a molecular weight of 7 kDa. Mojastin was previously shown to inhibit platelet aggregation in whole human blood at IC_{50} of 13.8 nM. However, mojastin was ineffective at inhibiting T24 cells, which exhibits the receptors $\alpha_v\beta_3$ and $\alpha_5\beta_1$ (Sanchez et al., 2006). The $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin receptors, have been linked to apoptosis, and bind both echistastin and rohodostomin. In order to alter mojastin's specificity the amino acids at the 54th and 55th amino acid were changed (Figure 2).

1.	Elegantin	$\mathbf{A}_{50}R_{51}G_{52}D_{53}\mathbf{N}_{54}P_{55}$
2.	Eg AM	A R G D **M** P
3.	Eg PN	**P** R G D N P
4.	Eg PM	**P** R G D **M** P

Figure 1. Partial sequences of the altered elegantin. The substitutions, Asn_{50} to Pro and Asn_{54} to Met, created in elegantin.

```
                    1         11        21        31        41        51        61        71
Mojastin 1          EAGEECDCGS PANPCCDAAT CKLRPGAQCA DGLCCDQCRF IKKGTVCRPA RGDWNDDTCT GQSADCPRNG LYG
Mojastin 2          ----GEECDCGS PANPCCDAAT CKLRPGAQCA DGLCCDQCRF IKKGTVCRPA RGDWNDDTCT GQSADCPRNG LYG
Recombinant Mojastin ----------NPCCDAAT CKLRPGAQCA DGLCCDQCRF IKKGTVCRPA RGDWNDDTCT GQSADCPRNG LYG
```

Figure 2. Amino acid sequence of mojastin 1 and recombinant mojastin (MOJ). The 54nd and 55rd amino acids that were mutated for this study are underlined.

In this study we used mutant mojastin constructs provided by the Soto lab, to express recombinant fusion proteins and test their ability to induce apoptosis.

1.4 *The specific aims of this study were to:*

1. Express and purify recombinant GST-MOJ wild-type and mutants.
2. Determine whether changing the 54th and 55th amino acid residues, □mmediately C-terminal to the RGD tripeptide, induced apoptosis in HUVEC cells.

2. Review of Literature

2.1 *Apoptosis: the Art of Cellular Death*

2.1.1 *Historical Perspective*

The morphology of cellular death was first observed by Carl Vogt in 1842 (Peter et al., 1997). However, the term apoptosis, a Greek description for falling off or dropping off, was introduced into literature in 1972 by Kerr et al. Apoptosis is characterized by cell shrinkage, detachment from surrounding cells, nuclear condensation, DNA fragmentation, membrane blebbing, and the formation of apoptotic bodies. These apoptotic bodies are eventually phagocytized by surrounding cells and macrophages or escape the cell lumen where they undergo necrosis (Wyllie, 1992). Apoptosis is critical for the development and structural organization of multicellular organisms. This observation is best exemplified in the embryonic development of *Caenorhabditis elegans.*

2.1.2 *A Simple Model of the Apoptosis Signaling Cascade*

In the development of hermaphroditic *C. elegans,* 131 of the 1090 cells had a predetermined time of death indicating that cell disposal was the result of genetic control (Peter et al., 1997). In *C. elegans*, *ced-3* and *ced-4* are proapoptotic genes and *ced-9* negatively regulates apoptosis. CED-3 is a cysteine protease and initiates apoptosis, when cleaved by CED-4. CED-9 binds to CED-4, which is in turn bound to the CED-3 zymogen, to form a complex that represses the proteolytic activity of CED-4. Binding of

CED-9 with EGL-1 displaces CED-4, releasing CED-4, and enabling it to activate the CED-3 zymogen, thus initiating apoptosis (Borner, 2003; Hengartner and Bryant, 2000; Kaufmann and Hengartner, 2001; Peter et al., 1997; Wyllie, 1997). The caspases, Apaf-1, and Bcl-2 are mammalian homologues of CED-3, 4, and 9, respectively. However, the apoptotic signaling mechanism in mammals differs from those of *C. elegans.*

2.1.3 *Starting from the Top: Extrinsic and Intrinsic Signaling*

Although the mammalian signaling cascade for apoptosis is much more complicated, the homologues of CED-3, 4, and 9, in mammals, still share the same general roles. Similar to CED-3, caspases are the initiators and effectors of apoptosis. Apaf-1, like CED-4, forms a complex that acts on the caspases. The members of the BCL-2 family of proteins, which are homologous to CED-9, regulate the apoptosis machinery. Initiator caspases-8 and 9 are activated by different signaling mechanisms but share commonalities in their downstream cascade and converge upon activation of caspase-3 and 7.

The signals that activate initiator caspases may be extrinsic or intrinsic. The extrinsic pathway starts with ligand binding, then clustering of death receptors (DR) belonging to the superfamily of tumor necrosis factor receptor (Kaufmann and Hengartner, 2001; Vermeulen et al., 2005). Members of this family include Fas, Tumor Necrosis Factor (TNF)-receptor-1, and other DRs, including DR-3, 4, 5 and 6. A common example of extrinsic signaling involves binding of the Fas receptor with Fas ligand. This causes trimerization of the Fas receptor leading to the recruitment of the

Fas-associated death domain protein (FADD) and procaspase-8 or 10, forming the death inducing signal complex (DISC). FADD is an adaptor molecule that has a death domain (DD), that binds to Fas and a death effector domain (DED) which in turn binds to the DED domain portion of procaspase-8. Due to its proximity to one another, procaspase-8 undergoes autoproteolysis (Hengartner, 2000) forming caspase-8 which then activates procaspase-3 and 7. Caspase-3 and 7 are responsible for the morphological changes that are characteristic of apoptosis.

DNA damage, oxidative stress, starvation, or chemotherapeutics (Borner, 2003) cause the releases cytochrome-c from the mitochondria into the cytoplasm, initiating the intrinsic pathway. Cytochrome-c binding in the presence of ATP causes a conformational change to the apoptotic protease activating factor-1 (Apaf-1), increasing its affinity for procaspase-9. Binding of the caspase recruitment domain (CARD) portion of Apaf-1 to the CARD of procaspase-9, results in the formation of a complex called the apoptosome. Activated caspase-9, within the apoptosome, can then act on caspase-3 and 7 leading toward apoptosis.

2.1.4 *Bcl-2 Family: Regulating Apoptosis*

As mentioned above, the Bcl-2 family of proteins in mammals is homologous to CED-9 of *C. elegans*. Proteins in the Bcl-2 family share one or more of four common Bcl-2 homology domains (BH1-BH4) and can be grouped according to their ability to either promote apoptosis or cell survival. Antiapoptotic Bcl-2 members include Bcl-2, Bcl-B, Bcl-X_l, Bcl-W, Bfl-1/A1, and Mcl-1, while proapoptotic Bcl-2 family members

can be further divided into the Bax-family (Bax, Bak, and Bok/Mtd) and the BH3 only family (Bid, Bad, Bmf, Hrk/DP5, Noxa, and Puma) (Vermeulen et al., 2005). Though the exact mechanism of regulation by the Bcl-2 family members is still under debate, the role of Bcl-2 as a regulator of apoptosis is clear.

Under homeostatic conditions Bax exists in the cytosol, and Bak in the mitochondria in their monomeric form. However, under stimuli such as DNA damage, starvation, or cell detachment, BH3-only member proteins are upregulated causing the tranfers of the death signal to the mitochondria. Bax and Bak are sequestered to the outer mitochondrial membrane where they homodimerize. It is unclear whether Bax/Bak, form membrane channels or whether they facilitate the opening of voltage gated channels, but their presence causes the permeabilization of the outer mitochondrial membrane. This results in the release of cytochrome-*c* into the cytoplasm (Borner, 2003; Chowdhury et al., 2006; Vermeulen et al., 2005) and the onset of the intrinsic signaling pathway of apoptosis. Antiapoptotic Bcl-2 family members such as Bcl-2 and Bcl-X_l are believed to regulate proapoptotic signals by maintaining outer mitochondria membrane integrity, though the mechanism has yet to be determined.

2.2 *Integrin Signaling and the Consequences of Extracellular Matrix Detachment*

In tissues, cells attach themselves to the extracellular matrix through receptor binding of integrins with cell adhesion molecules. The specific binding of integrins ($\alpha v\beta 3$, and $\alpha 5\beta 1$) leads to clustering of similar integrins on the cell membrane surface. This clustering recruits focal adhesion molecules such as talin, viniculin, Src, and FAK

(Matter and Ruoslahti, 2001). FAK, focal adhesion kinase, causes phosphorylation of phoshotidyl-inositol-1, 3, 4-diphosphate (PIP2) to form phoshotidyl-inositol-1, 3, 4, 5-triphosphate (PIP3) to act on PKB/Akt molecules which inhibit the activity of proapoptotic Bcl-2 family members such as Bad, Bim, and Bax, thus suppressing apoptosis. In the absence of this inhibition by PKB/Akt, due to detachment from the substratum or antagonistic binding of integrin receptors, the proapoptotic molecules translocate to the outer mitochondrial membrane, initiating the intrinsic apoptosis signaling pathway (Barja-Fidalgo et al., 2005; Bouchard et al., 2008; Grossmann, 2002).

2.3 *Detection of Apoptosis*

Annexin V binds with high affinity and specificity to phosphotidyl serine a membrane phospholipid. In viable cells phophotidyl serine faces the cytosol. During the early stages of apoptosis phosphotidyl serine flips and is exposed to the extracellular matrix (Vermes and Haanen, 1994). However the integrity of the membrane is maintained, differentiating these apoptotic cells from necrotic cells, which loose membrane integrity and exhibit membrane leakage. Using Annexin V attached to fluorescein isothiocyannate (FITC), a green fluorescence molecule, and a counter stain propidium iodide, a red fluorescence molecule, the number of apoptotic cells in a population may be quantified.

3. Methods

3.1 *Transformation of Escherichia coli*

PGEX-KG vectors containing the mutated mojastin constructs, developed by researchers in the Soto lab, were added to 100 ul of E.coli BL21-GOLD (Strategene) or NEB-DH5α (New England BioSciences) competent cells and the transformation was carried out according to the manufacturer's instructions. The cells were left to incubate for 30 min on ice, while SOC medium (Stratagene), was preheated in a 42°C waterbath. The transformation reaction was heat-pulsed at 42°C for 20 s and incubated on ice. After 2 min, 900 ul of warmed SOC medium was added to the reaction. The transformation reaction was then placed on a shaker and left to incubate at 37°C, 250 rpm for 1 h. Finally 20 ul of the tranformant was streaked on Lauria-Bertani agar (Fisher) with ampicillin plates and left to incubate at 37°C overnight.

3.2 *Fusion Protein Expression*

Eight colonies were cultured in 200 ml of 2x Yeast-Tryptophan (QBiogene) or Lauria-Bertani (LB) broth in the presence or absence of 10 ug/ml ampicillin. The culture was grown to an OD_{600} of 0.6-0.8 with vigorous agitation, 225 rpm, at 37°C. GST-MOJ was induced by adding 1 mM of isopropyl β-D-thioglalactoside (Amersham Biosciences) and left to incubate for an additional 2 h. The culture was then transferred to four 50 ml centrifuge tubes and centrifuged at 700x G for 10 min to obtain the pellet. The supernatant was discarded and the pellet was suspended in 1x PBS (Gibco).

3.3 *Lysis and Purification of GST-MOJ*

Cells were lysed using a sonicator, 20 bursts followed by 20 s on ice. The supernatant was retained after centrifugation for 10 min at 700x G and applied to a 1 ml GSTrap column (GE Biosciences) at 0.2-1 ml/min. The column was washed 5 ml with 1x PBS and eluted with 5 ml of reduced glutathione. Protein concentrations were determined using the Bradford assay.

3.4 *Molecular Weight Determination of GST-MOJ using SDS-PAGE Gels*

Proteins samples were mixed with 1x Laemmli Sample Buffer (BioRad) and boiled at 95°C for 5 min. The samples were cooled at room temperature and 40 ul was added to a NuPAGE 4-12 %, Bis-Tris gel (Invitrogen). The gel ran at 120 mA for 40 min in MES Buffer (Invitrogen). The gel was then stained with Coomassie Brilliant Blue (Sigma).

3.5 *Culturing HUVEC Cells*

HUVEC were a product of Lonza (C2517A) and cultured in 25 cm^2 flasks with endothelial cell basal medium (EMB-2 Basal Medium, CC3156) supplemented with: BBE, 2 ml; hEGF, 0.5 ml; Hydrocortisone, 0.5 ml; FBS, 10 ml; GA-1000, 0.5 ml. They were incubated at 37°C with 5% CO_2. The cells were subcultured once they reached 80% confluency. The process involved aspirating used media, washing with 5 ml of PBS, and detaching using 0.05% tryspin EDTA (Invitrogen). The action of trypsin was neutralized

with the complete culture media containing 2% bovine serum albumin. Cells were then split 1:2 and transferred to new 25 cm^2 flasks.

3.6 *Apoptosis Detection*

HUVEC cells were cultured to 80% confluency in a 75 cm^2 tissue culture flask (Fisher). Once the cells were confluent they were detached from the flask surface using 0.05% trypsin EDTA. Trypsin was neutralized with complete culture media. The cells seeded in 25 cm^2 flasks (Fisher) and left to attach for 4 h. The cells were then treated with 2.5, 5, 10, or 15 uM camptothecin; 10, 20, 50 or 100 uM echistatin; 1 or 10 uM GST; or 5 uM wild-type and mutant mojastin recombinant fusion proteins. The cells were observed and harvested after 18-20 h, with trypsin in the same manner as above. The cells were spun down and washed two times with cold PBS. Then they were resuspended in 1 ml of 1x PBS. Afterward, 100 ul was removed and exposed to 5 ul each of Annexin V-FITC and Propidium Iodide (BD Biosciences). The reaction was left at room temperature for 15 min and analyzed using the FACSCalibur after 400 ul of 1x binding buffer was added. Statistical analysis of the percent apoptotic cell population, using paired t-tests, was performed using InStat (GraphPad).

3.7 *Hoechst 33258 Staining*

HUVEC cells were cultured and collected as above and seeded 2.5 x 10^5 cells per slide well chamber (Nalgene). The cells were then fixed in 3% paraformaldehyde, then set in 100% cold methanol and stained with 10 ug/ml of Hoechst 33258 (Invitrogen).

4. Results

4.1 *Optimization of Culturing Conditions for Fusion Protein Expression*

The GST fusion protein expression in the *E. coli* NEB-DH5α strain was compared to those of the BL21 strain (Figure 3). At the same time the NEB-DH5α strain was cultured in either 2x Yeast Tryptophan or Lauria-Bertani broth. They were also cultured with ampicillin or without. After induction with IPTG, BL21 cells, grown in 2x YT broth with ampicillin (2x YTA), showed a significant increase in GST fusion protein expression (lane 4), whereas the NEB-DH5α cells in the same medium condition did not (lane 12). Moreover NEB-DH5α cells did not show an increase in GST fusion protein expression under any of the media conditions, 2x YT versus LB broth, and in the presence or absence of ampicillin. The expression of GST fusion proteins using the BL21 strain of *E. coli* in 2x YTA media, as seen from these results, was chosen for the continuation of this study.

4.2 *Detection of Purified GST-MOJ Fusion Protein Using SDS-PAGE*

After optimization of culture conditions, the GST-MOJ fusion proteins were expressed and purified using 1ml GSTrap affinity columns. SDS-PAGE was used to verify the purification of the GST fusion proteins from cell lysate. It was also determined that expression system *E. coli* was adequate for the production of GST-MOJ recombinants (Figure 4). As seen in lane 4, the pGEX-KG vector, without the mojastin

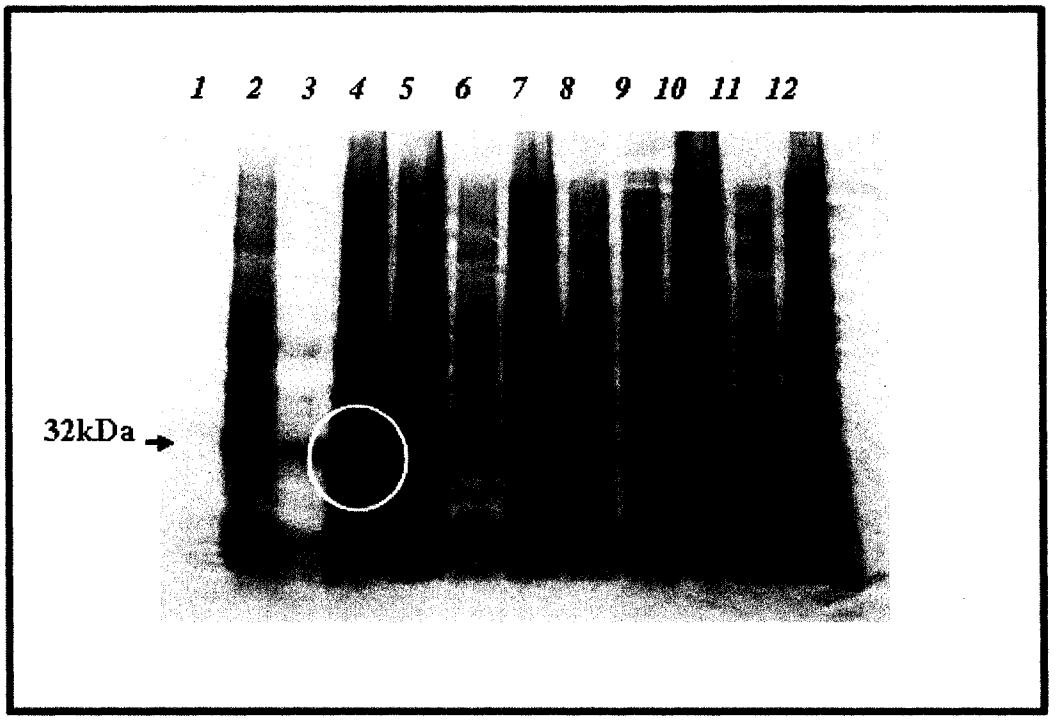

Figure 3. Comparative expression of GST-MOJ by BL21 and NEB-DH5α strains of *E. coli.* Different culture conditions were also tested. The strains were cultured in either 2x Yeast Tryptophan (YT) or Lauria-Bertani (LB) broth with or without the addition of ampicillin (A). Samples in lanes 4, 6, 8, 10, and 12 were induced with IPTG. Lanes 2, 5, 7, 9 and 11 were uninduced samples. Lane 1 contained the molecular weight standard (Biorad). Lane 2 & 4 were lysate samples from the BL21 strain, grown in 2x YTA broth. Lane 5 & 6 were lysate samples from the NEB-DH5α strain, grown in LB broth. Lane 7&8 were lysate samples from the NEB-DH5α strain, grown in LBA broth. Lane 9&10 were lysate samples from the NEB-DH5α strains, grown in 2x YT. Lane 11&12 were lysate samples from the NEB-DH5α strain, grown in 2x YTA. BL21 cells grown in 2x YTA showed a significant increase in GST-MOJ production (yellow oval).

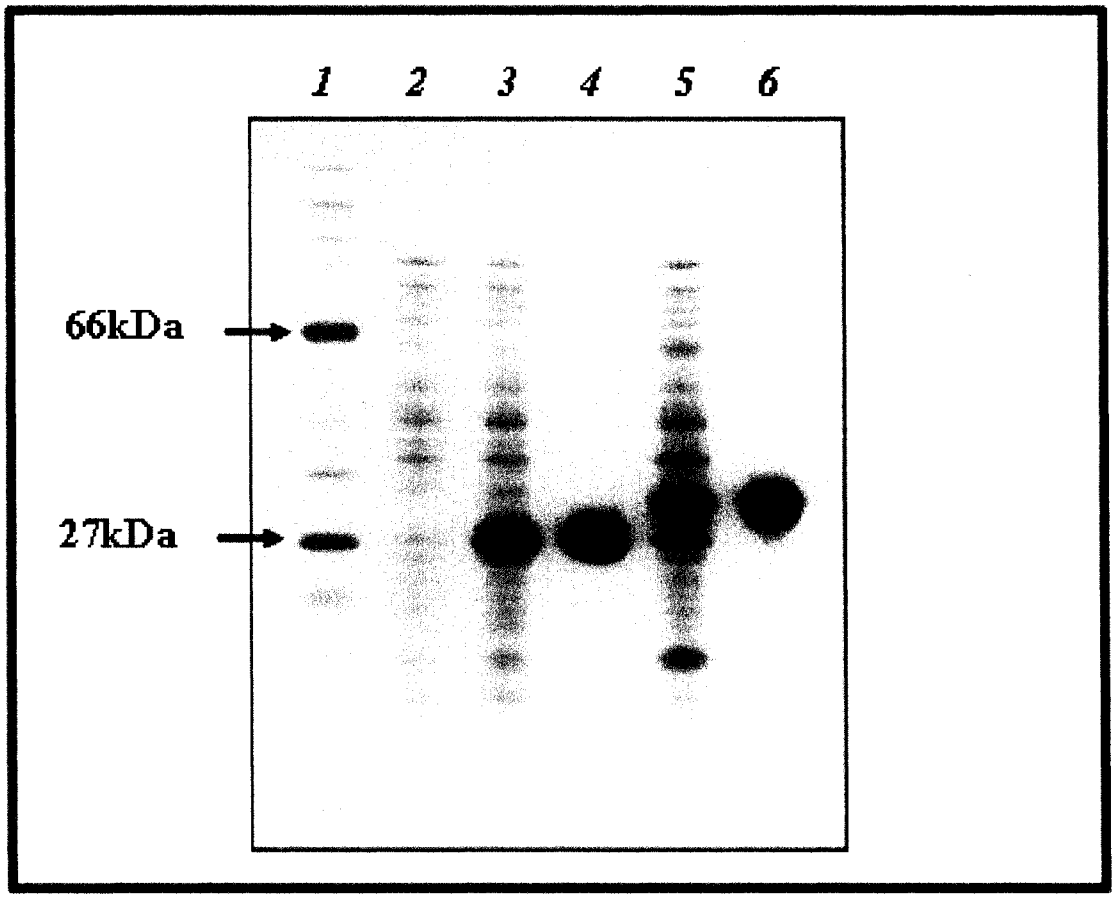

Figure 4. Purification of GST-MOJ visualized by SDS-PAGE. Lane 1: MW; Lane 2: GST un-induced; Lane 3: GST whole lysate; Lane 4: GST purified; Lane 5: GST-MOJWN whole lysate; Lane 6: GST-MOJWN purified.

insert, produced a band at 29kDa, the predicted size of the GST protein, whereas pGEX-KG with the mojastin insert produced a band at the predicted size of 34 kDa (lane 6).

Once it was established that the GST-MOJ fusion proteins were produced using this approach, the GST-MOJWP, NN, DM, and MP fusions proteins (Figure 5) were expressed and purified (lanes 4, 6, and 8 in panel A; lane 3 in panel B). Some of the samples also included GST by itself (blue arrow heads). This is the result of randomly selecting eight *E. coli* colonies, after transformation of BL21 cells, for culture. Cell lysates were also included in lanes 3, 5, and 7 of panel A and lane 2 of panel B.

4.3 *Optimization of Annexin V-FITC Detection of Apoptosis using FACSCalibur*

The ability to detect apoptosis, in an Annexin-V-FITC assay, was determined using camptothecin, a known inducer of apoptosis in HUVEC cells (Simak et al., 2002). This drug was added to HUVEC cells at 5, 10, and 15 uM concentrations and the cells were left to incubate for 24 h (Figure 6). Apoptosis was detected in the HUVEC cells exposed to camptothecin (panel B, C, and D in the lower right quadrant), however not in a dose dependent manner 5 uM (12.15%); 10 uM (17.21%); and 15 uM (14.53%). Also there was an abundance of late stage apoptotic and necrotic cells in the upper right quadrant and very few live cells in the lower left quadrant. This indicates that the dose range may be too high or that the incubation time was too long. For the succeeding experiments, 2.5 uM of camptothecin were used, as a positive control for apoptosis, allowing the visualization of the viable population of cells as well as apoptotic and necrotic cells. The commercially available disintegrin, echistatin, shown to induce

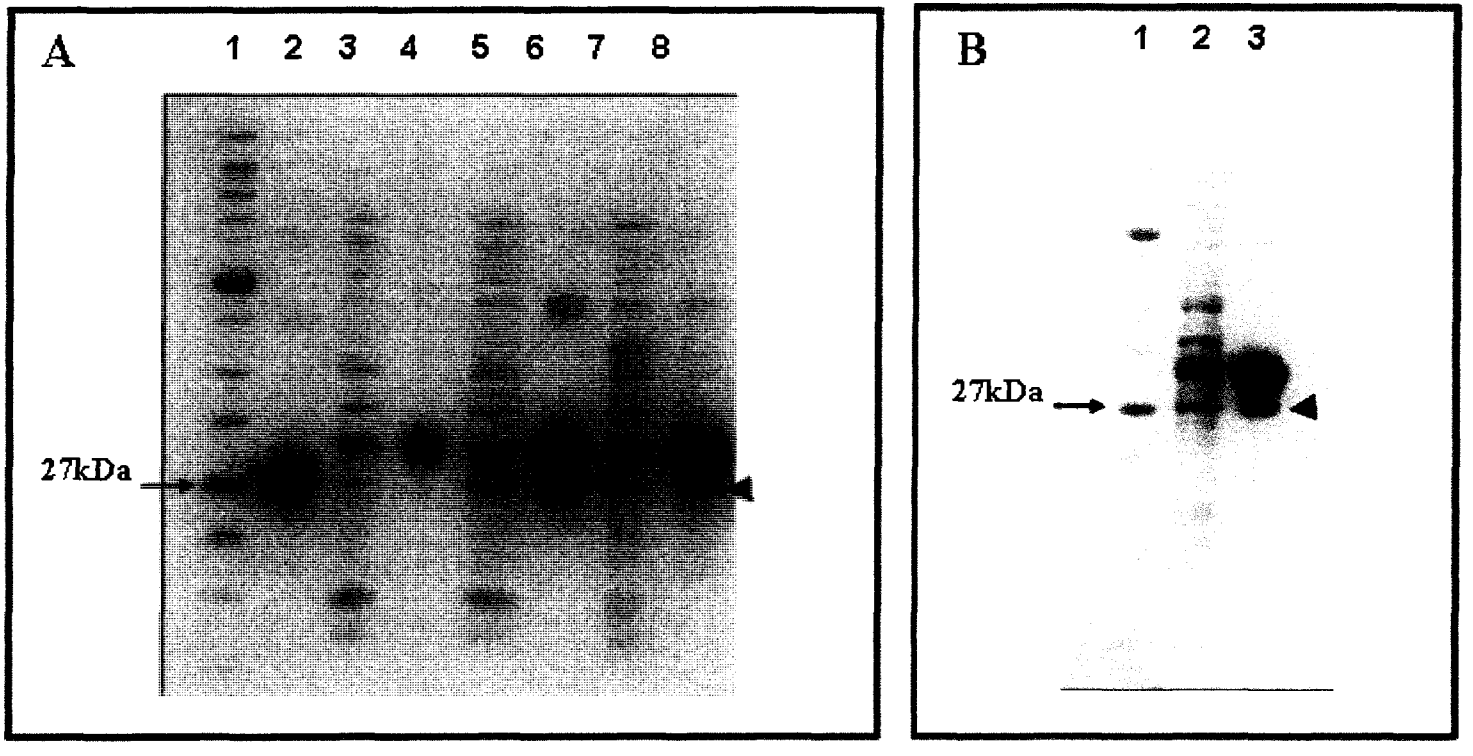

Figure 5. Purified GST-MOJ proteins detected by SDS-PAGE. (A) Lanes 3, 5 and 7 represent samples of whole lysates. Lanes 4, 6, and 8 represents the purified GST-MOJ fusion proteins. Production of GST due to random colony selection (blue arrow heads). Lane 1: MW; Lane 2: GST; Lane 3&4: GST-MOJDM; Lane 5&6: GST-GST-MOJNN; Lane 7&8: GST-MOJWP. (B) Lane 1: MW; Lane 2: GST-MOJMP whole lysates; Lane 3: GST-MOJMP purified.

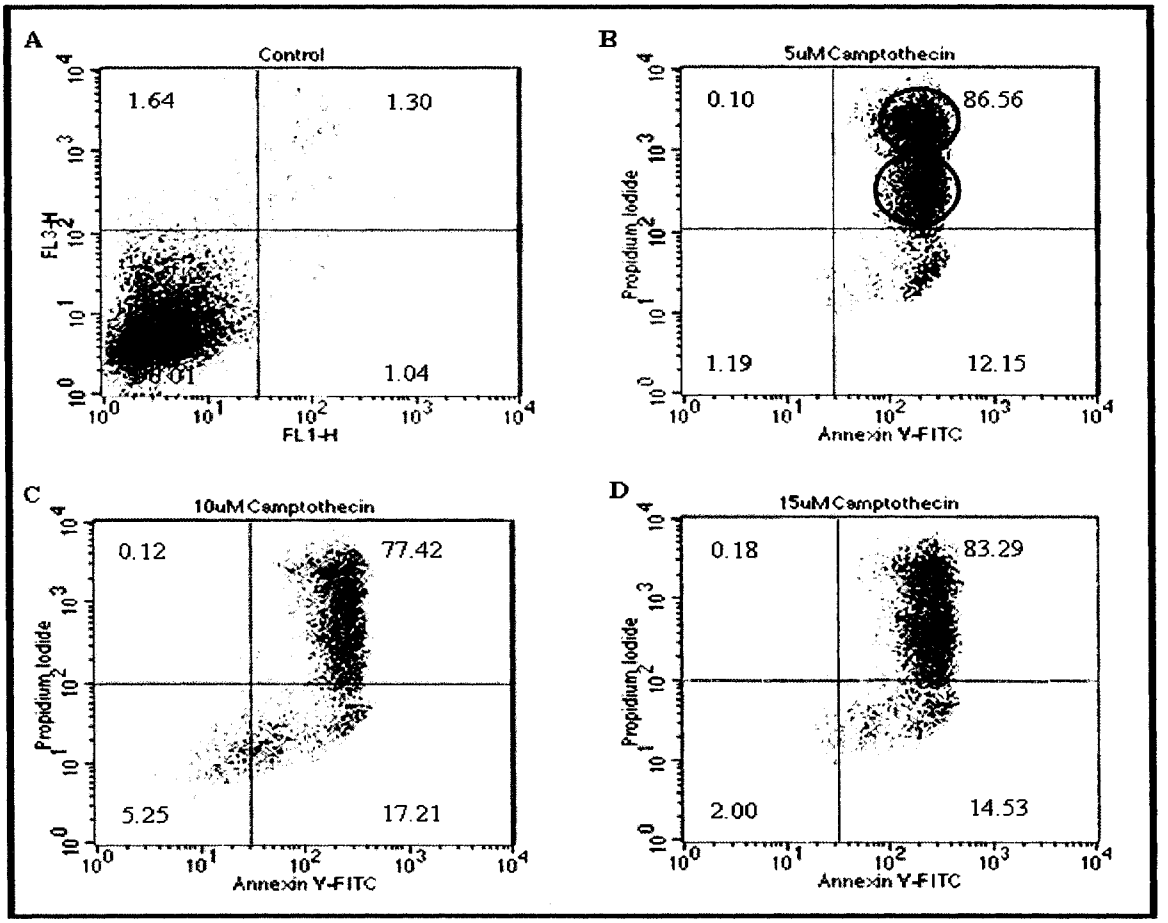

Figure 6. To determine the appropriate does for the positive control, HUVEC cells were exposed to 5, 10, and 15 uM of camptothecin. At 5 uM camptothecin 12.15% of the total population of gated cells were in early stage apoptosis (lower right quadrant, panel B), most of the cells (86.56%) were in late stage apoptosis or necrosis, and only 1.19% were viable. Most the cells were in the upper right quadrant for doses 10 uM (77.42%) and 15 uM (83.29%) as well (panel C and D), indicating that the dose at 5 uM was either too high or the incubation time was too long. For the positive control either exposure to 2 or 2.5 uM of camptothecin for 24 h should be used in order to obtain a larger population of viable cells (lower left quadrant). In the upper right quadrant of panel B there are two populations. The top population are necrotic cells (blue circle), while the bottom population are late stage apoptotic cells (yellow circle).

apoptosis in β_1 cells (Alimenti et al., 2004), was also tested as a positive control for apoptosis (Figure 7). Unfortunately, the apoptotic responses to echistatin at concentrations: 10 uM (2.00%), 20 uM (1.72%), 50 uM (2.98%), and 100 uM (2.88%); were similar to those of untreated HUVEC cells. Echistatin was chosen to be the other positive control, because it has been shown to induce apoptosis in endothelial cells and it was commercially available. Unfortunately, the echistatin purchased from Sigma showed no apoptotic activity. The manufacturers of echistatin were not forthcoming about the methods of echistatin production and Sigma, the distributing company, did not have any activity data on the disintegrin.

HUVEC cells were exposed to GST, as a negative control for apoptosis (Figure 8). There was no increase in apoptotic activity at concentrations 1uM (1.24%) and 10 uM (1.59%).

4.4 *Apoptosis Detection in HUVEC Cells after Exposure to GST-MOJ Fusion Proteins*

The mojastin disintegrin (RGDWN) has been shown to inhibit ADP-induced platelet aggregation at IC_{50} 13.8 nM (Sanchez et al., 2006). In this study wild-type recombinant fusion protein (GST-MOJWN) did not induce apoptosis at 5 uM (1.29 ± 0.3%) figure 9. The results show that 5 uM GST-MOJWP (1.01 ± 0.2%, $p > 0.05$) and 5 uM GST-MOJNN (1.58 ± 0.6%, $p > 0.05$) did not induce apoptosis in HUVEC cells. The same inactivity was observed for 5 uM GST-MOJDM (1.54 ± 0.1%, $p > 0.05$) and 5 uM GST-MOJMP (1.43 ± 0.3%, $p > 0.05$).

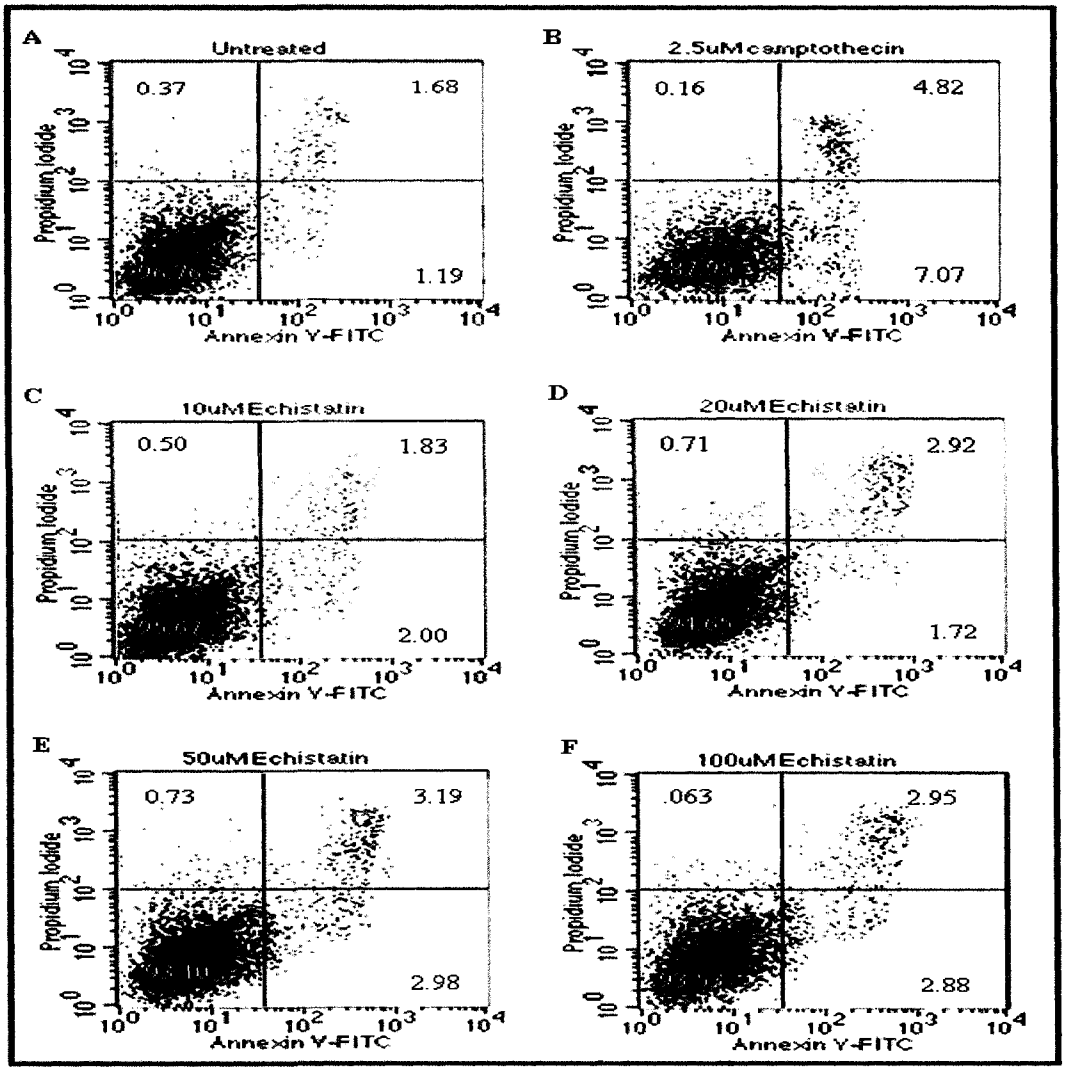

Figure 7. Exposure to echistatin did not illicit an apoptotic response in HUVEC cells. Varying concentrations of echistatin (panels C thru D) were added to flasks with HUVEC cells and incubated for 24 h at 37°C. Annexin V-FITC and PI staining showed that the population of apoptotic cells (lower right quadrant) for echistatin induced cells were the same as those of untreated HUVEC cells (panel A). Mean while in exposure to the positive control (camptothecin) showed that 7.07% of the gated population of HUVEC cells were apoptotic (panel B).

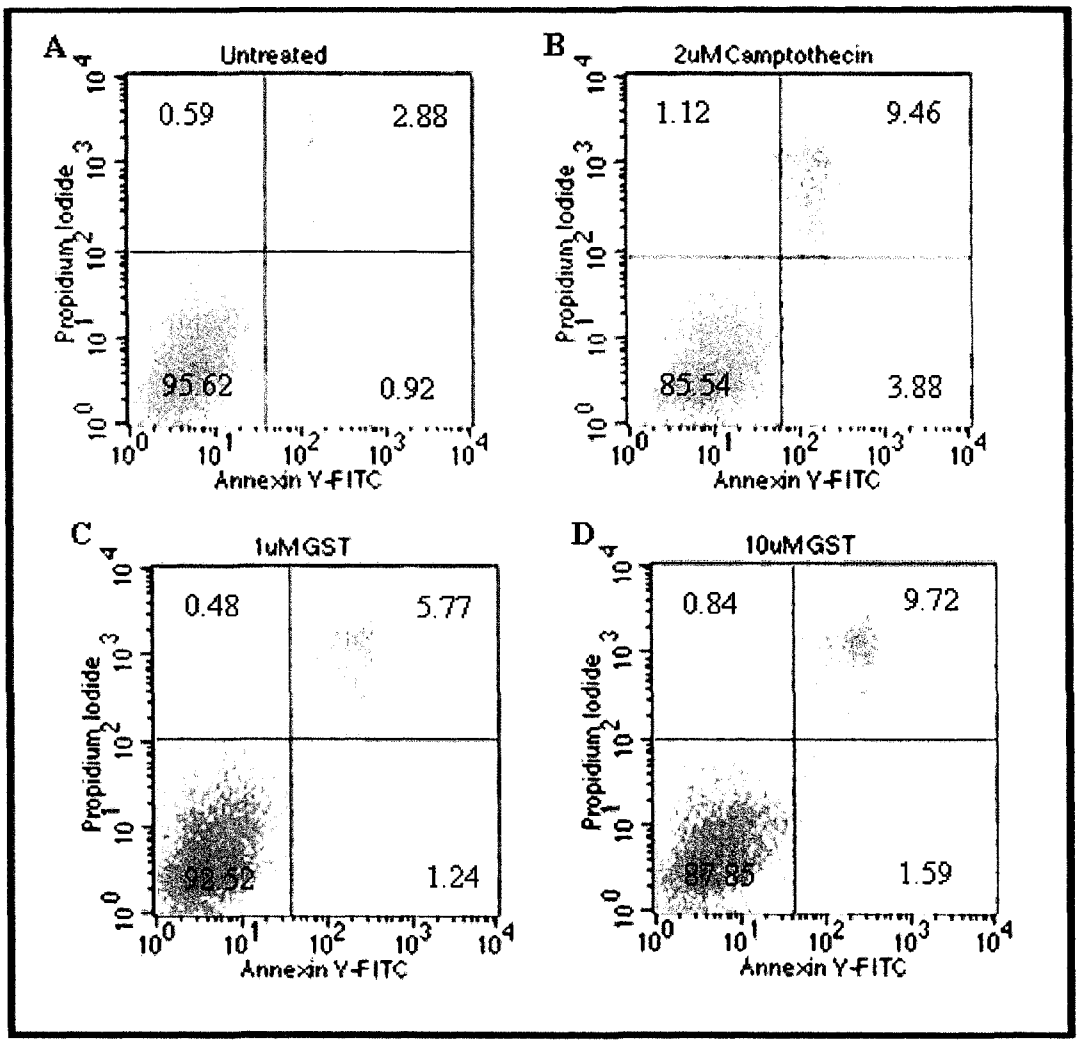

Figure 8. GST was tested at 1, 10 and 15 uM, for its affect on the viability of HUVEC cells. There was no change in the early stage apoptotic population (lower right quadrant; panels C, D, and E). However the late stage apoptotic and necrotic cell population did increase in a dose dependent manner; 5.77% at 1 uM (panel C) and 9.72% at 10 uM (panel D).

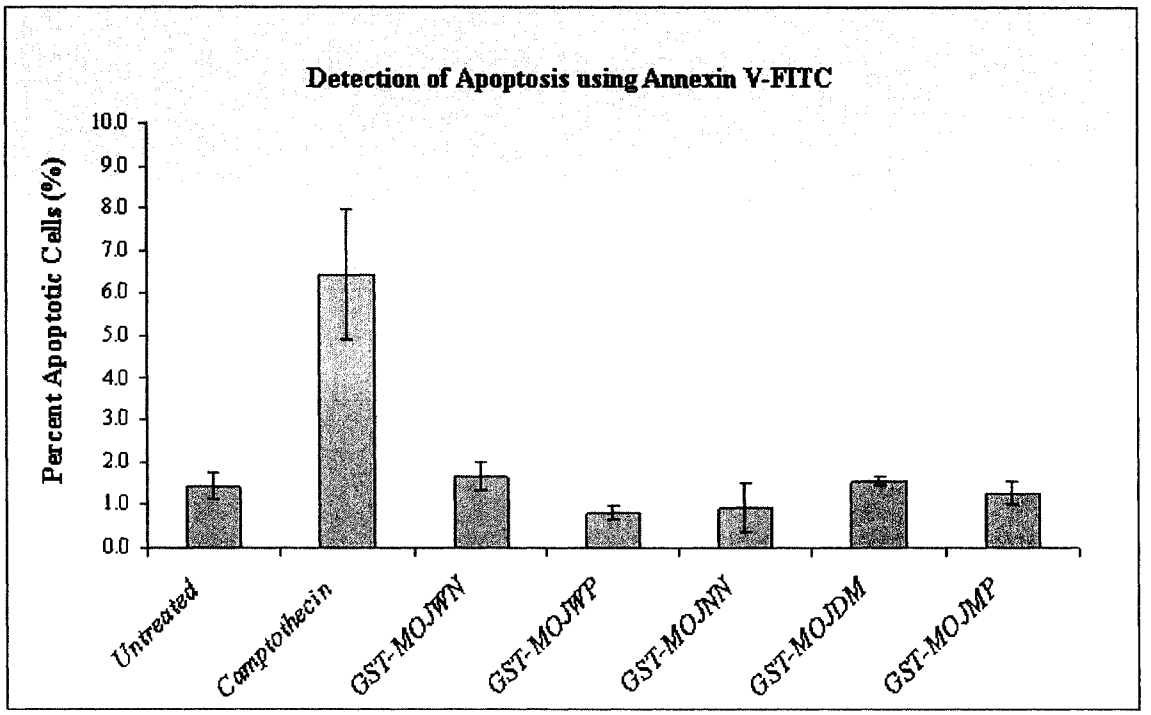

Figure 9. The apoptotic affect of 5 uM wild-type and mutant mojastin recombinant fusion proteins were tested on HUVEC cells. At 5 uM the percent of early stage apoptotic cells for each of the mutants and the wild-type were the similar as the untreated cells (1.41% ± 0.3). Statistical analysis using a paired t-test showed that the means were not significantly different for the wild-type and mutant mojastin recombinant fusion proteins as compared with the untreated sample ($P > 0.05$). This data reflects measurements taken from three different experiments. The bars represent ± SD.

Paired t-tests used to compare untreated samples and each of the other samples confirmed that the difference between the apoptotic cell population of wild-type and mutant mojastin recombinant fusion proteins and the untreated cells was not significant. However, when the apoptotic population of untreated cells was compared to those of camptothecin, there was a significant difference ($p < 0.001$)

4.5 *Hoechst 33258 Staining of HUVEC Cells Exposed to GST-MOJ Fusion Proteins*

Staining of the HUVEC cells after incubation with 2.5 uM Camptothecin, 5 uM GST-OJWN and GST-MOJNN verified the results above (Figure 10). Cells exposed to camptothecin exhibited nuclear condensation when stained with Hoechst 33258 (white arrows). Cells exposed to either GST-MOJWN and GST-MOJNN did not show any morphological changes that could be attributed to apoptosis.

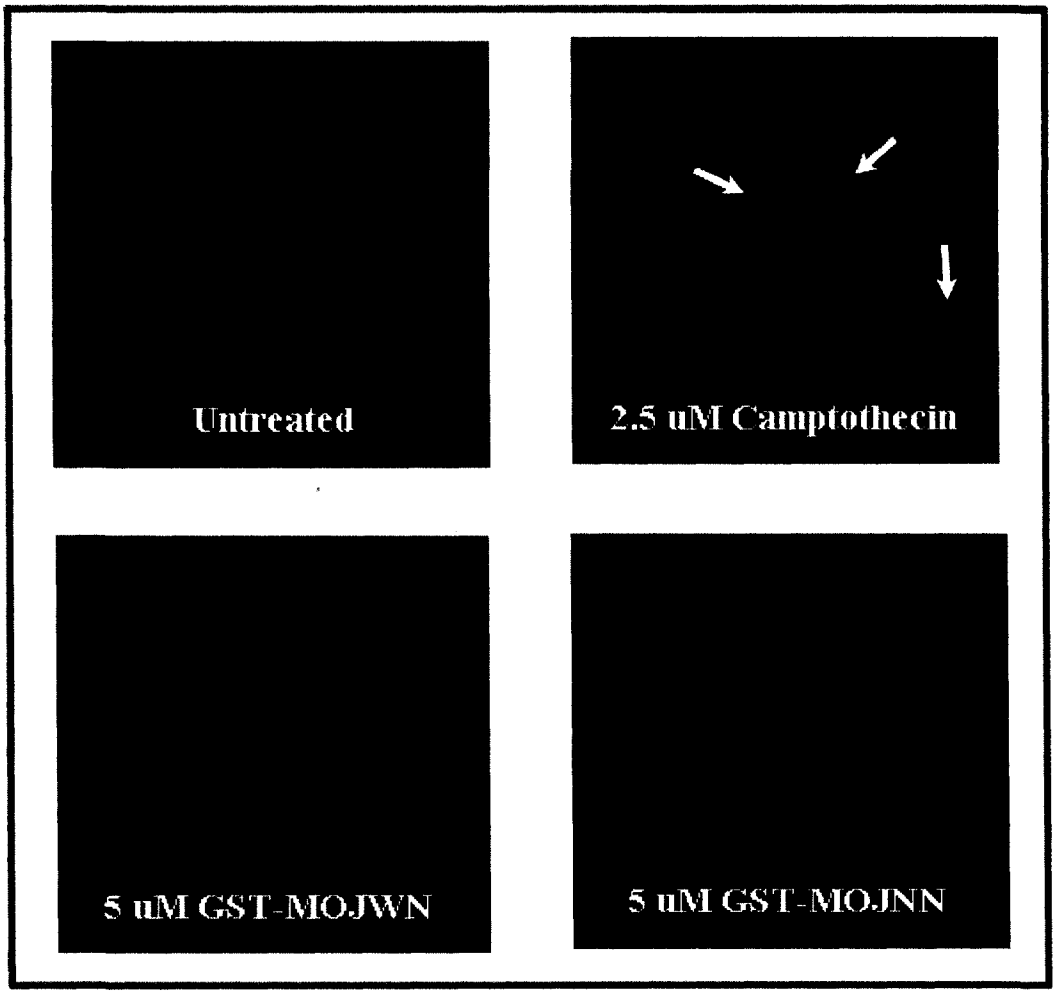

Figure 10. Apoptosis as characterized by nuclear condensed fragments were visualized using 10 ug/ml Hoechst 33258. HUVEC cells exposed to 5 uM GST-MOJWN and GST-MOJNN did not show an increase in nuclear condensation as compared with cells exposed to 2.5uM camptothecin (white arrows).

5. Conclusion

5.1 *Disintegrins as a Cancer Therapeutic*

Integrin binding of adhesion molecules in the extracellular matrix has been shown to induce signaling pathways that on the one hand result in cell migration and proliferation, while also suppressing signaling pathways that cause cells to undergo self destruction (Barja-Fidalgo et al., 2005; Zhang et al., 1995). Dysfunction in these signaling pathways causes tumor progression and metastasis. Thus it has been the aim of some cancer therapies to target ingterins and induce cell death in order to prevent the proliferation and migration of neoplastic cells. Disintegrins are natural antagonists of integrin binding with cell matrix proteins. Their binding of integrins causes cell detachment from the cell matrix, releasing the cell from antiapoptotic control and resulting in a special form of cell death called anoikis (Barja-Fidalgo et al., 2005; Morozevich et al., 2006). Recombinant proteins have been produced to test the binding affinity and specificity of disintegrins to integrins receptors (Chang et al., 2001).

5.2 *Protein Modeling: Predicting the Apoptosis Inducing Structure of Disintegrins*

Researchers in the Soto lab used site directed mutagenesis to change the 54th and/or 55th amino acid of the mojastin disintegrin to create ten mutants. In order to predict the activity of these mutants, three dimensional models were constructed using homology modeling. In this model a high resolution x-ray structure of trimestatin (Fujii et al., 2003) was used as a template for the disintegrin protein, due to the lack of

structural information available for mojastin. Trimestatin was chosen because it shared the highest sequence similarity to mojastin (81.7%), compared with any other medium length disintegrin. The mojastin sequence was aligned using trimestatin as the template by the program MODELLER. This produced five possible three dimensional models for mojastin. These models were analyzed using PROCHECK, a program that generates Ramachandran plots depicting the best stereochemistry for each of the models. The models chosen had 91% of their residues in favorable regions. This confirmed the reliability and stereochemical accuracy of the models produced. CHIMERA was then used to create a surface model which provided the best indicator of overall structure visually.

Using the three-dimensional structures created in this way researchers in the Soto lab were able to pick four mutants, from the ten, which were most likely to affect the induction of apoptosis. Two of the mutants contained the same 54th and 55th amino acids as echistatin (RGDDM) and rhodostomin (RGDMP). Echistatin (10 ug/ml) elicited an apoptotic response when exposed to GD25 cells (Alimenti et al., 2004) and 4 ug/ml of rhodostomin induced apoptosis in HUVEC cells (Wu et al., 2003). Two others mutants (NN and WP) were chosen for their structural similarity in the C-terminal region to MP because the models showed that changes in the 54th and 55th amino acids affect a conformational change in the C-terminal domain of disintegrins. Thus these four mutants along with wild-type mojastin (Figure 11) were chosen to be expressed as GST fusion proteins and assayed for their ability to induce apoptosis.

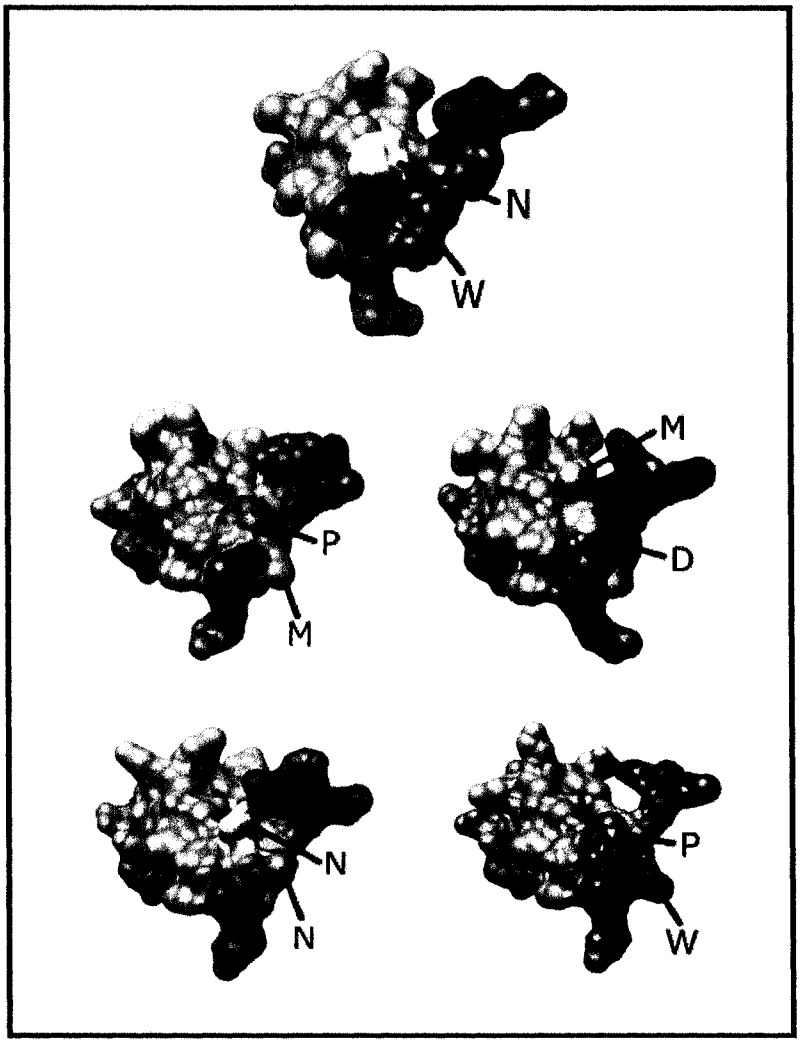

Figure 11. Surface models for mojastin and mutant sequences. Mojastin (WN); the two mutants with sequence similarity to rhodostomin (DM) and echistatin (MP); and the two mutants structurally similar to echistatin (NN and WP) are presented. Here the RGD-loop is shown in red and the C-terminus is shown in green. These models were created using MODELLER and visualized using CHIMERA. Each model was analyzed using PROCHECK to determine the most probable stereochemistry.

5.3 *Wild-Type and Mutant Recombinant Fusion Proteins: Inability to Induce Apoptosis*

Although, mojastin 1 and 2 inhibit platelet aggregation, the recombinant mojastin did not induce apoptosis in HUVEC cells, which is not surprising since mojastin did not inhibit T24 cells from binding fibronectin (Sanchez et al., 2006), the adhesion molecule bound by integrin receptors $\alpha_5\beta_1$ (Berman et al., 2003). However, it is surprising that GST-MOJDM and GST-MOJMP did not induce apoptosis in HUVEC cells. There are three possible explanations for my findings. Either the fusion proteins produced in this study were misfolded, apoptosis induced cell death due to exposure to disintegrins may be cell specific, or the RGD-loop recognition sequence produced was insufficient for $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin signaling of apoptosis.

Researchers have found that disintegrins produced *E. coli* have biological activity. Recombinant elegantin (Rahman et al., 1998) and rhodostomin (Chang et al., 1993), expressed by *E. coli* as glutathione-S-transferase fusion proteins, retained their structural morphology and biological activity. GST-Elegantin had the same inhibitory potency (IC_{50} 0.3 uM) as the purified toxin in ADP-induced platelet aggregation of platelet rich plasma (Rahman et al., 1998). Change et al. (1993) tested binding of human hepatoma cells to culture plates coated with GST-Rhodostomin (GST-RHO) or with GST by itself, and found that binding of human hepatoma cells to GST-RHO coated plates was 10 to 20 fold over those culture plates without. Two synthetic peptides were created to test whether the binding of human hepatoma cells was specific to the RGD-loop of GST-RHO. The first was GRGDSP, which retained the RGD sequence and the second was GRGESP with an Asp amino acid substituted with a Glu amino acid. Increasing amounts

of GRGDSP decreased the binding of human hepatoma cells to GST-RHO. However increasing amounts of GRGESP did not affect binding of human hepatoma cells, indicating that the cells were specifically binding to GST-RHO. The biological functionality of recombinant rhodostomin in these assays led Change *et al.* (1993) to conclude that the rhodostomin molecule bound to GST was properly folded. GST-MOJ and GST-MOJNN exhibited binding activity in adhesion assays performed using TD24 cells, while GST-MOJ, GST-MOJNN, and GST-MOJDM displayed binding activity in adhesion studies using skin melanoma cells (E. Sanchez, personal communication, March 6, 2008). This evidence shows that the wild-type and mutant mojastin recombinant fusion proteins were most likely folded properly.

Conforti et al. (1990) inserted the isolated vitronectin receptor ($\alpha_v\beta_3$) into different types of liposomes in order to test binding affinity of $\alpha_v\beta_3$ receptor to vitronectin The different types of liposomes were phosphotydilcholine (PC), PC + phosphotidylethanolamine (PE), and PC + PE + phosphotidylserine (PS) + phosphotidylinositol (PI) + cholesterol (chol). The results of this study showed that $\alpha_v\beta_3$ was able to recognize vitronectin more readily and bound to vitronectin with greater affinity as the liposome structure became more complex. Also $\alpha_v\beta_3$ receptors were able to bind other proteins such as von Willebrand factor and fibronectin. This suggests that integrins on different cells types may behave differently depending upon the cell membrane environment. This explanation may apply to GST-MOJDM. However, rhodostomin has been shown to induce apoptosis in HUVEC cells (Wu et al., 2003).

Thus some other factor must be influencing the lack in apoptotic activity of GST-MOJMP.

Since inhibition studies with kristin and dendroaspin, two disintegrins with the same sequence (PRGDMP), showed that they bound with equal avidity to $\alpha_{IIb}\beta_3$, it was surprising that the mutants GST-MOJDM and GST-MOJMP with C-terminal residues adjacent to the RGD-loop similar to echistatin (RGDDM) and rhodostomin (RGDMP) did not induce apoptosis (Rahman et al., 1995). Kristin and dendroaspin also competed with each other for the same receptor binding site on $\alpha_{IIb}\beta_3$ integrins (Rahman et al., 1995), suggesting that the target for the two disintegrins is the same. Exploration of the N-terminal residues adjacent to the RGD-loop may produce a mojastin mutant recombinant fusion protein that can induce apoptosis in endothelial cells.

Studies done with recombinant echistatin point out the C-terminal domain of the disintegrin to be a contributing factor in $\alpha_5\beta_1$ binding (Wierzbicka-Patynowski et al., 1999). In their study Wierzbicka-Paynowski et al. (1999) examined at conformation changes in the integrin model due to ligand binding using antibodies, called anti-ligand-induced binding site (LIBS) antibodies, to the new binding site. They substituted the C-terminal sequence of eristostatin (WNG) with those of echistatin (HKGPAT) and found that inhibitory potency of the hybrid increase was over 4-fold greater than that of the wild-type. This suggests that C-terminal region of the mojastin should be considered when producing mutant mojastin recombinants to affect apoptosis.

The amino acids N-terminal to the RGD-loop are **PA**RGDDM for echistatin and **IP**RGDMP for rhodostomin. Binding studies done with elegantin showed that

replacement of the Pro residue, N-terminal to the RGD-loop, with Ala produced peptides that bound with greater avidity to $\alpha_5\beta_1$, emphasizing that not only does the C-terminal residues flanking the RGD-loop, contribute to the binding specificity of integrins, but the residues N-terminal residues to the RGD-loop contribute as well (Rahman et al., 1995).

In this mutant mojastin recombinant fusion proteins were used to study the ability of the 54th and 55th amino acids, C-terminal to the RGD-loop, to induce apoptosis signaling in HUVEC cells, containing the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins. Wild-type and mutant mojastin recombinant fusion proteins were expressed with a molecular weight of 34 kDA. Though these recombinant fusion proteins did not affect apoptosis signaling in HUVEC cells, this inactivity from exposure to the recombinant fusion proteins may be the result of a partial recognition sequence. The recombinant fusion proteins produced here only contained sequence similarity of the 54th and 55th amino acid C-terminal, to the RGD-loop. However, the amino acids N-terminal to the RGD-loop and the C-terminal region outside of the RGD-loop have also been shown to affect inhibition of platelet aggregation. Thus the mutant mojastin recombinant fusion proteins with the correct amino acids C-terminal to the RGD-loop may not be sufficient to induce apoptosis in HUVEC cells.

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IRB Approval Letter

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From: Pamela Stacks, Ph.D.
Associate Vice President
Graduate Studies and Research

Date: October 11, 2006

The Human Subjects-Institutional Review Board has approved your request to use secondary data related to human subjects in the study entitled:

> "Induction of apoptosis by mutations in the binding loop of the Mojastin disintegrin protein"

This approval, which provides exempt status under Category D, is contingent upon the subjects included in your research project being appropriately protected from risk. Specifically, protection of the anonymity of the subjects' identity with regard to all data that may be collected about the subjects from your secondary sources needs to be ensured.

The approval includes continued monitoring of your research by the Board to assure that the subjects are being adequately and properly protected from such risks. If at any time a subject becomes injured or complains of injury, you must notify Dr. Pamela Stacks, Ph.D. immediately. Injury includes but is not limited to bodily harm, psychological trauma, and release of potentially damaging personal information. This approval for the human subject's portion of your project is in effect for one year, and data collection beyond October 11, 2007 requires an extension request.

If you have any questions, please contact me at (408) 924-2480.

cc. Julio Soto, DH 439-0100

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