CANCER PROCESSES PROBED BY MULTIVALENCY: INVESTIGATIONS WITH GALECTIN-3 AND LACTOSE FUNCTIONALIZED DENDRIMERS

by

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ABSTRACT

Cancer has become a prevalent disease that is the second leading cause of death in the United States. Various cancers have been identified as either over or under expressing a sugar binding protein: galectin-3. The target of this research is to investigate cancerous events that are impacted by galectin-3 and mediate these events through the use of a multivalent binding partner to galectin-3. This binding partner is lactose functionalized PAMAM dendrimers.

Apoptosis has been reported as another phenomenon that galectin-3 impacts. By using a reporter assay, viability, cytotoxicity and apoptosis were observed for cancer cell line A549 in the presence of exogenously added galectin-3 and/or lactose functionalized dendrimers. It was found that exogenous galectin-3 and glycodendrimers did not have any significant impact on these cell viability tests. Therefore, glycodendrimers can be used to probe multivalent effects without threat of toxicity.

Metastasis was investigated through a modified *in vitro* scratch assay. By monitoring the migration of cancer cells, it was found that exogenously added galectin-3 retarded cell migration. When glycodendrimers were included, migration was partially restored. This revealed the implications of exogenous galectin-3 regarding the metastatic potential of carcinomas. When the implications of the domains of galectin-3 were investigated, it was found that the truncated galectin-3 containing only the carbohydrate recognition domain (CRD) was unable to replicate the same effects observed in full length galectin-3. Immunofluorescence microscopy was used to locate the multivalent binding partner and galectin-3 in the assay. While endocytosis of galectin-3 was observed, no colocalization with the multivalent binding partner was observed intracellularly, supporting the hypothesis of an extracellular interaction mediating the results. Multivalent interactions between glycodendrimers and galectin-3 impacted cellular migration.

Angiogenesis revealed that exogenous galectin-3 induced neovascularization. Glycodendrimers impacted galectin-3 mediated angiogenesis. Glycodendrimers alone could elicit effects either enhancing or negating angiogenesis depending on the dendrimer generation. Fluorescent tags revealed glycodendrimer accumulation on or inside the cells and galectin-3 on the surface of cell groups.

Overall, these studies show that glycodendrimers can interact multivalently and affect cellular processes.

CHAPTER ONE

DENDRIMERS IMPACT GALECTIN-3 MEDIATED CANCER CELL MIGRATION *IN VITRO*

Introduction

The fields of biochemistry and supramolecular chemistry do not seem to be fields of overlapping research. But the truth is that a lot of important biochemistry would also fall under the category of supramolecular chemistry. In recent years, the lines between many different disciplines has become blurred as we continue to collaborate in an attempt to attain a better understanding of important science. This has led to exciting developments that begin to encompass more and more of the big picture.

Supramolecular chemistry

Since its naming in the 1980s, the field of supramolecular chemistry has gathered a wide variety of research under its broad discipline of study. Simply put, this field is defined as the study and design of non-covalent chemistry on much larger complexes. While many chemistries, such as guest-host complexes, hydrogels, signalosomes and drug delivery systems may fall into this field, at the core, this field combines many branches of research under the terms of understanding intermolecular effects[1]. These investigated effects can be any combination of non-covalent interactions such as hydrogen bonding, the hydrophobic effect, metal-ion complexation, Van der Waals interactions and others. In fact, these interesting non-covalent factors that contribute to

supramolecular interactions have been reported to be highly dependent on Van der Waals forces, which was previously thought to be mostly non-contributing[2].

Much early research in this field came from the discovery of hydrogen bonds in DNA and later led to an understanding that enabled synthetic complex systems such as crown ethers that hosted cations of varying valencies to form stable compounds[3]. More recent research has evolved to more sophistication in the design of complexes that perform a desired action such as self-assembly. Some well-studied examples of self-assembly come from nature. One example is the Tobacco Mosaic Virus which forms from a self-assembly of a large number of subunits into a helical structure to protect the RNA strand contained within[4]. By studying natural systems, scientists have learned many of the valuable properties and techniques in molecular recognition necessary to design, initiate and afford a self-assembling system. By understanding the equilibrium, thermodynamics, cooperativity and efficiency in the self-assembling systems, greater and more complex systems have been designed.

Biological Interest

Biology has always been at the forefront of supramolecular systems; one classic example of this is the process of protein folding. Post-translationally, a primary sequence of a protein must undergo proper folding in order to obtain its designed function. Sometimes with the use of a chaperone protein, secondary structure is formed from hydrogen bonds, and then tertiary structure is formed from other non-covalent interactions such as ionic interactions, pi-pi stacking and hydrophobic effects. Although

many proteins are folded with the use of chaperone proteins, the step-wise contribution of non-covalent interactions stays the same[5].

Even though our understanding of protein folding is far from complete, there is enough understanding to design synthetic peptides, called foldamers, for specific functions. Through careful selection of the framework, foldamers have been designed to bind their target even with energies rivaling the native affinities[6]. With current understanding and prediction of non-covalent interactions, larger and more complex foldamers have been designed and synthesized for specific functions successfully. It has been hypothesized that these complexes can be designed for a great number of conformations and functions with increased understanding and prediction of the interactions created[7]. Researchers used these techniques to design foldamers such as a mixed α/β peptide that bound B-cell lymphoma-extra large (Bcl-XL) with ten times greater affinity than the natural peptide[6].

Research like this has continued to produce more compounds that are "biomimetic." Investigations into anticoagulation began in a similar way with the natural polysaccharide heparin. By forming smaller polysaccharide fragments, it was found that anticoagulation could be controlled. This has further led to *de novo* synthesis of complex glycans that are now pharmaceutically available and have been optimized to elicit the greatest non-covalent interaction for thrombosis[8]. This complex polysaccharide scaffold has been investigated in many different diseases for uses such as infections, cancer and inflammation. Research within this sphere of glycomics led to anti-malarial drug erythropoietin which stimulated red blood cell production[8]. Other research like this

investigated wound healing and fibronectin which is known to promote cell migration and adhesion. Using peptide fragments of fibronectin and by a single amino acid substitution, the group could control inhibition or promotion of wound healing in different models[9].

Often within biological supramolecular interactions, multivalency is a utilized property. In general, any ligand- receptor interaction is non-covalent in nature. In order to attain more biologically relevant interactions, often nature utilizes multiple binding interactions. For example, when an influenza virus attaches to an epithelial cell, it must bind to terminal sialic acid groups on the cell surface multivalently via hemagglutinin trimers on the viral capsid[10]. Mimicking viral glycan recognition has become a strategy to combat viruses such as HIV and Ebola[11].

Some modes of multivalency that are investigated here are statistical effects, chelation, receptor clustering and crosslinking, but these described properties are not all encompassing. When there are multiple ligands available to bind a receptor, there is a statistical increase in the amount of time the receptor stays bound. If a ligand unbinds, there is a close ligand to take its place (Figure 1A)[12]. The strength of the interaction is not increased but the unbound time is decreased. Chelating is an entropic argument, when a binding interaction takes place, there is an entropic penalty. But if a bivalent ligand binds a bivalent receptor, the entropic cost is lower than if a bivalent receptor binds two monovalent ligands. Therefore, the overall interaction is more favorable than the counterpart (Figure 1B)[10]. Receptor clustering is a biological effect where a multivalent ligand brings multiple receptors in closer proximity to each other (Figure 1C)[13]. This can

lead to an activated signal, such as where two receptors are required for the activated complex, or a weak signal can be intensified. Another major impact of multivalency in biological systems can be the ability to crosslink and form higher order aggregates (Figure 1D)[14]. These crosslinking effects can be either with neighboring cells, with other receptors on the same cell or binding the surrounding factors such as the ECM.

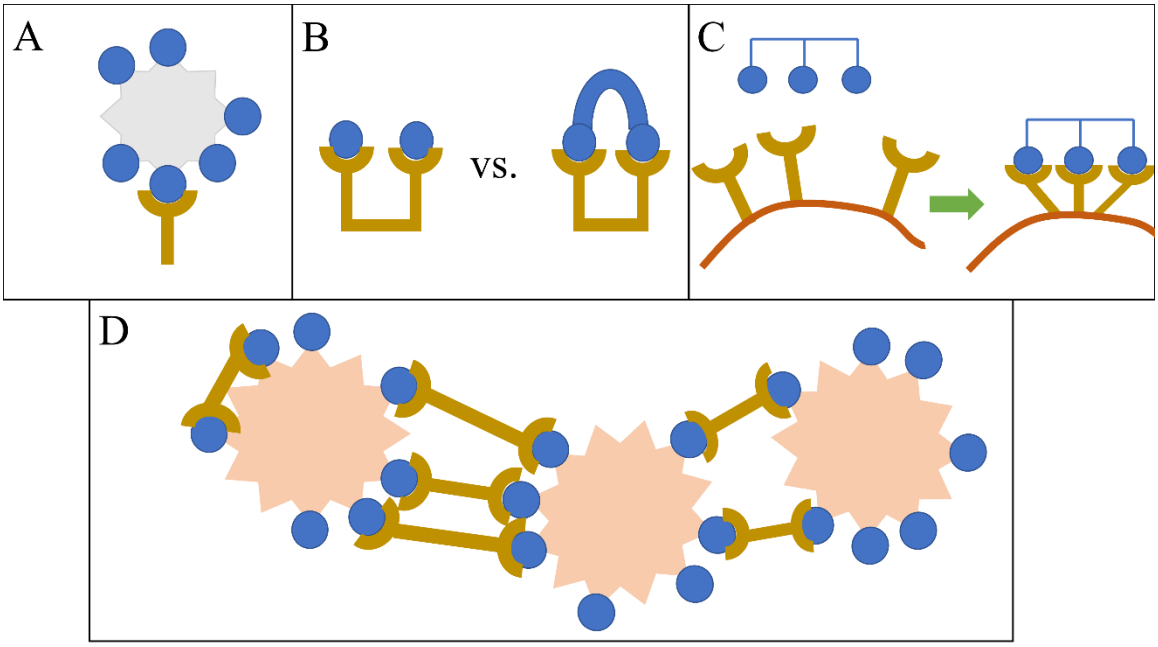

Figure 1. Modes of multivalency. Statistical binding effects (A), chelating effect (B), receptor clustering (C), and cross-linking (D).

Further investigations into multivalent design have shown that linker length between ligands directly impacts the strength of the multivalent interaction. In work done by the Whitesides group, they reported an increase in the free energy of the bivalent interaction as the linker was lengthened until an optimum binding energy was reached, after that point, increasing the length of the linker decreased the strength of the

interaction[15]. Multivalent interactions can be synergistic, additive or negative in cooperativity[10]. Generally, multivalent interactions are of greater functional affinity (avidity) than the monovalent interaction[16]. An increase in the understanding of multivalent interactions and improved design of multivalent systems has shown these tools being used to influence a variety of targets such as cancer, fertility and immune responses[16].

This chapter will describe the role of galectin-3 in cancer processes and the impact of multivalently designed scaffolds to interact with these processes. Our investigations have taken us to explore the contributions of multivalency in cancer processes such as toxicity, apoptosis, angiogenesis and metastasis.

Galectin-3

Galectin-3 is a β-galactoside binding protein in the galectin family, of which 15 have been identified in humans. They are all characterized by a conserved carbohydrate recognition domain (CRD) and have been categorized into three subgroups. The largest subgroup is prototype galectins which contain a single CRD. The second group is tandem repeats which consist of two CRDs connected by a linker domain. The final group only contains galectin-3 and is named the chimera galectin because of its single CRD and unique N-terminal domain which is disordered[17]. Because of its unique characteristics within the family, galectin-3 has become the most studied of the galectins. Galectin-3 is essential for early development and organogenesis but it has also been reported to be important for cancer progression[18–21]. The oncogenic and tumor suppressive activities of

galectin-3 appear to be directly dependent on its cellular localization and these localizations are not inclusive; galectin-3 provides different functions in different cancers (Figure 2, Table 1). For example, there is evidence that cytoplasmic galectin-3 acts as an antiapoptotic factor by preventing mitochondrial damage or cytochrome c release[22]. Galectin-3 can also mimic the function of B-cell lymphoma 2 (Bcl-2) and bind to Bcl-2-associated death promoter (BAD) and Bcl-2 homologous killer (BAK) which when localized to the mitochondria, promote the release of cytochrome c and progress apoptosis[22]. But when localized to the nucleus, galectin-3 can promote apoptosis through transcription regulation[23].

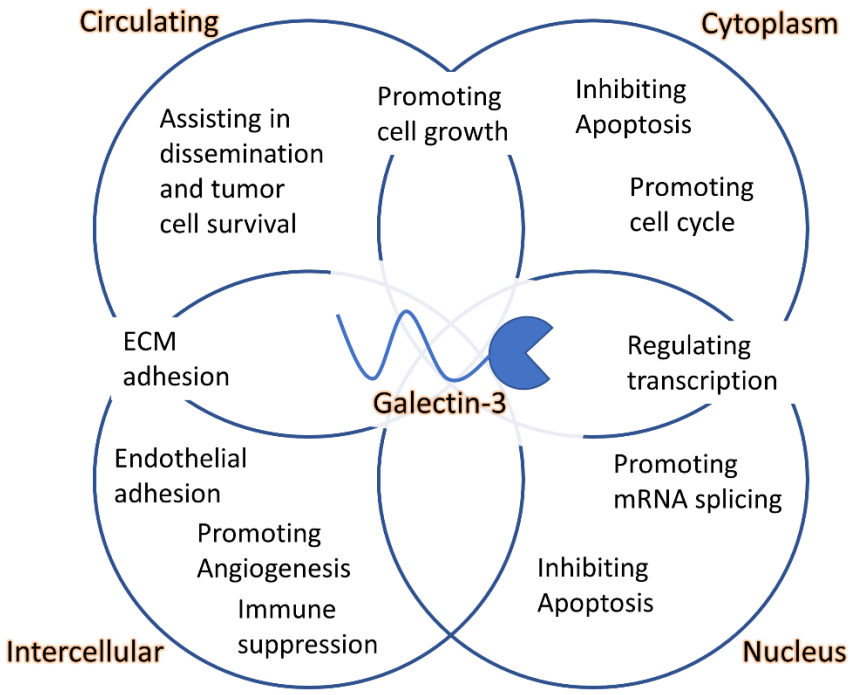

Figure 2. Relative cellular locations of galectin-3 and the cancerous processes modulated. Adapted from reference[23].

Cancer	Location	Reference
Adenocarcinoma of the endometrium	Nucleus	Vladoiu, M., et al.
Melanoma	Nucleus	Vladoiu, M., et al.
Thyroid carcinoma	Nucleus	Vladoiu, M., et al.
Colorectal adenoma and carcinoma	Cytoplasm	Vladoiu, M., et al.
Follicular and papillary thyroid carcinoma	Cytoplasm	Vladoiu, M., et al.
Adenocarcinoma of the endometrium	Cytoplasm	Vladoiu, M., et al.
Melanoma	Cytoplasm	Vladoiu, M., et al.
Thyroid carcinoma	Cytoplasm	Vladoiu, M., et al.
Squamous cell carcinoma of the tongue	Cytoplasm	Vladoiu, M., et al.
Colorectal adenocarcinoma	Mitochondria	Vladoiu, M., et al.
Breast adenocarcinoma	Endosome	Vladoiu, M., et al.
Colorectal adenocarcinoma	Plasma Membrane	Vladoiu, M., et al.
Renal cell carcinoma	mRNA expression	Dong, R., et al.
Thyroid Carcinoma	Extracellular	Dong, R., et al.
Hepatocellular carcinoma	Extracellular/ mRNA expression	Dong, R., et al.
Prostate cancer	Extracellular/ decreased cytoplasm and nucleus	Dong, R., et al.
Pancreatic carcinoma	Extracellular/ tissue expression	Dong, R., et al.
Colorectal cancer	Extracellular	Dong, R., et al.
Breast cancer	Extracellular/tissue expression/ mRNA expression	Dong, R., et al.
Bladder cancer	Extracellular/ tissue expression	Dong, R., et al.
Gastric cancer	Extracellular	Dong, R., et al.
Lymphoma	Extracellular	Dong, R., et al.

Table 1. Locations of galectin-3 in various cancers. Adapted from references[24,25].

Galectin-3 can also affect a wider range of processes because of its ability to oligomerize through its N-terminal domain. When found outside the cell, galectin-3 has been reported to form higher order multimers, up to pentamers[26]. This function is dependent on the CRD and the N-terminal domain in coordination[27]. Galectin-3 does not

contain an export sequence, so its excretion to the extracellular region must be through a non-classical secretion mechanism[27]. Once in the extracellular space, there are a great number of glycosylated extracellular ligands available for interactions[18]. These interactions can lead to lattice formation and redistribution of glycoconjugates on the cell surface[28,29]. This can lead to either signaling enhancement, protecting receptors from being activated or prevention of endocytosis[30]. Many of these glycoreceptors for galectin-3 are involved in cell adhesion such as laminin, fibronectin, and other extracellular matrix components[17]. There also is indication of an endocytosis/ exocytosis balance to extracellular galectin-3 levels. In macrophage like cells, endocytosis occurred rapidly, within 5-10 minutes and was dependent on the CRD and N-terminal domain and could be blocked by lactose[31].

Higher affinity ligands for galectin-3 have been reported such as Thomsen-Friedenreich (TF) antigen which is revealed on aberrantly glycosylated Mucin 1 receptor on the surface of cancer cells. This interaction has reported an equilibrium dissociation constant (K_d) of 47 µM from galectin-3 to TF antigen[32]. Other galectin-3 ligand interactions have been described with a K_d of 61 ± 2 µM to lactose, 67 µM to N-Acetyllactosamine[33]. But some of the highest affinity ligands for galectin-3 are either natural or designed multivalent interactions, for example, in one review, a polylactosamine chain was the best reported ligand[18]. Other experiments have revealed that pectin-derived polysaccharides had dissociation constants (K_d) of up to 49 nM[34].

Apoptosis

Early in the development of cancer, a neoplastic tumor must gain the ability to bypass encoded cell suicide called apoptosis. Apoptosis is a highly regulated and complex process that can be activated by many different mechanisms. Apoptosis is characterized by a coordinated and systematic dismantling of the cell, without inflammation, and eventual phagocytosis by macrophages. DNA mutations, cytokines and death receptors can activate apoptosis through extrinsic and intrinsic pathways[35]. These pathways lead to the initiation of the caspase cascade which activates specific proteases to begin the process of apoptosis: condensing DNA, protein crosslinking and forming apoptotic bodies[36]. A common trait of cancer cells to bypass apoptosis is through loss of the p53 protein which has been termed "the guardian of the genome." P53 functions as a DNA damage sensor and can turn on intrinsic apoptosis at signs of DNA damage[37]. Up or down regulating different players can have great impacts on the cascade of apoptosis within a cell.

Multivalency has become an active area of research in regard to activating apoptosis in cancer cells. Of particular interest is the TNF-related apoptosis-inducing ligand (TRAIL) which is highly selective against cancer cells but has few side impacts. This ligand activates Death Receptor (DR) 4 and 5 which induces caspase dependent apoptosis. Different scaffolds such as potato virus x have been tagged with up to 490 TRAIL monomers. Due the flexibility of the virus, this complex has been shown to be between 3-10 times more effective than the TRAIL monomer in an *in vitro* model[38]. A multivalently modified protein proved to be an effective agonist against the death

receptors, up to 2 fold more effective than the TRAIL monomer[39]. Additional apoptosis inducing techniques have included a complex polymeric system of an amphipathic dendron that carries multiple functional units. There is a long hydrophobic chain that can insert into the cell membrane, and the researchers are investigating linking this region to different toxic compounds. The dendron also contains many lysine residues which present a high concentration of cationic charges to the plasma membrane. This dendron is presented in an inactivated form, but upon exposure to slightly acidic conditions such as would be present in a tumor microenvironment, the aggregates will disperse and become active[40].

Galectin-3 has been reportedly involved in apoptosis, especially in the context of immune suppression. Galectin-3 can bind to CD98 on the surface of T-cells which is involved in the regulation of apoptosis[19]. There are also reports that overexpression of galectin-3 can protect breast cancer cells from drug induced apoptosis by improving cell adhesion properties[41]. The multimeric properties of galectin-3 have been noted to make it more efficient than galectin-1 at signaling apoptosis in T cells[42].

Since galectin-3 reportedly enhanced cancer cell survival after exposure to apoptotic factors, Aykaç and coworkers designed gold nanoparticles with β-cyclodextrin to target galectin-3 for delivery of methotrexate to a tumor (Figure 3). This would improve delivery of hydrophobic methotrexate and increase selective toxicity for cancer cells[43]. Chang and coworkers reported another design of glyco-nanofibers that self-assembled around siRNA coupled with doxorubicin to create a complex that was membrane penetrating. Enhanced targeting of doxorubicin and silencing RNA to cancer

cells was demonstrated over normal cells. The load was released due to high glutathione content in cancer cells which reacted to the ferrocenium in the cationic ferrocenium-modified lactose derivative[44].

More elaborately designed supramolecular complexes have been reported recently to combat these issues of apoptosis and toxicity in cancer cells[45].

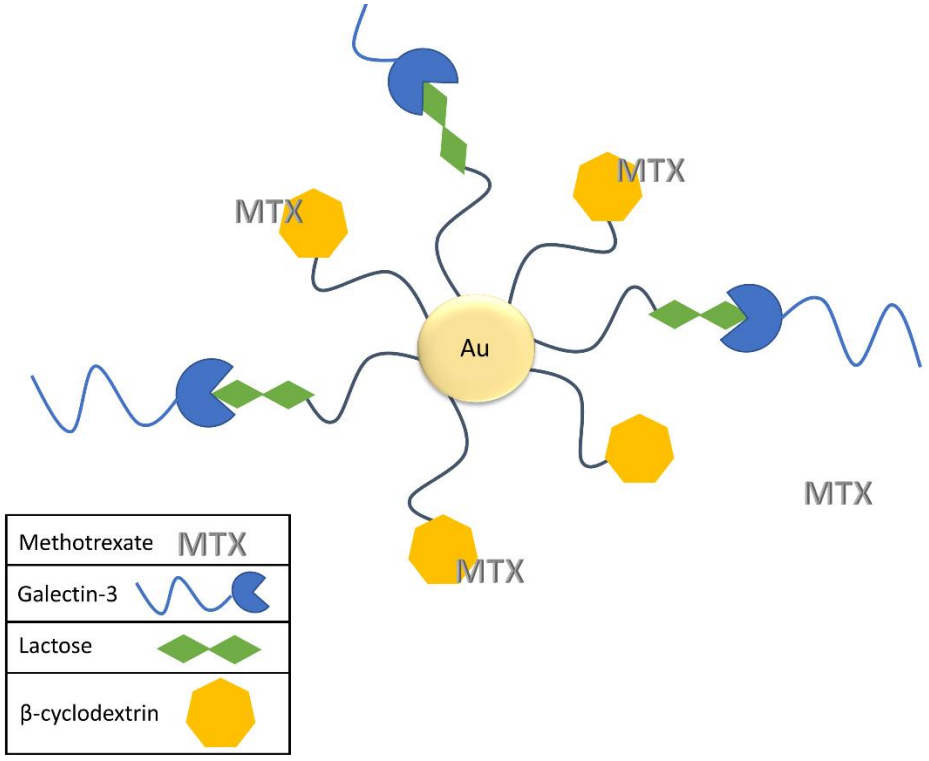

Figure 3. Drug delivery scaffold for methotrexate (MTX) to target to lectins such as galectin-3 which have a higher local concentration around tumors. Adapted from reference 41.

Metastasis

When a tumor has more fully developed, another devastating process is metastasis. Tumor cells begin to disseminate from the primary tumor, inhibiting the signals for anoikis as it becomes detached from the ECM and intercellular contacts. The tumor cells will invade a nearby circulatory system and, in that system, maintain tight intercellular contacts for survival. Then there will be extravasation into a new tissue environment where the secondary tumor will begin colonization[37].

Galectin-3 is known to play parts in many of these steps of the metastatic cascade[46]. From early on, it was known that galectin-3 mediated cell-cell adhesion through glycoconjugates[47]. There are several reports of galectin-3 binding to mucin 1 extracellularly and activating Epidermal Growth Factor Receptor (EGFR) in human epithelial cancer cells[48]. This has been reported to activate Extracellular Signal-Regulated Kinase 1/2 (ERK 1/2) which leads to cell migration[49,50]. And this signaling was dependent on the multivalent properties of galectin-3, signaling was diminished when collagenase was used to cleave the N-terminal domain[50]. Galectin-3 can also play a role in the binding interactions necessary for migration. When bound to N-cadherin, this can lead to destabilization of the intercellular interactions leading to fewer interactions preventing migration[51]. Galectin-3 can also bind to cell surface integrins and cluster them, activating their endocytosis and further destabilizing cell-ECM contacts[52]. It appears that not only does galectin-3 assist in signaling cell migration, but it assists in destabilizing the interactions that keep the cell bound to that location within the extracellular matrix. In fact, the oligomerization potential has become so vital to the

metastatic activity of galectin-3, that there have been attempts to use the CRD of galectin-3 as a therapeutic since it no longer can interact multivalently[53].

Matrix Metalloproteases (MMP) contribute an important role in the extracellular modulation of metastasis by galectin-3. It was shown that MMP-2/-9 can cleave galectin-3 within the N-terminal domain, inhibiting its ability to oligomerize. Interestingly, when this happens, the affinity of the CRD for ligands increases up to two-fold[19,54]. It was reported that MMP-7 also cleaves galectin-3, and MMP-7 abrogated galectin-3 mediated migration in a wound healing assay (Table 2)[55].

To continue the fight to inhibit metastasis, designs with multivalency have shown promise. A study reported that Laminin-332, an ECM component involved in cell motility, could be modified by N-acetylglucosaminyltransferase to display N-acetylglucosamine. Further developments revealed that galectin-3 could not bind to these residues and therefore could not support cell interactions through integrins that led to migration[56]. Synthesis of trivalent β-lactosyl clusters inhibited the formation of lung metastases in mice[57].

A few naturally derived products have been used to inhibit galectin-3's metastatic potential. The Raz group investigated a modified citrus pectin and found that there was a reduction in the metastatic and angiogenic behavior of breast cancer cells[46]. There has been extensive work done to design a higher affinity ligand for galectin-3 and therefore, interfere with its cancerous functions. Once such example was designed to be a TF-antigen- galectin-3 binding mimetic. A synthetic disaccharide-amino acid conjugate for galectin-3 was designed and found to decrease lung metastases in a xenograft model[58].

Designed higher affinity ligands have the potential to impact metastasis in the transport phase of cancer cells through a circulatory system. Modified BSA with glycoligands was reported with high affinity for galectin-3, the complex with 21 glycans reported a binding enhancement of 100 fold[59]. A highly exotic system was designed bearing cyclodextrins functionalized with lactosides similar to beads on a string. These self-assembled pseudopolyrotaxanes elicited significant effects in a galectin-1 mediated hemagluttination assay with T-cells. They reported an aggregation dependence on the concentration of lactosides and the number of beads (cyclodextrins) on the string (Figure 4)[60]. These examples further demonstrate the task at hand to combat metastases. This highly complex process has many links that are being targeted for therapeutics as our understanding is further expanded.

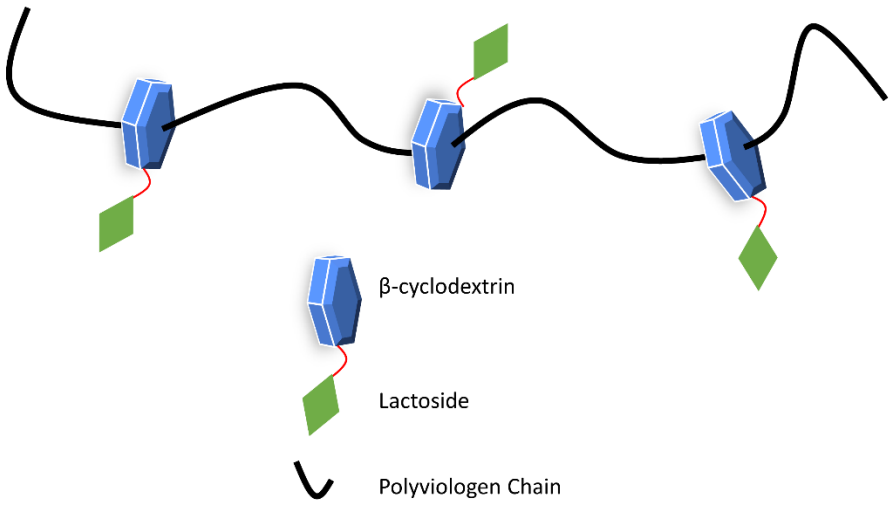

Figure 4. Self-assembling supramolecular complex with lactoside functionalized β-cyclodextrin on a polyviologen chain inhibited galectin-1 mediated agglutination. Adapted from reference 57.

Angiogenesis

The process of angiogenesis, or blood vessel formation, can be activated by external signals and therefore makes it a mostly extracellularly initiated process. These signaling molecules, usually cytokines, can be released for either paracrine or autocrine signaling. These cytokines can illicit processes such as endothelial cell migration, tubulogenesis, remodeling of the cell surface, and cell proliferation. Some particular cytokines such as Interluken-6 (IL-6) and Granulocyte-colony stimulating factor (G-CSF) which are known to induce migration in endothelial cells have been reported to be secreted from endothelial cells through a galectin-3 mediated process. In particular, interaction of galectin-3 with cell-surface glycoprotein CD146, also called mucin 18, promotes cytokine release[61]. Exogenous full-length galectin-3 at concentrations found in cancer patients was sufficient to induce secretion of these cytokines and others involved in intercellular interactions[62]. The expression of specific cytokines ICAM-1, integrin $\alpha_v\beta_1$, E-selectin and VCAM-1 increased heterotypic cellular adhesion, migration and tubulogenesis[62]. This interaction of galectin-3 with the cell surface of endothelial cells is dependent on its carbohydrate binding ability, as this activity is inhibited when a competitive disaccharide is added[19]. The Raz group reported enhanced migration of endothelial cells when galectin-3 was cleaved at H[64] by MMPs-2/-9[63]. When galectin-3 was cleaved by these proteases in the presence of breast cancer cells, migration and morphogenesis of the endothelial cells was enhanced (Table 2)

FORM OF GALECTIN-3	PATHWAY IMPACTED	EFFECT	LOCATION OF GALECTIN-3	EXPERIMENTAL METHOD	REFERENCE
$A_{62} - I_{250}$	Metastasis	Inhibits galectin-3 mediated migration	Extracellular	Wound healing in T84	Puthenedam, M., et al.
$P_{76} - I_{250}$	Metastasis	Inhibits galectin-3 mediated migration	Extracellular	Wound healing in T84	Puthenedam, M., et al.
$P_{113} - I_{250}$	Metastasis	Inhibits galectin-3 mediated migration	Extracellular	Wound healing in T84	Puthenedam, M., et al.
$Y_{63} - I_{250}$	Angiogenesis	Increases migration and interaction with endothelial cells	Extracellular, total cell lysates, nuclear fraction	Chemotaxis and capillary formation assay	Nangia-Makker, P., et al.

Table 2. Table of truncated versions of galectin-3 and their impact on cancerous processes[55,63]

There is much more reported work describing the impact of galectin-1 on angiogenesis. Researchers reported that galectin-1 released by cancer cells can promote H-Ras signaling and the Mitogen-Activated Protein Kinase (MAPK)/ Extracellular signal-Regulated Kinases (ERK) pathway which leads to cell proliferation and migration[64].

The Yu group reported tube formation of endothelial cells in media from another batch of galectin-3 treated endothelial cells. When this conditioned media was added to the endothelial cells, significantly greater capillary formation was observed. This was mitigated by the addition of lactose or the antibodies of cytokines such as integrin $\alpha_v\beta_1$[62]. Further reports showed increased tube formation as a result of Vascular Endothelial Growth Receptor 1/2 (VEGFR1/2) activation. When endothelial cells were incubated

with either galectin-1 or galectin-3, enhanced tube formation was reported. When the endothelial cells were incubated with both galectin-1 and galectin-3, greater tube forming activity was observed[65]. Early reports by the Panjwani group showed that galectin-3 was a mediator of a VEGF and basic Fibroblast Growth Factor (bFGF) angiogenic response. They reported this activity was mediated through the interaction of galectin-3 with these factors and the N-glycans present on integrin $\alpha_v\beta3$ which caused clustering of the integrin and activation of focal adhesion kinase which is involved in cellular adhesion and spreading[66]. This correlation between galectin-3 and VEGF mediated angiogenesis has revealed a clear reason for this interaction. The N-glycosylated extracellular domain of VEGFR2 binds galectin-3 and has been reported to increase its cell surface retention[67]. Angiogenesis is normally a heavily regulated process that coordinates and mitigates signals. When VEGFR2 is activated by VEGF-A, this leads to endocytosis and redistribution of VEGFR2 on the cell surface[68]. By retaining VEGFR2 on the cell surface, this can lead to heightened signaling. Stallcup and coworkers reported endothelial cell surface interactions with galectin-3 which revealed NG2, a proteoglycan found to be involved in the coordination between endothelial cells and pericytes. They reported a cell surface complex consisting of NG2, integrin $\alpha_3\beta1$ and galectin-3. This activated complex enhanced motility and morphology of the endothelial cells[69]. An additional pathway was recently reported as a result of extracellular galectin-3. Galectin-3 can be secreted by cancer cells in hypoxic conditions. When galectin-3 was bound to endothelial cells, Jagged-1 (JAG1)/Notch signaling was activated and led to angiogenic sprouting[70].

Galectin-3 was also reportedly rapidly endocytosed and targeted to either be recycled or destined to lysosomes for degradation in endothelial cells. Endocytosis was dependent on the CRD but destination to the lysosomes was dependent on the N-terminal domain of galectin-3[71]. This reveals that not only does galectin-3 contribute to surface interactions, but there is also the aspect of uptake that could impact angiogenesis.

In vitro studies have shown that galectin-3 mediated angiogenesis can be inhibited with lactose treatment. Some small molecules have been designed to diminish the effect of galectin-3 on angiogenesis. A small molecular weight inhibitor, 33DFTG, has shown promise revealing a K_d of 68 nM to galectin-3's CRD[72]. In endothelial sprouting studies, this molecule inhibited galectin-3 mediated VEGF migration. *In vivo* studies revealed reduced corneal angiogenesis[73]. Researchers reported a few selectively de-sulfated heparin derivatives as inhibitors for galectin-3. In an endothelial tube formation assay, all of the heparin derivatives inhibited galectin-3 mediated tube formation, and some were able to reduce tube formation beyond the control[74].

Other, more elaborate designs have been investigated. The Caporali group reported the assembly of carbon nanotubes to safely deliver microRNAs into cells. This delivery was enhanced by functionalizing the nanotubes with a cationic polymer which increased the binding of the microRNAs. In a tube formation assay, this supramolecular complex decreased tube formation of endothelial cells[75]. These reports show promise as we begin to further elucidate the pathways and processes activated by cancer cells in the angiogenic cascade.

PAMAM Dendrimers

Armed with these important prior reports, further investigation of galectin-3 mediated apoptosis, toxicity, metastasis and angiogenesis is the goal of the research reported in this thesis. Because of the oligomeric properties of galectin-3, a multivalent scaffold was selected with the ability to functionalize the end groups with lactose. Poly-amidoamine (PAMAM) dendrimers have repeating amidoamine branches, and the number of branches doubles with each increasing generation. Generations 2, 3, 4 and 6 (G2, G3, G4, G6) were selected for these studies (Figure 5). The terminal amine of the branch is functionalized with lactoside endgroups via a thiourea linkage. A G2 is functionalized on average with 12 lactosides, a G3 with 15 lactosides, a G4 with 45 lactosides, and a G6 with 100 lactosides per dendrimer. While a G2 dendrimer is more circular and amorphous, the larger G4 and G6 are more akin to a globular sphere. Lactoside functionalization increases the aqueous solubility of the dendrimers, making them easy to use in a physiological buffer. The sizes of these dendrimers are analogous to other biomolecules, making them generally non-immunogenic[76]. Branches bearing lactosides are quite flexible and able to accommodate different steric requirements in a tumor environment[77]. Therefore, carbohydrate functionalized PAMAM dendrimers are good tools to study the multivalent aspects of galecin-3 in cancer processes.

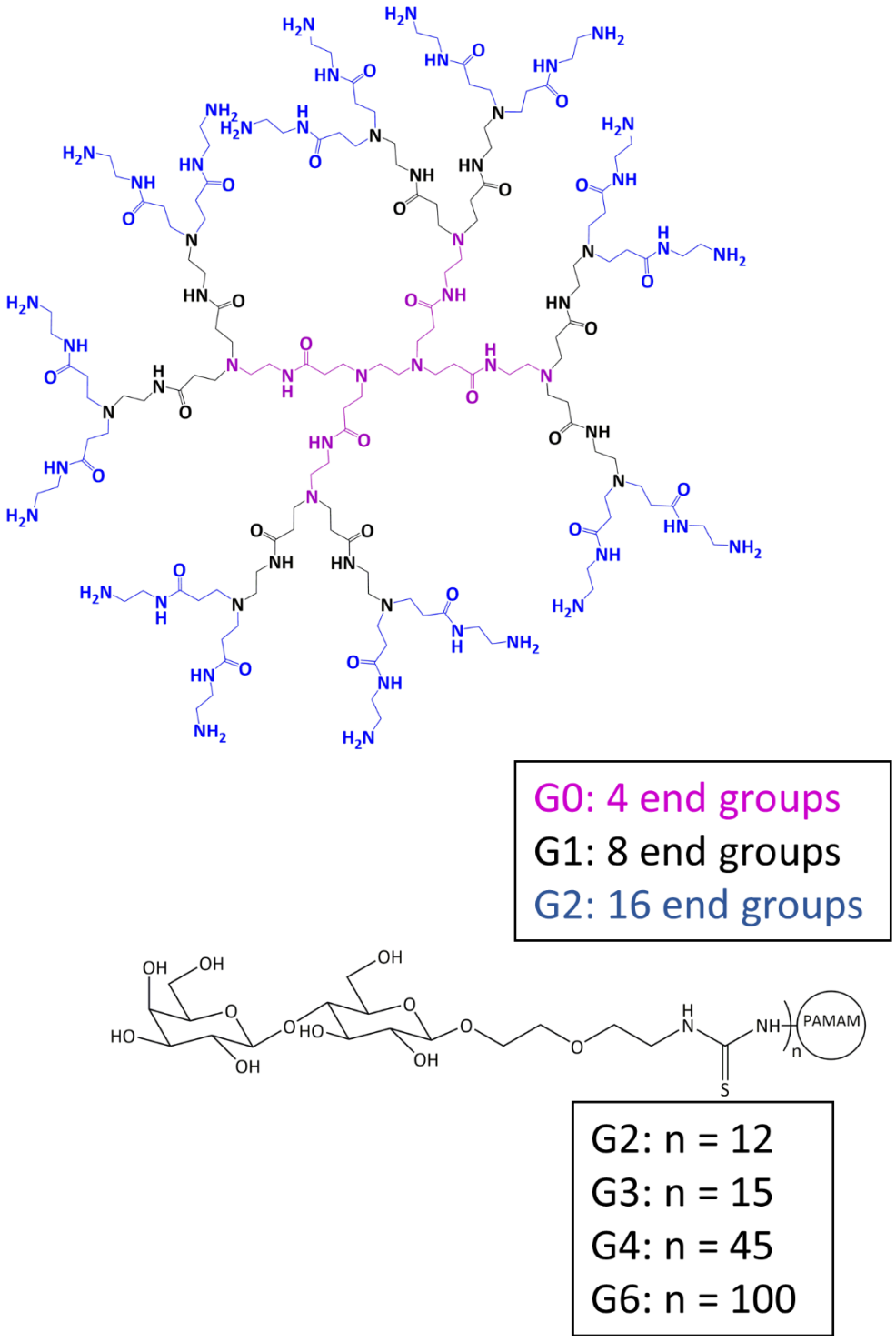

Figure 5. PAMAM dendrimers. The number of end groups doubles with each subsequent generation. The average amount of functionalization with lactose for the glycodendrimers used in this thesis is shown.

Conclusion

The research presented here will first be directed toward assessing the utility of the dendrimers in cellular systems. The toxicity of the dendrimers will be assessed to determine whether they induce necrotic effects. Next, the dendrimers will be tested with and without exogenous galectin-3 to determine if apoptotic effects are induced. Uptake assays will be utilized to determine the localization and potential interactions of the dendrimers. These results together reveal that the glycodendrimers are a useful tool for investigating further cellular effects without detrimental side effects.

Next, galectin-3 mediated migration and angiogenesis will be assessed with the glycodendrimers. These results will show the impact of multivalency on these cancerous processes and will show the utility of lactose functionalized dendrimers as tools to further probe multivalent biological processes, especially in the context of cancer progression.

Multivalent interactions can be applied to many processes, and the understanding of multivalency can lead to the development of new systems that intercept biological processes, especially when the processes are extracellularly mediated. Extended glycosylation of cellular networks shows the importance of understanding the mechanisms of glycosylation, the interactions, and the signals that are initiated from it.

CHAPTER TWO

THE TOXICITY, UPTAKE, AND IMPACT ON GALECTIN-3 MEDIATED APOPTOSIS OF LACTOSE FUNCTIONALIZED DENDRIMERS

Contribution of Authors and Co-Authors

Manuscript in Chapter 2

Author: Mackenzie S. Fricke

Contributions: Conducted toxicity, viability and apoptosis studies. Wrote manuscript.

Author: Samuel P. Bernhard

Contributions: Functionalized dendrimers, obtained fluorescent micrographs for uptake, determined hydrodynamic radii and surface charges of dendrimers. Wrote manuscript.

Co-Author: Willy Totten

Contributions: Collected AO/EB fluorescent micrographs.

Co-Author: Katarina Achazi

Contributions: Cell maintenance, experiment and setup for uptake confocal micrographs.

Co-Author: Paul Hillman

Contributions: Collected FACS results.

Co-Author: Rainer Haag

Contributions: Used his microscope and FACS instrumentation

Corresponding Author: Mary J. Cloninger

Contributions: Collected viability, toxicity and apoptosis results with dendrimers alone and edited manuscript.

Manuscript Information

Mackenzie S. Fricke, Samuel P. Bernhard, Willy Totten, Katarina Achazi, Paul Hillman, Rainer Haag, Mary J. Cloninger*

Biomolecules Special Issue: Moving Forward with Dendrimers

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Introduction

Many biological processes are mediated by glycan-lectin interactions and induce signal transduction. One of the first such discoveries by Nowell, P.C. (1960) showed that lectins from red kidney beans could induce mitosis in lymphocytes[78]. Since then, many studies have reported on the vast influence of cell-surface carbohydrates and their receptors[79]. Although most of these carbohydrate-receptor interactions are relatively weak, many heavily influence biological processes such as glycan mediated cell attachment and endocytosis of a virus[80,81]. To enhance the selectivity and specificity of these weak carbohydrate-mediated interactions, multivalency is commonly invoked[13]. Since the entropic cost of binding is paid by the first ligand receptor interaction in a multivalent system, each subsequent interaction benefits enthalpically. Overall, multivalency increases the binding avidity of the system[13].

To investigate a multivalent system, a hyperbranched polymer functionalized with lactosides was utilized[82]. Poly(amidoamine) (PAMAM) dendrimers are hyperbranched polymers with repeating amidoamine units emanating from a central core (Table 3). When the amino end-groups are functionalized with lactose via a thiourea linkage, the dendrimers present a multivalent ligand system[82–84].

Another attractive feature of PAMAM dendrimers is their potential utility in drug delivery. The ability to present therapeutics multivalently to a target can increase the efficacy of the drug[76]. Many novel drugs are plagued with physical limitations such as low specificity, low solubility, high system rejection and side effects. Dendrimers and

other nanoparticles can be designed so as to release cargo at a specific target[43,85]. Dendrimers present many ways of attaching drugs either covalently or non-covalently, for example, through sequestering the drug on the interior of the dendrimer[86] or by designing a functionalized drug with a cleavable bond via a specific target[76]. Dendritic display of a therapeutic agent can also mitigate its low solubility. Drug resistance is a large problem especially in the antimicrobial industry, and the multivalent presentation of therapeutics can alter the development of bacterial resistance[87].

Of particular importance for the research described in this chapter, the decreased cellular organization of tumors can be used to specifically target cancerous cells. Dendrimers can be quite large, and because of this they do not as readily penetrate healthy tissues. The decreased cellular junctions and unorganized vasculature of tumors allows for large dendrimers to penetrate this space, an effect known as the enhanced permeation and retention (EPR) effect[88,89]. The ERP effect could lead to fewer side effects from nonspecific delivery[76].

The receptor target chosen for our research is galectin-3. Human galectin-3 is a 26 kDa protein with a β-galactoside binding domain that plays roles in processes including embryogenesis, heart disease, inflammation, and tumor progression[90]. All galectins contain a highly conserved carbohydrate binding domain. Unlike the other galectins in this family, galectin-3 possesses a non-carbohydrate-binding N-terminal domain that has been described as collagen-like[91]. The N-terminal domain allows galectin-3 to oligomerize, which allows its receptor function to be multivalent[30]. When galectin-3 and

lactose functionalized dendrimers associate, a host of multivalent cross-linking interactions and consequences are enabled[42,66,92–94].

The investigations described in this chapter are to give greater understanding of the efficacy of dendrimers as drug delivery systems and their ability to probe multivalent interactions with galectin-3. Toxicity can be defined as a substance's ability to induce necrosis in a cell. Acute toxicity is the ability of a substance to break up the cell membrane and cause an undirected, proinflammatory result. Toxicity is an important trait to examine in therapeutics research because toxicity paired with non-specific interactions are highly undesirable characteristics. If a substance has been designed to induce specific interactions, toxicity is an off-target effect that must be mitigated.

Uptake in a cellular system represents the ability of a substance to be endocytosed. These results would indicate if a substance has the ability to penetrate the cell and induce intracellular interactions. When designing a scaffold for therapeutics, different concessions must be made depending on if the substance stays in the extracellular space or is endocytosed.

Inducing apoptosis in cancer cells represents the gold standard for cancer therapeutics. Apoptosis is highly regulated and anti-inflammatory; activating this process in cancer cells is a difficult task since many cancers have deleted or altered expression of apoptosis regulating proteins.

To understand the potential interactions of a substance, the toxicity, uptake, and apoptotic potential of the compounds of interest must be assessed. Galectin-3 is involved in many cancer processes,[19,95–97] and the results reported in this chapter afford a greater

understanding of the properties of the glycodendrimers that are used to study galectin-3 mediated cancer processes.

Results

Toxicity Studies Using Lactose Functionalized Dendrimers

Lactose functionalized dendrimers are designed to present binding ligands to galectin-3 and induce effects through this multivalent carbohydrate-receptor interaction. Glycodendrimers are emerging as important tools for the study of galectin-3 mediated cancer processes. To use lactose functionalized dendrimers effectively in cell-based assays, first their toxicity had to be determined. If the multivalent glycopolymers are toxic, then the information garnered from studies of cellular processes will be considerably different than results obtained using nontoxic polymers.

The toxicity was measured using a fluorescent viability dye. Three cell lines were investigated: A549 human lung carcinoma cells, DU-145 human prostate carcinoma, and HT-1080 human fibrosarcoma cells. These cell lines were selected for their robustness and endogenous expression of galectin-3 in order from greatest to least: DU-145, A549, then HT-1080. Each cell line was incubated in high concentrations of each generation of lactose functionalized dendrimers (G2, G3, G4 or G6) and Celltox™ dye that fluoresces in the presence of DNA. Fluorescence was recorded at 2 and 24 hours. The results were averaged from six replicates of each experiment. The concentration of lactose functionalized dendrimer in the assay was at least 20 and up to 400 times greater than used in any of the assays used for understanding cellular aggregation, migration, or

angiogenesis. These concentrations were selected so that the concentration of lactose was equivalent in each assay, even when a different generation of dendrimer was used with different numbers of lactosides per framework. Even at these high concentrations, there was no significant dendrimer toxicity reported in any of the cell lines tested (Figure *6*).

Other researchers have reported cytotoxicity in human epithelial colorectal adenocarcinoma cells at concentrations above 700 μM for cationic or unfunctionalized G2 dendrimers and cytotoxicity at all concentrations for G3 and G4 cationic or unfunctionalized dendrimers[98]. These researchers also report a significant decrease in cytotoxicity when the surface of the dendrimers was functionalized with lauroyl or PEG chains. They hypothesized that this decrease in toxicity was due to the reduced level of positive charge on the surface of the lauroyl and PEG functionalized dendrimers relative to the unfunctionalized dendrimers with amino termini. We conclude that this same type of shielding effect is observed when PAMAM dendrimers are functionalized with lactosides[99].

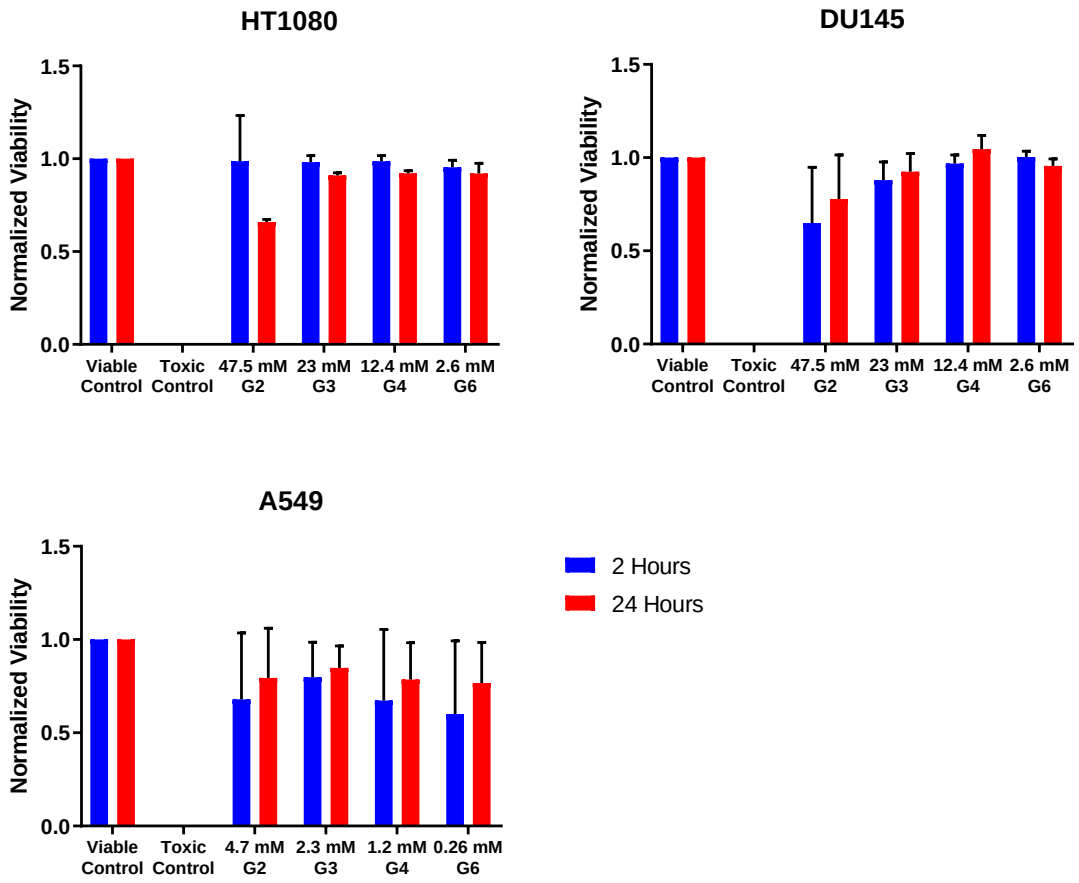

Figure 6. Toxicity assay using DU145, HT1080 and A549 cells. Toxicity was compared to untreated cells ("viable control") and toxic control cells. Blue bars are measured after 2 hours, and red bars are measurements after 24 hours of incubation. Error bars demonstrate the standard deviation of 6 replicates.

Toxicity was assessed using two additional tests on A549 cells using all four generations of lactose functionalized glycodendrimers (G2, G3, G4, and G6). A viability test was used in which a substrate is only able to enter viable cells; once the substrate is intercellular, proteases are able to cleave and activate a fluorescent tag. Thus, the fluorescence emission is proportional to the number of living cells in the sample. A toxicity test was also performed in which the substrate is only able to be cleaved and

activated by proteases released from a dead cell. Ionomycin was used as the toxic control in the toxicity test, and SDS was used as the control for non-viable cells in the viability test. These two tests represent a more complete picture of the toxicity profile on the cancer cells. Lactose functionalized dendrimers had no impact on cellular viability or toxicity compared to the viable cell controls (Figure 7).

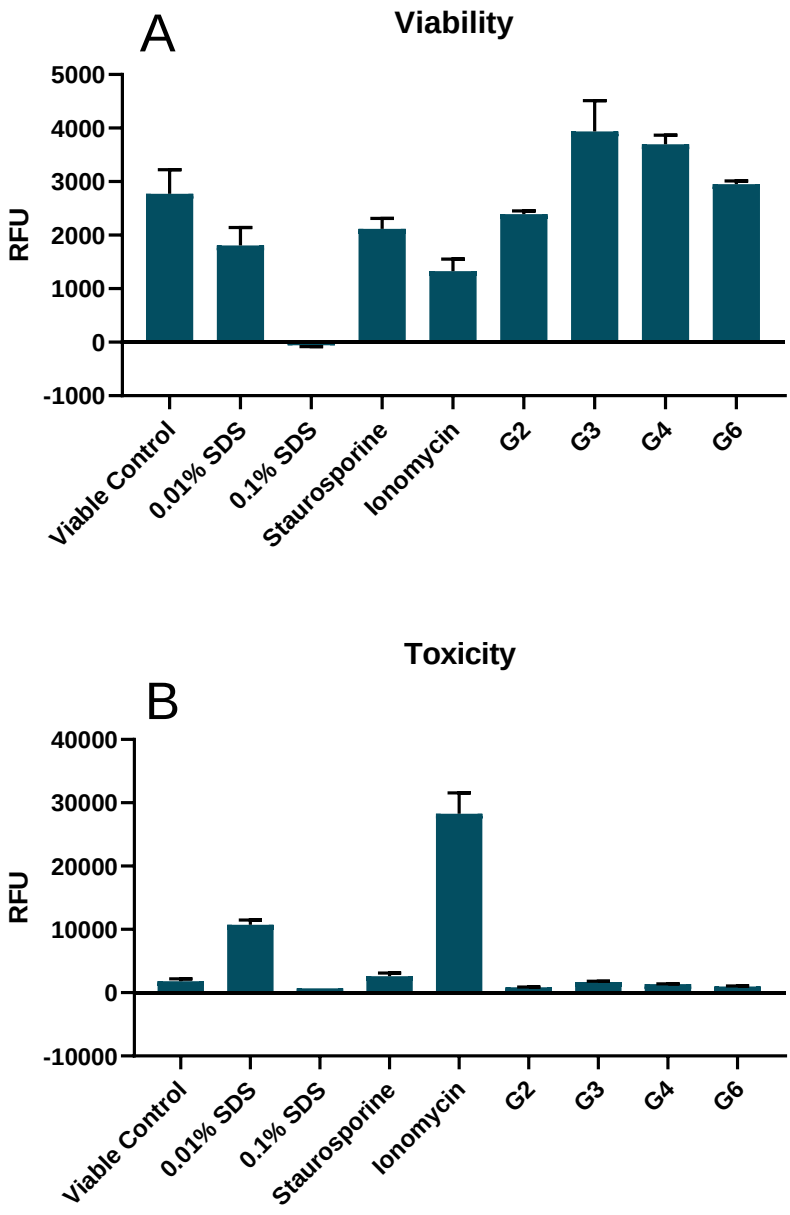

Figure 7. Viability (a) and Toxicity (b) of A549 cells after 5-hour incubation with PBS (viable control), lactose functionalized dendrimers (G2, G3, G4, G6), or control compounds as a measure of relative fluorescent units. Error bars represent standard deviation from up to six replicates.

The same tests were also conducted with exogenously added galectin-3 to assess whether the interaction between the glycodendrimers and galectin-3 impacts the cellular

viability. Results of the live cell fluorescence assay described above indicate a slight decrease in cellular viability when the cells were incubated with galectin-3 and dendrimers for five hours (Figure 8A). The decrease in viability is comparable for galectin-3 and for co-additions of glycodendrimer and galectin-3, indicating that the slight reduction in cellular viability is caused by galectin-3 rather than by galectin-3/glycodendrimer aggregates (Figure 8A). No significant toxicity was observed in the dead cell fluorescence assay when cells were incubated with galectin-3 and dendrimers compared to the viable control (Figure 8B).

Other reports have shown that galectin-3 can be involved in the toxicity or viability of cells through extracellular interactions. It was recently reported, for example, that the secretion of galectin-3 by surrounding cells contributes to renal failure and kidney disease in patients receiving chemotherapy. Inhibiting galectin-3 through the use of modified citrus pectin (MCP) afforded a reduction of tissue damage in mouse models[100]. Our results here show that galectin-3 does not have a significant adverse effect on cancer cellular viability. The difference between our results and the prior published results is most likely attributable to the differences between studies at the cellular level and the systems (murine) level.

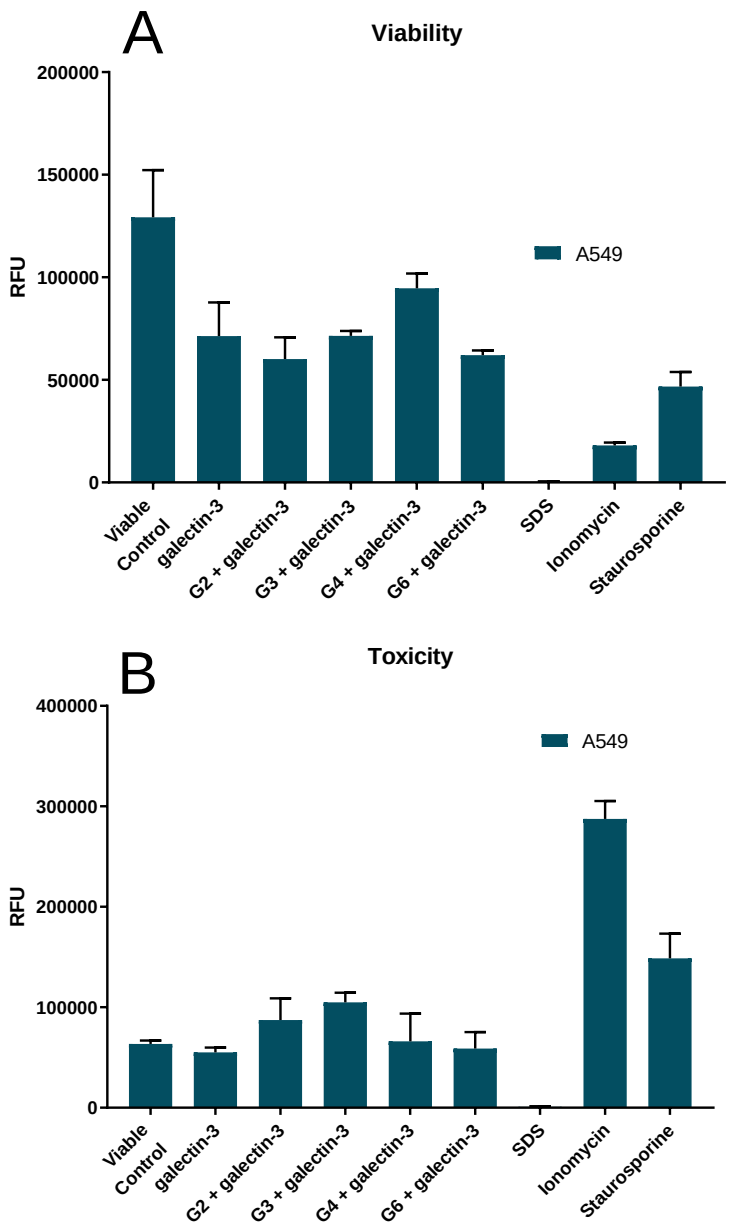

Figure 8. Viability (A) and Toxicity (B) of A549 cells after incubation for 5 hours with control compounds or galectin-3 (6.6 µM) and glycodendrimers (G2, G3, G4, G6). Results reported as relative fluorescent units. Error bars represent standard deviation from six replicate experiments.

Cellular Uptake of lactose functionalized dendrimers

As noted above, glycodendrimers do not exhibit toxic effects in the three cancer cell lines that were tested. In addition to toxicity, the location of the dendrimers needed to be determined to rationalize their potential effects and interactions with the cells. This is especially important because galectin-3 has both intracellular and extracellular roles in cancer progression[23,101]. Multivalent binding partners for galectin-3 are envisioned to be useful for studies of both extracellular and intracellular functions of the protein, but if cellular uptake of the glycodendrimers is not observed, then modifications will need to be made for studies of intracellular galectin-3[102].

Quantitative and qualitative observations of the cellular uptake of lactose functionalized PAMAM dendrimers (G2, G3, G4, G6) into A549 adenocarcinoma human alveolar basal epithelial cells was monitored using a glycosidic-C6 hydrozone linked Alexafluor 647 tag during confocal microscopy and FACS flow cell cytometry experiments.

Glycodendrimer generation	M_w (kDa)	Lactose endgroups	Hydrodynamic diameter (nm)
G2	7.7	12	--
G3	14.1	24	2.0±0.4
G4	32.7	57	6.2±1.1
G6	91.6	130	10.6±2.2

Table 3. Characteristics of lactose functionalized PAMAM dendrimers. Weighted mass averages (M_W) were determined by MALDI-TOF. Effective diameters for glycodendrimers were determined by DLS at 11.5 μM in PBS. G2 glycodendrimer signal was too small for reliable cumulants data fit.

Quantitative studies using flow cytometry show concentration and time-dependent results and demonstrate enhanced uptake (Figure 9A, B). Uptake of all four generations of lactose functionalized dendrimers was observed. Uptake of lactose functionalized G2 dendrimer was comparable to uptake of the monomeric dye. A statistically significant increase in uptake was observed for the lactose functionalized G3 dendrimer relative to monomeric dye. Although significant uptake of lactose functionalized G4 and G6 dendrimers was observed, the monomeric dye and the smaller glycodendrimers were more efficiently taken up by the cells. This alludes to a sweet spot for glycodendrimer uptake centered around the 2.0±0.4 nm hydrodynamic radius of the G3 glycodendrimer (Table 3), which suggests that the mechanism of uptake relies on an optimum glycodendrimer size. Higher generation dendrimers may be too large to efficiently permeate the cell while small dendrimers may not be reliably retained.

There is some suggestion that small polymeric nanoparticles (<100 nm), such as the glycodendrimers described here, are brought into the cell via pinocytosis (i.e. fluid endocytosis) or receptor mediated endocytosis[103]. Qualitative observations through confocal microscopy yield critical information on the final resting place of these dendrimers (Figure 9C). Co-localization of the Alexafluor 647 fluorescent signal with a lysosomal GFP stain had increased intensity at 24 hours compared to 5 hours, which suggests that over time, cellular uptake and intracellular trafficking targets lysosomal disposal. The observation of the lactose functionalized dendrimers in the lysosome at 24 hours supports the hypothesis of uptake mechanisms such as pinocytosis or receptor mediated endocytosis[103].

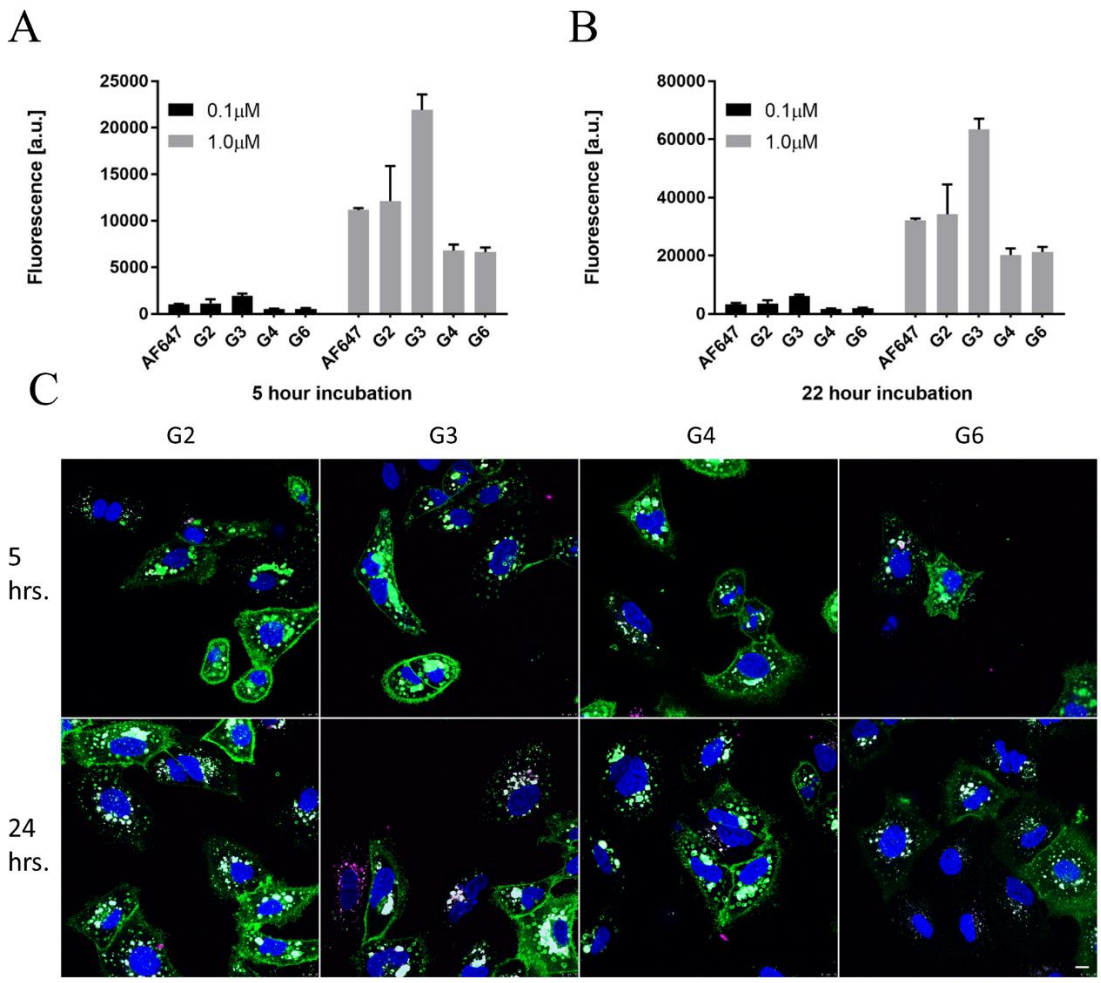

Figure 9. Cellular uptake of lactose functionalized PAMAM dendrimers by A549 cells is dependent on concentration, exposure time, and dendrimer generation. Uptake of dendrimers at 5 hours (A) and 22 hours (B) of incubation for a series of dendrimer generations at 0.1 μM and 1.0 μM. (C) Confocal microscopy images of A549 cells at comparable time points, where green, magenta, and white coloring represent the lysosome, glycodendrimer and co-localized signal respectively. Scale bar is 10 μm.

Apoptosis Assays Using Glycodendrimers

Lactose functionalized dendrimers have been designed to interact with β-lactoside binding proteins such as galectin-3. In the beginning of this investigation, it was determined that toxicity is not a mode of action. Therefore, signaling effects may be a

mechanism by which these dendrimers can impact cellular systems. One such well documented signaling mechanism is induction of apoptosis. Extrinsic signals are well documented in inducing apoptosis in human cell lines[104]. A reported example is that of the many receptors of the tumor necrosis factor (TNF) family, which initiate signaling through the clustering of receptors that bind a respective signaling ligand. This further transduces a signal to the cytoplasmic domain, where activation occurs in a cascade-like fashion to the point of forming the death-inducing signaling complex (DISC) which activates procaspase 8, making apoptosis irreversible[36].

Galectin-3 has been reported to be involved in apoptosis in many different cell systems. Galectin-3 can be pro- or antiapoptotic depending on its localization within the tumor environment[105]. Intracellularly, it has been reported that galectin-3 generally is an antiapoptotic factor, and there are many functions within the cell that cytoplasmic galectin-3 can affect. For example, galectin-3 can interact with the mitochondria and decrease mitochondrial damage and reduce release of cytochrome c to the cytoplasm, which may be enhanced by its association with Bcl-2[105]. Extracellular galectin-3 is largely pro-apoptotic[105]. The Raz research group proposed an inverse correlation between intracellular galectin-3 and the ability of extracellular galectin-3 to induce apoptosis[106]. Additionally, extracellular galectin-3 can provide a defense against drug therapy by interacting with Na+/K+ ATPase, which can act as an anti-apoptotic transporter. In addition, galectin-3 activates P-gp, which is a toxic molecule pump in cancer cells[107,108]. In colon cancer cells, extracellular galectin-3 was protective against apoptosis by

clustering TNF receptors and thereby blocking their internalization and eliminating their signaling effect[109].

Exogenous galectin-3 alone has also been reported to induce apoptosis through extracellular signaling mechanisms in various epithelial cells as well as T-cells[106,109,110]. It has been reported that high concentrations of extracellular galectin-3 (3-10 μM) induce apoptosis in various human T-cell lines[42,106]. In colorectal adenocarcinoma, exogenous galectin-3 was reported to induce apoptosis but was inhibited when integrins were sialylated[110]. We tested the induction of apoptosis in A549 cells in the presence of high concentrations of galectin-3 to determine its ability to influence cell signaling transduction and therefore abrogate that signal with lactose functionalized dendrimers.

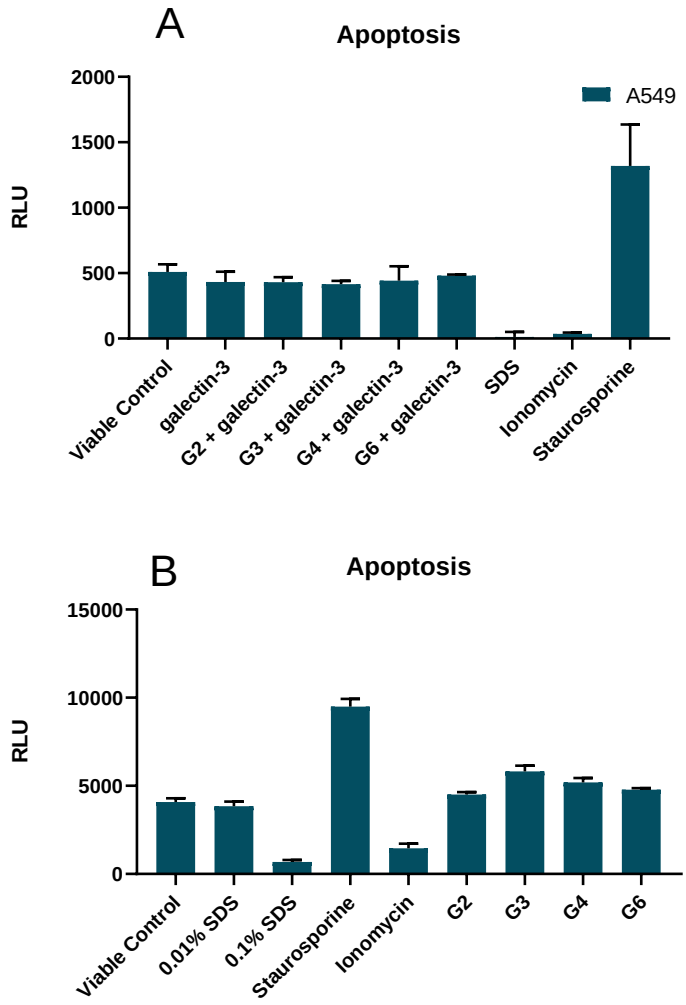

Figure 10. Apoptosis was reported for A549 cells in the presence of control compounds SDS, Ionomycin, Staurosporine or lactose functionalized dendrimers and galectin-3 or buffer PBS after 5 hours of incubation (A). Apoptosis for cells treated with glycodendrimers, or control compounds (B). Units are in relative luminescent units for apoptosis. Error bars represent standard deviation from six replicate experiments.

Lactose functionalized dendrimers were incubated with A549 cells, and apoptosis was measured. No significant deviation from cells with PBS (viable control) was observed when glycodendrimers were added. (Figure 10B).

Galectin-3 was exogenously added to adhered A549 cells and allowed to incubate for 5 hours. Apoptosis was measured using a luminogenic substance that is activated when cleaved by caspase 3/7 which are well-known players in the apoptotic cascade. No significant difference was observed between galectin-3 and the PBS control (viable control), and significantly less apoptosis occurred than for the staurosporine positive control (Figure 10A).

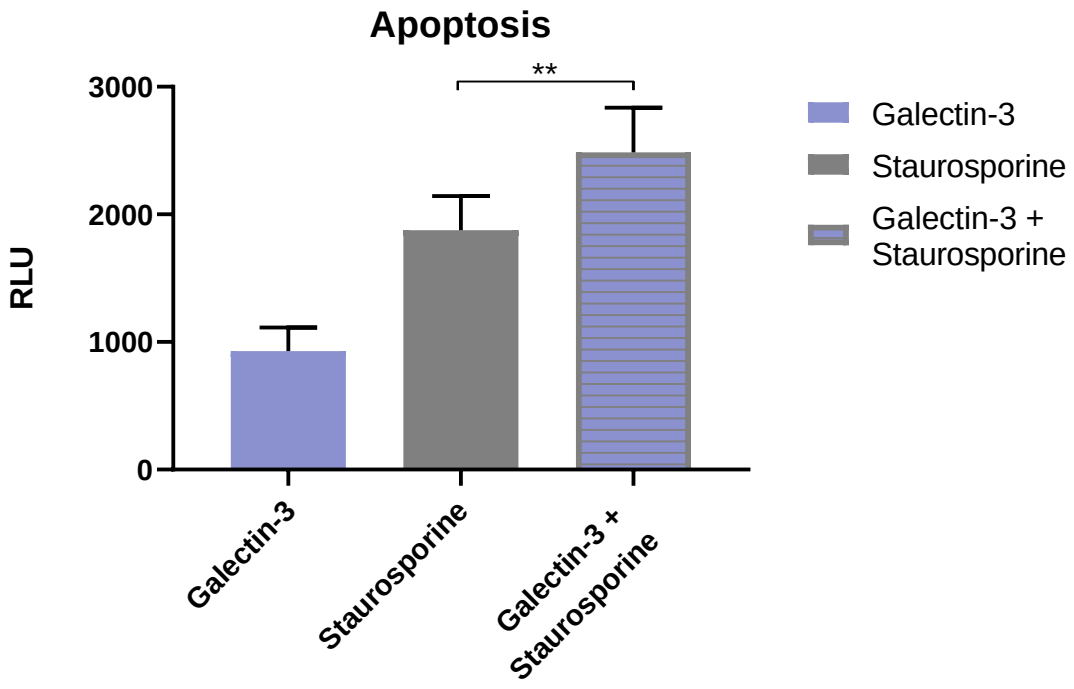

Figure 11. Induction of apoptosis in A549 cells after incubation for 5 hours with galectin-3, staurosporine, or galectin-3 and staurosporine.

To determine if exogenous galectin-3 plays a role in the efficacy of staurosporine to induce apoptosis, apoptosis was compared for samples containing exogenously added galectin-3, staurosporine, or both together (Figure 11). While staurosporine alone showed

increased apoptosis after 5 hours of incubation, adding galectin-3 with the staurosporine significantly increased that induction of apoptosis even though galectin-3 alone did not significantly impact apoptosis (Figure 10A). There are reports that galectin-3 can interfere with drug treatments, and galectin-3 has become a target for investigating drug resistance. While many reports show decreased cell death as a result of galectin-3[51,109,111–113], our results show increased drug induced apoptosis because of galectin-3. A potential hypothesis is that galectin-3 is undergoing the receptor clustering activity that has already been reported by us and others[29,93,114]. This could indicate that staurosporine activity is increased upon receptor exposure, which can be revealed by galectin-3 receptor clustering.

Acridine orange and ethidium bromide cellular investigations by confocal microscopy

Staining with acridine orange and ethidium bromine (AO/EB) was conducted to visually confirm the results of the apoptosis experiment. A549 cells were adhered to glass slides overnight and then incubated with control or test compounds for five hours. The samples were stained with AO/EB and visualized on a confocal microscope. Viable cells displayed green staining throughout the cell, with relatively homogenous fluorescence. Necrotic cells displayed red ethidium bromine staining on their nucleus due to plasma membrane degradation. Apoptotic cells were stained green but with punctate fluorescence in the nucleus due to condensation of DNA and blebbing.

Initially, to validate the procedure and identify phenotypes, controls of viability using SDS, staurosporine, and galectin-3 were collected. The results of these controls

appropriately displayed necrosis and apoptosis (Figure 12C, D), and the cells with galectin-3 or PBS had a healthy appearance (Figure 12A, B). These results highlight the main staining and morphological differences used to characterize the cells in this experiment.

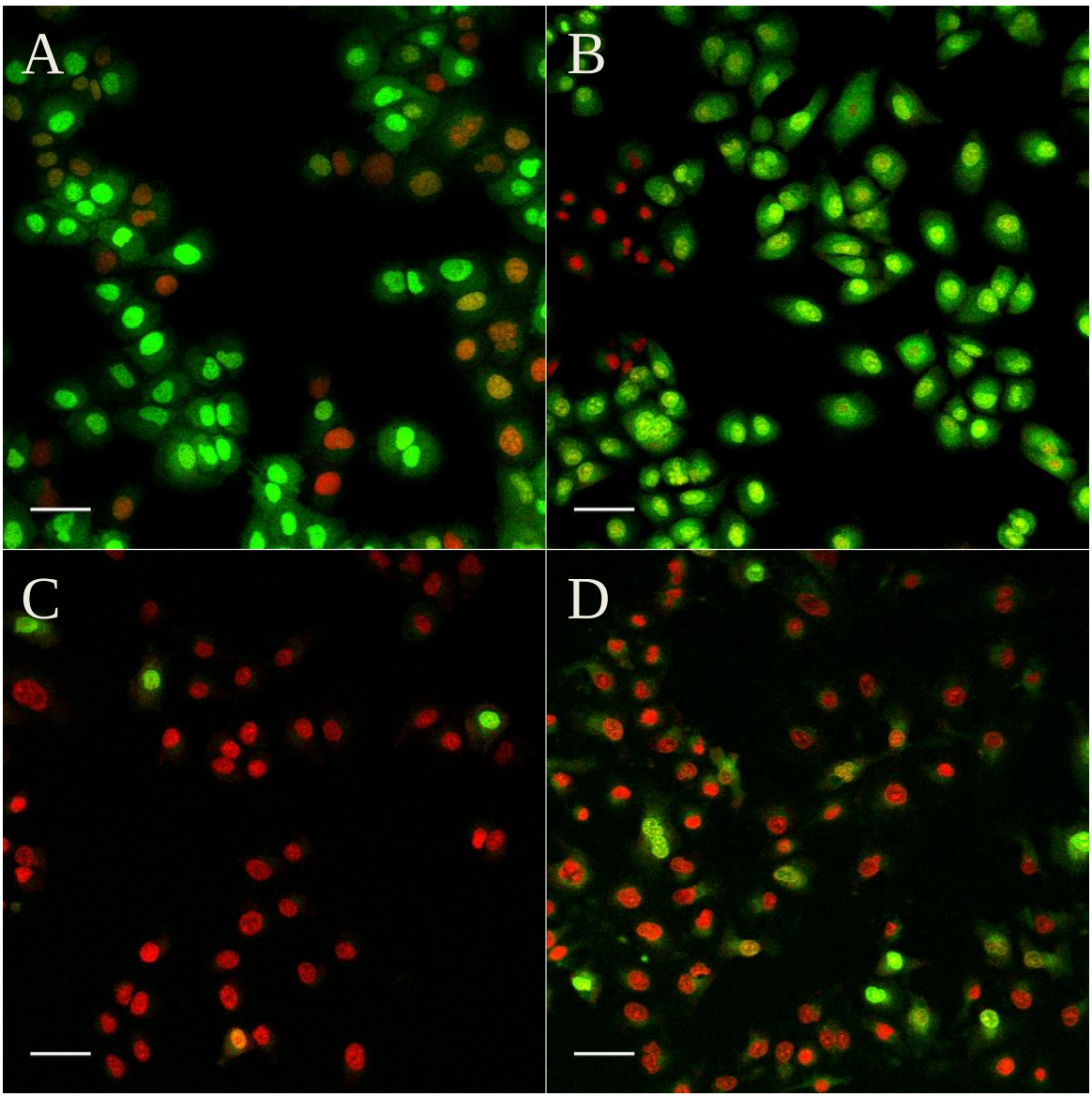

Figure 12. Confocal microcopy images of A549 after 5 hours of incubation with: (A) Galectin-3 control conditions, (B) cells under healthy conditions, (C) necrosis control SDS 0.01% conditions, (D) apoptosis control conditions 100 µM staurosporine. All samples were stained with 100 µg/mL acridine orange (green) and ethidium bromide (red). Scale bar is 50 µm.

Cells in the presence of galectin-3 and one of the dendrimers G2, G3, G4, or G6 all appeared to have similar phenotypic distributions. All stages of cell health were

observed regardless of dendrimer generation (Figure 13). This agrees with previous results of toxicity and apoptosis where there was no increase in toxicity or apoptosis reported when exogenous galectin-3 was added with the lactose functionalized dendrimers. Therefore, it can be concluded that adding galectin-3 and lactose functionalized dendrimers does not increase apoptosis above the normal cellular homeostatic processes.

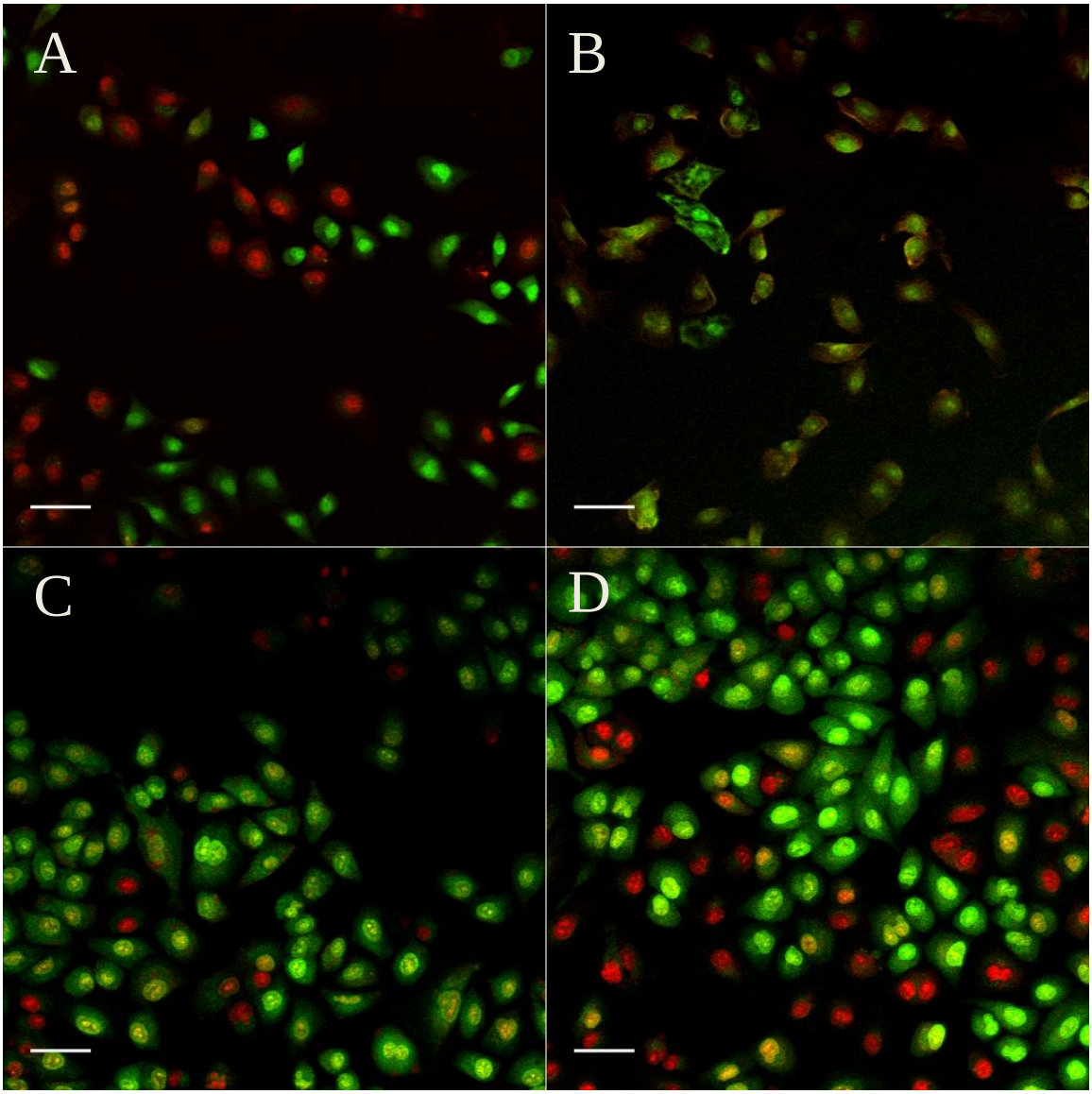

Figure 13. Confocal microscopy images of A549 cell incubated for 5 hours with galectin-3 and G2 (A), G3 (B), G4 (C) or G6 (D) lactose functionalized dendrimers. All samples were stained with 100 µg/mL acridine orange (green) and ethidium bromide (red). Scale bar is 50 µm.

To visualize dendrimer interaction in these trends, G2 and G6 lactose functionalized dendrimers were also functionalized with Alexa Fluor 647. Fluorescence was found both closely around the cell and in the intercellular space (Figure 14A, Figure

15A). Penetration by the dendrimers appeared to be more intense using the lactose functionalized G2 dendrimer than the G6 glycodendrimer (Figure 14 vs. Figure 15), which would support the theory of a “sweet spot” for best uptake dependence on dendrimer size. When cell viability is compromised, uptake of the dendrimers is increased (Figure 14, cells with the most EB stain show the most G2). While not precise enough to correlate dendrimer localization with cell viability, these results do further confirm the interaction of lactose functionalized dendrimers with cancer cells. Uptake of the dendrimers appears to remain constant regardless of whether galectin-3 is exogenously added or not (compare Figure 14 and Figure 15 to Figure 9).

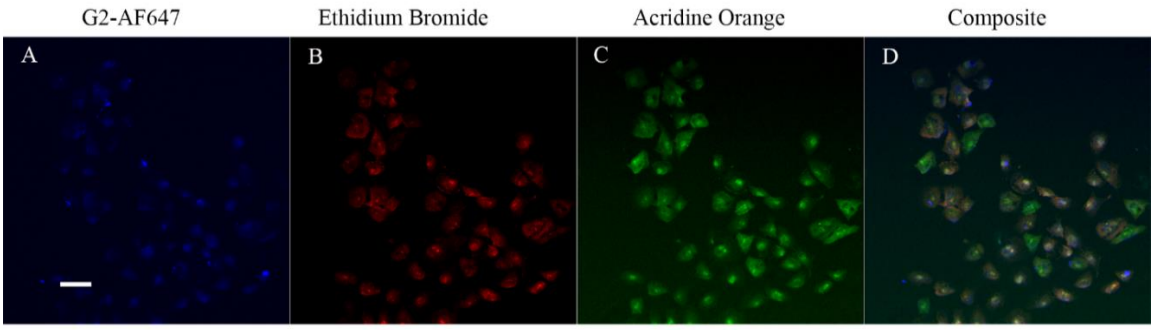

Figure 14. A549 cells after 5 hours of incubation with G2-AF647 and galectin-3. Cells stained with AO/EB and visualized by confocal microscopy. Scale bar 50 µm.

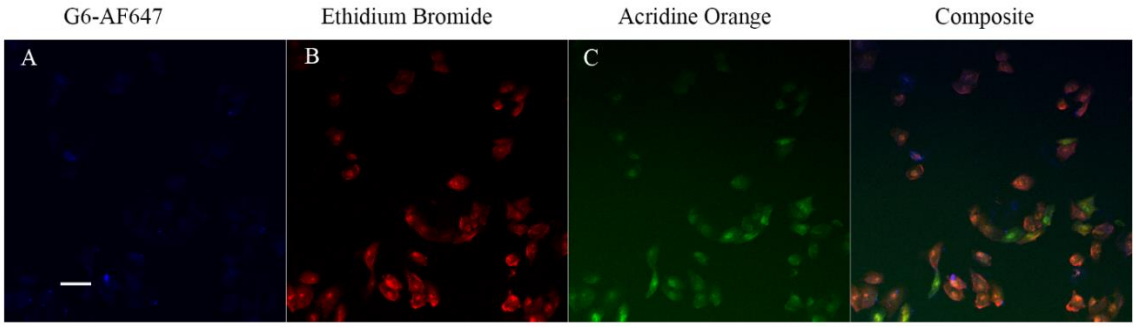

Figure 15. A549 cells stained with AO/EB after 5 hours incubation with G6-AF647 and galectin-3. Scale bar 50 µm.

Discussion

Functionalized PAMAM dendrimers are attractive substrates for drug delivery and biological applications because of their commercial availability, ease of functionalization and size selectivity[76,86,115,116]. Multiple end groups for functionalization create the availability for multiple compounds attached to one dendrimer, which can utilize multivalency as well as targeting and release strategies[89,99]. Our system uses a multivalent probe to elucidate biological functions by functionalizing PAMAM dendrimers with lactose, a known binding partner for galectin-3[23,97,117]. The utility of the glycodendrimers in assays to discern and mediate cellular processes depends on the uptake and toxicity of the compounds. Even at concentrations of glycodendrimers much higher than would be expected to be used in cell-based assays, no toxic effects were observed. Even after incubation for 24 hours, significant toxicity was not observed in A549 cells (Figure 6).

Further investigation of lactose functionalized dendrimer characteristics led to questions about its localization. Details such as cellular location were determined by uptake assays. Unfunctionalized AF647 was readily taken up by A549 cells, and glycodendrimers functionalized with AF647 were also taken up well by the cells. Visualization by confocal microscopy revealed accumulation in lysosomal compartments in as little as 5 hours. The results demonstrated a size dependency in dendrimer uptake, with the smallest dendrimers (G2 and G3) revealing more fluorescence within the cells than the higher generation dendrimers (G4 and G6). Even the largest glycodendrimer studied, G6, was taken up by the cells. Extended incubation revealed higher fluorescent accumulation in the cell, which could indicate recycling to the cell surface or slower uptake mechanisms involved. Clathrin mediated uptake mechanisms are most commonly reported, but clathrin independent uptake has also been reported for galectin-3[118]. Galectin-3 has been noted for its ability to mediate endocytosis of extracellular glycosylated proteins[28,118,119]. Researchers have reported a mechanism that includes monomeric galectin-3 binding to proteins such as CD44 and β_1-integrins which further recruit glycosphingolipids for carbohydrate binding and galectin-3 oligomerization. This eventually leads to a mechanistic bending of the membrane to create a clathrin independent carrier[119]. The endogenous presence of galectin-3 in A549 cells could enable dendrimer uptake via this mechanism. Extracellular galectin-3 is also reported to play pivotal roles in clathrin independent endocytosis of CD59 and MHCI (major histocompatibility complex class I)[118]. It is hypothesized that uptake of receptors can be tipped to favor or inhibit uptake depending on extracellular galectin concentrations[97]. The

variation in microenvironment concentrations and receptor glycosylation could very likely explain the many different effects, and sometimes counteracting effects, that galectins have in different cell lines. Variation of higher-order galectin lattices could explain why apoptosis is induced in T-cells but not in the A549 lung cancer cell line studied here.

Uptake into the lysosome has been reported to induce apoptosis in an intrinsic manner[120]. This occurs through the process of lysosomal membrane permeabilization (LMP) in which the contents of the lysosome, including proteases and hydrolases such as cathepsins, are leaked into the cytosol. Even though these hydrolases act in low pH environments, some such as cathepsin S, a cysteine protease, can last for hours in the cytosolic environment. There is also evidence of cytosolic acidification, which can further perpetuate the activity of the leaked hydrolases. Even though the mechanism of the LMP is poorly understood, in general, the proton pump is blocked which inhibits degradation of the lysosomal contents. Some of these contents, such as L-leucyl-L-leucine methyl ester (LeuLeuOMe), can polymerize and act as a detergent to degrade the lysosomal membrane[120]. In fact, the apoptosis inducing control staurosporine acts similarly to the mechanism with LeuLeuOMe. It has been reported that exposure of staurosporine to human foreskin fibroblasts leads to cathepsin D accumulation in the cytosol, which brings about the release of cytochrome c from the mitochondria, activating caspase-9 and caspase-3 and further perpetuating the apoptotic cascade[121]. If galectin-3 assists in the uptake of staurosporine, this would explain why our results show an

increase in activated caspase-3 which is part of the apoptosis indicator in our experiment (Figure 11).

Our results have shown that galectin-3 and/or lactose functionalized dendrimers do not induce apoptosis in A549 cells. Our results, and others, have shown that extracellular galectin-3 and lactose functionalized dendrimers are endocytosed[31,71,95,96]. One likely explanation for the lack of apoptosis in our experiments is that no LMP occurs, and therefore no induction of signaling and apoptosis is triggered. It may also be that the interaction of galectin-3 with the dendrimers is unable to impact such a highly regulated mechanism as apoptosis.

Reports have shown that extracellular galectin-3 can protect cells from apoptosis inducing drugs. In cells treated with doxorubicin, adding exogenous galectin-3 partially rescued cell viability[108]. Our results display that exogenous galectin-3 further elevates apoptosis in A549 cells in the presence of staurosporine, an apoptosis inducer. An explanation could be that extracellular galectin-3 tends to cluster large extracellular receptors, such as aberrantly glycosylated mucin 1[93,114]. This reveals other smaller receptors that can be involved in processes such as homotypic aggregation or endocytosis.

Confirmation by AO/EB staining did not reveal induction of apoptosis in A549 cells due to exogenously added galectin-3 and/or lactose functionalized dendrimers even though exogenous galectin-3 has been reported to influence many cancerous activities such as angiogenesis, migration and aggregation[49,63,93].

Conclusions

Galectin-3 can multimerize and engage in multivalent behavior, presenting a favorable receptor for lactose functionalized dendrimers. The results reported here could further reveal opportunities for drug design of systems that utilize this type of cellular uptake in coordination with the expected extracellular interactions. The lactose functionalized glycodendrimers were demonstrated to be non-toxic and non-apoptosis-inducing. Without the threat of these non-desirable effects, these dendrimers could be further functionalized with endgroups such as targeting receptors or fluorescent probes to study signal colocalization or to understand therapeutic effects. There are a myriad of biological multivalent interactions, especially in diseases, and glycodendrimers are emerging as important systems to probe and discern these biological interactions.

Experimental Procedures

Toxicity Assay

Whole media was poured off cells and covered in 0.25% Trypsin, 0.02% EDTA solution and incubated for 5-10 minutes. Cells were gently aspirated using a disposable pipette, collected in a 15 mL falcon tube and centrifuged for 5 minutes at 2000 rpm. Trypsin/EDTA solution was poured off and the cell pellet was resuspended in 1 mL whole media. 10 μL cell solution was transferred to sterile tube and diluted with 90 μL media and mixed. 10 μL of this solution was transferred to another sterile tube and

combined with 10 μL Trypan Blue. 10 μL of this cell/dye solution was placed on a hemocytometer, and cells were counted to determine total cell count. From this count the cells were diluted to a 200,000 cells/mL solution with whole media. Two solutions of controls were made from this cell solution: toxic control and viable control. The viable control contained cell solution with Celltox dye at a ratio of 1:500 in whole media. Toxic control contained cell solution, lysis buffer in 1:25, Celltox dye at 1:500. A media control was made with whole media and Celltox dye at 1:1000. Dendrimer solutions were concentrated to 8 mg/mL in whole media. Into a 96 well plate, the solutions were aliquoted with six replicates. Total volume in each well was 100 μL. For each of the dendrimer wells, 50 μL of dendrimer solution was combined with 50 μL of viable cells; G2: 47478 μM, G3: 23043 μM, 12448 μM, G6: 2620 μM; in A549 cell assay: G2: 4748 μM, G3: 2304 μM, G4: 1245 μM, G6: 262 μM. For the toxic control wells: 50 μL toxic control solution was combined with 50 μL of whole media. The viable control wells: 50 μL viable cell solution (1:500 Celltox dye with 200,000 cells/mL) and 50 μL of whole media. Media control wells: 100 μL media control solution. Plate was covered with foil to protect dye from light and placed in an incubator at 37 ºC, 5 % CO_2. At 2 and 24 hours, the plate was removed from the incubator and fluorescence was recorded using a fluorescent plate reader.

Materials

General reagents were purchased from Acros and Sigma Aldrich Chemical Companies. PAMAM dendrimers were purchased from Dendritech. Alexafluor 647

hydrazide and cell staining reagents were obtained from ThermoFisher Scientific. The Apotox-Glo Triplex Assay was purchased from Promega.

Cell Culture

Three immortalized cancer cell lines were selected for analysis based on their robustness and expression of galectin-3. DU 145 (ATCC®: HTB-81™) prostate carcinomas were cultured in media consisting of Dulbecco's modified Eagle's medium (DMEM: Gibco, 61100) with 1.8 g/L $NaHCO_3$ and supplemented with MEM vitamin (Gibco, 11126), Penn/strep (Gibco, 15140), NEAA (Gibco, 11140), EAA (Gibco, 11130) and 10% v/v fetal bovine serum (FBS). HT-1080 (ATCC®: CCL-121™) fibrosarcomas were cultured in media consisting of Dulbecco's modified Eagle's medium (DMEM: Gibco, 61100) with 1.8 g/L $NaHCO_3$ and supplemented with MEM vitamin (Gibco, 11126), Penn/strep (Gibco, 15140), NEAA (Gibco, 11140), EAA (Gibco, 11130) and 5% v/v fetal bovine serum (FBS). A549 (ATCC® CCL-185™) lung carcinomas were cultured in F-12K Medium (Kaighn's Modification of Ham's F-12 Medium) (ATCC® 30-2004™) supplemented with Penn/strep (Gibco, 15140) and 10% v/v fetal bovine serum (FBS). Cells were cultured in a humidified incubator with 5% CO_2 and 37 °C. Before confluency, cells were subcultured using 0.25% (w/v) Trypsin- 0.53 mM EDTA.

General procedure for the synthesis of Alexafluor 647 functionalized glycodendrimers

Lactose functionalized PAMAM dendrimers were synthesized as previously described[82]. Glycodendrimers, each generation obtained as a white solid, were dissolved

to form a 2 mg/mL solution in filtered Milli-Q water. $NaIO_4$ (2 equiv. per glycodendrimer) was introduced and allowed to stir for 2 h. Subsequent addition of the Alexafluor 647 hydrazide (1 equiv. per glycodendrimer) was performed by introducing an aliquot from a 2 mg/mL aqueous solution under low-light conditions and allowed to react for 45 min. Purification was accomplished by dialysis (1000 MWCO, 3 x 1 L, 90 minutes each), and confirmed by TLC.

FACS flow cell cytometry

A549 cells were seeded and incubated for 24 hours. Cells were treated with Alexafluor 647 functionalized glycodendrimers and incubated for 5 and 22 hours at a final concentration of 0.1 μM and 1.0 μM. Culture medium was removed, and cells were washed with PBS and harvested with trypsin. Cellular uptake was recorded using a FACS instrument.

Confocal Microscopy

A549 cells were seeded in a glass bottom petri dish and cultured overnight with CellLight reagents targeting lysosomal or early endosomal GFP transfection. Cells were treated with Alexafluor 647 functionalized glycodendrimers and incubated for 5 and 24 hours at a final concentration of 0.1 μM and 1.0 μM. The nucleus was stained with Hoechst 33342 for 30 minutes and rinsed with PBS to remove residual components. Cells were imaged and analyzed using a confocal microscope.

Apoptosis Assay

A549 cells were lifted and counted using a hemocytometer. Cells were added to wells of a 96 well plate so each well contained 9,000 cells. The plate was placed in a humidified incubator at 37°C, 5% CO_2 and >80% humidity to allow cells to adhere overnight. The next day, test compounds and vehicle controls were added for a final volume of 100 µL per well. A viability control was provided by 0.1% SDS, a cytotoxicity control was provided by ionomycin at a final concentration of 100 µM, and the control for apoptosis was staurosporine at a final concentration of 10 µM. Test compounds were galectin-3 at a final concentration of 6.609 µM, lactose functionalized dendrimers were 224.3, 142.04, 61.15, and 21.84 µM, for G2, G3, G4, and G6, respectively. Cells were incubated for 5 hours at 37°C, 5% CO_2 and >80% humidity, as per the manufacturer's instructions for the Apotox-Glo Triplex Assay. 20 µL of Viability/ Cytotoxicity Reagent containing both GF-AFC Substrate and bis-AAF-R110 were added to each well. The plate was further incubated for 80 min, and fluorescence was measured at $400_{Ex}/505_{Em}$ for viability and $485_{Ex}/520_{Em}$ for cytotoxicity using a Biotek hybrid 1 plate reader. 100 µL of Caspase-Glo 3/7 reagent was added to all wells, and the plate was briefly shaken. Incubation for 30 min was carried out at room temperature protected from light, and luminescence was then measured on the plate reader.

Acridine Orange/ Ethidium Bromide

A549 cells were suspended to 50,000 cells/mL. The 8 well plate was seeded with 300 µL of suspended cell solution (15,000 cells per well) and allowed to adhere and

incubate at 37 °C and 5% CO_2 overnight. For all experiments containing galectin-3, a 5-hour incubation was used. Compounds, media and/or dendrimers and galectin-3 were added to the wells for a final volume of 300 µL per well (30 µL dendrimers in milli-Q water or control compounds, 90 µL galectin-3 in PBS, 180 µL whole media). After the 5-hour incubation period at 37 °C and 5% CO_2, the media in the wells was removed. Wells were gently washed with PBS, stained with 20 µL of the 1:1 AO/EB solution, and the wells themselves were removed from the glass bottom. A drop of ProLong™ Gold Antifade Mountant with DAPI was placed in the center of each slide area and a coverslip was placed on top. The sample was protected from light and imaged immediately using a Zeiss LSM 510 Meta microscope with a 20x objective and filters set for acridine orange and ethidium bromide[122]. Acridine orange: Excitation: 488 nm, Emission range: 521-542 nm and Ethidium Bromide: Ex 514 nm, Emissions range: 606-692 nm. Acridine orange (AO) and ethidium bromide (EB) were prepared in milli-Q water to a concentration of 100 µg/mL separately and combined into a 1:1 solution. The AO/EB solution was protected from light at all times and stored at 4 °C for no longer than a week. Additionally, a set of control compounds including; ionomycin 100 µM in 0.001% DMSO, staurosporine 100 µM, and SDS 0.01% were prepared in milli-Q water and stored at 4 °C for use in initial control experiments. Staurosporine and 0.01% SDS were run in extra wells whenever possible as additional controls.

CHAPTER THREE

LACTOSE FUNCTIONALIZED DENDRIMERS IMPACT GALECTIN-3 MEDIATED CANCER CELL MIGRATION *IN VITRO*

Contribution of Authors and Co-Authors

Manuscript in Chapter 3

Author: Mackenzie S. Fricke

Contributions: Conducted scratch assays, functionalized dendrimers with fluorescent tag, collected fluorescent micrographs, expressed galectin-3 and CRD.

Co-Author: Kyle Tweedy

Contributions: Helped design and optimize scratch assays.

Corresponding Author: Mary J. Cloninger

Contributions: Edited manuscript.

Manuscript Information

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____ Accepted by a peer-reviewed journal
____ Published in a peer-reviewed journal

Introduction

Galectin-3 is a chimeric, β-galactoside binding lectin that is upregulated in many cancers[21]. Galectin-3 can be found in the nucleus, cytoplasm and at the cell's surface. Galectin-3 is necessary for many homeostatic cellular processes, but the roles of galectin-3 in transformed cells are not well understood. The localization of galectin-3 can potentially indicate if it is involved in oncogenic or tumour suppressive processes[23]. For example, intracellular galectin-3 has anti-apoptotic effects when localized in the cytosol but has pro-apoptotic effects inside the nucleus[23]. When localized at the cell surface, galectin-3 can be involved in many cancer processes such as immune suppression, matrix adhesion, cell-cell adhesion, endocytosis and exocytosis, tumor metastasis and angiogenesis[23,46,61,123,124]. Intracellularly, galectin-3 can be involved in cell growth, apoptosis, transcription regulation, angiogenesis and cancer cell adhesion[23,125].

Galectin-3 binds to beta-galactosides with relatively weak monovalent affinity[18]; multivalent protein-carbohydrate interactions are required for galectin-3 mediated cellular recognition processes[126]. Galectin-3 contains two different domains; the highly conserved domain among the 15 known mammalian galectins[32] is a galactoside-binding carbohydrate recognition domain (CRD)[127]. Unlike all other galectins, in addition to the CRD, galectin-3 also contains an intrinsically disordered "collagen like" N-terminal domain with a repeating sequence of proline, glycine, alanine and tyrosine[128]. The N-terminal domain is believed to be critical for protein multimerization and also for

signalling mechanisms which are believed to bestow galectin-3 with many of its unique characteristics[18,63,92].

Through a non-classical secretion mechanism, galectin-3 can be found at the cell's surface. This excretion is dependent on the N-terminal domain, and it is thought that this excretion occurs through the use of exosomes[129]. When in the extracellular space, there are several glycosylated surface proteins available as potential binding partners for galectin-3[18,51]. Some notable examples are N-cadherin, which is involved in cell-cell adhesion, and mucin 1, a heavily glycosylated transmembrane protein that becomes aberrantly glycosylated with terminal galactosides on cancer cells[114,130].

Within the tumour microenvironment, galectin-3 is present at high extracellular concentrations (in the micromolar range), allowing for protein aggregation to induce cellular crosslinking[52,131–133]. Galectin-3 mediated cellular crosslinking directly influences the homotypic aggregation of cancer cells in suspension[93] and has also been shown to be an important step in the metastatic cascade[46]. Prior work in our lab and others has shown that exogenously added galectin-3 increases homotypic aggregation of suspended cancer cells[47,93,114].

Monovalent protein-carbohydrate interactions are generally weak. Many lectins rely on multivalency to increase their overall binding avidity by decreasing the entropic cost and increasing the enthalpic contribution[13,83]. Here, we propose to use a multivalent polymer scaffold functionalized with lactosides to investigate the impacts of multivalency on cellular migration.

Poly amidoamine (PAMAM) dendrimers are symmetrically branched polymers with end groups that can be functionalized with carbohydrates, such as lactose, through a variety of linkages[134]. Increasing generations of this dendrimer double the number of amino end-groups available for functionalization. Our lab has successfully functionalized and characterized generations 2, 3, 4 and 6 (G2, G3, G4 and G6) lactose functionalized dendrimers[82]. The glycodendrimers used in the studies reported here were functionalized with an average of 15, 20, 45 and 100 lactosides, respectively (Figure 16). PAMAM dendrimers have been highly investigated as of late, due to their utility in drug delivery systems. These dendrimers present multiple modes of ligand attachment, have low toxicity, and have good solubility. Because of their many end groups, targeted delivery can be probed[76,115,116,135].

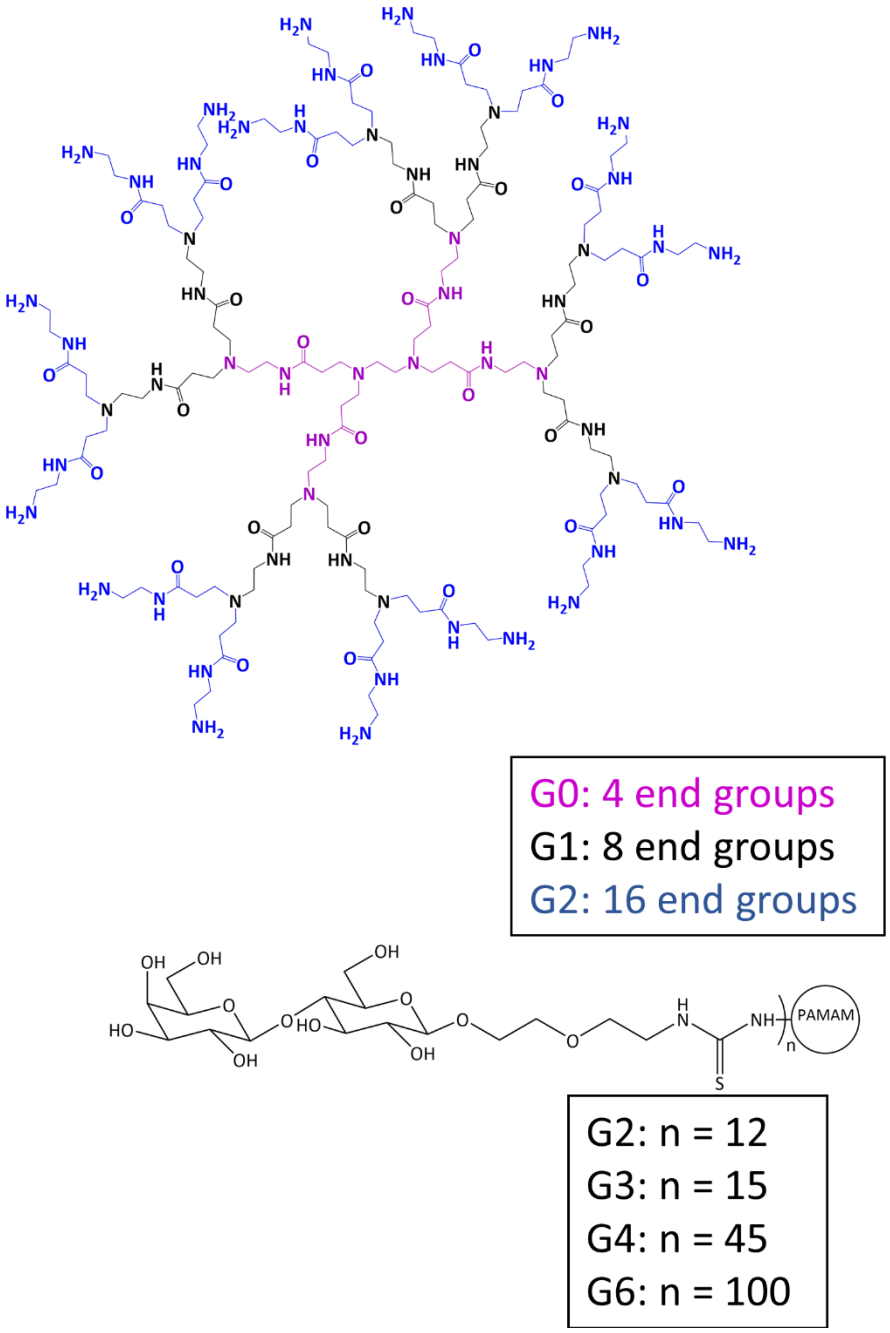

Figure 16. PAMAM dendrimers demonstrating the exponential increase in terminal end groups with each subsequent generation. Lactose is functionalized to the terminal amino groups through a thiourea linkage.

Here, lactose functionalized G2, G3, G4, and G6-PAMAM dendrimers were used to investigate galectin-3 mediated chemotaxis using two cancer cell lines. A modified *in vitro* scratch assay was used to investigate the migratory potential of these cancer cells. A migration trend was identified based on the effects of exogenously added galectin-3 and glycodendrimers. The role of galectin-3 in the metastatic cascade is not well understood, and our ability to attenuate migration using glycodendrimers enables us to advance our understanding of the role of carbohydrate-protein interactions in directing cancer cellular migration.

Results

Galectin-3 has been implicated in many cancerous pathways[136]. One of the most devastating diagnostically is metastasis, is a multistep process in which tumor cells migrate to a distant site and form a secondary tumor[137]. In order to metastasize, a tumor has to break the cell-matrix interactions and invade a nearby circulatory system, usually by increasing its cell-cell adhesions[51]. Extracellularly, galectin-3 can impact cell-cell adhesion and cell-matrix interactions[18,138].

Here, a scratch assay was used to assess the impacts of galectin-3 and glycodendrimers on cancer cell migration. Two cancer cell lines were selected for this investigation based on their relative endogenous production of galectin-3. DU145 prostate cancer cell lines produce comparatively high levels of galectin-3, while HT1080 fibrosarcoma cells produce low amounts of galectin-3[93,139]. Endogenous galectin-3 indicates an increasing likelihood of existing galectin-3 mediated processes, and the

impacts of excess galectin-3 were therefore hypothesized to be more prominent in DU145 cells and less impactful in HT1080 cells.

Scratch Assay with DU145 and glycodendrimers with and without excess galectin-3

A scratch assay was designed to investigate the effect of lactose functionalized dendrimers and exogenous galectin-3 on the migration of DU145 cells, which are prostate carcinomas[140]. These cells exhibit the highest concentration of endogenously produced galectin-3[93] for the two cell lines tested. A scratch in the cell population was created using a two well insert (Ibidi). The extent of migration into the scratch was recorded every 4 hours for 16 hours total. Cell migration into the scratch at each subsequent timepoint was normalized to the cell-free area at the initial time point and graphed. The assays were performed in both the presence and absence of exogenously added galectin-3. It was anticipated that there might be enough endogenous galectin-3 to interact with the lactose functionalized dendrimers and have an impact on migration when using DU145 cells. However, in the presence of lactose functionalized G2, G3, G4 and G6 dendrimers, there was no impact on the migration rate of DU145 cells compared to the galectin-3 buffer control, PBS (Figure 17).

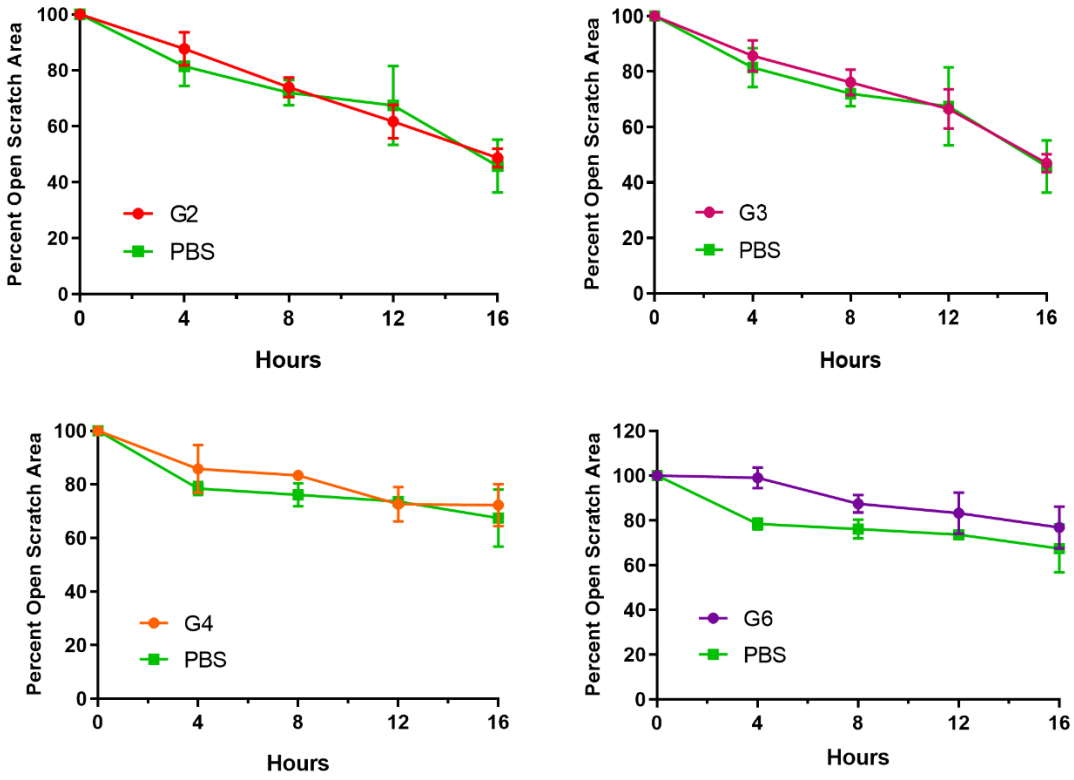

Figure 17. Migration of DU145 cells in the presence of PBS (green) or lactose functionalized dendrimers. G2 (red), G3 (pink), G4 (orange), or G6 (purple).

When galectin-3 was added exogenously to the scratch, the migration of the cells was decreased, if not halted (Figure 18, blue). When each generation of lactose functionalized dendrimer was added to the scratch in the presence of excess galectin-3, G2 and G3 dendrimers, comparatively smaller and more amorphous in shape, exhibited a migration trend similar to the PBS standard at the end of 16 hours (Figure 18, red for G2; Figure 18, pink for G3). Although migration was initially retarded in the presence of G2 and G3, by 16 hours the cellular migration was fully restored in the presence of small glycodendrimers. When the scratch contained G4 or G6 lactose functionalized dendrimer

with exogenously added galectin-3, the scratch closure was not as slow as the galectin-3 control or as fast as the PBS control (Figure 18, orange for G4; Figure 18, purple for G6). One likely explanation for the observed effect is as follows: as lactose functionalized dendrimers are added, they present a competitive binding partner for galectin-3 that can bind up some of the galectin-3. This would decrease the extent of cell-cell crosslinking mediated by galectin-3. This seems likely since galectin-3 is often proposed to oligomerize and induce cross-linking[21,26]. The large glycodendrimers G4 and G6 bind more galectin-3 than the smaller glycodendrimers[82], which may explain the observed differences in migration for small and large generation dendrimers.

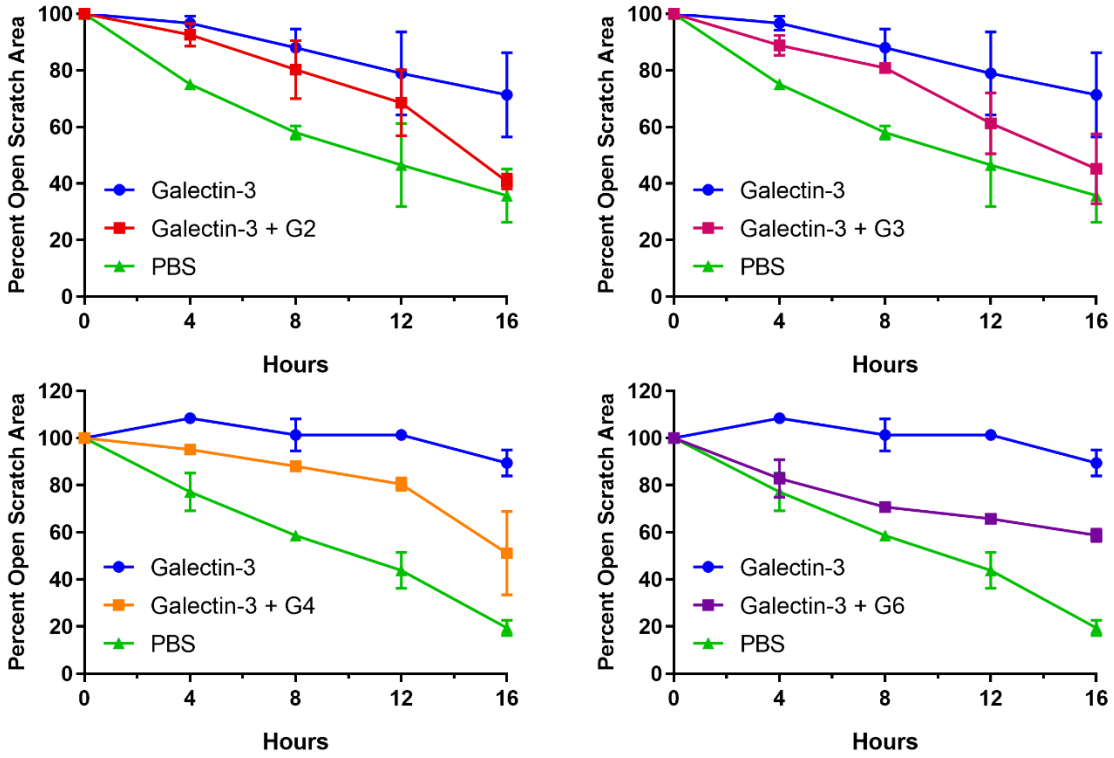

Figure 18. DU-145 prostate carcinoma cell migration into the scratch in the presence of, PBS (green triangles), exogenous galectin-3 (blue squares) and each generation of lactose functionalized dendrimer: G2 lactose functionalized dendrimer with exogenous galectin-3 (red circles), G3 dendrimer and exogenous galectin-3 (pink circles), G4 dendrimer and exogenous galectin-3 (orange circles), G6 dendrimer and exogenous galectin-3 (purple circles). The assay was run for 16 hours, and scratch area was recorded by image acquisition every four hours and normalized to the open scratch area at 0 hours.

Immunofluorescence in the Scratch Assay of DU145 cells

Extracellular galectin-3 can interact with many different cell adhesion molecules (CAMs), leading to the ability to improve cell junctions and to greater attachment to the extracellular matrix (ECM)[141]. These properties can directly impact the metastatic cascade[141]. In endothelial cells, extracellular galectin-3 can be rapidly endocytosed and would then be destined for either recycling to the plasma membrane or targeted to

lysosomes[71,142]. Insight into which processes in cancer cellular migration are significantly affected by galectin-3 was gained by observing the localization of galectin-3 during the scratch assay.

To visualize the localization of galectin-3, immunofluorescence was performed. Alexa Fluor 488 conjugated galectin-3 antibody was added at 4 or 12 hours in the scratch assay, and the scratch was fixed and mounted after 1 hour of incubation. The samples were visualized using a Zeiss LSM 510 scanning confocal microscope. When excess galectin-3 was added to the scratch, a smooth webbing-like pattern of the intercellular area was clearly visible (Figure 19A, B). Also observed was the presence of small, spotted features indicating aggregates of galectin-3. When PBS was added to the scratch, very faint fluorescence was observed, indicating the presence of endogenous galectin-3 with DU145 cells (as expected, Figure 19C, D).

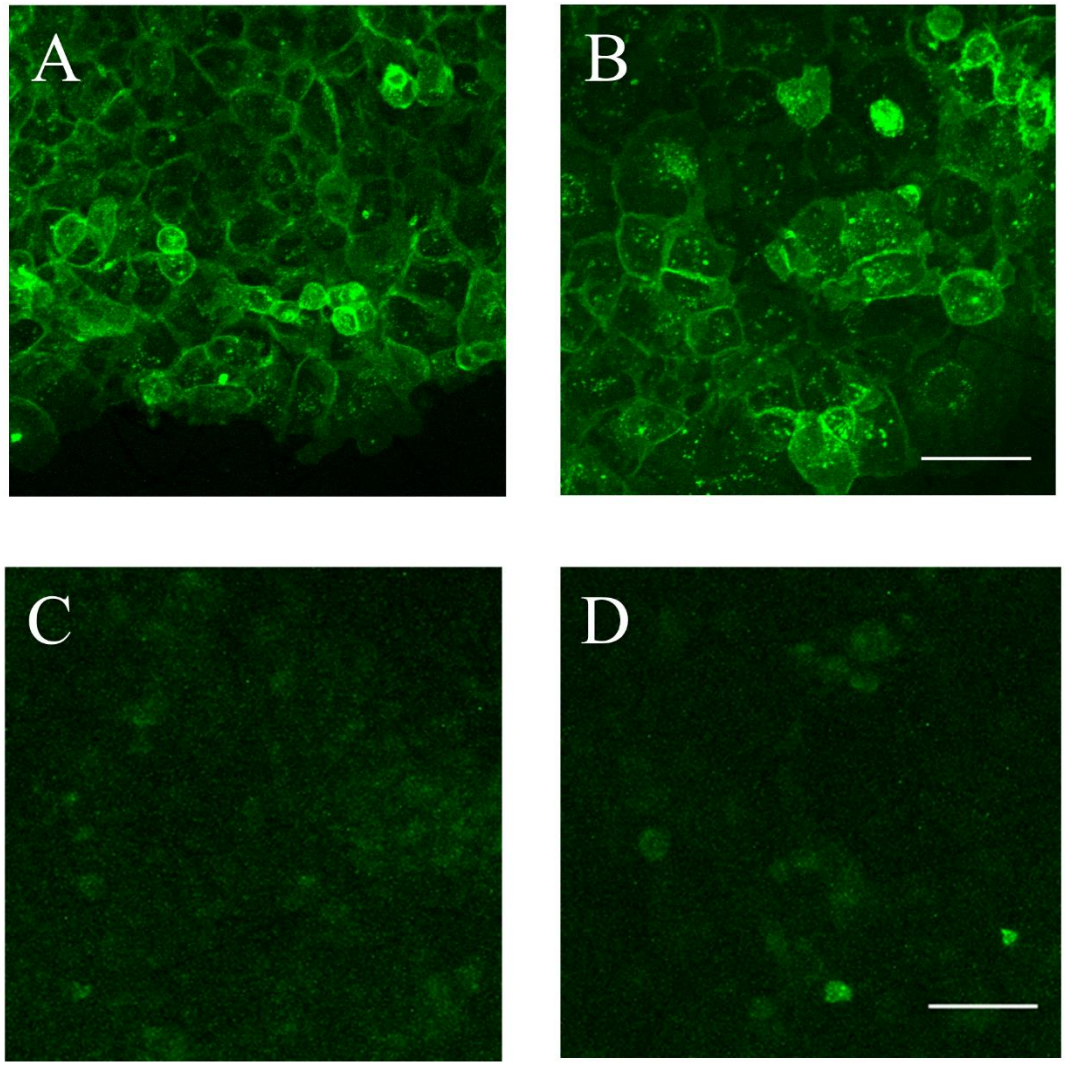

Figure 19. Scratch Assay labelling galectin-3 with anti-galectin-3-Alexa Fluor 488. A) DU145 cells after 4 hours of migration with exogenously added galectin-3. B) DU145 cells after 12 hours of migration with exogenously added galectin-3. C) Cells after 4 hours of migration with only endogenous galectin-3. D) Cells after 12 hours of migration with only endogenous galectin-3. Scale bar 50 μm.

When lactose functionalized G6 glycodendrimer was added along with exogenous galectin-3, webbing and widespread spotting was still observed (Figure 20A). Although slightly less pronounced, the same spots along the cells were observed for lactose

functionalized G2 dendrimers, indicating that in both cases galectin-3 aggregates are detectable with the cells (Figure 21B). The intercellular webbing-like feature was observed for both the large (G6) and the small (G2) glycodendrimers.

Immunofluorescence with inhibition of endocytosis

To determine whether the galectin-3/glycodendrimer aggregates were endocytosed or were externally bound to the plasma membrane, incubation with the fluorescently-labeled galectin-3 antibody was carried out at both 37 °C (Figure 20A, C) and at 4 °C (to inhibit endocytosis of the antibody, Figure 20B, D). First, galectin-3 was added to DU145 scratched cells and incubated at 37 °C for 4 hours, as in the beginning of the scratch assay described above. The reagents were then removed, and the antibody solution was added. Immediately, the cells were placed on ice in the dark at 4 °C for 1 hour. After 1 hour, the antibody solution was removed, and the cells were fixed and mounted as previously described. The subsequent images showed smooth webbing features on the intercellular space for all samples. Significantly less green spots were observed in/on the cells (between the webbings) in the samples at 4 °C than in the samples at 37 °C (compare Figure 20C to Figure 20D).

Scratches in the presence of both G6 lactose functionalized dendrimer and galectin-3 showed many green spots that must correspond to aggregation of galectin-3 upon incubation at 37 °C (Figure 20A). This would support other reports that galectin-3 self-associates into oligomers[26,29]. When this sample was compared to a sample where the antibody binding was conducted at 4 °C, there appeared to be fewer spotted features, but

webbing was still observed in the sample that was incubated at 4°C (when endocytosis was blocked, compare Figure 20A to Figure 20B). These results indicate that galectin-3 is likely being rapidly endocytosed during this experiment and also reveals that endocytosis still readily occurs even when large dendrimers, such as G6, are present in the assay.

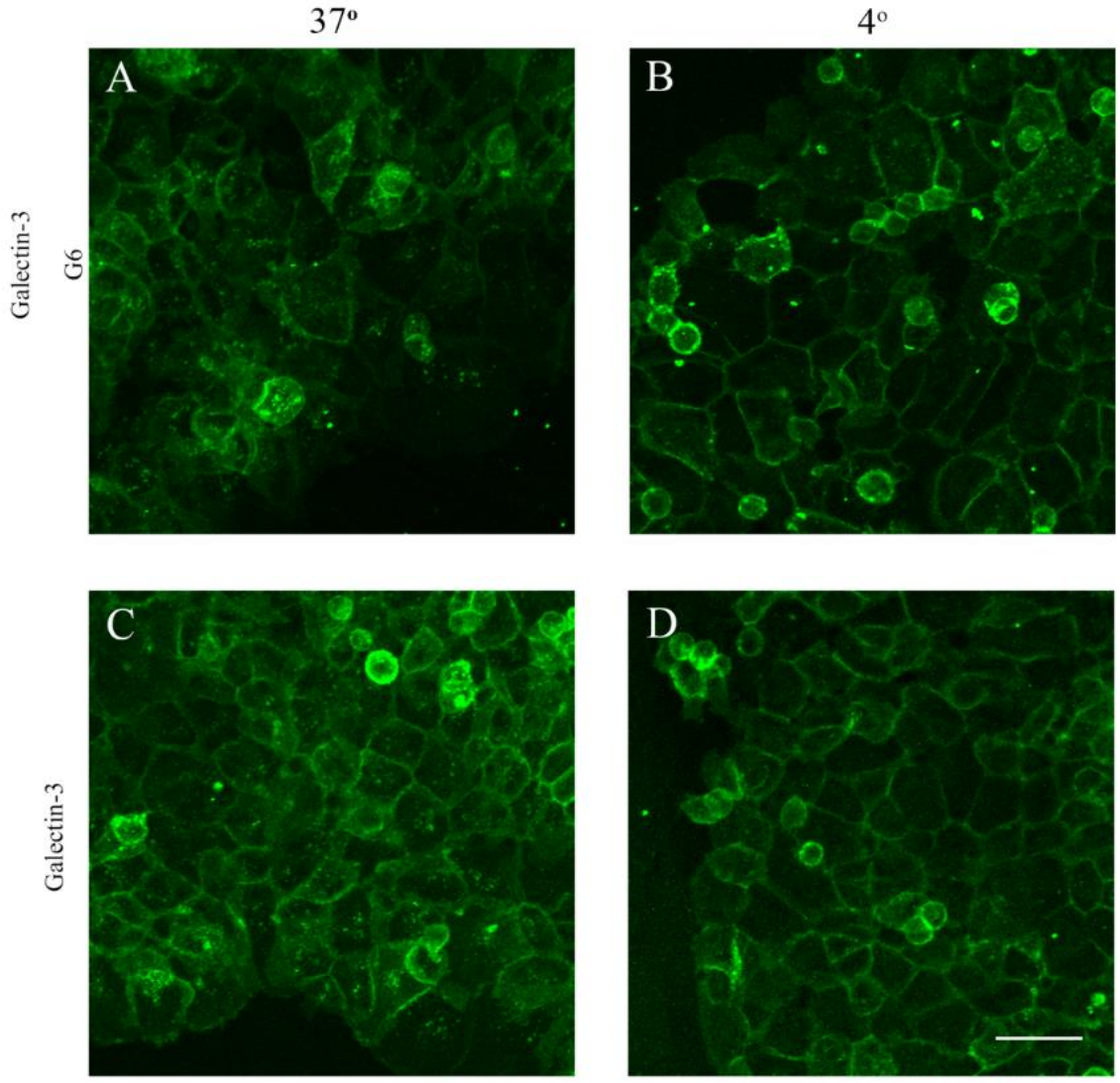

Figure 20. Scratch Assay with antibody incubation at 4 °C or 37 °C. A) DU145 cells after migration for 4 hours with excess galectin-3 and G6 lactose functionalized dendrimer with antibody binding at 37 °C. B) Cells after 4 hour migration with galectin-3 and G6 dendrimer with antibody incubation at 4 °C. C) 4 hours of cell migration with exogenously added galectin-3 and antibody incubation at 37 °C. D) DU145 cells after 4 hours of migration with excess galectin-3 and antibody binding at 4 °C. Scale bar 50 μm.

Immunofluorescence with fluorescently labelled glycodendrimers

To understand the localization of glycodendrimers during the migration assay, lactose functionalized G2 and G6 dendrimers were functionalized with Alexa Fluor 647 in a 1:1 labeling ratio. The inserts were removed, and fluorescently labeled dendrimers were incubated in the scratch with exogenously added galectin-3. After incubation for 4 hours, galectin-3 antibody binding was conducted at 37 °C and at 4 °C to compare localization of the dendrimers and galectin-3 when endocytosis was allowed (37 °C) or inhibited (4 °C). Antibody incubation at 4 °C again revealed galectin-3 rimming the exterior of the cell but fewer aggregates present within the cell (compare Figure 21A to B and Figure 21C to D). Fluorescently labeled G2 was both bound to the cell and localized inside the cell at 37 °C and at 4 °C (Figure 21A and B). Antibody incubation at 37 °C revealed rimming of the cells by galectin-3, uptake of galectin-3, and lactose functionalized G2 inside the cell. However, significant colocalization of the fluorescently labeled G2 dendrimer and galectin-3 within the cell was not observed (Figure 21B). Fluorescently labeled G6 also appeared to be spotted over the sample. However, at 37 °C there again appeared to be little colocalization between G6 and galectin-3 (Figure 21D). This suggests that, if glycodendrimer/galectin-3 aggregates enter the cell concomitantly, then their association does not appear to be sustained intracellularly. Thus, the use of glycodendrimers to nucleate formation of galectin-3 aggregates for mediation of cellular processes may be impactful at the cell surface while intercellular processes may not be affected by galectin-3/glycodendrimer association.

Scratch Assay with HT1080 and dendrimers with and without excess galectin-3

A different cancer cell line was also examined to observe the effect of dendrimers and galectin-3 on cell migration: HT1080 human fibrosarcoma cells. Since this cell line migrated much more quickly than the previous one, the assay was adjusted to be monitored every two hours for 12 hours. As reported using DU145 cells, adding exogenous galectin-3 to the scratch resulted in significantly decreased migration into the scratch (Figure 22, blue line). Adding an identical amount of the buffer (PBS) resulted in rapid scratch closure during the 12-hour assay. When G4 lactose functionalized dendrimer was added alone to the scratch, the resulting migration was between that of the galectin-3 control and PBS (Figure 23). The other glycodendrimers alone were unable to significantly impact the migration rate (Figure 23). The different effect of a G4 glycodendrimer on cellular migration was an unexpected result. Since all the other results show similar effects from all generations of the dendrimers, the reason for this outlier result is unclear. When each glycodendrimers was added in the presence of excess galectin-3, migration into the scratch was greater than with galectin-3 alone but was not as rapid as with PBS alone (Figure 22).

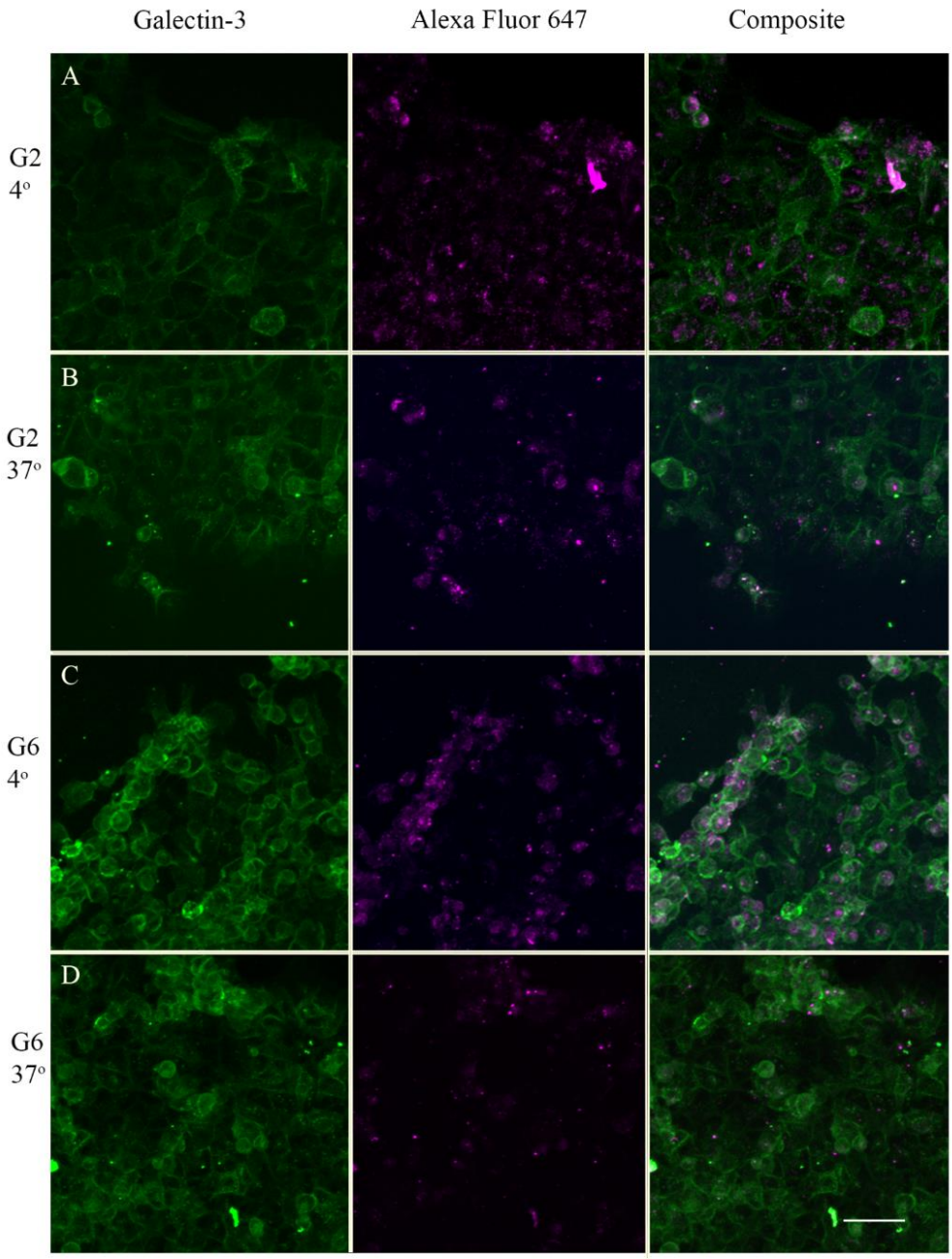

Figure 21. Scratch Assay after 4 hours incubation with excess galectin-3 and AF647 labelled dendrimer. A) DU145 cells with G2-AF647 and galectin-3 antibody incubated at 4°C. B) DU145 cells with G2-AF647 and galectin-3 antibody incubated at 37°C. C) DU145 cells with G6-AF647 and galectin-3 antibody incubated at 4°C. D) DU145 cells with G6-AF647 and galectin-3 antibody incubated at 37°C. Scale bar 50 µm.

Of the two cell lines reported here, HT1080 cells produce the least endogenous galectin-3. Thus, excess galectin-3 was not expected to have the dramatic impact on migration trends that was observed. HT1080 cells likely rely less on galectin-mediated cellular processes for binding, but the results here suggest that galectin-3 can become a major component of the migration process even for cells such as HT1080 cells that lack significant amounts of endogenous galectin-3. Moreover, lactose functionalized dendrimers can significantly impact this galectin-3 mediated migration even for cells such as HT1080s cells. There are distinct differences between HT1080 and DU145 cells. The time necessary to complete this wound healing experiment differs significantly. While the HT1080 cell scratch is completely closed by 12 hours, DU145 cells required 16 hours for complete migration into the scratch. This could indicate that different pathways are predominantly responsible for the cellular mechanisms of migration in the two cell lines. Even though G2 and G3 glycodendrimers were more effective at restoring migration in DU145s, those same concentrations were not as effective at restoring migration in HT1080s. Since G2 and G3 glycodendrimers should be providing the same action in both experiments, i.e. binding up galectin-3, it may be that the impact of galectin-3 binding is less dramatic on the migration response of HT1080 cells. Because of different pathways contributing to migration and lower endogenous levels of galectin-3, it seems likely that galectin-3 is not as involved in the migration of HT1080 cells. Thus, when glycodendrimers are added, effectively sequestering galectin-3, there must be other migration mechanisms that compensate for the loss of extracellular galectin-3 for HT1080 cells.

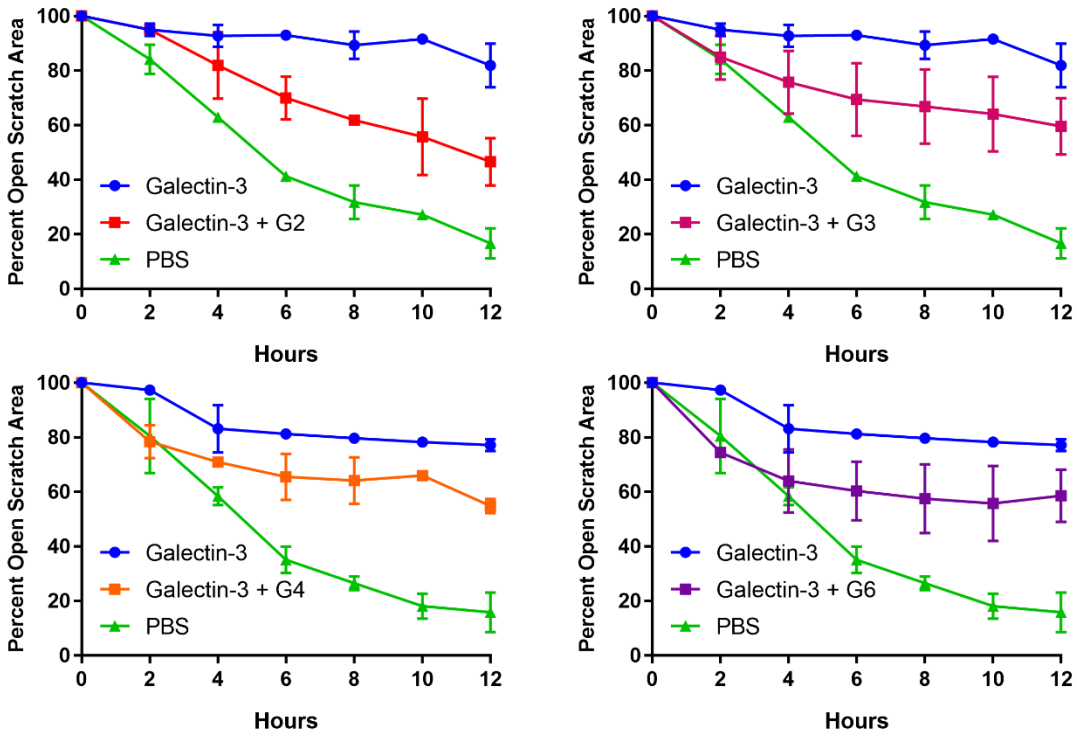

Figure 22. HT1080 human fibrosarcoma cells migration rate in the presence of PBS (green triangles), exogenous galectin-3 (blue squares), and each generation of lactose functionalized dendrimers: G2 dendrimers with exogenous galectin-3 (red circles), G3 dendrimer and exogenous galectin-3 (pink circles), G4 dendrimer with exogenous galectin-3 (orange circles), G6 dendrimer and exogenous galectin-3 (purple circles).

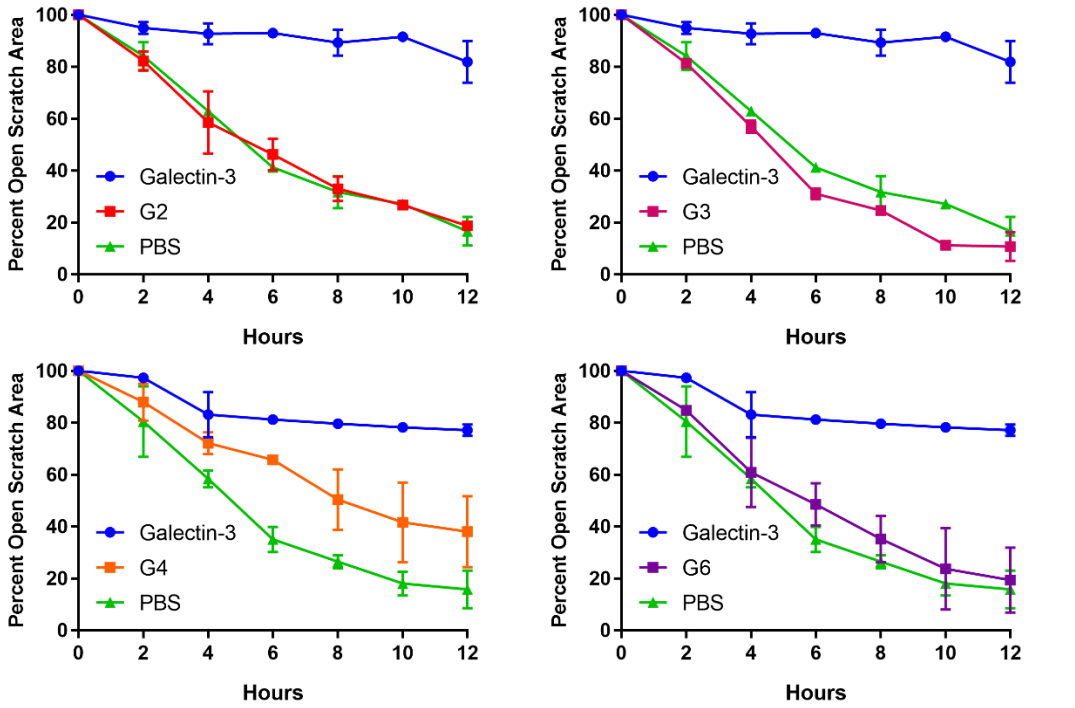

Figure 23. Migration of HT1080 cells in the presence of PBS (green), galectin-3 (blue) or lactose functionalized dendrimers: G2 (red), G3 (pink), G4 (orange), or G6 (purple).

Comparison of scratch assay results for full length galectin-3 and the carbohydrate recognition domain (CRD) using DU145 cells

The different domains of galectin-3 are known to exhibit different effects[18,63,79,143]. The CRD of galectins binds β-galactosides, and the disordered N-terminal domain of galectin-3 contains the function of self-association. Our next investigation was to determine if the N-terminal domain had an impact on the observed migration result. The CRD construct was successfully expressed, verified through SDS-PAGE and concentrated in the same manner as the full-length protein. The CRD construct was added to DU145 scratches and compared to scratches in the presence of the full-length protein. The CRD construct was unable to impact the migration of DU145s in

the same manner as full-length galectin-3 (Figure 24). The CRD migration trend had no significant variation from the migration of cells in the presence of PBS. When lactose functionalized G6 dendrimer was added with the CRD in the scratch, no significant change was observed from the PBS control. As reported previously, cells incubated with lactose functionalized G6 dendrimer in the presence of full-length galectin-3 showed a significantly different migration trend than cells incubated in PBS (Figure 24).

These results show that the N-terminal domain was essential for the arrested cellular migration arising from addition of galectin-3. Thus, the self-associating N-terminal domain on the full-length protein impacted cancer cell migration significantly and reproducibly.

In addition, experiments were performed to determine the multivalent effect of glycodendrimers in this assay. When monomeric lactose was added with exogenous galectin-3, 3 times the concentration of lactose on a G2 glycodendrimer was required to have the same effect on migration that G2 glycodendrimers demonstrated (Figure 25: red line). These results show that in this biological assay, multivalency is more effective at impacting galectin-3 mediated cellular migration than the monomeric interaction.

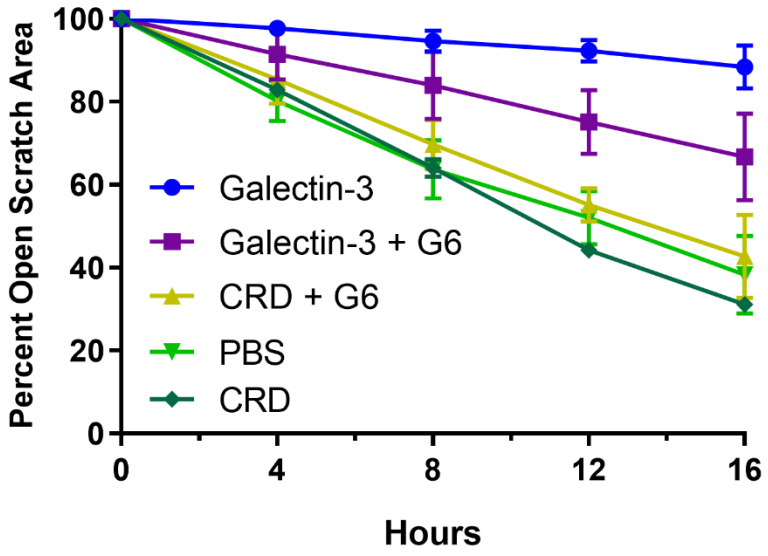

Figure 24. Migration of DU-145 cells into the scratch area over 16 hours. With exogenously added galectin-3 (blue squares), exogenous galectin-3 and G6 lactose functionalized dendrimer (purple circles), PBS (green diamonds), the CRD (brown triangles), and the CRD with G6 lactose functionalized dendrimer (yellow triangles).

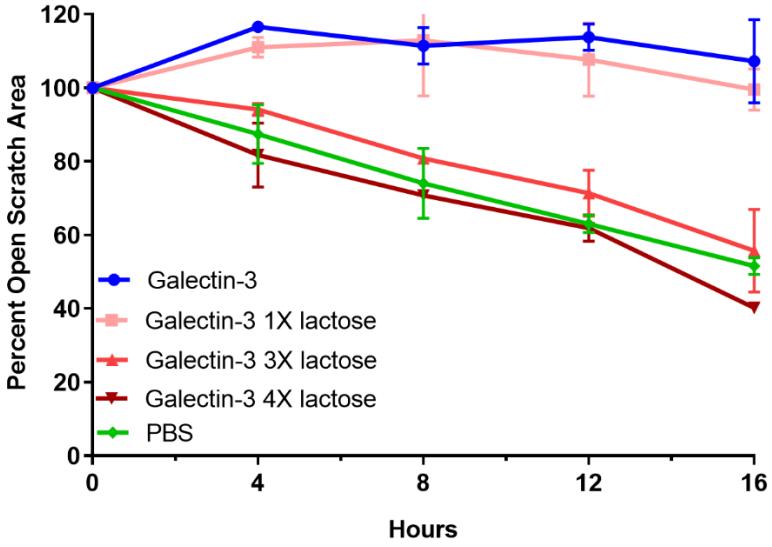

Figure 25. Migration of DU145 cells with exogenously added galectin-3 and increasing concentrations of monomeric lactose. Lactose concentrations were equivalent to G2 scratch experiments.

Discussion

Cancer progression is highly dependent on the metastatic potential of the cancerous cells[144]. This motility results from decreased cell-matrix interactions and varying cell-cell homotypic and heterotypic interactions[145]. In a metastatic tumor, the cells must break the interactions with the basement membrane and with the surrounding cells and avoid anoikis as a result. A cascade of events leads to migration of these cells away from the primary tumor[37]. After intravasation into a circulatory system, homotypic aggregation is vital to the survival of the tumor cells[46]. Many of the highly controlled processes involved in metastasis depend on the characteristics of the tumor microenvironment including extracellular galectin-3[51,138]. The tumor microenvironment is dynamic and varies greatly between cancers, and there exist many irregularly glycosylated receptors to interact with galectin-3[146]. Interaction with these receptors can bring about transmembrane signaling and clustering of the receptors, leading to exposure of other membrane receptors[93]. Understanding these interactions that are taking place at the cell membrane is vital to understanding the mechanisms involved in tumor metastasis.

Previous work has shown that homotypic aggregation of cancer cells can be attenuated by exogenous galectin-3 and lactose functionalized PAMAM dendrimers[93]. Exogenously added galectin-3 induces homotypic aggregation in a cancer cell suspension. A lactose functionalized G2 PAMAM dendrimer inhibits galectin-3 mediated homotypic cellular aggregation. A lactose functionalized G6 dendrimer, on the other hand, induces cellular aggregation[93].

The impactful properties of these materials were again demonstrated in this *in vitro* scratch assay. This modified *in vitro* scratch assay mimics some of the *in vivo* effects observed in chemotaxis[140]. It was found that at micromolar concentrations, exogenously added galectin-3 slowed cancer cell migration into the scratch in both cell lines tested. When a multivalent ligand scaffold was introduced alone to the scratch, in the form of lactose functionalized glycodendrimers, there was no significant impact on migration. Glycodendrimers with exogenously added galectin-3, on the other hand, showed improved migration as compared to exogenous galectin-3 alone. By supplying a competitive binding partner for galectin-3, significantly faster migration was observed than when galectin-3 was present without the glycodendrimers. These results show that the glycodendrimers are an effective probe to interact with galectin-3 and impact cellular migration. These glycodendrimers are more effective than the corresponding monovalent lactoside ligand, and galectin-3 is only an effective interactor in cellular migration when the full, intact protein is present. These results indicate that the glycodendrimers interact efficiently with galectin-3 and thereby mitigate the effect of galectin-3 on cellular migration.

Immunofluorescence was used to visualize the localization of galectin-3 by adding fluorescently labeled galectin-3 antibody. Two distinctive features were observed from the results. First, a smooth webbing pattern of galectin-3 on the periphery of the cells was much more prevalent on scratch samples with exogenously added galectin-3. This indicates that the galectin-3 is bound to the cells and is available to interact in the intercellular space. The second feature was observable spots that indicate aggregates of

galectin-3. These features were observed in samples with galectin-3 alone and also when lactose functionalized dendrimers were present. When endocytosis of the antibody was inhibited by incubation at 4 °C, the same webbed feature was observed as at 37 °C, but the spotting was much less prominent. Thus, our results and other published results indicate that some galectin-3 is involved at the intercellular space, and some is undergoing rapid endocytosis and possible recycling back to the cellular surface[71,95].

This indicates two fronts on which exogenously added galectin-3 is interacting and potentially driving the observed results. The front involving endocytosis could indicate a signal that endocytosed galectin-3 is transducing[30,147]. We further investigated this by labelling G2 and G6 glycodendrimers with Alexa Fluor 647 to visualize the location of the dendrimers in this assay. The results did not support the colocalization of galectin-3 and dendrimers when endocytosed into the cell with either labelled glycodendrimer. If signal transduction is impacted by the addition of galectin-3 and glycodendrimers, then intracellular colocalization would be expected. Alternatively, intercellular galectin-3 is involved in binding different glycosylated receptors, and the glycodendrimers appear to mitigate this interaction[51].

Galectin-3 domains contribute different roles to the migration results reported here. When the N-terminal domain is not present, the truncated protein only has the capability to bind carbohydrates, and no significant effect is seen in the migration behavior of DU145 cells. This indicates that the N-terminal domain is necessary for the retardation of DU145 migration. Whether this domain only impacts extracellular processes or also impacts endocytosis remains to be investigated[31,71].

Conclusions

The results reported here show that exogenous galectin-3 can impact the migration of cancer cells. Moreover, lactose functionalized dendrimers can significantly mitigate galectin-3 mediated effects on cellular migration. In a modified scratch assay, lactose functionalized dendrimers were shown to restore the galectin-3 inhibited migratory propensity of both DU145 and HT1080 cancer cells. Smaller glycodendrimers (G2 and G3) were more effective than larger glycodendrimers (G4 and G6). Fluorescence micrographs of labeled materials indicate that galectin-3 is localized in the intercellular space and is also readily endocytosed by the cells. The lack of observable colocalization of the internal galectin-3 and internal glycodendrimers suggests that extracellular mechanisms rather than internal signal transduction pathways are targeted by glycodendrimer/galectin-3 aggregates. Reduction of cellular migration is only seen with the intact protein including the N-terminal domain; the CRD alone does not alter cellular migration. By inhibiting the impact of galectin-3 with glycodendrimers, it is clear that multivalency is a potent component of galectin-3's effect on cellular migration. These results show that galectin-3 is pivotally involved in cancer cell migration. In this assay, galectin-3 drives homotypic interactions which anchor cells in their locations. With these results, it is clear that cancer cell migration can be mitigated by carbohydrate functionalized multivalent frameworks such as glycodendrimers that target galectin-3.

Experimental Procedures

Cell Culture

Two immortalized cancer cell lines were selected for analysis based on their robustness and expression of galectin-3. DU 145 (ATCC®: HTB-81™) prostate carcinomas were cultured in media consisting of Dulbecco's modified Eagle's medium (DMEM: Gibco, 61100) with 1.8 g/L $NaHCO_3$ and supplemented with MEM vitamin (Gibco, 11126), Penn/strep (Gibco, 15140), NEAA (Gibco, 11140), EAA (Gibco, 11130) and 10% v/v fetal bovine serum (FBS). HT-1080 (ATCC®: CCL-121™) fibrosarcomas were cultured in media consisting of Dulbecco's modified Eagle's medium (DMEM: Gibco, 61100) with 1.8 g/L $NaHCO_3$ and supplemented with MEM vitamin (Gibco, 11126), Penn/strep (Gibco, 15140), NEAA (Gibco, 11140), EAA (Gibco, 11130) and 5% v/v fetal bovine serum (FBS). Cells were cultured in a humidified incubator with 5% CO_2 and 37 °C. Before confluency, cells were subcultured using 0.25% (w/v) Trypsin- 0.53 mM EDTA.

Expressing and purifying galectin-3 and the CRD

Human galectin-3 plasmid transformed *E. coli* cells were provided by the Avraham Raz lab. The *E. coli* cells were cultured to an OD_{600} = 1.2, and galectin-3 expression was induced by adding IPTG to a final concentration of 0.1 mM. The protein was selected out after microfluidization using Glutathione Sepharose 4B slurry in PBS.

The tether was cleaved using Prescission Protease. The sample was purified by washings, and the ligand was removed by dialysis. The purity of the protein was tested using gel electrophoresis, and the concentration was verified by BCA assay and absorbance at 280 nm[133] where $E^{1\%} = 6.1$ when $A_{280} = E^{1\%} \times C$.

Scratch Assay

When plates reached approximately 80% confluency, complete media was poured off, and the plate was washed with 1X PBS. Plates were covered in 0.25% Trypsin, 0.02% EDTA solution and were incubated for 5-10 minutes at 37°C to detach cells from the plate. Cells were gently aspirated using a disposable pipette along with some complete media, collected in a 15 mL falcon tube and centrifuged for 5 min at 2000 rpm. The trypsin/EDTA/complete media solution was poured off, and the cell pellet was resuspended in 1 mL of complete media. 10 µL of this cell solution was transferred to a sterile Eppendorf tube and diluted with 90 µL of media and mixed. 10 µL of this solution was transferred to another sterile Eppendorf tube and combined with 10 µL of Trypan Blue. 10 µL of this cell/dye solution was placed on a hemocytometer, and cells were counted to determine total cell count. Cells were diluted to 700,000 x 10^6 cells/mL in complete media. Inside the well of a 24 well plate, a 2-well culture insert (Ibidi: 80209) was attached to the bottom of the well. 70 µL of the cell solution was aliquoted to each well of a 2-well insert and briefly shaken. The plate was then placed in the humidified incubator with 5% CO_2 and 37 °C until cells adhered to the plate in a mono-confluent layer, about 8-12 hours. The plate was removed from the incubator, and inserts were

gently removed using sterile tweezers. Each well was gently washed with about 1 mL of complete media. Galectin-3 was diluted in PBS to 0.5- 0.6 mg/mL. The CRD was diluted in PBS to 0.6 mg/mL. The total volume in each well was 250 μL. Dendrimers were functionalized as previously reported[82]. Dendrimers and protein were aliquoted into respective wells to a final concentration of: CRD = 0.397 mg/mL, 28.3 μM, galectin-3 = 0.243 mg/mL, 7.35 μM, G2 = 94 μM, G3 = 56.6 μM, G4 = 20.64 μM, G6 = 10.34 μM. Concentrations of monomeric lactose: 1X = 0.848 mM, 2X = 1.696 mM, 3X = 2.54 mM, 4X = 3.39 mM. Plates were then stored in the incubator and removed every 2 or 4 hours to take photos of the length of the scratch. Photographs were later stitched together using GIMP2 or ImageJ[148], cropped, and the scratch area was quantified using T-scratch.

Immunofluorescence Microscopy

To replicate the surface that the cells bind to in the scratch assay, slides were cut from a TC 24 well plate and then placed on the bottom of the wells of another 24 well plate. To this slide, an insert was attached, and cells were added in the same method as the scratch assay. After the assay had run for either 4 or 12 hours, the wells were rinsed with whole media and filled with 250 μL of 1:50 MAb (Alexa Fluor 488 conjugated galectin-3 antibody, BioLegend) in whole media and incubated for 1 hour at 37 °C or 4 °C to inhibit endocytosis. The antibody solution was then removed, and the well was covered with 4% paraformaldehyde for 15 minutes. Finally, the well was covered with PBS-T for 3 minutes protected from light. When the PBS-T was removed, the slides were mounted to a No 1 glass cover slip with Prolong Gold with DAPI and allowed to cure

overnight at RT. When cured, the edges were sealed with clear nail polish and the samples were stored at 4 °C until they could be visualized by fluorescent microscopy on a Zeiss 510 inverted confocal microscope.

Alexa Fluor 647 labeled lactose functionalized dendrimer

1 mg of Alexa Fluor 647 Hydrazide was dissolved in 500 µL Millipore water to make a 2 mg/mL solution. Generations 2 and 6 lactose functionalized dendrimers were dissolved in Millipore water to make a 2 mg/mL solution. To the dendrimer solution was added 2 equiv. of $NaIO_4$ per dendrimer, and the mixture was stirred at RT for 2 hours. After 2 hours, Alexa Fluor 647 solution was added at 1 equiv. per dendrimer and allowed to react for 30 min at RT. The reaction mixture was then dialyzed against Millipore water in 1 kDa MWCO dialysis membrane (Spectrum Laboratories, Inc., 6 Spectra/Por Dialysis Membrane) with the water replaced every hour to three hours and then left overnight. The dialyzed mixture was then lyophilized to dryness and reconstituted in PBS to afford a 2 mg/mL solution. Characterization of labeling was conducted using a UV-Vis spectrophotometer by measuring the absorbance at 650 nm, and concentration of dye was calculated using the extinction coefficient of 250,000 cm^{-1} M^{-1} giving a labeled G6-AF647 to dendrimer ratio of 1:1.

CHAPTER FOUR

LACTOSE FUNCTIONALIZED DENDRIMERS INFLUENCE GALECTIN-3 MEDIATED ANGIOGENESIS

Introduction

Angiogenesis is a highly regulated process that tumors utilize in later stages of cancer progression. Embryogenesis extensively utilizes this process of elongating tubes and creating appropriate branching of vasculature. Angiogenesis is sometimes turned on in wound healing, hypoxia and female reproductive cycling, but it is always highly regulated and only activated for a short period of time. In contrast, the process of angiogenesis is continually being activated by tumors[37].

Vasculature is important to tissues because it supplies necessary oxygen and nutrients but also serves as a waste clearance route. When a solid tumor has reached a certain size, usually 1 mm^3, the normal vasculature is no longer sufficient to provide nutrients for the tumor[149]. But there is increasing evidence of angiogenesis playing a part in cancer progression much earlier than expected[37]. When a so called "angiogenesis switch" is flipped, the cancer cells can send out signals which can bind to their respective receptors on the surface of endothelial cells (Figure 26A, Figure 26B). When this occurs, the endothelial cells are activated and begin neovascularization, which includes proliferation and migration[150] (Figure 26C, Figure 26D). After formation of the new capillaries, recruitment and interaction with pericytes control the matrix reassembly and stabilize the newly formed vasculature. There are some signals that are proangiogenic

stimulators such as Vascular Endothelial Growth Factor (VEGF) and Basic Fibroblast Growth Factor (bFGF), and can be sequestered in the extracellular matrix (ECM) until liberated by matrix degrading proteases[37]. There are also anti-angiogenic signals, such as Thrombospondin-1 (TSP-1), which need to be outweighed before an angiogenic response can initiate. Because of the constant stimulation of these endothelial cells by cancer signals, the resulting vasculature is less refined and regulated than healthy endothelial activation. This abnormal vasculature allows for enhanced permeability and retention (EPR) and has become a common target of therapeutic research[76].

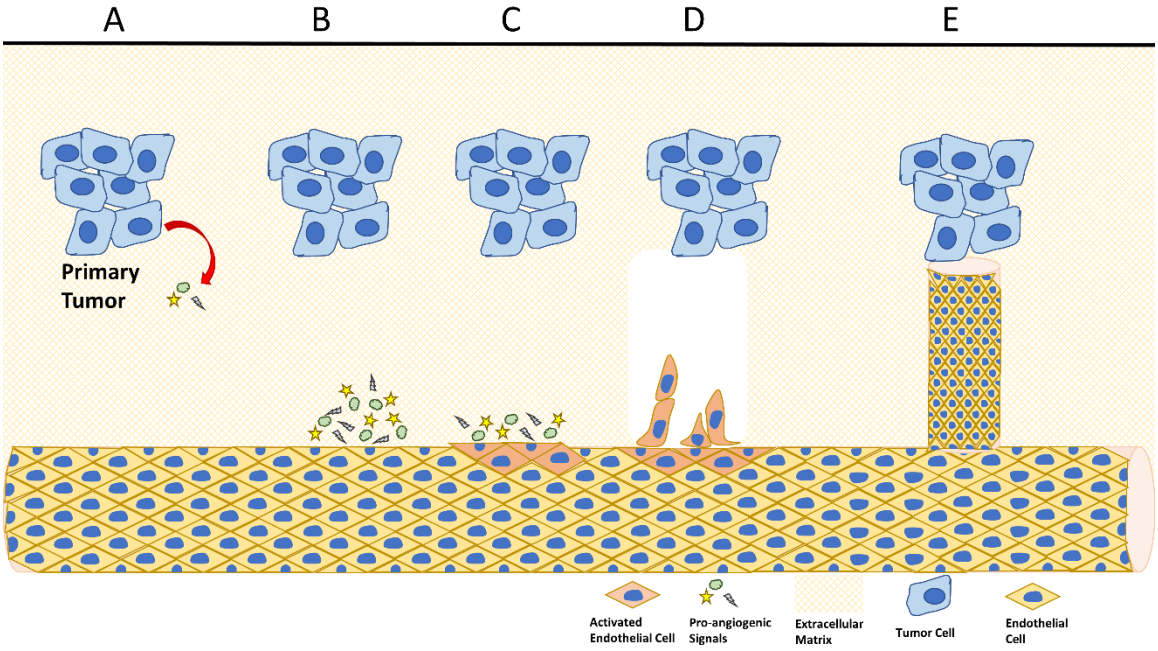

Figure 26. The angiogenic cascade. When a tumor reaches approximately 1 mm^2 it no longer has enough nutrients for survival. The "angiogenic switch" is flipped and the tumor sends out pro-angiogenic signals into the surrounding matrix (A) and epithelium (B). When these signals bind to their respective angiogenic receptors (C), internal pathways are activated to begin proliferation and migration toward the signal (D). Proteases are released to degrade the extracellular matrix, and neovascularization brings blood supply to the tumor (E). Adapted from reference[17].

When these proangiogenic signaling molecules bind to receptors such as Vascular Endothelial Growth Factor Receptor 2 (VEGFR2), signal transduction turns the cytoplasmic domain of VEGFR2 into a tyrosine kinase domain which initiates a cascade of signals leading to endothelial cell gene expression, cell proliferation, increased cell adhesion, cell migration, and actin remodeling[68]. Based on this method of signal transduction, there are some main points of attack for targeted therapies. Some molecules, such as Aflibercept, have been designed to bind the extracellular domain of VEGFR2 and inhibit further signal transduction by natural ligands. There are also tyrosine kinase inhibitors that act to mitigate the kinase and pathway cascade of VEGFR2. These VEGRF2 targeted drugs have been approved for treatment and are effective in combination with chemotherapy[68]. Other treatments such as Pegaptanib (Macugen) target circulating VEGF-A and bind selectively to the heparin binding domain, inhibiting the bioactivity of VEGF-A as a signaling ligand[68].

Galectins 1, 3, and 8 modulate angiogenesis through interactions with different cell surface receptors. For example, galectin-8 induces endothelial migration through interaction with the CD166 receptor[136]. Galectin-3 has been reported to bind to VEGFR2 and increase surface retention time, which increases cytosolic phosphorylation initiating the angiogenic cascade[67]. In normal angiogenesis, VEGFR2 is endocytosed to balance the signal initiation that it provides. Occasionally, particular galectins can have stimulatory and inhibitory effects that are concentration dependent. Exogenous galectin-9 can have pro- or antiangiogenic effects depending on the concentration[150]. Galectin-7 has been reported to be necessary in the wound healing process through endothelial cell migration

and proliferation in an *in vitro* model[136]. Furthermore, mice with knocked-out galectin-7 displayed reduced epithelial healing following wounding[136]. Early on, it was reported that chimeric galectin-3 can enhance the process of angiogenesis and that the carbohydrate binding domain is essential to that activity[151]. Further investigations have revealed that the ability of extracellular galectin-3 to be cleaved by Matrix metalloproteases (MMPs) is important to capillary formation. On Matrigel plugs, transfected endothelial cells with either native or uncleavable galectin-3 revealed that galectin-3 cleaved at A^{33} or H^{64} produced greater neovascularization than uncleavable galectin-3[63]. Galectin-3 has also been reported to be valuable in epithelial repair.

Other investigations have led to the discovery of additional angiogenic pathways that galectin-3 impacts. Under hypoxic conditions, galectin-3 can bind to endothelial cells and promote JAG1/Notch signaling which activates angiogenesis[70]. The importance of galectin-3 in capillary formation by endothelial cells has been demonstrated by blocking that formation through the use of antibodies and lactose[18]. Galectin-1 has also drawn attention for its part in angiogenesis. Galectin-1 is similar to galectin-3 in that it contains a carbohydrate binding domain but does not contain the unstructured N-terminal domain. Galectin-1 is of the class of prototypical galectins containing one binding domain with the ability to form a homodimer (Figure 27D).

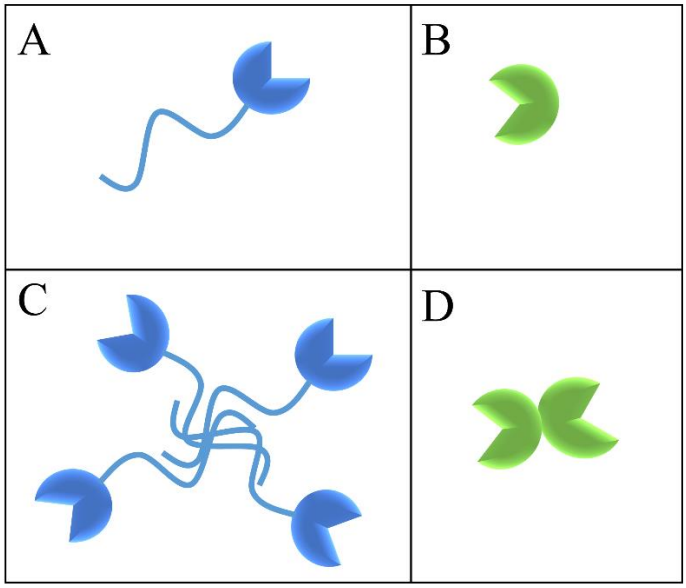

Figure 27. Schematic representations of galectin-3 (A) and galectin-1 (B) and their potential self-associations as a multimer (C) and a dimer(D).

Researchers found that, in galectin-1 knocked-down endothelial cells, migration was completely restored by application of exogenous galectin-1[64]. They further reported that application of exogenous galectin-1 significantly increased phosphorylation of Raf, Mek and Erk. This leads to enhanced Ras signaling and cell proliferation[64]. Similar results have been reported in our own lab revealing that exogenous galectin-1 significantly enhanced capillary formation in endothelial cells[152].

Much of the enhanced angiogenic activity of galectins is dependent upon extracellular carbohydrate binding and oligomerization. We propose that lactoside functionalized dendrimers will serve as a competitive binding partner for galectin-3 that will impact the downstream effects of neovascularization observed in galectin-3 mediated capillary formation. Previous reports from our lab have shown the success of lactose

functionalized PAMAM dendrimers to impact galectin-1 induced capillary formation[152]. These experiments represent an important step in understanding and interrupting the interactions that lead to neovascularization.

Results

Capillary Formation Assay

To assess initiation of angiogenesis, a capillary formation assay was used. Human Umbilical Vein Endothelial cells (HUVECs) were used as a model to monitor capillary formation. A 96 well plate was coated with 4.5 mg/mL Matrigel and allowed to polymerize at 37 °C. Cell suspensions were prepared with combinations of galectin-3 (10 µg/mL), glycodendrimers and a challenge media (F12K-CM) so the final volume in each well was 70 µL. Each well contained 50,000 cells and was incubated for 5 hours before image acquisition on an inverted phase contrast microscope. Images were analyzed using Image J Angiogenesis Analyzer (NIH) for nodes, junctions and length, to describe the extent of capillary formation (Figure 28). Length is the total pixel length of segments, elements and branches; a node is a pixel with three neighbors; a junction is a group of nodes. These elements of the captured image contain three particular attributes of capillary formation: the branching of tubes, the connections to tubes, and the total length of the new tubes.

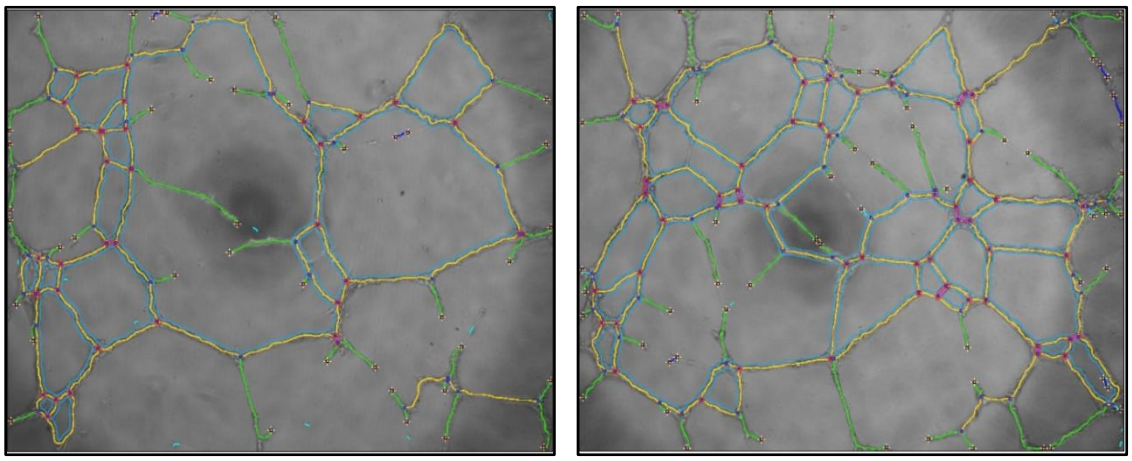

Figure 28. Representative images of Angiogenesis Analyzer in HUVEC with 10 µg/mL exogenously added galectin-3 (B) or untreated (A). Yellow tracing indicates a tube, dark blue circles are nodes and red circle around the nodes are junctions.

When galectin-3 was added exogenously to an activated cell suspension, more extended capillary formation was visible (Figure 28). Two different concentrations of galectin-3 were assessed for greatest impact on capillary formation, and three different timepoints were used to determine the time of greatest significance. It was determined that 10 µg/mL of galectin-3 after 5 hours of incubation gave the most reproducible and most significant results. Quantifying the number of pixels of nodes, junctions and length of capillary formation produced quantification of results in a significant manner (Figure 29). Based on these results, it was determined that exogenous galectin-3 at concentrations of 10 µg/mL produced the most significant augmentation of capillary formation. This is in accordance with other published data, which states that 10 µg/mL exogenous galectin-3 was able to increase capillary formation[65,66,151]. Results within our lab have shown that galectin-3 is able to bind up and cluster cell surface receptors in cancer cells, and this same activity may be a galectin-3 mediated effect seen in this capillary formation assay[93].

When extracellular galectin-3 binds to extracellular glycoreceptors and clusters them, this may expose other previously concealed receptors that are involved in angiogenic signaling. This could support the previously mentioned research where galectin-3 can bind to VEGFR2 and increase cell surface retention time and therefore increase signaling[67].

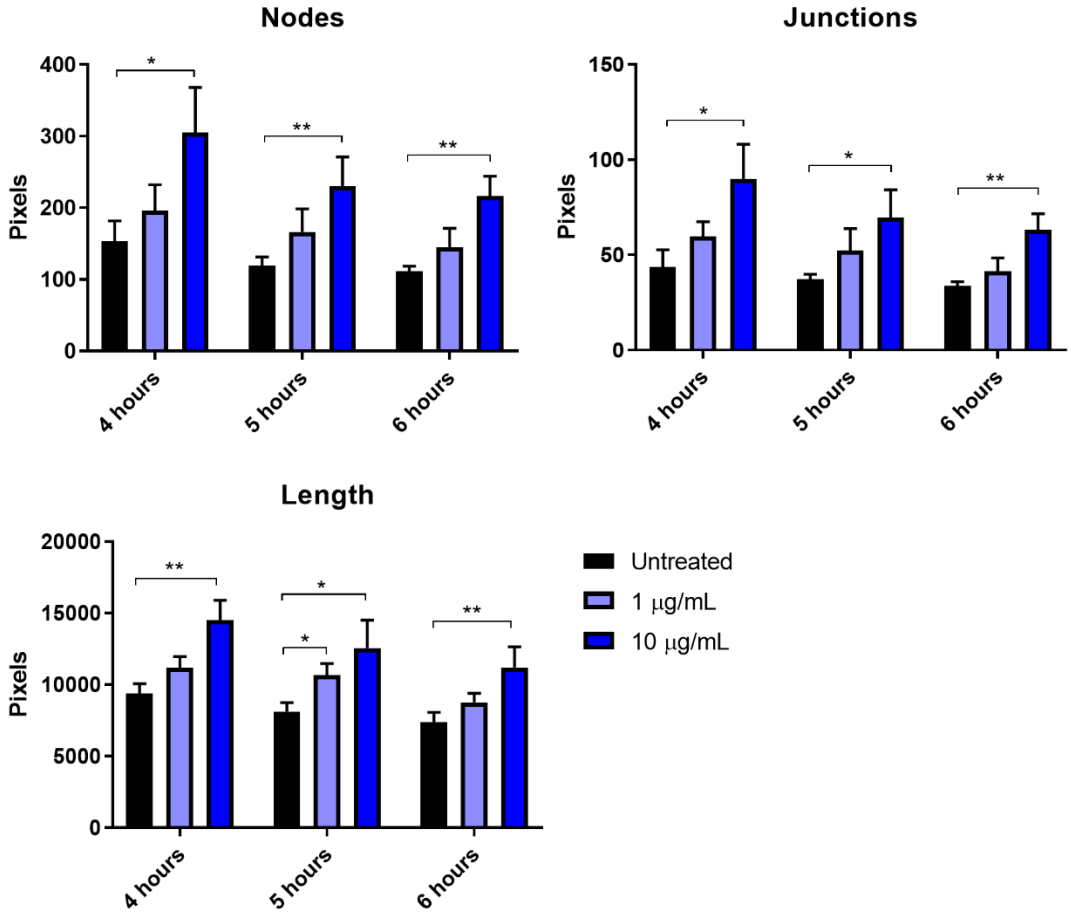

Figure 29. Pixel count of nodes, junctions and length of untreated HUVEC, or with 1 µg/mL galectin-3, or 10 µg/mL galectin-3 after 4, 5, and 6 hours of incubation. P-values were determined using an unpaired t-test using Welch's correction (* = $p < 0.0332$).

Since addition of exogenous galectin-3 augments capillary formation, next we determined whether the addition of a multivalent binding partner, lactose functionalized dendrimers, would significantly affect these results. Lactose functionalized dendrimers were added in concentrations of increasing factors of 10 to HUVECs incubated with 10 μg/mL galectin-3. The concentrations of dendrimers that were chosen were at similar ratios of galectin-3/lactose to the ratios that were shown to be effective in the previous migration experiment (Chapter 3).

When G2 lactose functionalized dendrimers were added in addition to exogenous galectin-3, lower concentrations of dendrimer appear to assist in galectin-3 mediated formation of nodes, junctions and tube length (Figure 30: pink bars). These results show that G2 lactose functionalized dendrimers augment galectin-3 induced capillary formation in this assay. Higher concentrations of G2 glycodendrimer (3.2 μM, 32 μM) do not elicit the same effects. This might be attributed to lower concentrations of G2 accessing a more monomeric-like carbohydrate effect while this pathway is not accessible with higher concentrations of G2 which elicit a more overwhelmingly multivalent effect.

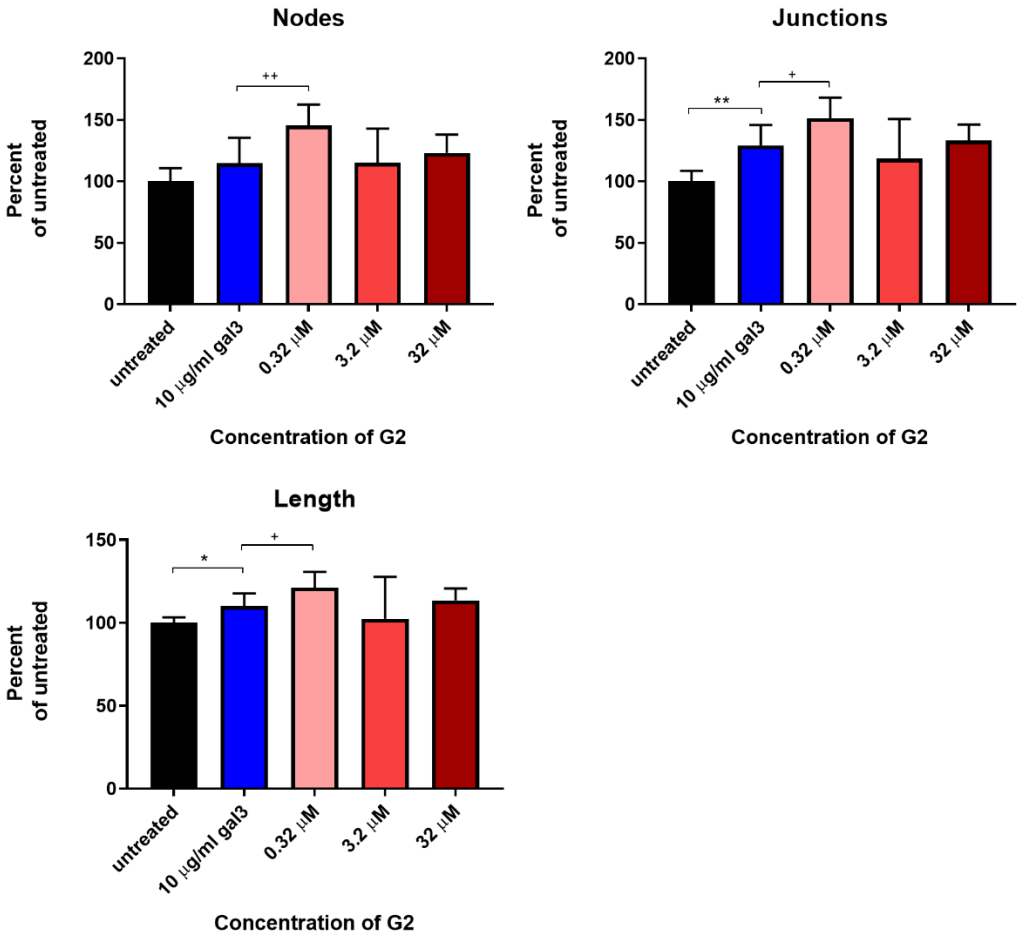

Figure 30. Capillary formation of HUVECs incubated in the presence of 10 µg/mL galectin-3 and G2 lactose functionalized dendrimer or untreated cells. Images of capillary formation were acquired after 5 hours of incubation. Nodes, junctions and length calculated as a percent of the untreated cells. Number of replicates: n = 4-8. P-values were determined using an unpaired t-test using Welch's correction (* = $p < 0.0332$).

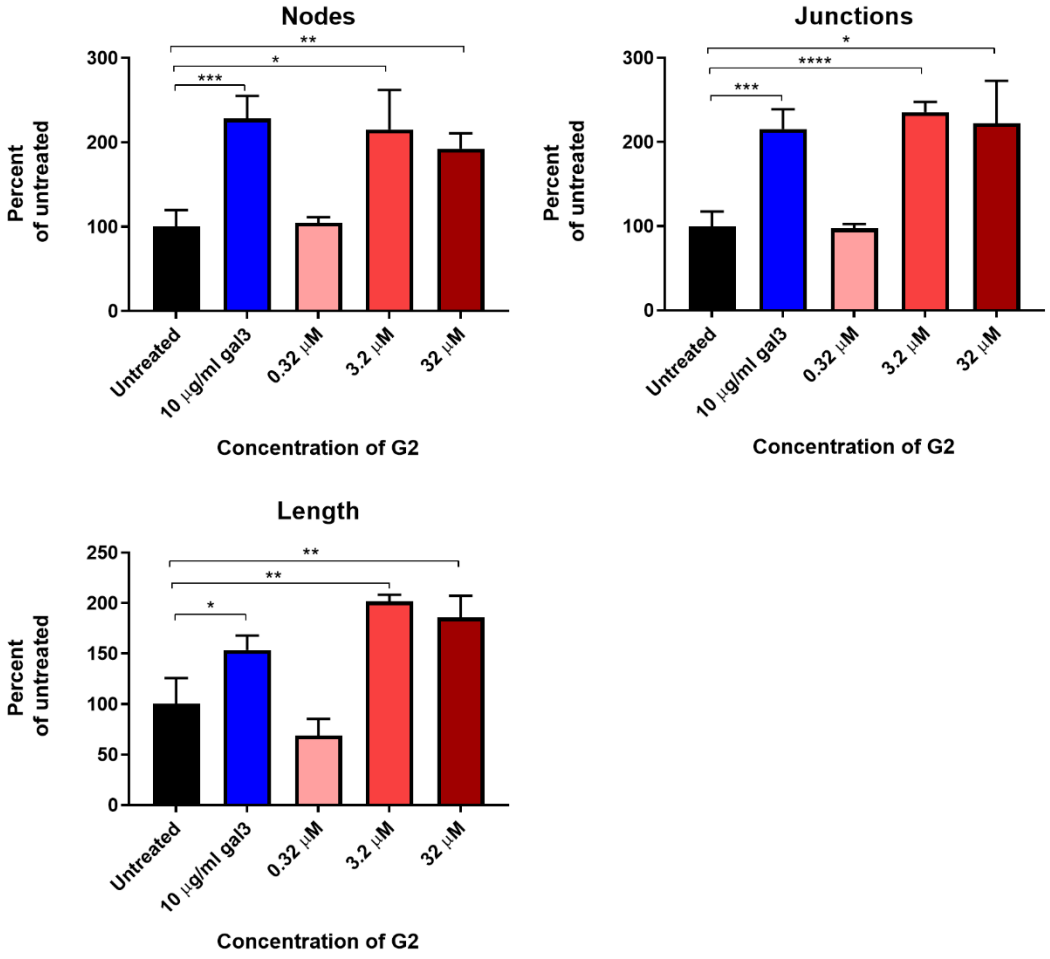

Figure 31. Capillary formation of HUVECs as quantified by extent of nodes, junctions and length after 5 hours of incubation with G2 lactose functionalized dendrimers. Images acquired after five-hour incubation with components. Number of replicates: n =2-4. P-values were determined using an unpaired t-test using Welch's correction (* = $p < 0.0332$).

To determine if lactose functionalized dendrimers were interacting with a galectin-3 mediated process or if they interact with other receptors, the same experiment was done without exogenously added galectin-3. When G2 lactose functionalized dendrimer was added in the smallest concentration, neovascularization was significantly lower than the control with exogenously added galectin-3 (Figure 31: light red bars).

Higher concentrations of G2 (3.2 μM, 32 μM) showed capillary formation that was significantly greater than the untreated control (Figure 31: red and dark red bars). This might be explained by the fact that a small amount of endogenous galectin-3 is present in these endothelial cells[66,153]. Small concentrations of G2 are not present with high enough quantities of galectin-3 to have any effect on neovascularization and thus the results show the capillary formation similar to the untreated control. But when greater concentrations of G2 are added, their quantities are large enough that they can undergo some level of crosslinking with the endogenous galectin-3, therefore mediating vasculature formation. The two highest concentrations of G2 (3.2 μM, 32 μM) showed significantly greater nodes, junctions and length than the untreated control. This would appear to agree with previous results where smaller amounts of G2 mediated vasculature formation when additional galectin-3 was added (Figure 30). In this experiment with only endogenous galectin-3 to interact with, larger concentrations of G2 are necessary to augment capillary formation.

G3 lactose functionalized dendrimers were tested in the same manner to assess their impact on galectin-3 mediated capillary formation. After 5 hours of incubation, there was a significant increase in nodes, junctions and length of tubes in cells treated with galectin-3 and G3 lactose functionalized dendrimers (Figure 32: green bars).

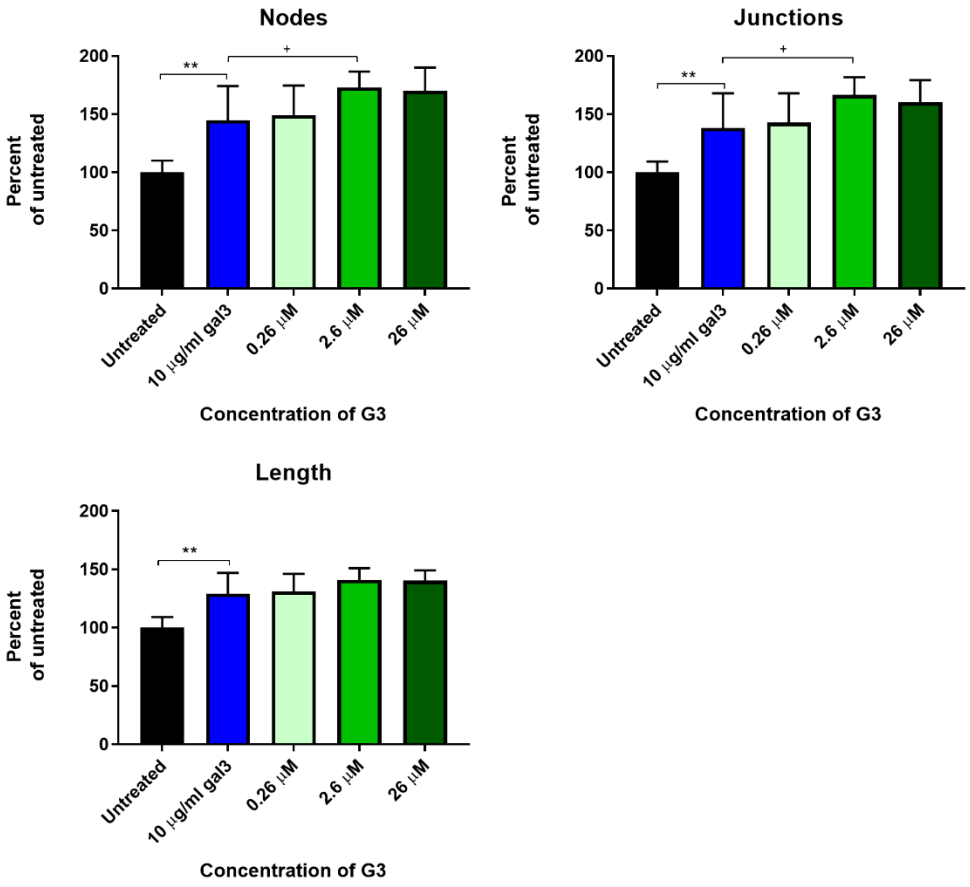

Figure 32. Capillary formation of HUVEC in the presence of 10 µg/mL galectin-3 and G3 lactose functionalized dendrimers as a percentage of untreated cells after 5 hours of incubation. Number of replicates: $n \leq 8$. P-values were determined using an unpaired t-test using Welch's correction (* = $p < 0.0332$).

G3 lactose functionalized dendrimers also showed interesting results in augmenting neovascularization when galectin-3 was added exogenously (Figure 32). However, when no additional galectin-3 was added and G3 glycodendrimer was added, capillary formation was not significantly different than the untreated control even at the highest concentrations tested (Figure 33: green and dark green bars). Only the lowest

concentration of G3 showed a slight increase in nodes, junctions and length from the untreated control (Figure 33: light green bars).

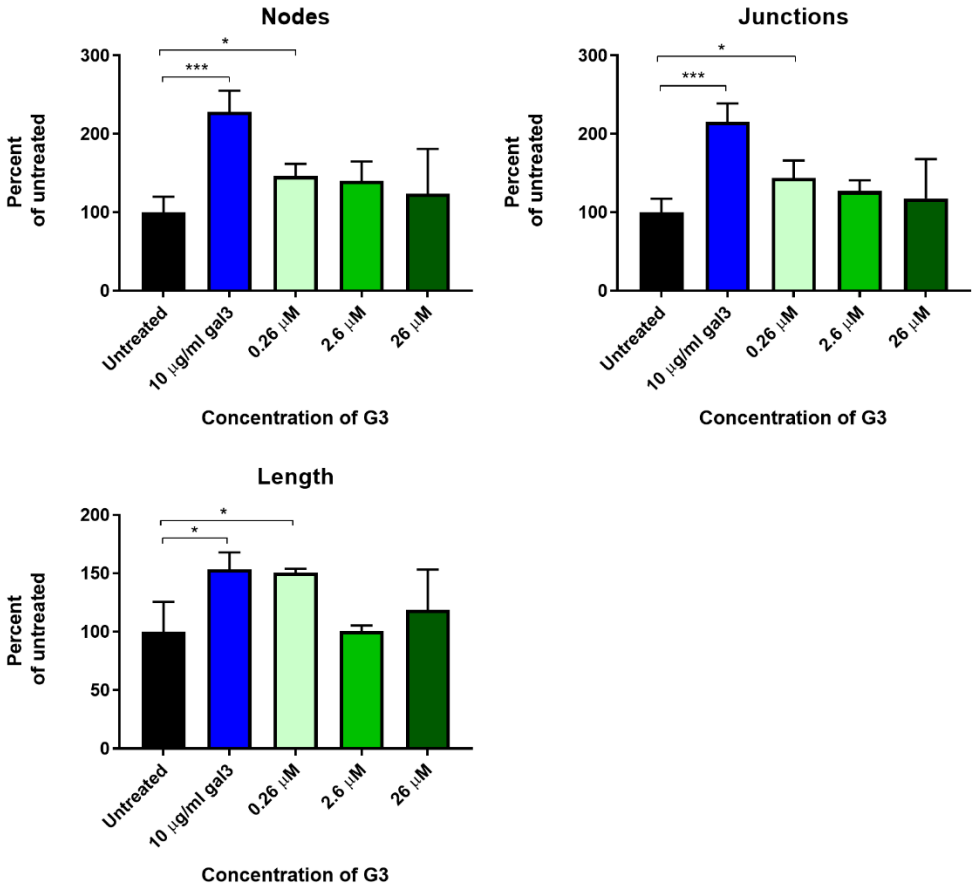

Figure 33. Neovascularization of untreated HUVECs with G3 lactose functionalized dendrimer after five hours of incubation. Results presented with nodes, junctions and length as a percentage of the untreated control. Images acquired after five-hour incubation with components. Number of replicates: $n \leq 4$. P-values were determined using an unpaired t-test using Welch's correction (* = $p < 0.0332$).

These results of augmented neovascularization of HUVECs in the presence of galectin-3 and lactose functionalized dendrimers, G2 and G3, may be partially explained by sterics and size. G2 and G3 are the smallest of the generations of lactose

functionalized dendrimers that were studied, and they present fewer lactosides per dendrimer. G2 is functionalized on average with 12 lactosides, and a G3 on average displays 15 lactosides per dendrimer. These smaller sizes and lactoside loadings may facilitate cross-linking between galectin-3 and glycoreceptors which could be useful for capillary formation. The differences in concentrations of G2 and G3 that augment galectin-3 mediated capillary formation may indicate that a highly specific lactoside and multivalent display is required for mediating galectin-3 induced capillary formation. Additionally, there are reports of carbohydrates eliciting angiogenic effects on endothelial cells[154–156]. These smaller glycodendrimers, G2 and G3, might be able to bind to these receptors, such as CD44, with their lactosides and exhibit carbohydrate dependent effects. This could elicit an alternative signaling effect and pathway that could enhance capillary formation. In untreated cells, a lower concentration of G2 may be able to access these receptors and these pathways. A larger concentration of G3 is required in untreated cells because this larger dendrimer would not be expected to be able to efficiently fit to access these receptors.

In untreated cells, greater concentrations of G3 glycodendrimer may bind more galectin-3, but since only endogenous galectin-3 is present, this might not be sufficient to mediate receptor cross-linking. Therefore, capillary formation is reduced to that of the untreated control. Because G3 glycodendrimers are larger than G2, they might also be too large to fit into the space required to crosslink glycosylated receptors involved in capillary formation. These results may also indicate a “tipping point” at which concentration of glycodendrimer acts more to “grab up” extracellular galectin-3 and not

be involved in crosslinking. This argument may indicate G2 as an ideal multivalent partner to facilitate galectin-3 mediated crosslinking of glycosylated receptors, such as integrin αvβ3 and VEGFR2, therefore, slightly larger dendrimer G3 may only be able to minimally facilitate crosslinking and therefore does not show much impact when exogenous galectin-3 is not present[66,67]. Other results from our lab have shown that G3 is the dendrimer most highly taken up into cancer cells, this may indicate higher rates of endocytosis which could affect capillary formation.

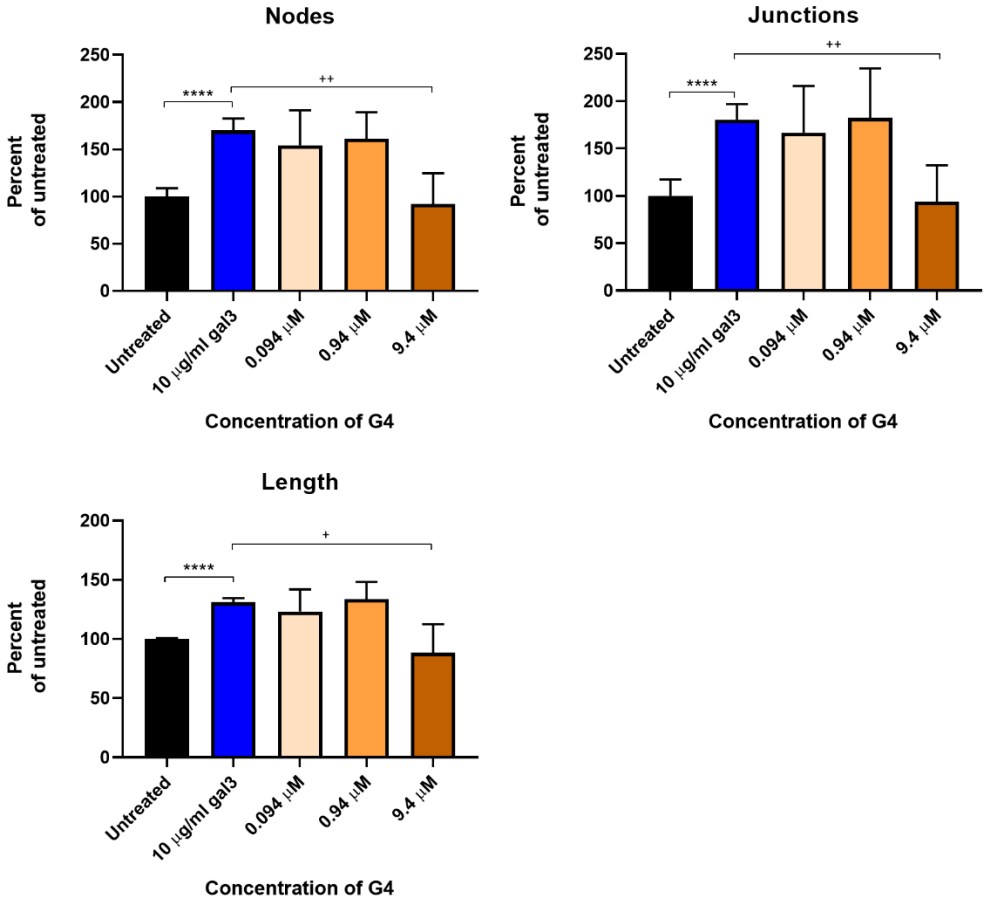

Figure 34. Capillary formation of HUVECs in the presence of 10 µg/mL galectin-3 and G4 lactose functionalized dendrimer after five hours of incubation normalized to the results for untreated cell nodes, junctions and tube length. Number of replicates: $n \leq 8$. P-values were determined using an unpaired t-test using Welch's correction (* = $p < 0.0332$).

When G4 lactose functionalized dendrimers were added to HUVECs incubated with exogenous galectin-3, high concentrations of dendrimer reduced capillary formation (Figure 34: dark orange bars). The formation of nodes, junctions and tube length were similar to untreated cells when high concentrations (9.4 µM) of G4 lactose functionalized dendrimer was added with exogenous galectin-3 to HUVECs.

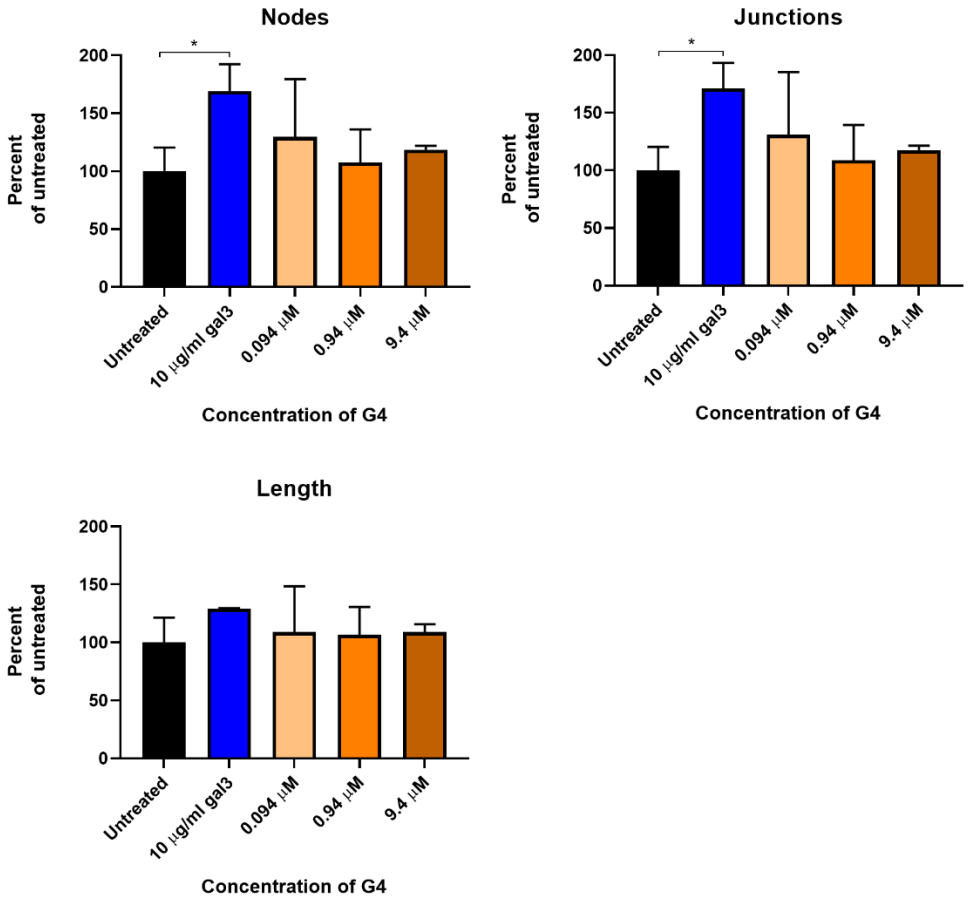

Figure 35. Capillary formation in untreated HUVECs with G4 lactose functionalized dendrimer after 5 hours of incubation. Results presented as a percentage of untreated cells in terms of nodes, junctions and length. Images acquired after five-hour incubation with components. Number of replicates: $n \leq 4$. P-values were determined using an unpaired t-test using Welch's correction (* = $p < 0.0332$).

When G4 lactose functionalized dendrimers were incubated with HUVECs over the course of five hours, there was no significant difference in nodes, junctions and tubes in the vasculature compared to untreated cells (Figure 35: all bars). Since the greatest concentration of G4 with exogenously added galectin-3 decreased neovascularization, it agrees that when exogenous galectin-3 is not present, there is not enough galectin-3

present to have an angiogenic impact from G4 dendrimers alone. Therefore, the results do not show any effect from G4 and endogenous galectin-3.

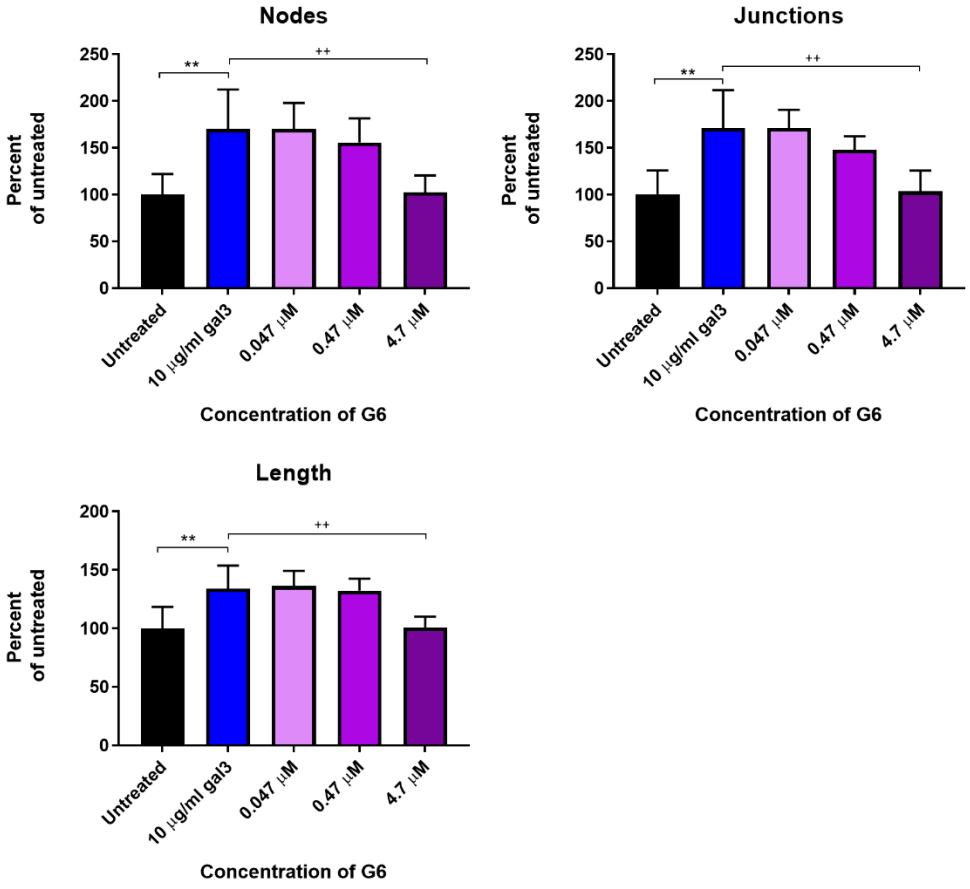

Figure 36. Capillary formation of HUVECs in the presence of exogenous galectin-3 (10 µg/mL) with G6 lactose functionalized dendrimer. Results calculated as a percent of untreated cells in nodes, junctions and length of tubes. Images acquired after five-hour incubation with components. Number of replicates: $n \leq 8$. P-values were determined using an unpaired t-test using Welch's correction (* = $p < 0.0332$).

When G6 lactose functionalized dendrimers were added to HUVECs treated with galectin-3, inhibition of capillary formation was also observed in a significant manner

(Figure 36: dark purple bars). The highest concentration of G6 glycodendrimer with galectin-3 reduced capillary formation to that of untreated cells.

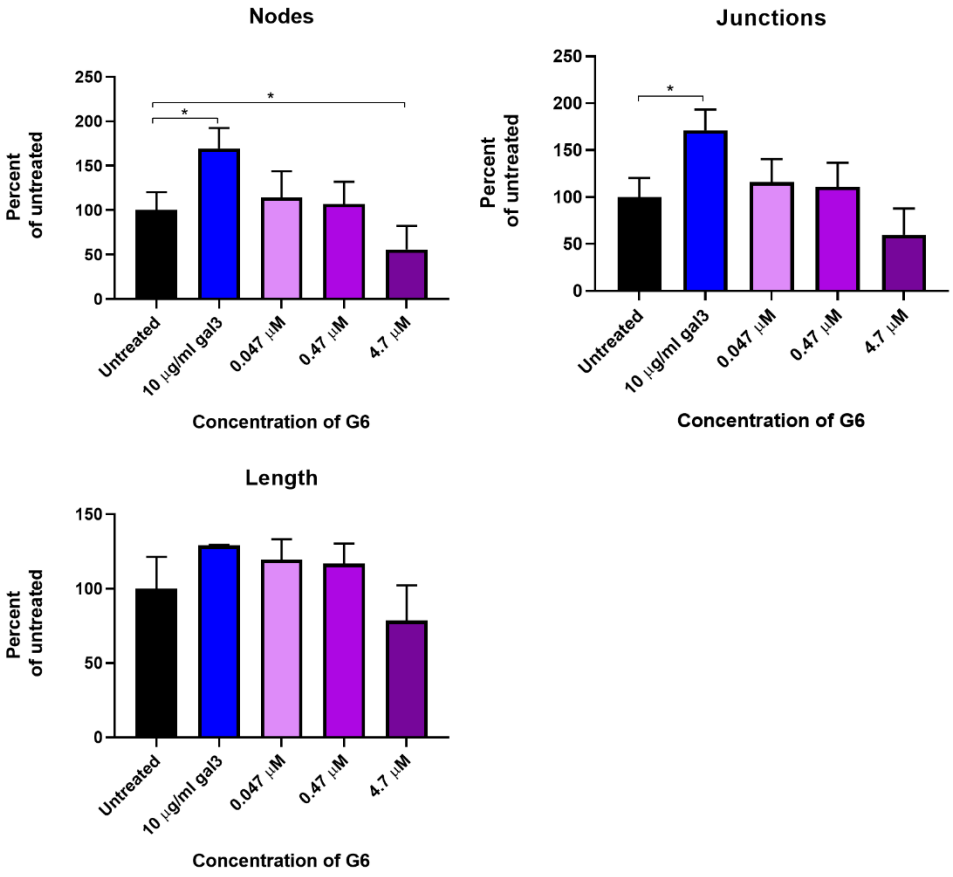

Figure 37. Extent of nodes, junctions and length in untreated HUVECs after 5 hours incubation with G6 lactose functionalized dendrimer as a percentage of untreated cells. Number of replicates: $n \leq 4$. P-values were determined using an unpaired t-test using Welch's correction (* = $p < 0.0332$).

When G6 was added without exogenous galectin-3, only the highest concentration showed decreased node formation from the untreated control (Figure 37: Nodes, dark purple bar). Even though junctions and length don't show a significant difference from

the untreated control, there is an obvious inverse relationship between addition of higher concentrations of G6 and capillary formation. Since this relationship was also observed when galectin-3 was added exogenously, we can hypothesize that G6 is the most effective at mediating and decreasing capillary formation in these endothelial cells from galectin-3 mediated neovascularization. G6 effectively binds and subverts galectin-3 whether exogenous or endogenously sourced.

These results indicate that the larger G4 and G6 glycodendrimers have a significantly different effect on capillary formation than the smaller G2 and G3 glycodendrimers. G4 glycodendrimers on average display 41 lactosides, and a G6 is functionalized on average with 82 lactosides. This assay is performed such that the concentration of lactose remains constant across the dendrimer generations, thus causing the concentrations of the glycodendrimers to vary across the generations. Therefore, this difference between observed effects for small and large glycodendrimers is not due to differences in lactose concentration. One rationale for the differences could be that sterics are impactful; even though G4 and G6 glycodendrimers have more lactosides per dendrimer, some lactosides are likely to be displayed in a sterically inaccessible manner. This would be especially true when multiple galectin-3 proteins are bound to a glycodendrimer. Inaccessibility of some of the lactosides on the large glycodendrimers could decrease the effective binding of galectin-3 in a manner that facilitates crosslinking between galectin-3 and glycoreceptors. Another likely explanation is that the size difference in the dendrimers plays a critical role even before considering the accessibility of the lactosides. G2 and G3 glycodendrimers are most similar in molecular weight and

both have open, amorphous structures. G4 and G6 glycodendrimers both are larger, more spherical macromolecules. These larger glycodendrimers bind galectin-3, but perhaps their larger size less effectively supports crosslinking of galectin-3 to angiogenic receptors. This would lead to an effect where extracellular galectin-3 is sequestered by G4 and G6 dendrimers, “distracting” galectin-3 from crosslinking activity.

Immunofluorescence

To further investigate the impact these exogenous factors have on inducing capillary formation in the assay, fluorescent labeling was done. G2 and G6 glycodendrimers were functionalized with AlexaFluor 647 in a 1:20 and 1:2 dye to dendrimer to dye ratio, respectively. After 4 hours of incubation with dye functionalized dendrimer and 10 µg/mL galectin-3, AlexaFluor 488 tagged galectin-3 monoclonal antibody (Biolegend) at 1:100 in PBS was added and incubated for another hour. Samples were then fixed with cold methanol for 10 minutes, covered with mountant with DAPI and imaged on a Zeiss Meta 510 laser confocal microscope. Untreated cells and cells incubated with exogenous galectin-3 were first analyzed to confirm endogenous and exogenous detection of galectin-3.

Both the untreated cells and the cells treated with 10 µg/mL galectin-3 revealed fluorescence rimming the edge of cell groups (Figure 38A, B). When exogenous galectin-3 was added (Figure 28), it did not appear that there was much fluorescence rimming the edges of individual cells, as seen in a previous immunofluorescent experiment (Figure 19). While galectin-3 was revealed to be covering the surface of the cell groups, the areas

of budding showed greater fluorescence than other areas of the cell sample. This suggests that galectin-3 is most important for the sprouting and support of endothelial cell migration.

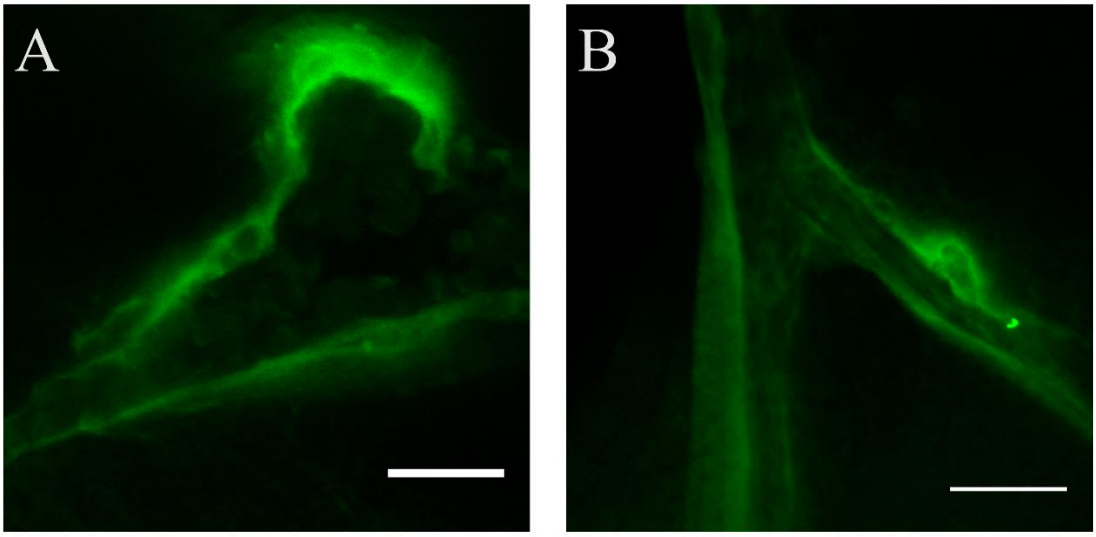

Figure 38. Immunofluorescence of HUVEC undergoing neovascularization in untreated (A) or cells treated with 10 µg/mL of galectin-3 (B) after 5 hours of incubation. Galectin-3 visualized by anti-galectin-3-AF488. Scale bar 50 µm.

To assess what impact the dendrimers had on the capillary formation results, fluorescently tagged G2 and G6 were used. The two lowest concentrations of G2 from the capillary formation experiment were used (0.32 µM, 3.2 µM) since with exogenously added galectin-3, there was augmentation of capillary formation.

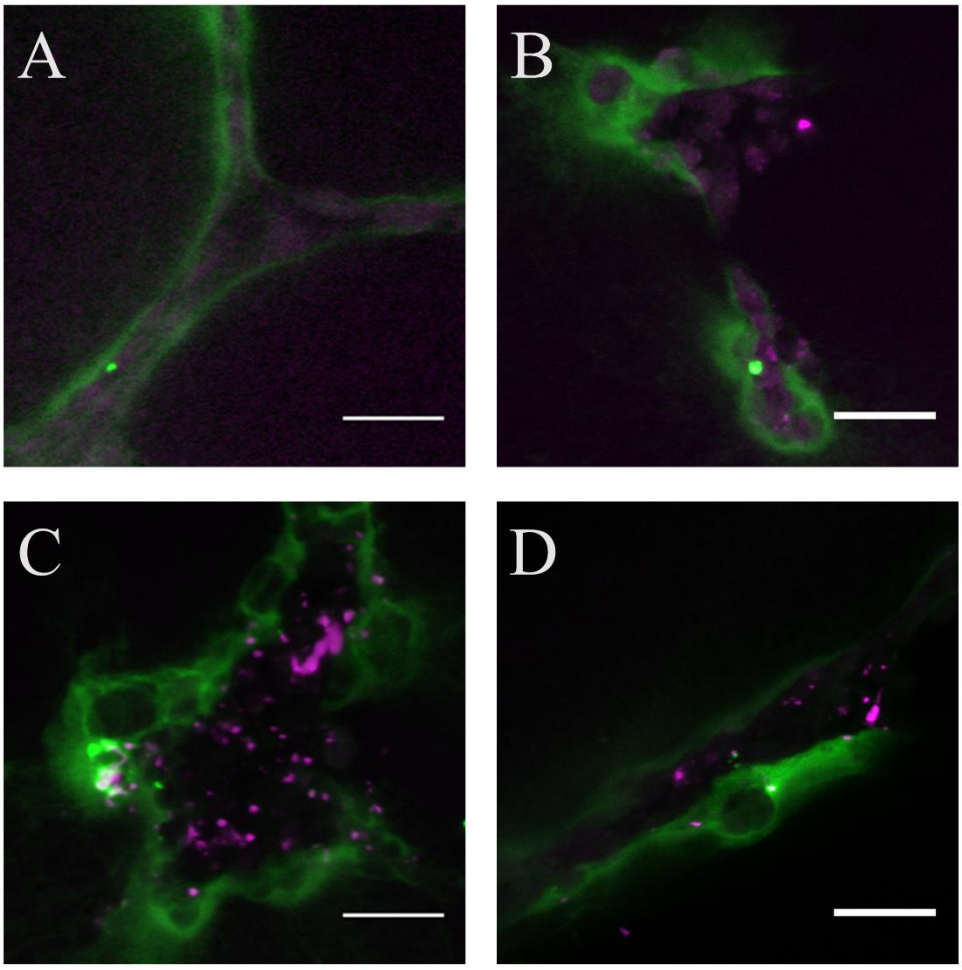

Figure 39. Fluorescent microscopy of HUVECs undergoing neovascularization in the presence of 10 µg/mL galectin-3 and AF647 labeled G2 lactose functionalized dendrimers. 0.32 µM G2 (A), 3.2 µM G2 (B) and 4.7 µM G6 (C, D) after five hours of incubation. Scale bar 50 µm.

Results revealed that G2 tagged dendrimer was taken up into the cell or bound tightly to the cell's surface (Figure 39A, B). The localization of galectin-3 did not appear to vary because of the presence of the dendrimer (Compare Figure 38B to Figure 39A, B). There was no evidence of colocalization of dendrimer with galectin-3. Since lower concentrations of G2 augment neovascularization with exogenous galectin-3, results were

expected to help elucidate that interaction. The results obtained here did not appear to support or disprove the hypothesis that G2 is involved in crosslinking galectin-3 and glycoreceptors.

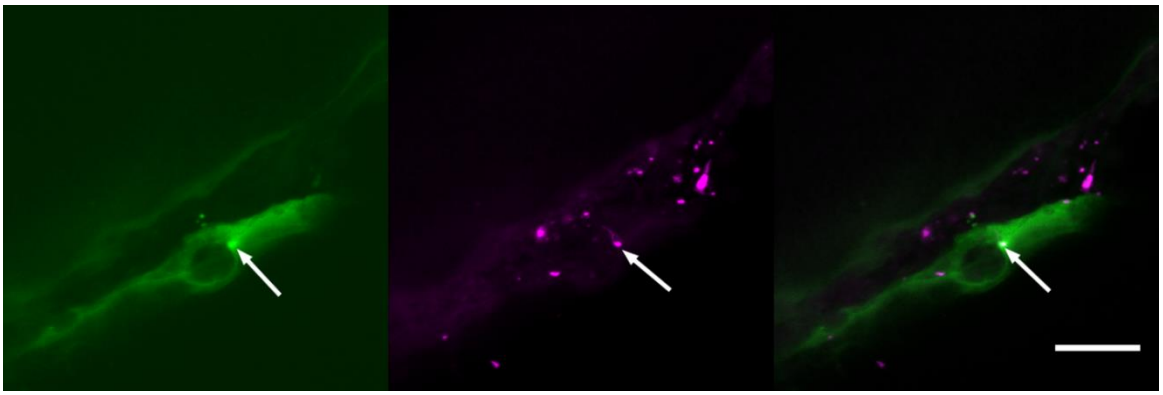

Figure 40. Fluorescent images of HUVEC with 10 µg/mL galectin-3 and 4.7 µM G6-AF647. Arrows added to show area of colocalization. Scale bar 50 µm.

Fluorescent microscopy was also done to determine the localization of G6 and to assess how G6 decreased capillary formation with exogenously added galectin-3. Results showed that G6 is also either taken up by HUVECs or is bound to the cell's surface in aggregates. There was some indication of colocalization of G6 with galectin-3 but only present in a few spots (Figure 40). Localization of galectin-3 still revealed smooth fluorescence on the edge of cell groups. If, as discussed above, G6 acts to negate the effect of galectin-3 in this assay by sequestering galectin-3 into inactive particles, the glycodendrimer/galectin-3 aggregates could easily have been washed away and would not be detected in the micrographs[82].

Discussion

This capillary formation assay confirmed previous literature that exogenous galectin-3 does significantly augment neovascularization. This may be due to a variety of factors including receptor binding that releases angiogenic cytokines[62]. The Yu group found that the primary endothelial receptors bound by galectin-3 were CD146/MUC18 through N-glycans, and suppressing expression of this receptor inhibited the release of the angiogenic cytokines[61]. Others have reported that galectin-3 binds glycosylated VEGFR2 and increases cell surface retention. In the presence of activating ligands, angiogenesis activation is extended[67]. The results presented here also agree with capillary formation reported from our lab with galectin-1[152].

When lactose functionalized G2 dendrimer was added with exogenous galectin-3, the lowest concentrations augmented galectin-3 mediated capillary formation (Figure 30: pink bars). This was also observed for a ten-fold higher concentration of lactosides from G3 with exogenously added galectin-3 (Figure 32: green bars). Fluorescent results reveal that G2 is endocytosed into endothelial cells or bound to the cells, and there is no significant indication of colocalization of galectin-3 with G2 (Figure 39A, B). These results could indicate that G2 and G3 mediate a “sweet spot” of lactoside concentration for the enhancement of galectin-3 mediated neovascularization (Figure 41). Previously, reported uptake results showed that G3 was taken up more by cancer cells than other dendrimers examined. This endocytosis of G2 and G3 could help to regulate the

extracellular concentration of galectin-3 to an optimal level to augment capillary formation.

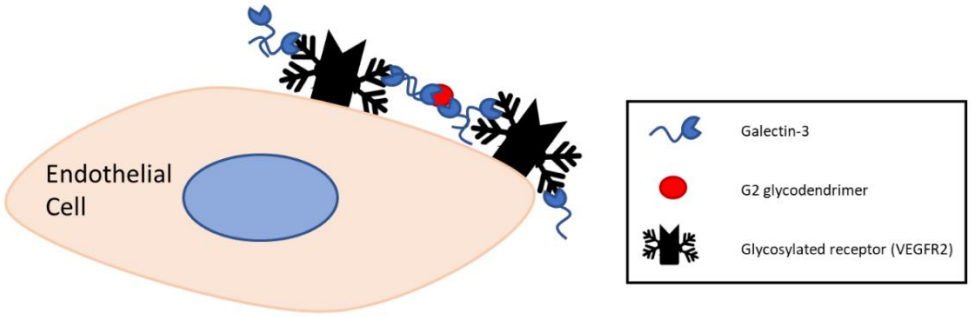

Figure 41. Expected interaction of G2 and G3 dendrimers and galectin-3 with glycosylated extracellular receptors involved in angiogenesis such as VEGFR2. Extracellular interactions lead to extended angiogenic activation.

Control experiments were conducted to confirm a multivalent lactoside effect from the dendrimers on the extent of neovascularization. Monomeric lactose was added in concentrations similar to lactose from a G2 (3.2 μM, 32 μM). With exogenously added galectin-3, capillary formation was augmented in the highest concentration of monomeric lactose used (3.85 mM) (Figure 42: grey bars). These results support our findings with G2 lactose functionalized dendrimers (Figure 30). Similar results with monomeric lactose are expected for processes mediated by specific carbohydrate/protein interactions, but at a higher concentration to confirm that multivalent enhancement occurs using glycodendrimers. The monomeric results might also be explained by a carbohydrate dependent enhancement of capillary formation in endothelial cells. Researchers have

reported that 2'-fucosyllactose induces expression of intercellular adhesion molecule-1 (ICAM-1) and induces release of angiogenic factors like bFGF and VEGF through JAK2 signaling[154]. Other reports have shown that lectin galactoside-binding soluble 3 binding protein (LGALS3BP) enhances angiogenesis through a galectin-3 dependent mechanism by producing VEGF through PI3k/Akt pathways[157]. These results could point to a carbohydrate binding mechanism being involved in the galectin-3 mediated angiogenic process. In the results presented here, this might explain why both the lower concentration of lactose functionalized G2 dendrimer and the lower concentration of lactose monomer enhanced capillary formation whereas higher concentrations in both cases cause inhibition (Figure 30) and (Figure 42: 50 mM)[151]. This would also provide a logical explanation for why G2 without exogenous galectin-3 augments neovascularization. A smaller valency of lactosides can still benefit capillary formation in these endothelial cells.

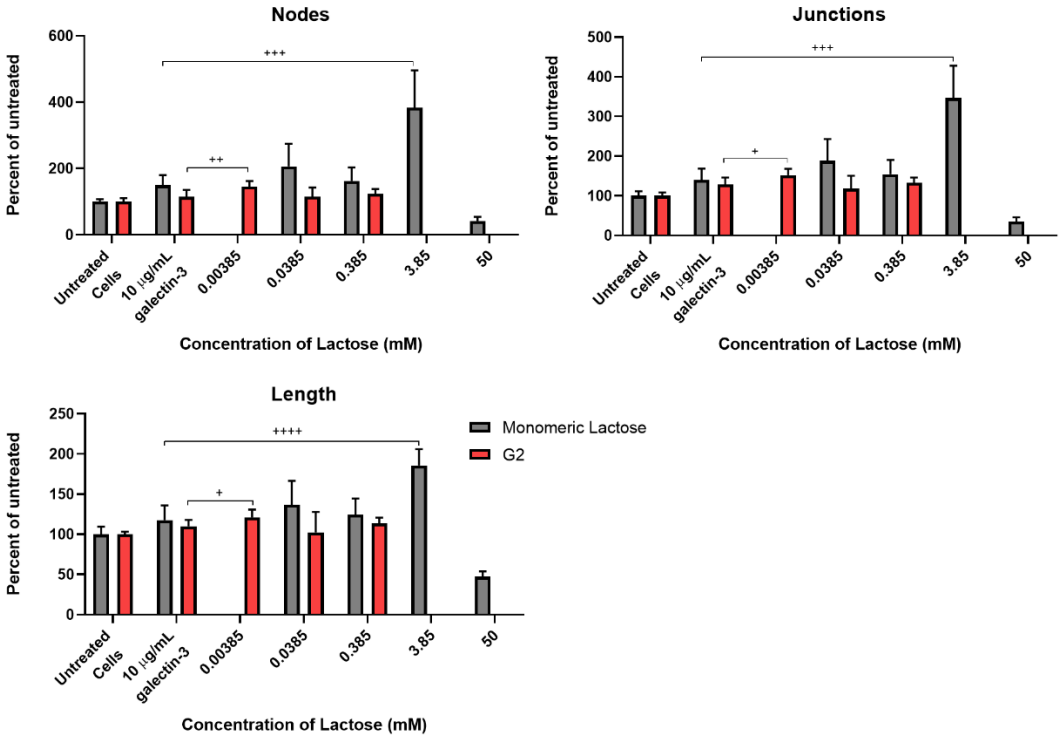

Figure 42. Capillary formation of HUVECs treated with 10 µg/mL galectin-3 and increasing concentrations of monomeric lactose. Concentrations of lactose similar to highest concentrations of lactose from G2 (Figure 30: red and dark red bars). Images taken after 5 hours of incubation. Number of replicates: n ≤8. P-values were determined using an unpaired t-test using Welch's correction (* = $p < 0.0332$).

When G4 was added with exogenous galectin-3, the highest concentration of G4 showed a decrease in capillary formation similar to the untreated control (Figure 34: dark orange bars). But this result was not observed when G4 was present with endogenous galectin-3, there was no change from the untreated control (Figure 35). This result could be explained by nanoparticle formation of G4 and galectin-3 when there are high enough concentrations of galectin-3 available.

Experiments with G6 lactose functionalized dendrimers showed similar trends whether galectin-3 was added exogenously or not. Immunofluorescence showed a few

spots of colocalization (Figure 40). This could be discrete nanoparticles of galectin-3 and G6. This demonstrates that G6, and to a smaller effect G4, sequester available extracellular galectin-3 (either endogenously sourced or exogenously added) and nucleate the formation of aggregates that can be bound to the cell's membrane or could be washed away in the sample preparation before image acquisition (Figure 43).

These results reveal that galectin-3 mediated capillary formation is inhibited by large lactose functionalized dendrimers with multivalently presented lactosides. Extracellular galectin-3 enhances capillary formation of endothelial cells by localizing on the budding areas of cell groupings. This could either point to receptor clustering and signaling, further activation of the endothelial cells, or supporting the migrating front of activated endothelial cells. Glycodendrimers present what appears to be a dual mechanism for impacting neovascularization. When glycodendrimers are present with galectin-3, there can be a reduction of endothelial cell growth or enhancement depending on generation of glycodendrimer. Because the smaller glycodendrimer can also elicit enhancements without exogenous galectin-3, this points to an independent mechanism that is not completely dependent on multivalency since these results are not observed in the other glycodendrimer generations. But small concentrations of smaller glycodendrimers with galectin-3 further enhances capillary formation indicating that there is a sweet spot of support that smaller glycodendrimers can provide to galectin-3 mediated neovascularization. This could potentially be through supporting galectin-3 crosslinking of glycosylated angiogenic receptors. When larger generations of glycodendrimers inhibit galectin-3 mediated neovascularization, this indicates the

effectiveness of the glycodendrimers to interact with and prevent the angiogenic effects of galectin-3. These findings can contribute to the work being done to understand angiogenesis and its implications in cancer development.

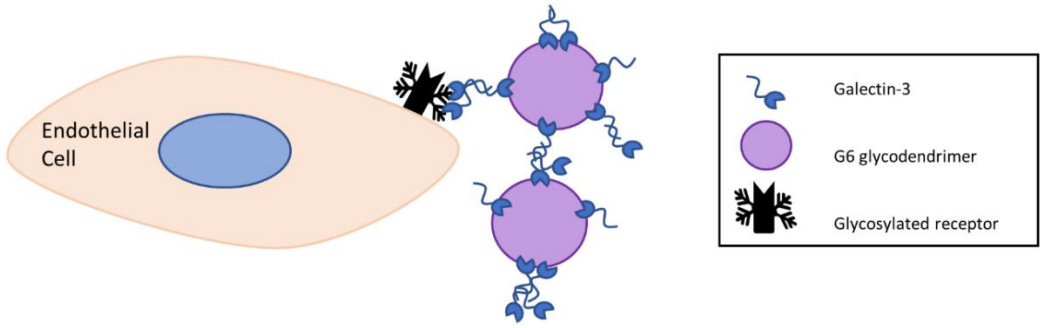

Figure 43. Anticipated interaction of G6 glycodendrimers with extracellular galectin-3 in capillary formation. Glycodendrimers can nucleate nanoparticle formation which can divert galectin-3 from interacting with the glycans on the endothelial cell, leading to decreased activation of neovascularization. Schematic is not drawn to scale.

Conclusions

The findings reported here represent a powerful method to investigate the effects of angiogenesis. Since angiogenesis is usually initiated by extracellular factors, investigations to impede these factors make a positive contribution to the understanding of the role of galectin-3 in the advancement of angiogenesis. Galectin-3 has been named as a mediator of angiogenesis, even at the initial stages. Therefore, inhibitors of galectin-3 significantly affect angiogenesis. Multivalent interactions with galectin-3 have been

shown to be effective in biological contexts. Multivalently presented lactosides interact with galectin-3 and cause different biological outcomes which are particularly important to cancer research. The results here confirm that galectin-3 does augment capillary formation by enhancing nodes, junctions and length of tube formation. Smaller dendrimers such as a G2 and G3 show a small enhancement of capillary formation in galectin-3 mediated tube formation assays at relatively low concentrations of glycodendrimers. Larger dendrimers such as G4 and G6 show inhibition of capillary formation in galectin-3 mediated tube formation assays. These results indicate that galectin-3 does interact with these dendrimers in the context of this capillary formation assay and that a multivalent display of lactosides is effective at interceding in galectin-3 interactions.

These studies with galectin-3 have revealed that it is a powerful inducer of capillary formation. Although its specific purpose has yet to be determined in these studies, this multifunctional protein plays a large role in inducing angiogenic behavior in HUVECs. This function of galectin-3 is predicted to be largely extracellular indicating that the ability of galectin-3 to bind carbohydrates in a multivalent fashion is what drives these resulting functions. From these studies, galectin-3 has been revealed as a powerful target in angiogenic research and further studies to modulate the function of galectin-3 in larger systems must be assessed. Together, these results present effective tools for further understanding the process of angiogenesis through the use of glycodendrimers and galectin-3.

Experimental Procedures

Cell culture

Cells were received from (ATCC® PCS-100-013™) and initially seeded as per manufacturer's instructions in TC treated plates (Fisher Scientific) at 2,500 – 5,000 cells per cm^2. Complete Media was comprised of Vascular Cell Basal Medium (ATCC® PCS-100-030™) supplemented with Endothelial Cell Growth Kit-BBE (ATCC® PCS-100-040™) (Bovine Brain Extract: 0.2% v/v, rhEGF: 5 ng/mL, L-glutamine: 10 mM, Heparin sulfate: 0.75 U/mL, Hydrocortisone hemisuccinate: 1 µg/mL, Fetal Bovine Serum: 2% v/v, Ascorbic Acid: 50 µg/mL) and Penicillin-Streptomycin-Amphotericin B Solution (ATCC® PCS-999-002™) (Penicillin: 10 U/mL, Streptomycin: 10 µg/mL, Amphotericin B: 25 ng/mL). To subculture confluent cells, Trypsin-EDTA for Primary Cells (ATCC® PCS-999-003™) was used to lift cells, Trypsin Neutralizing Solution (ATCC® PCS-999-004™) was used to inhibit further trypsinization. Cells were counted using trypan blue and a hemocytometer.

For use in the microtubule formation assay, F12K-CM was formulated as a challenge media. F12K medium was supplied from Fisher scientific and was supplemented with 0.1 mg/mL Heparin (Sigma), 0.05 mg/mL ECGS (Sigma) and 10% FBS (Atlanta Biologicals).

Capillary Formation Assay

Matrigel (Fisher Scientific) was thawed at 4 °C and diluted to 4.5 mg/mL with PBS. 30 µL diluted Matrigel was plated into each well of a 96 well plate and allowed to polymerize in a 37 °C incubator for at least 30 minutes. Cell suspensions were prepared to add 70 µL to each well. To each well was added 50,000 cells, 10 µg/mL (0.33 µM) galectin-3 and/ or lactose functionalized dendrimers in F12K CM with F12K to 70 µL. Each experiment was placed in four well replicates. After the 70 µL cell suspension was added on solidified Matrigel, the plate was incubated for 5 hours at 37 °C. Images were collected on an inverted scope with a 4X objective. Images were analyzed for nodes, junctions and length using Angiogenesis Analyzer in Image J. Significance was determined using an unpaired t-test using Welch's correction.

Alexa Fluor 647 labeled lactose functionalized dendrimer

1 mg of Alexa Fluor 647 Hydrazide (Fisher Scientific) was dissolved in 500 µL Millipore water to make a 2 mg/mL solution. Generations 2 and 6 lactose functionalized dendrimers[82] were dissolved in Millipore water to make a 2 mg/mL solution. To the dendrimer solution was added 2 equivalents of $NaIO_4$ per dendrimer, and the mixture was stirred at RT for 2 h. After 2 h, Alexa Fluor 647 solution was added at 1 equiv. per dendrimer and allowed to react for 30 min at RT. The reaction mixture was then dialyzed against Millipore water in 1 kDa MWCO dialysis membrane (Spectrum Laboratories, Inc., 6 Spectra/Por Dialysis Membrane) with the water replaced every hour to three hours and then left overnight. The dialyzed mixture was then lyophilized to dryness and

reconstituted in PBS to afford a 2 mg/mL solution. Characterization of labeling was conducted using a UV-Vis spectrophotometer by measuring the absorbance at 650 nm, and concentration of dye was calculated using the extinction coefficient of 250,000 cm^{-1} M^{-1} giving a labeled G6: dye ratio of 2:1. A labeling ratio of G2: dye ratio was determined to be 21:1.

Immunofluorescence Microscopy

To create samples of the capillary formation assay for fluorescent detection, a 15 well µ-slide plate (Ibidi: 81506) was used with 10.6 µL of Matrigel per well at a concentration of 4.5 mg/mL and allowed to polymerize in the incubator for 30 minutes at 37° C. Alexa Fluor 647 conjugated G2 (0.32, 3.2 µM) and G6 (4.7 µM) were added to each well with 10 µg/mL galectin-3 in triplicate. After 4 hours of incubation, the wells were rinsed and filled with 25 µL of 1:100 MAb (Alexa Fluor 488 conjugated galectin-3 antibody, BioLegend) and placed in the incubator for an additional hour. The plate was removed from the incubator, rinsed with PBS and covered with cold methanol for 10 minutes. Methanol was removed, the wells were rinsed with PBS and covered with Prolong Gold with DAPI (Invitrogen) and images were acquired on a Zeiss 510 inverted confocal microscope.

CHAPTER FIVE

SUMMARY AND CONCLUSIONS

Carbohydrate interactions are highly relevant to biological systems, and therefore multivalency is a tool often used to study these systems. Multivalent probes have been reported and used in these chapters. Lactose functionalized PAMAM dendrimers are a valuable tool to probe multivalency in biological systems. Utilizing generations 2, 3, 4 and 6, a variety of properties such as size, three dimensionality, and extent of functionalization with the biologically relevant ligand lactose were assessed for their impact on galectin-3 mediated cancer cellular processes including angiogenesis and migration. These dendrimers interact with oligomeric galectin-3 and do impact cancerous processes *in vitro*.

The reported results here show that these glycodendrimers do not elicit toxic effects on cancerous epithelial cells, nor do they impede viability. With and without exogenously added galectin-3, the glycodendrimers do not induce apoptosis in A549, lung carcinomas. These results demonstrate the potential of these glycodendrimers to interact with cells multivalently while not eliciting off-target effects.

Also reported here, exogenous galectin-3 retards migration of DU145 prostate carcinomas and HT1080 fibrosarcoma cells. When the glycodendrimers are added, a restored migration rate is observed for the cells. This indicates that the glycodendrimers are effectively able to interact with exogenous galectin-3 and reverse the galectin-3 induced retardation of migration. Further investigations have revealed that endocytosis of

galectin-3 and the glycodendrimers may play a role in the observed effects, but it is not evident that colocalization of the receptor and multivalent ligand occurs intracellularly. Results using truncated galectin-3 have also shown that the N-terminal domain is necessary for the retardation effects elicited by galectin-3. Taken together, these results show that lactose functionalized dendrimers are an effective interactor with galectin-3 even to the point of mitigating galectin-3 impacted cellular migration. These studies have also revealed the large impact of extracellular galectin-3 on the coordinated process of migration. As a protein with implications in many processes and pathways, these results have determined that in high concentrations, galectin-3 can modulate cancer chemotaxis through restricting migration.

The final results presented here investigate the multivalent impact of galectin-3 and glycodendrimers in a capillary formation assay. Exogenously added galectin-3 was shown to significantly increase capillary formation in activated human umbilical vein endothelial cells (HUVECs). Generations 2 and 3 of the glycodendrimers were able to augment this galectin-3 mediated neovascularization. Generations 4 and 6 of the glycodendrimers inhibited galectin-3 mediated capillary formation. Further investigations showed that glycodendrimers alone were sufficient to impact neovascularization. Generations 2 and 3 enhanced capillary formation in untreated HUVECs. Generation 6 of the glycodendrimers inhibited capillary formation in HUVECs and generation 4 had no impact in untreated HUVECs. Fluorescent labeling revealed endocytosis or close association of the glycodendrimers with the HUVECs and rimming of galectin-3 around a cell group. These studies have revealed that extracellular galectin-3 has large implications

in a highly regulated process such as angiogenesis. This agrees with other reports that the multifunctional protein galectin-3 can impact another important cancer process. Taken together, these results show that multivalent glycodendrimers can impact even a signaling mediated cellular process such as angiogenesis through interaction with oligomeric galectin-3.

The part played by galectin-3 in the mediation of these cancer processes further implicates it as a valuable target in the progression of cancer research. Since many proteins are highly specific in their function, galectin-3 presents a unique and ubiquitous target for cancer research. Since galectin-3 has been implicated in numerous additional cancerous processes, targeting this protein for future therapeutic research may present the ability to inhibit many cancerous pathways through one target.

This thesis describes the results of a promising multivalent interactor for the multitude of glycan mediated cellular events, not limited to galectins. Further investigations could uncover other extracellularly mediated glycan events that drive other cellular processes for investigations into immune research, diseases and aging. These results are likely to inspire multivalent research as a tool against many of the health problems we are confronted with as a society.

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APPENDIX A

Primary Data

Apoptosis Assay Data

	1	2	3	4	5	6	7	8	9	10	11	12	
A													400,505
													485,520
B		34212	113115	118352	98683	171119	16715	44896	29495	29559	25659		400,505
		3810	114628	105744	103023	66288	2243	7009	7618	12300	6224		485,520
C		14617	109120	129448	81786	158305	14910	42671	28401	30521	26415		400,505
		2648	100682	57980	120065	69000	2191	6896	7662	13924	6464		485,520
D		13942	109066	115895	79118	126325	10910	29247	21939	19102	16050		400,505
		2645	119258	58055	76938	73146	2051	6087	8058	12004	5438		485,520
E		26561	55179	82890	84846	53433	8003	15219	13147	16426	12904		400,505
		303023	142256	82918	65575	34638	2419	5079	1808	11281	3186		485,520
F		25920	68578	80354	102394	50092	7110	13156	21312	12863	10724		400,505
		296967	136461	53558	70302	37287	2378	4791	6704	10047	2775		485,520
G		23865	57837	78191	69769	48040	7003	12911	20931	13542	10167		400,505
		269407	181742	55038	60934	22563	2354	4690	6541	10094	2605		485,520
H													400,505
													485,520

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		SDS	G3 gal3	G4 gal3	G2 gal3	PBS	SDS	G3 gal3	G4 gal3	G2 Gal3	PBS	
C		SDS	G3 gal3	G4 gal3	G2 gal3	PBS	SDS	G3 gal3	G4 gal3	G2 Gal3	PBS	
D		SDS	G3 gal3	G4 gal3	G2 gal3	PBS	SDS	G3 gal3	G4 gal3	G2 Gal3	PBS	
E		Ionomyc	Staurosp	G6 gal3	Gal3		Ionomyc	Staurosp	G6 gal3	Gal3		
F		Ionomyc	Staurosp	G6 gal3	Gal3		Ionomyc	Staurosp	G6 gal3	Gal3		
G		Ionomyc	Staurosp	G6 gal3	Gal3		Ionomyc	Staurosp	G6 gal3	Gal3		
H												

Figure 44. Plate reading for A549 cells with added reagents after 5 hours of incubation. Viability measured at 400_{ex} 505_{em}. Toxicity measured at 485_{ex} 520_{em}.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		32	442	531	518	551	38	59	38	47	36	
C		33	470	364	481	508	41	47	46	48	52	
D		98	492	572	440	624	45	53	56	53	68	
E		92	1661	517	497	708	63	57	41	54	57	
F		108	1427	532	555	633	78	58	47	48	53	
G		112	1034	533	398	654	64	51	54	53	60	
H												

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		SDS	G3 gal3	G4 gal3	G2 gal3	PBS	SDS	G3 gal3	G4 gal3	G2 Gal3	PBS	
C		SDS	G3 gal3	G4 gal3	G2 gal3	PBS	SDS	G3 gal3	G4 gal3	G2 Gal3	PBS	
D		SDS	G3 gal3	G4 gal3	G2 gal3	PBS	SDS	G3 gal3	G4 gal3	G2 Gal3	PBS	
E		Ionomyc	Staurosp	G6 gal3	Gal3		Ionomyc	Staurosp	G6 gal3	Gal3		
F		Ionomyc	Staurosp	G6 gal3	Gal3		Ionomyc	Staurosp	G6 gal3	Gal3		
G		Ionomyc	Staurosp	G6 gal3	Gal3		Ionomyc	Staurosp	G6 gal3	Gal3		
H												

Figure 45. Plate reading for A549 cells with added reagents after 5 hours of incubation. Luminescence readings indicative of apoptosis.

Scratch Assay Data

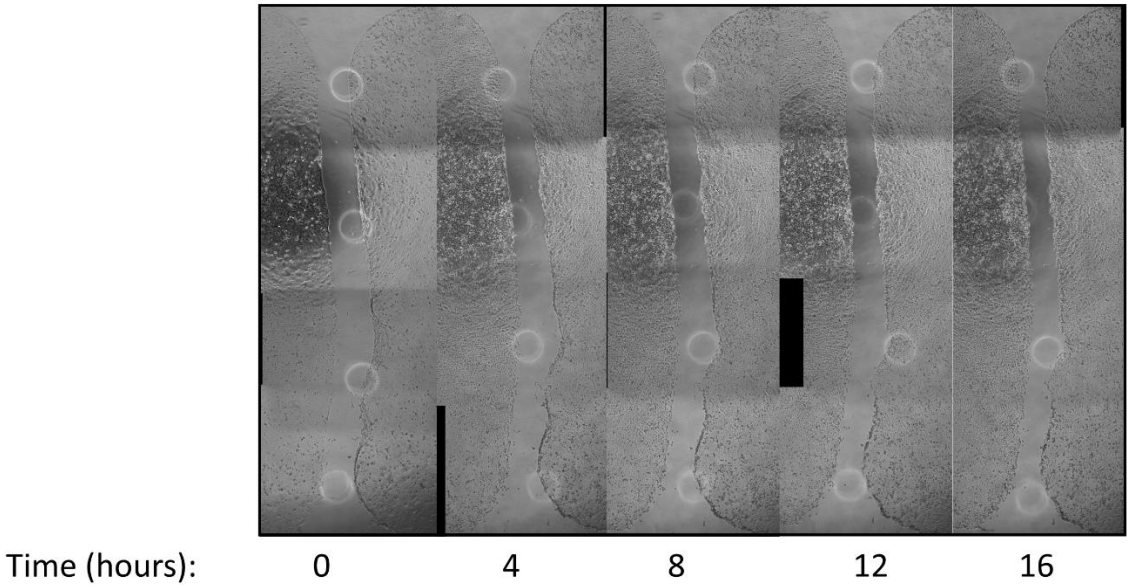

Figure 46. DU145 migration into the scratch over 16 hours with exogenously added galectin-3.

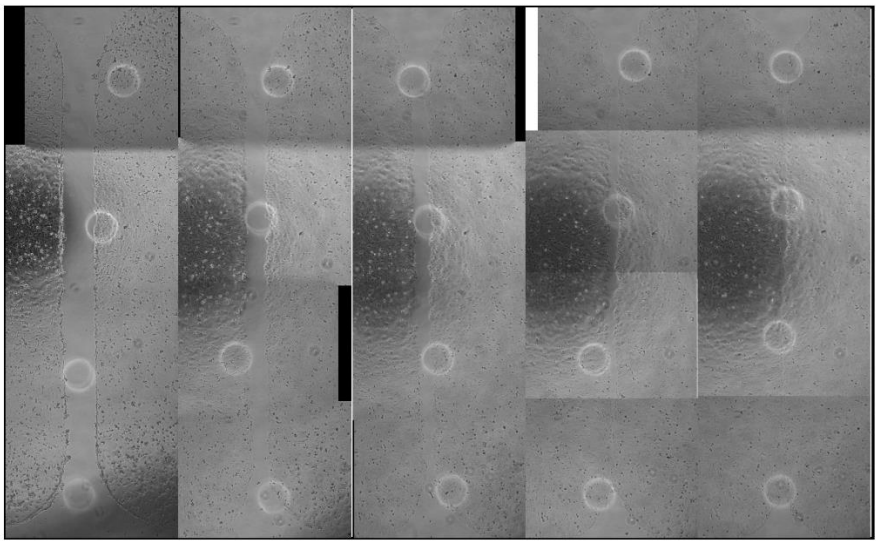

Figure 47. DU145 migration into the scratch over 16 hours with PBS.

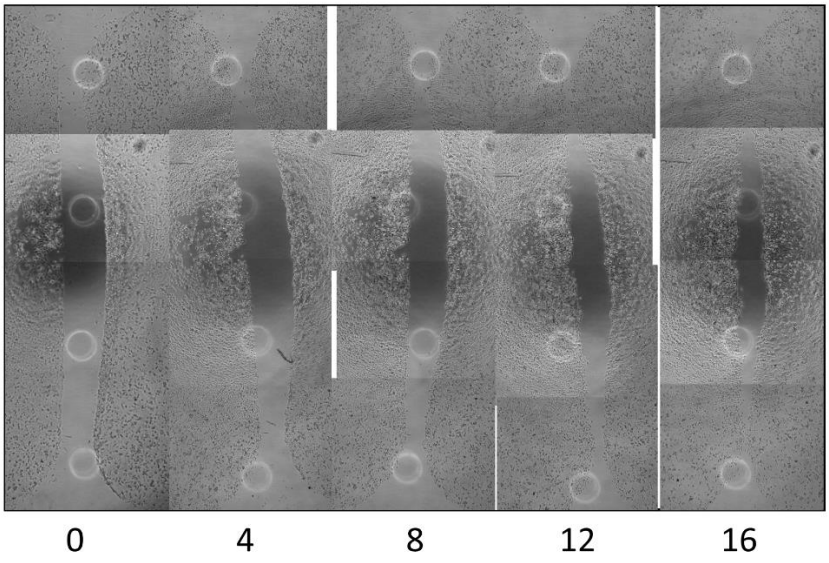

Figure 48. DU145 migration into the scratch with exogenous galectin-3 and G2.

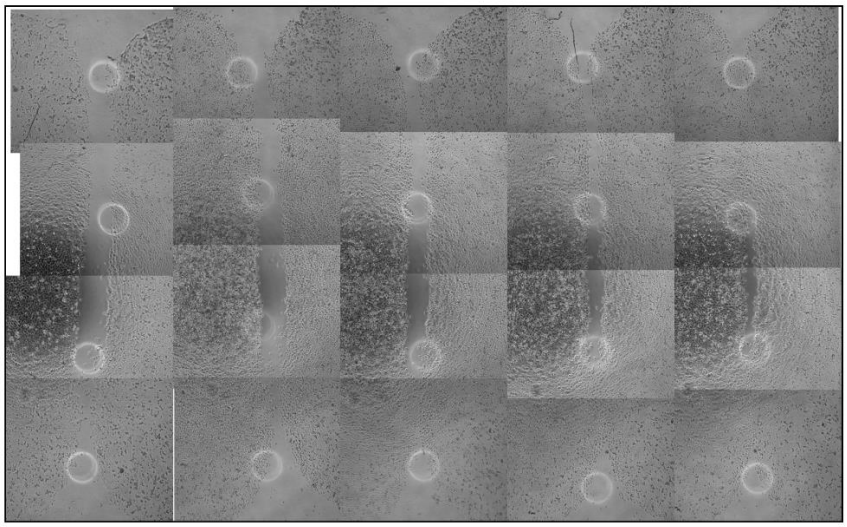

Figure 49. DU145 migration into the scratch with galectin-3 and G3.

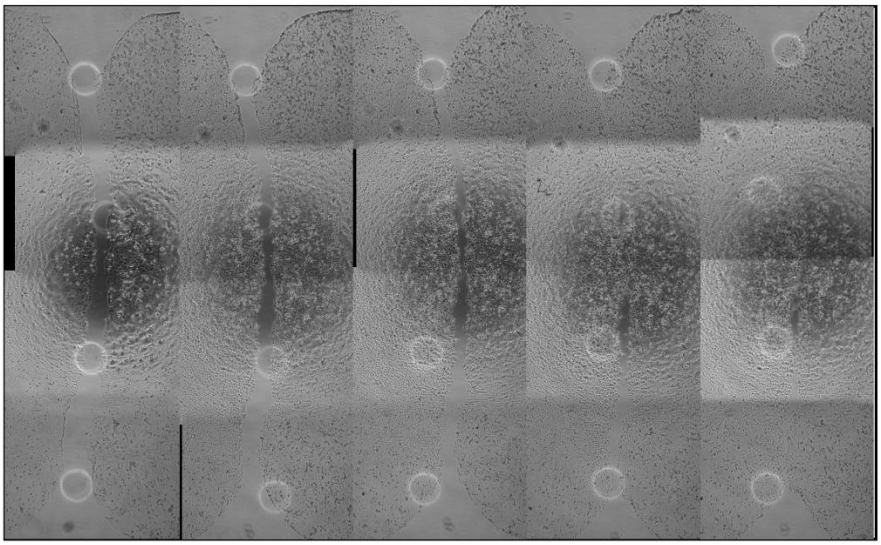

Figure 50. DU145 migration with galectin-3 and G4.

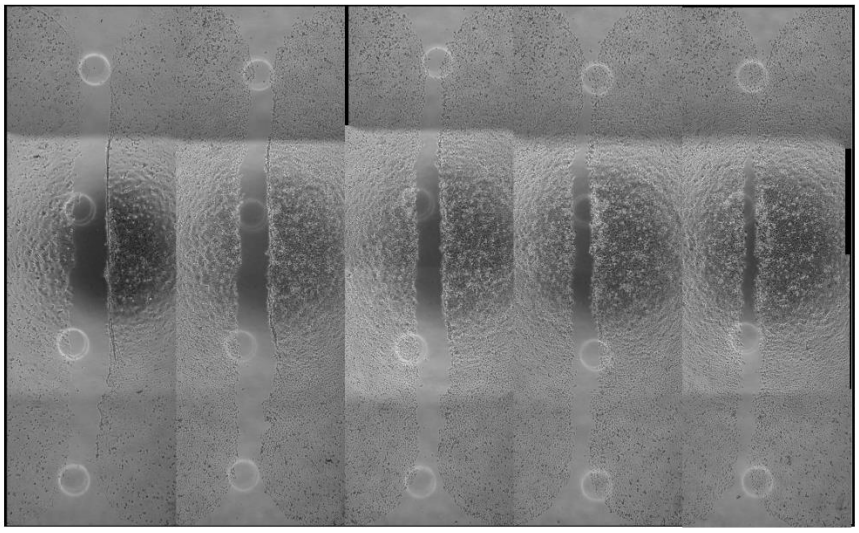

Figure 51. DU145 migration with galectin-3 and G6.

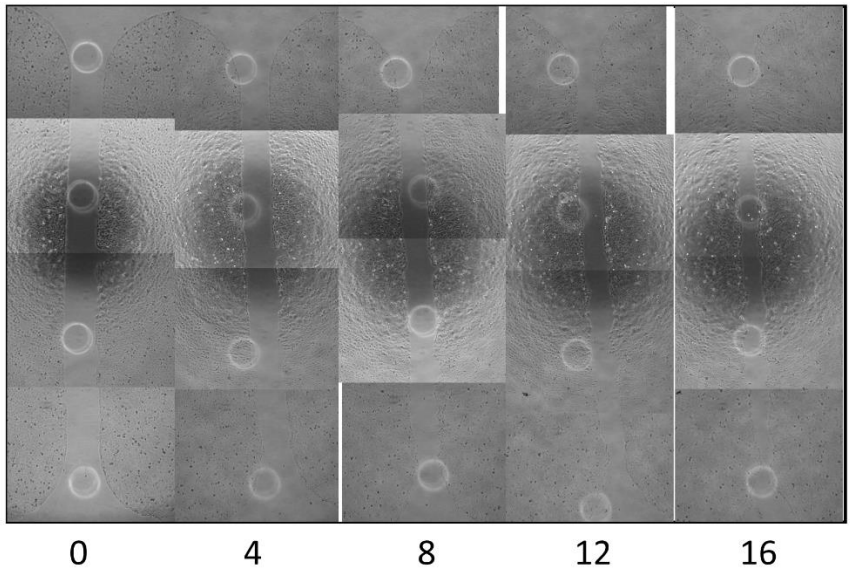

Figure 52. DU145 migration with G2.

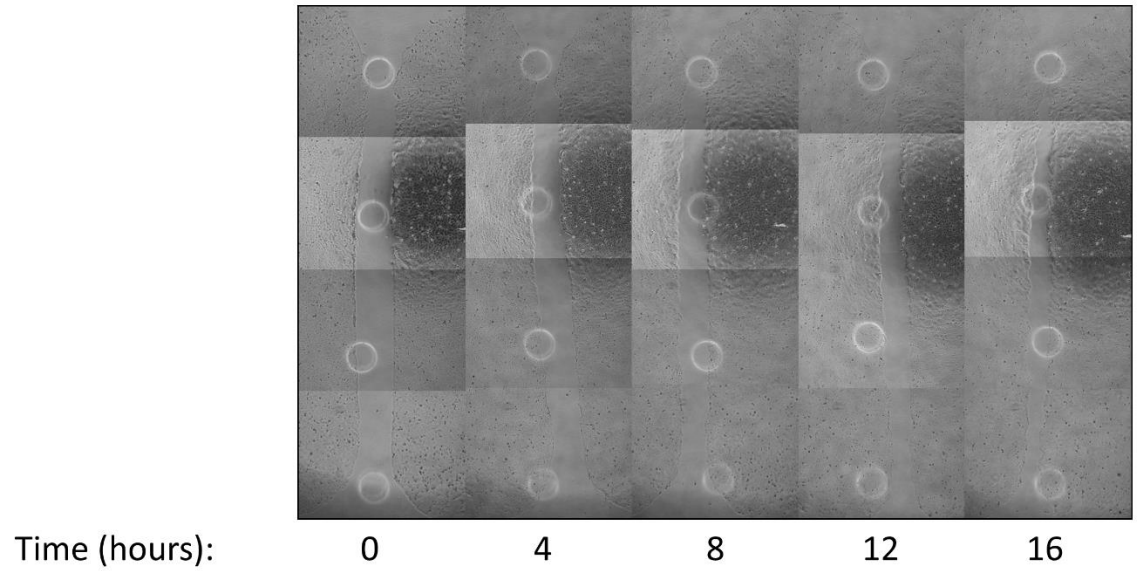

Figure 53. DU145 migration into the scratch with G3.

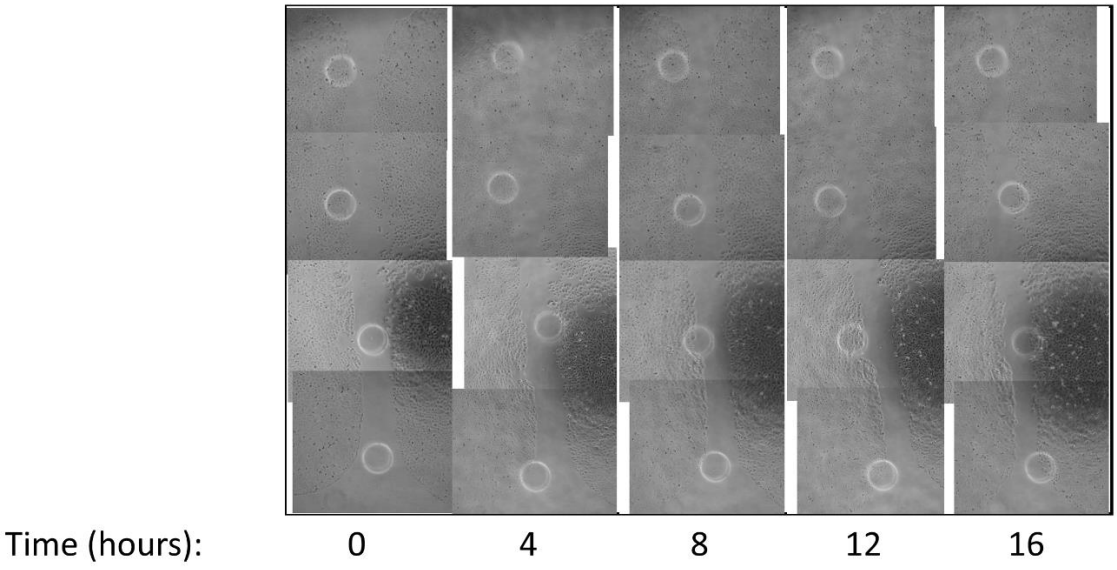

Figure 54. DU145 migration with G4.

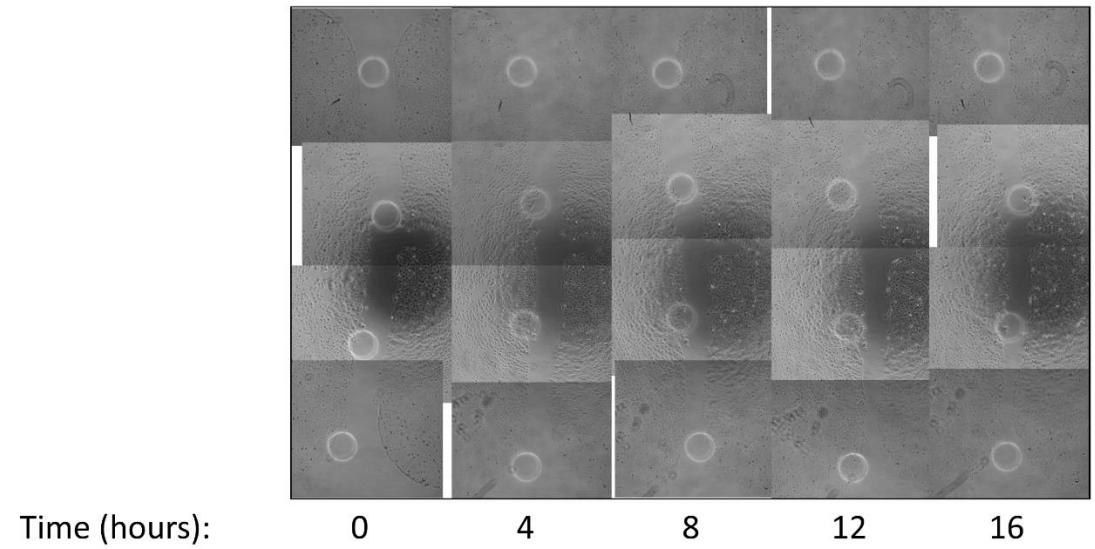

Figure 55. DU145 migration with G6.

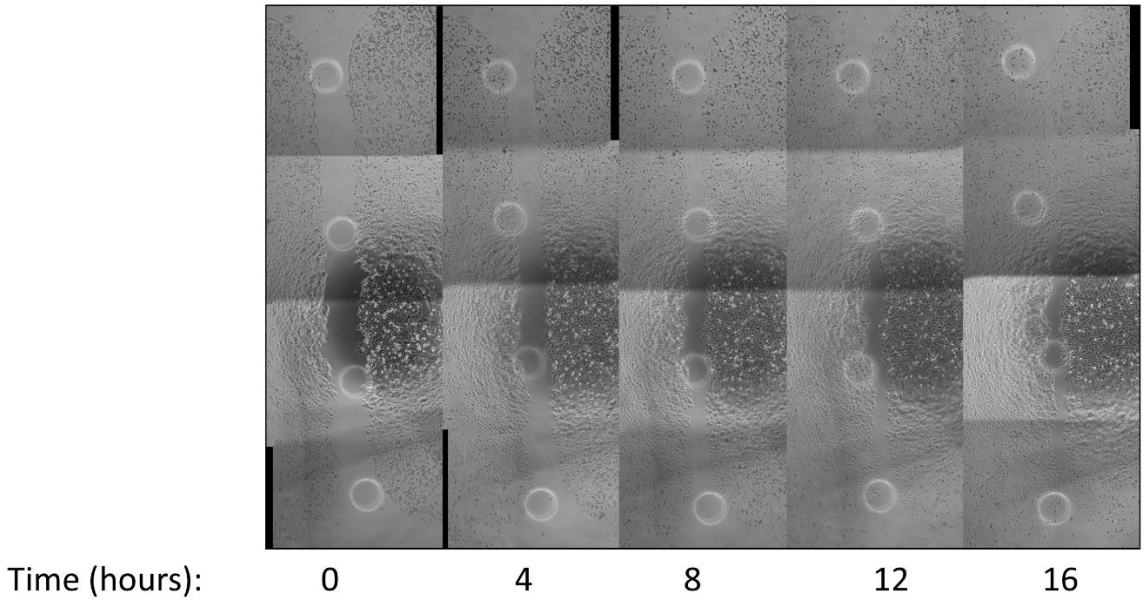

Figure 56. DU145 migration into the scratch with the CRD.

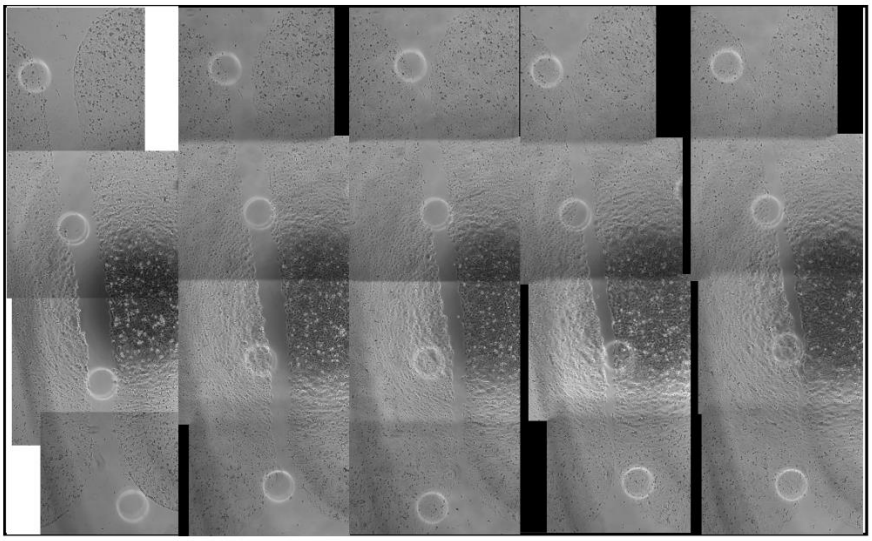

Figure 57. DU145 migration into the scratch with the CRD and G6.

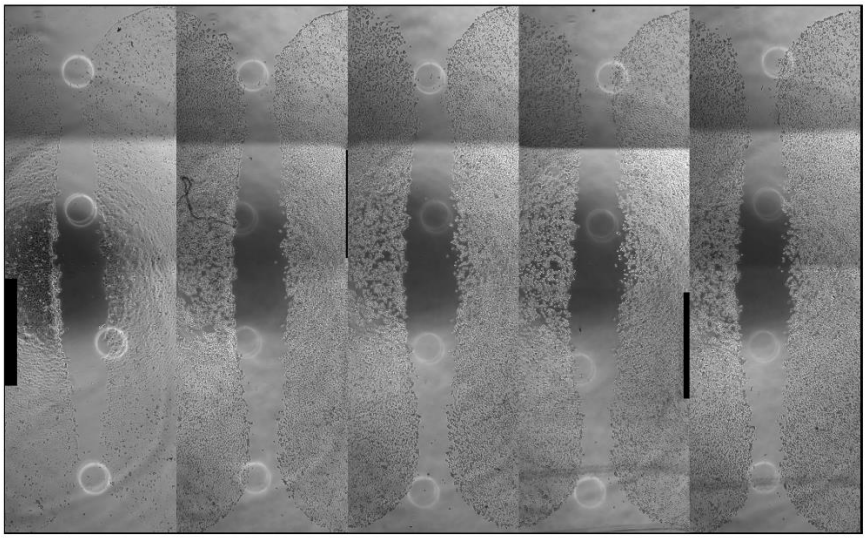

Figure 58. DU145 migration with galectin-3 and 1X monomeric lactose (0.848 mM).

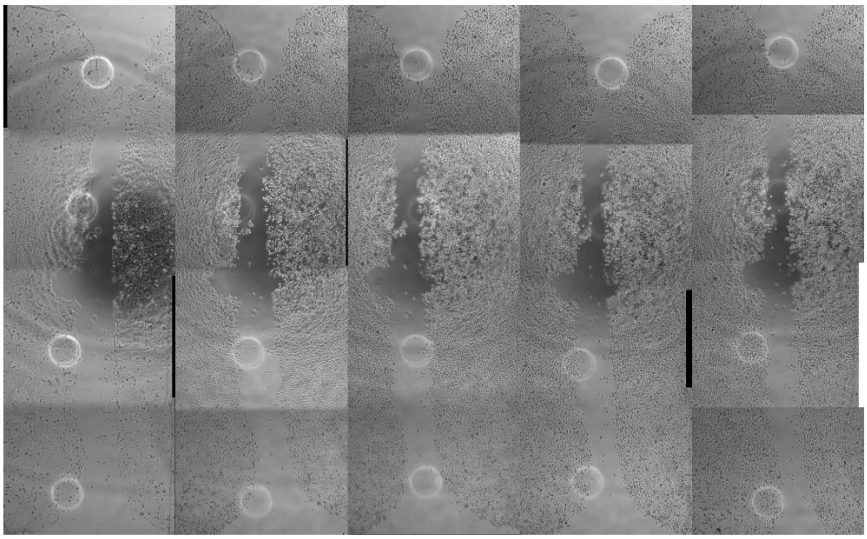

Figure 59. DU145 migration with galectin-3 and 2X monomeric lactose (1.69 mM).

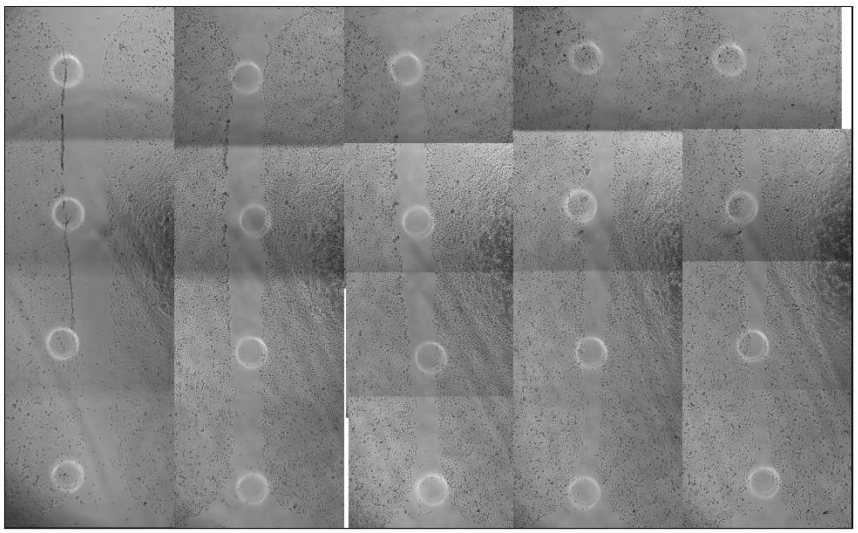

Figure 60. DU145 migration with galectin-3 and 3X monomeric lactose (2.54 mM).

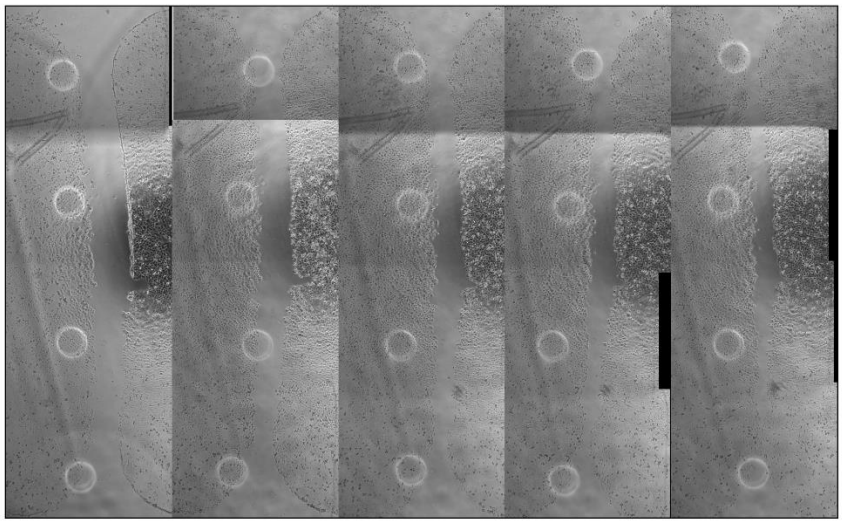

Figure 61. DU145 migration with galectin-3 and 4X monomeric lactose (3.39 mM).

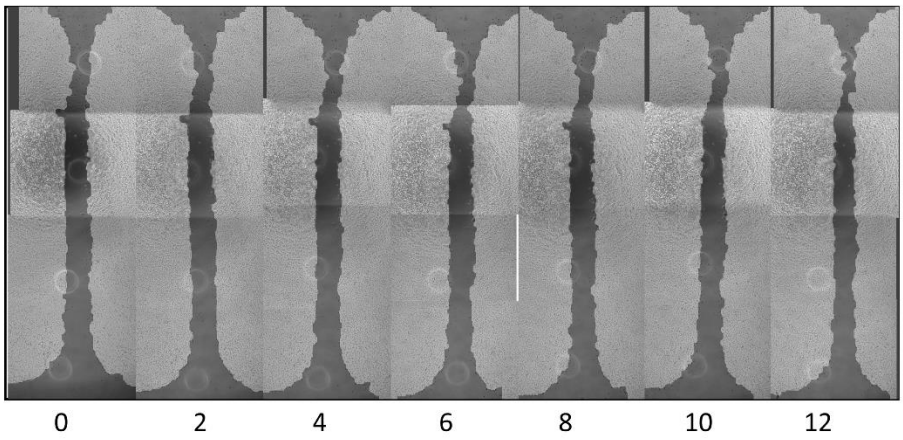

Figure 62. HT1080 migration with galectin-3 over 12 hours.

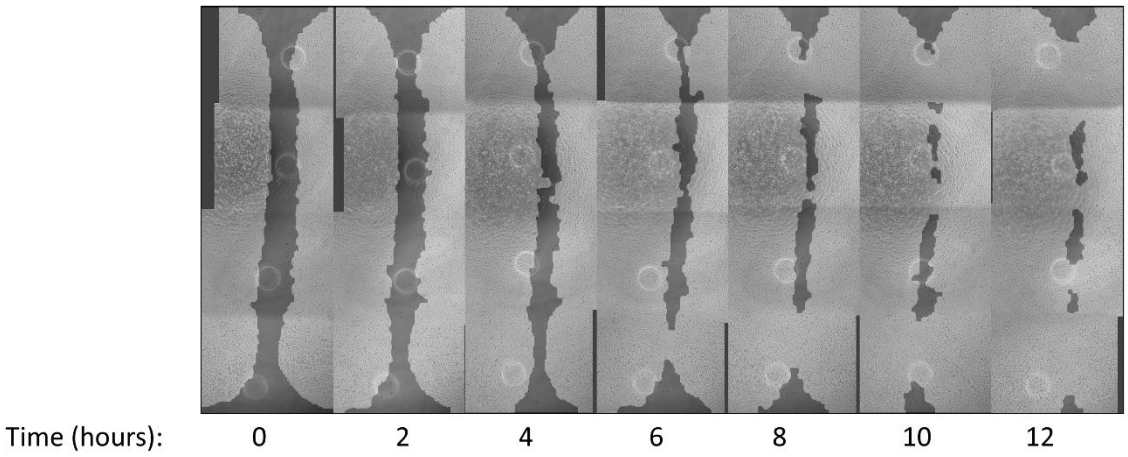

Figure 63. HT1080 migration with PBS.

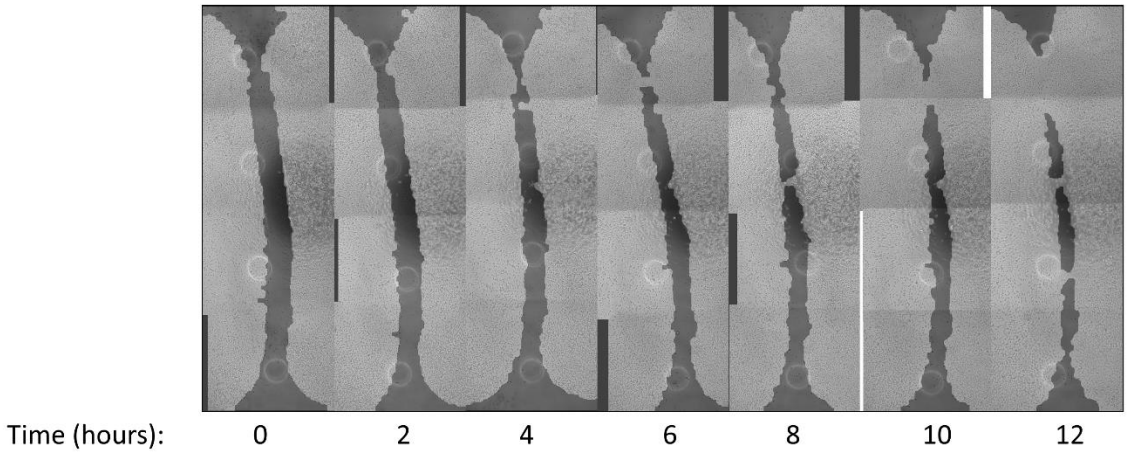

Figure 64. HT1080 migration with galectin-3 and G2.

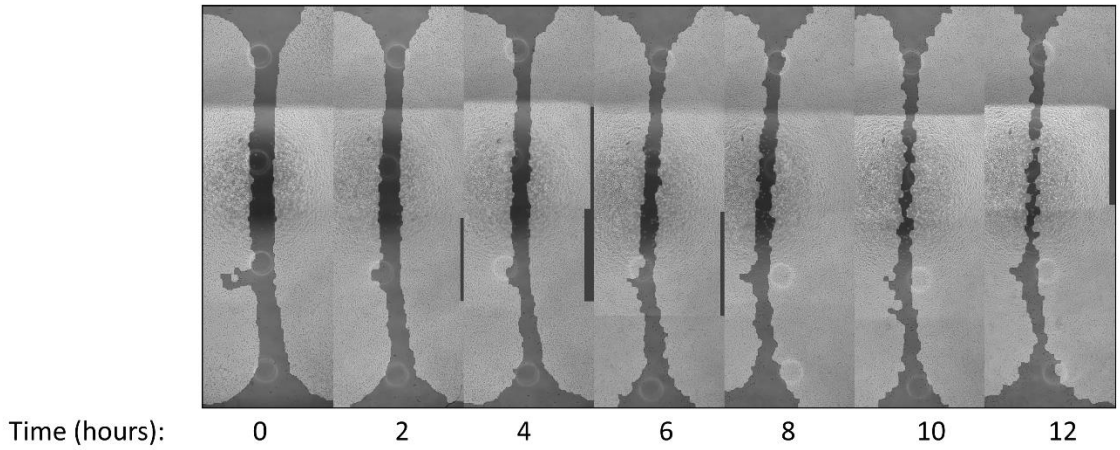

Figure 65. HT1080 migration with galectin-3 and G3.

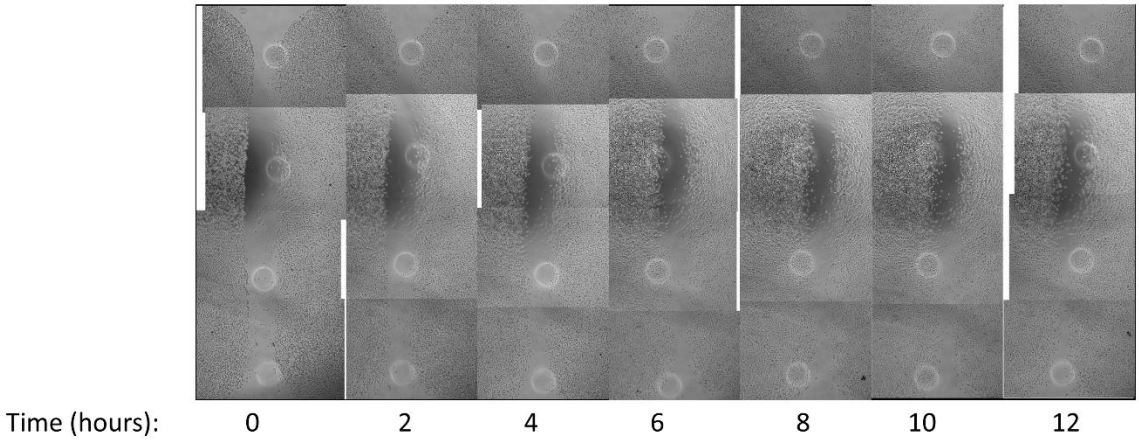

Figure 66. HT1080 migration with G4 and galectin-3.

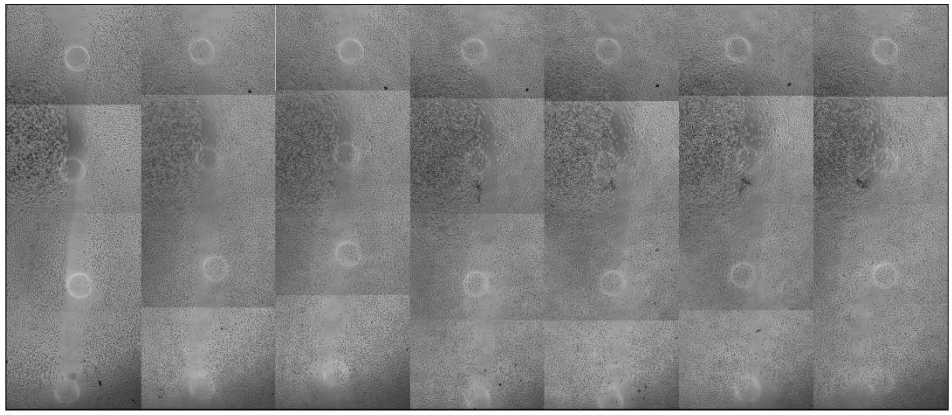

Figure 67. HT1080 migration with galectin-3 and G6.

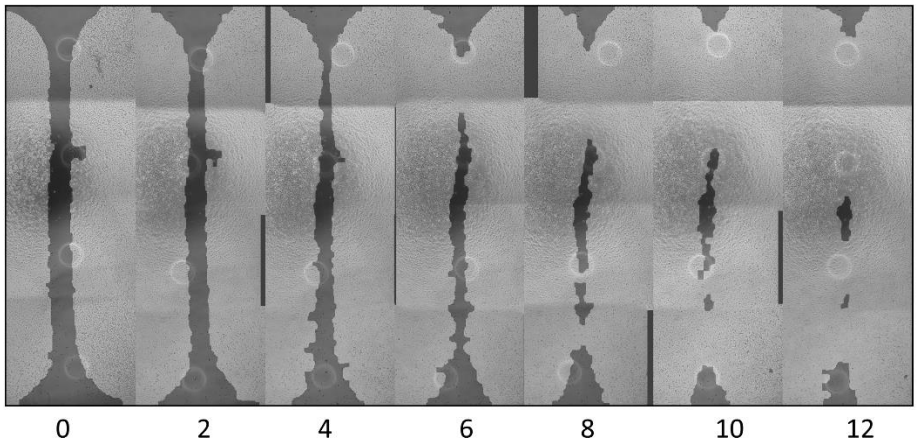

Figure 68. HT1080 migration with G2.

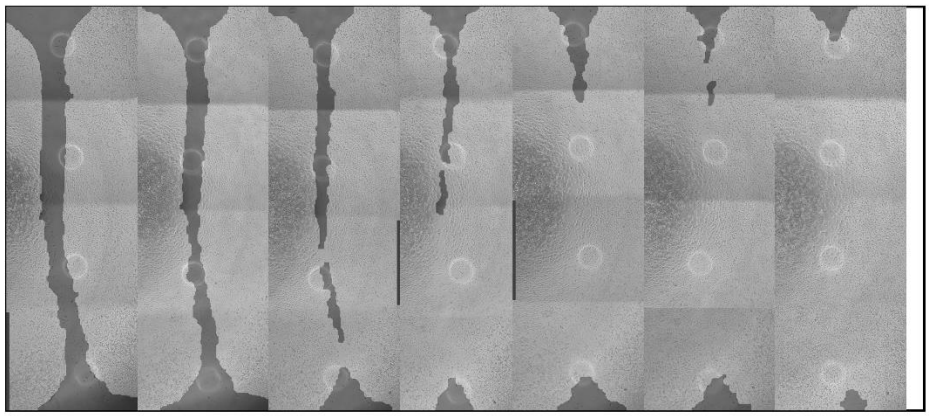

Figure 69. HT1080 migration with G3.

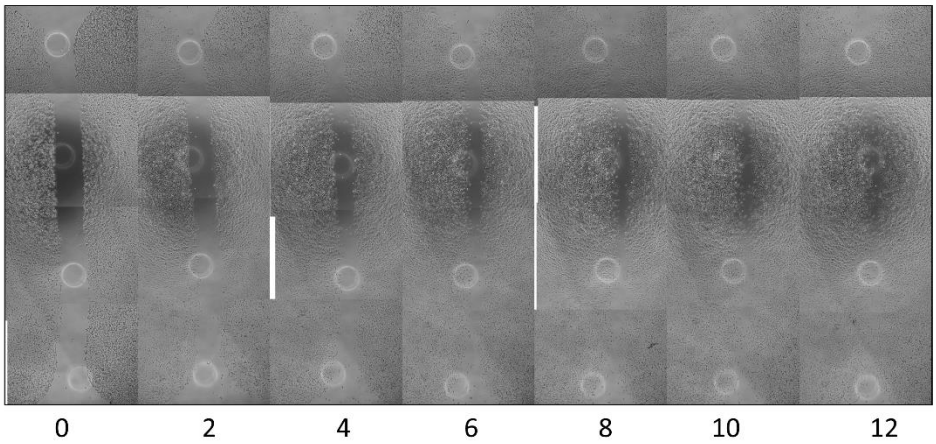

Figure 70. HT1080 migration with G4.

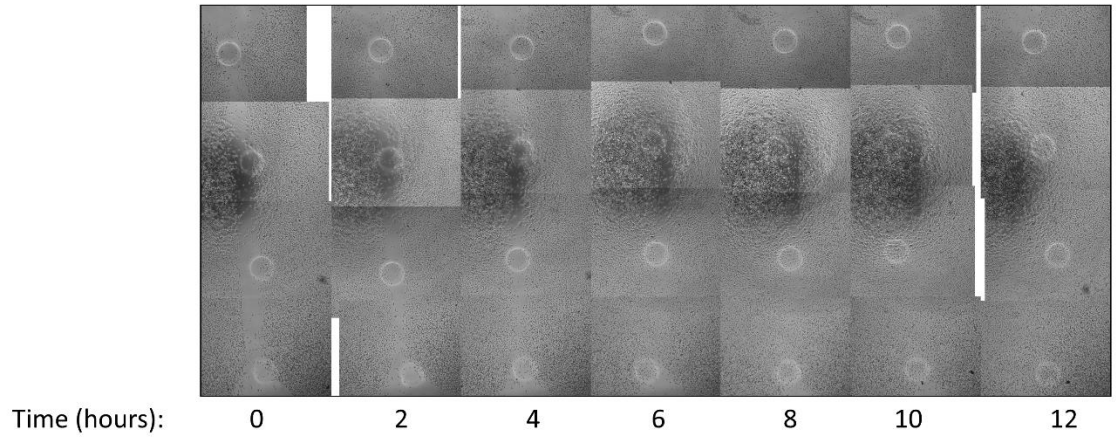

Figure 71. HT1080 migration with G6.

Angiogenesis Assay

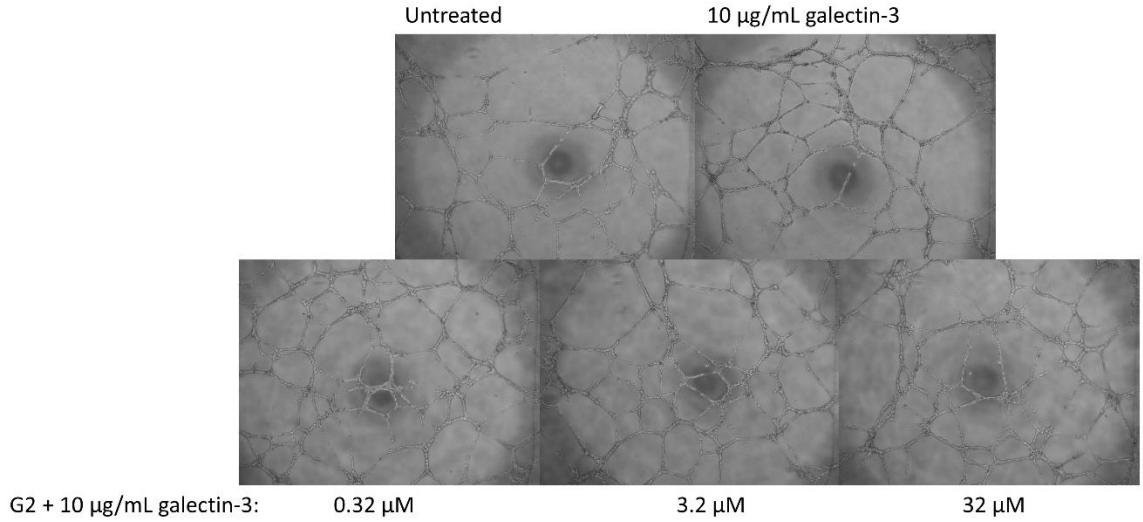

Figure 72. Capillary formation of HUVECs with exogenous galectin-3 and G2 after 5 hours incubation.

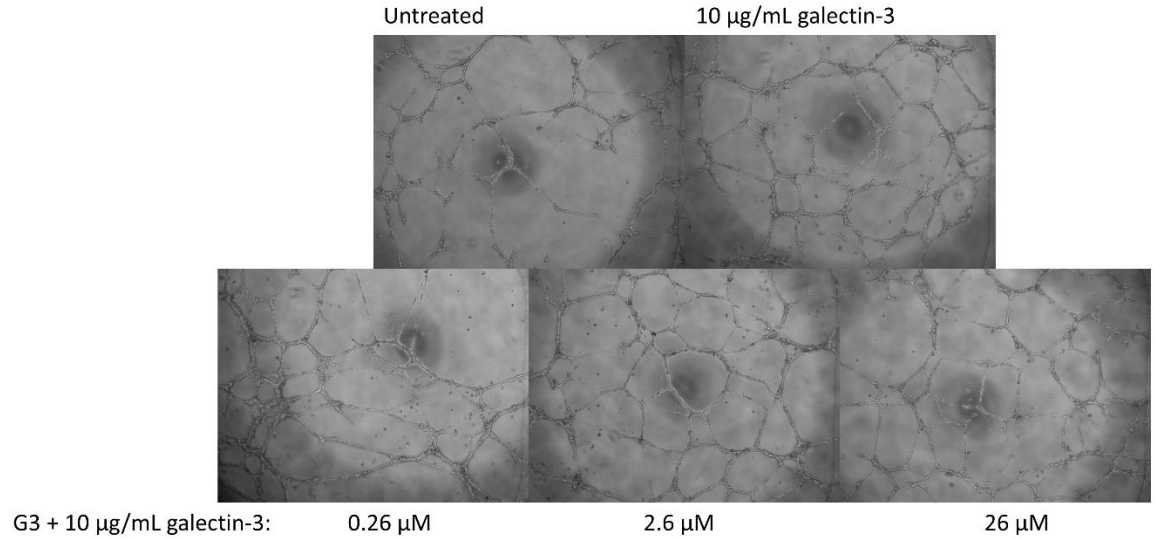

Figure 73. Capillary formation of HUVECs with exogenous galectin-3 and G3 after 5 hours incubation.

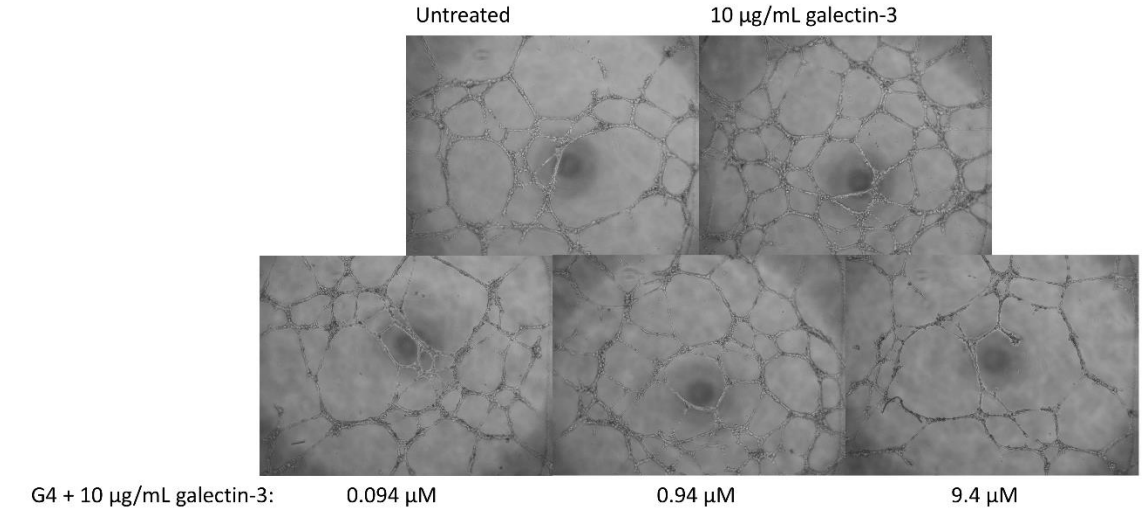

Figure 74. Capillary formation of HUVECs with exogenous galectin-3 and G4 after 5 hours incubation.

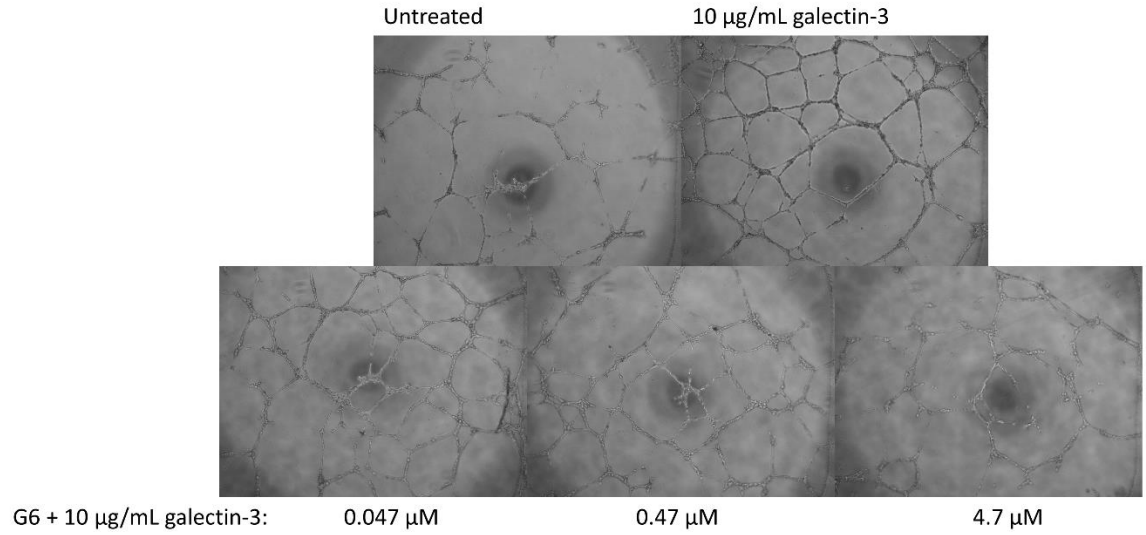

Figure 75. Capillary formation of HUVECs with exogenous galectin-3 and G6 after 5 hours incubation.

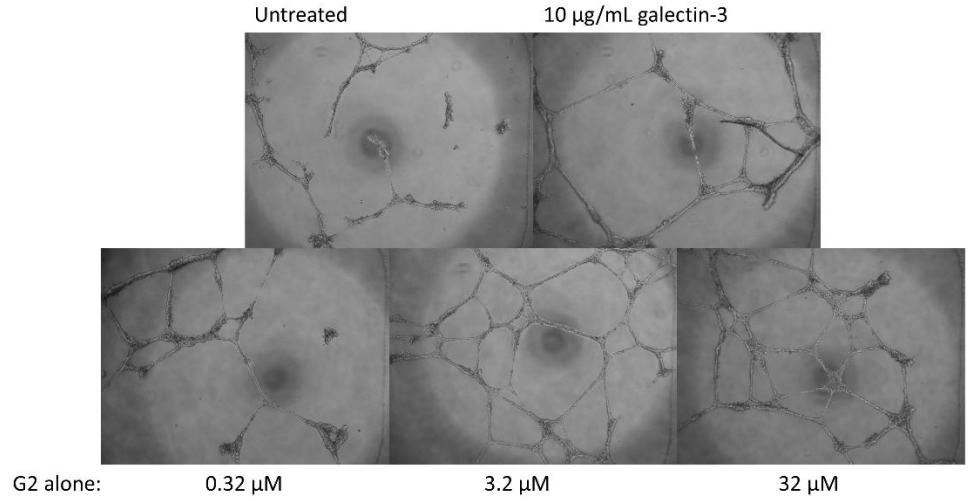

Figure 76. Capillary formation of HUVECs with G2 alone after 5 hours incubation.

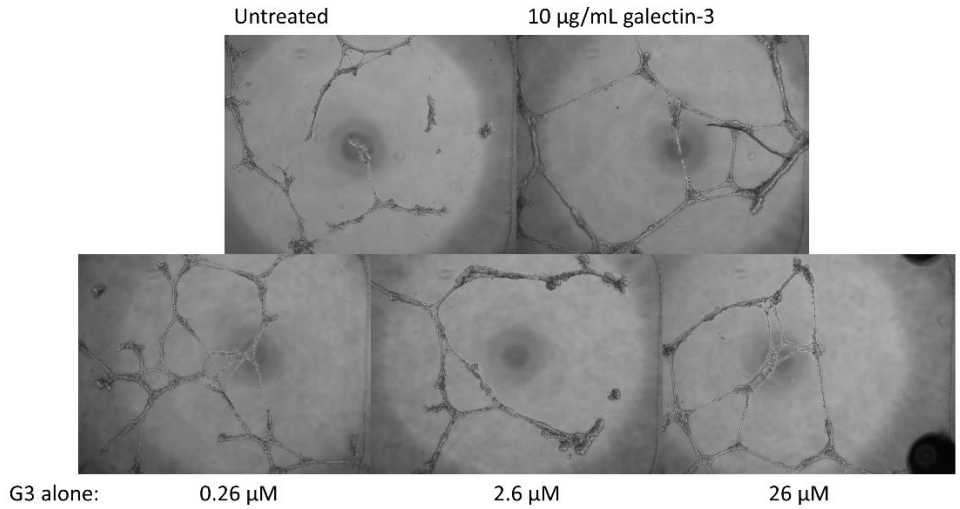

Figure 77. Capillary formation of HUVECs with G3 alone after 5 hours incubation.

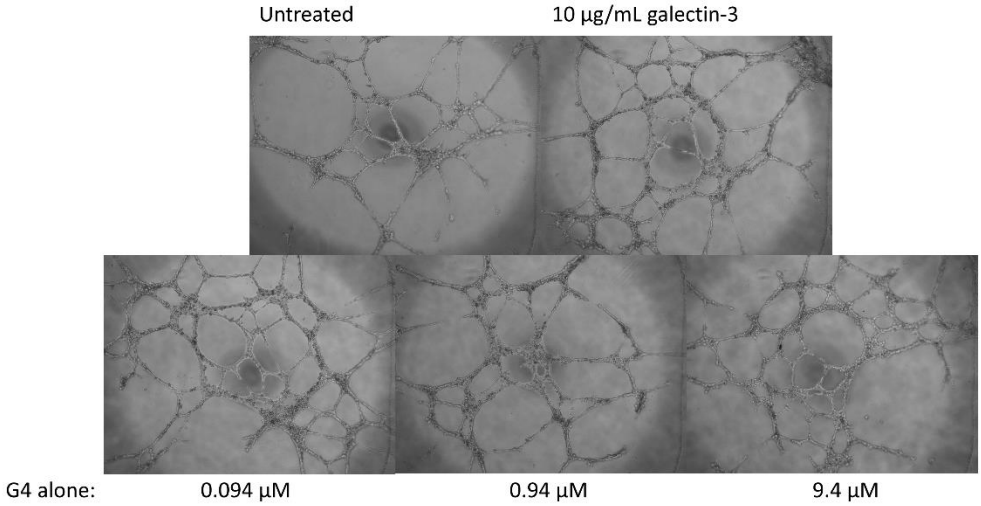

Figure 78. Capillary formation of HUVECs with G4 alone after 5 hours incubation.

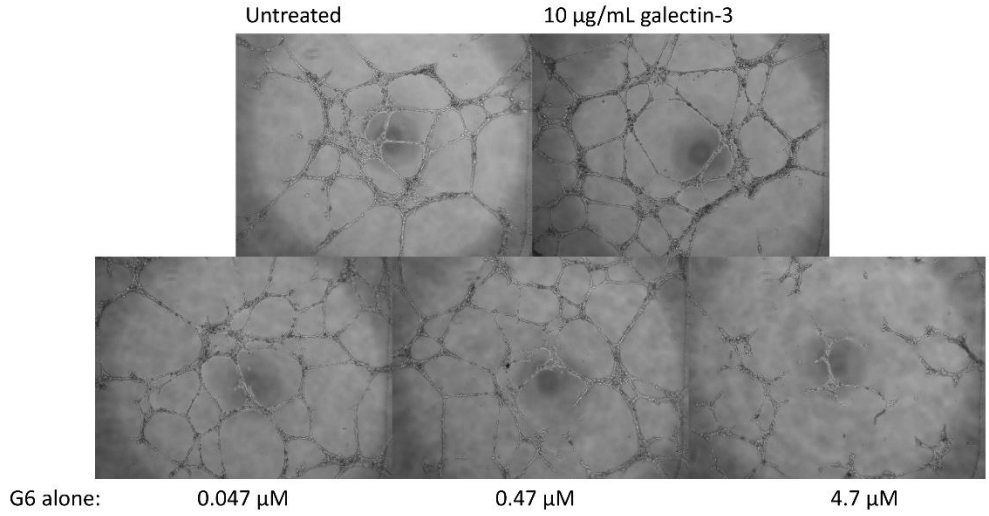

Figure 79. Capillary formation of HUVECs with G6 alone after 5 hours incubation.

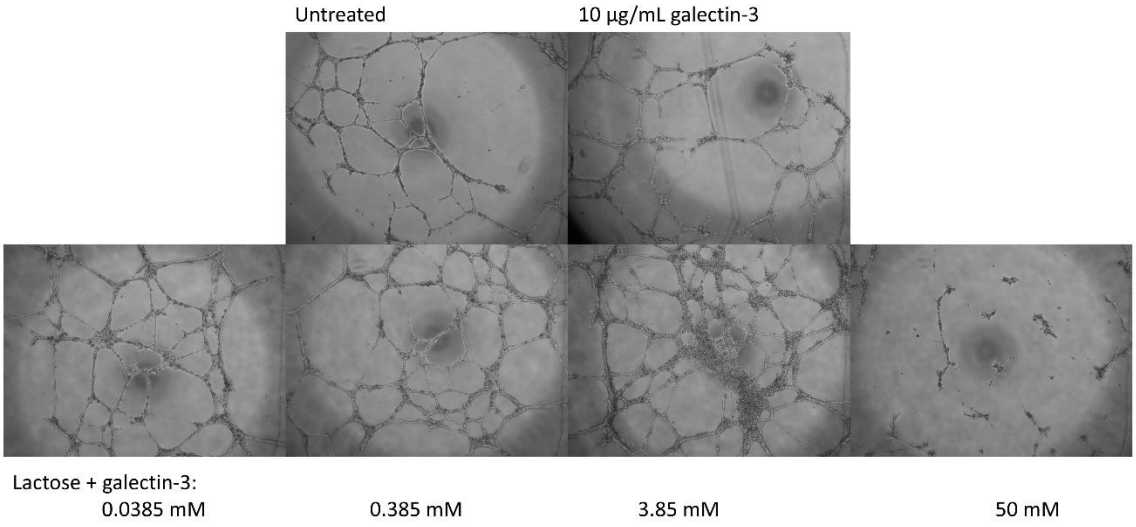

Figure 80. Capillary formation of HUVECs with galectin-3 and monomeric lactose after 5 hours incubation.