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Birk Evavold April 9, 2019

Oxidative Regulation of Galectin-1 Antimicrobial Properties

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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Galectin-1 (Gal-1) is a protein that binds carbohydrates with β-galactoside linkages. Through this binding, Gal-1 exerts a variety of immune modulatory effects. Gal-1, however, has a high propensity to oxidative inactivation. Here, we demonstrate that Gal-1 cysteine residues can be altered by alkylation with iodoacetamide. This alteration inhibits oxidation of Gal-1 as measured by column affinity binding and HL-60 cell agglutination. Additionally, we show that Gal-1 possesses antimicrobial properties and can directly kill *Providencia alcalifaciens* O5 (PAO5), a pathogenic bacteria species known to cause traveler's diarrhea. Furthermore, we demonstrate that oxidation regulates Gal-1's antimicrobial properties as alkylated Gal-1 exhibits stronger bactericidal activity despite similar binding potential. We also studied the oxidation of Gal-1 to understand structural changes between reduced and oxidized Gal-1. While we do not presently have a crystal structure of oxidized Gal-1, we successfully optimized oxidation conditions such that we can produce purely oxidized monomeric Gal-1. We intend to use this optimization to make crystals of oxidized Gal-1 and elucidate atomic level structural changes of Gal-1 oxidation.

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I would like to thank Dr. Sean Stowell for his support on my project in designing experiments and fostering my curiosity on galectin oxidation. Additionally, I would like to thank Dr. Connie Arthur for her input and expertise on galectin. Finally, I would like to thank my committee members, Dr. Sean Stowell, Dr. William Kelly, and Dr. Tracy McGill for their support and input on this thesis.

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Background:

Immune System

The immune system largely exists to recognize foreign pathogens and eliminate them from the host. Simultaneously, pathogens, in an evolutionary arms race, coevolve with hosts to evade immune system activation and recognition.[1] The immune system is broadly divided into two arms: the adaptive immune system and the innate immune system.[2]

Adaptive Immunity

The adaptive immune system provides a host the ability to detect and eliminate pathogens with plasticity throughout the host's life. Most notably, the adaptive immune system consists of B cells, T cells, and antibodies that in concert with other cell types like dendritic cells can mount immune responses to pathogens after exposure. Critically, the adaptive immune system must be exposed to the pathogen for immune activation. However, once exposed, a host will develop immunologic memory where future infections of the same pathogen can lead to stronger and quicker immune responses. This concept underlies vaccination.[3]

Innate Immunity

In contrast, the innate system, as the name suggests, conveys host defense without previous pathogen exposure. Innate immunity frequently relies on recognition of evolutionarily conserved pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs) to provide immune response.[4] Cells such as

macrophages are able to sense bacteria and pathogens in the host. These cells, relying on PAMPs, then phagocytose the pathogens to protect the host. Similarly, dendritic cells can sense PAMPs and activate T cells in the adaptive immune system and are key to linking the adaptive and innate immune systems.[5]

Antimicrobial Peptides

Beyond cell types associated with the innate system, hosts also express a repertoire of antimicrobial peptides that convey the innate ability to recognize and kill pathogenic microbes.[5] Common antimicrobial peptides include: defensins, which are expressed in neutrophils and epithelial cells[6]; cathelicidins, which are expressed in macrophages[7]; protegrins, which are expressed in leukocytes[8]; and granulysin, which is expressed in natural killer (NK) cells and cytolytic T lymphocytes (CTLs).[9] These peptides can directly interact with intracellular or extracellular microbes and limit microbial viability.

Carbohydrates and Lectins

Carbohydrates serve many biological functions beyond acting as molecular energy stores; they also play a vital role in cellular identification. Lectins, proteins with sugar-binding properties, are responsible for deciphering and differentiating various carbohydrate structures.[10] Lectins were first identified in plants during the early 20th century when researchers were fascinated by certain plant extracts that resulted in agglutination of blood (known as hemagglutination).[11] These extracts became known as lectins. The effect the researchers witnessed was a result of lectins binding to carbohydrate ligands on blood cells.

Galectins

Galectins are an important family of lectins named because of their known ability to bind β-galactoside sugars (Figure 1).[12] Several types of galectin exist and are found in animals to regulate immune responses, making their study both biologically and medically relevant.[13] Members of this protein family, like all lectins, bind carbohydrates through a carbohydrate recognition domain (CRD) with distinct carbohydrate binding specificity. Galectins are divided into three classes based on distinct structural characteristics: prototypical galectins consisting of a single CRD; chimeric galectins having one CRD and an N-terminal linking extension; and tandem repeat galectins possessing two distinct CRDs linked by a peptide structure (Table 1).[14]

	Prototypical	Tandem-Repeat	Chimeric
Monomer			
In Solution (beyond $K_{Dimerization}$)			
Galectins:	-1, -2, -7, -10, -13, -14	-4, -8, -9, -12	-3

Table 1: The three galectin classes with cartoon representations of quaternary structure differences[14]

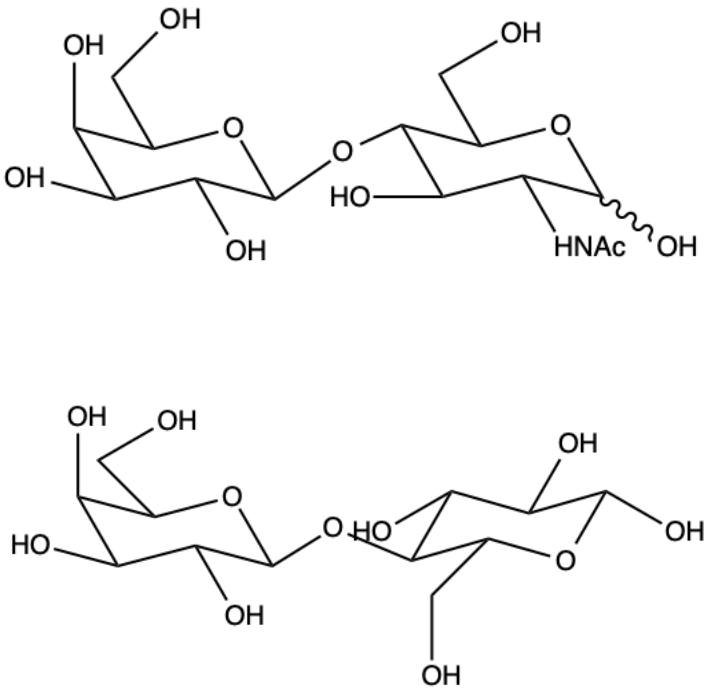


Figure 1: Common carbohydrate ligands used in galectin research

Top: N-Acetyllactosamine (β 1-4), and Bottom: Lactose (β 1-4)[14]

Galectin-1

Galectin-1 (Gal-1) is a member of the galectin family and was the first galectin that was discovered.[15] Monomeric Gal-1 is 14.5 kDa and is 135 amino acids in length.[16] In solution, Gal-1 exists as a homodimer in a monomer-dimer equilibrium with a K_d of 7 uM.[17] Gal-1 is encoded by the gene LSGALS1, which is located on chromosome 22q12.[16] The protein is widely expressed across various cell types and tissues in the human body and is notably upregulated in embryonic development. [16]

Gal-1, with such a broad tissue expression, has been implicated in a wide variety of biological functions. Studies have shown that Gal-1 regulates cell growth, cell migration by altering adhesion,[18] motility and invasion,[16] hematopoietic differentiation, nerve development and repair,[19] T cell positive and negative selection,[20] and host immunity.[21] Furthermore, recent research has focused on Gal-1's role in cancer as the protein is frequently upregulated in tumors and may pose a target for cancer treatment therapies.[22]

Gal-1, like many members of the galectin family, has a propensity to oxidize through the formation of intermolecular disulfide bonds between cysteine residues. Upon oxidation, the protein loses lectin carbohydrate-binding properties. Relatively few studies have examined Gal-1 oxidation and the functional implications of oxidation. That being said, it has been demonstrated that oxidized Gal-1 uniquely promotes axonal regeneration through macrophage activation in injured neurons.[19] Additionally, of the six cysteine residues in Gal-1, Cys16 and Cys88 are the critical residues for intramolecular Gal-1 disulfide bond formation, and Cys2 can oxidize intermolecularly and form oxidized Gal-1 oligomers (Figure 2). [23]

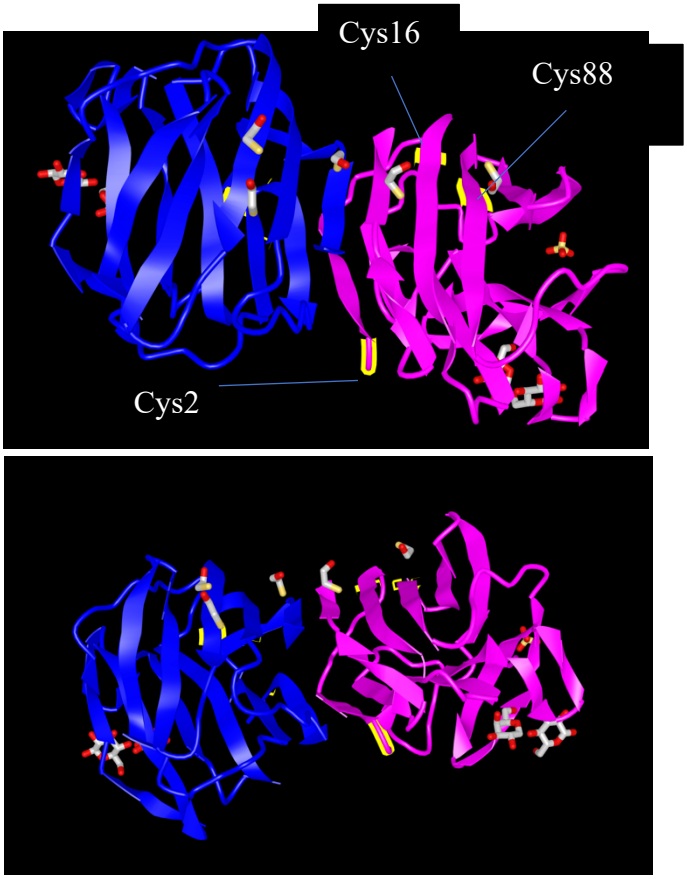


Figure 2: Crystal structure of Gal-1[24]

Reduced Gal-1 acts as a homodimer in solution. Cys-2, -16, and -88 are highlighted in yellow on the pink subunit.

Providencia alcalifaciens

Providencia alcalifaciens is a species of gram-negative bacteria that belongs to the *Enterobacteriaceae* family. This species of *Providencia* commonly causes traveler's diarrhea and is therefore considered pathogenic.[25] Within the *Providencia alcalifaciens* species, many strains exist based on their surface glycosylation patterns. PAO5, one strain of *Providencia alcalifaciens,* possesses an O-Glycan structure that can act as a ligand for Gal-1.[26]

Context of this work

While galectins have been shown to exhibit antimicrobial properties, prototypical galectins have not yet demonstrated bactericidal activity. This work aims to characterize Gal-1's antimicrobial properties and how oxidation regulates this killing. Furthermore, this work optimizes the conditions for structural studies of Gal-1's oxidation.

Methods

Lactosyl-Sepharose Bead Synthesis

This process covalently attaches lactose to Sepharose-4B beads to be used in affinity chromatography. 100 mL of Sepharaose-4B beads is brought to a pH of 11 using a 0.5 M sodium carbonate buffer. 10 mL of divinyl-sulfone is reacted with the beads for 70 minutes to add cross-linking sites for the lactose. Excess divinyl sulfone is then removed and the beads are subsequently incubated for 15 hours in a 10% lactose solution. Upon completion of the reaction, the excess lactose is removed by washing the beads, and they are stored in Phosphate Buffered Saline (PBS) with sodium azide.

Carbohydrate-Binding Activity Assay

To assess the galectin for carbohydrate binding activity and thus oxidation, affinity chromatography with lactosyl-Sepharose beads is used. A 1 mL column of lactosyl-Sepharose is equilibrated with PBS. The protein solution is then run over the column and 0.5 mL fractions are collected. The column is then washed with 5 mL of PBS, again with 0.5 mL fractions collected. Finally, the protein is eluted in 100 mM lactose in PBS, again with 0.5 mL fractions collected (Figure 3). The protein concentration in each

fraction is determined through spectrophotometry. Fractions collected in the flow-through or wash portions are considered inactive, and the fractions in the elution portion are considered active protein.

Purification of Galectin

Galectin-1 is cloned by expressing the LGALS1 gene via a plasmid vector in *Escherichia coli* (*E. coli*). This plasmid also contains an ampicillin antibiotic resistance gene. The bacteria are grown in Luria Broth with 50 mg/L of ampicillin to select for bacterial cells with the galectin plasmid. Once the cultures reach an optical density (OD) of 0.45 at 600 nm, the cultures are induced with 0.28g/L Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4-5 hours. The cells are then centrifuged and lysed through sonication. The resulting proteinacious supernatant is run over a lactosyl-Sepharose affinity column and eluted with 100mM lactose in PBS (Figure 3). Protein concentrations from eluted fractions are determined through protein spectrophotometry at 280 nm wavelength.

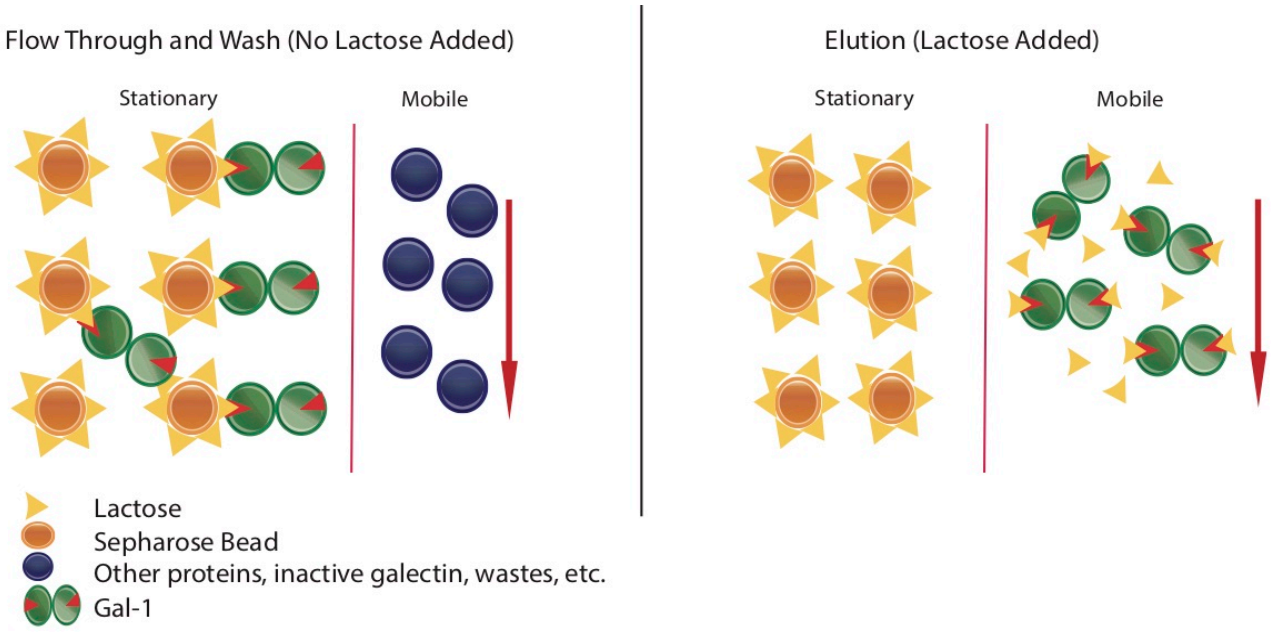


Figure 3: Measurement of galectin activity and purification of galectin through affinity chromatography

Left: Protein solution added to column without soluble lactose. Any active protein will bind with the lactosyl-Sepharose in the stationary phase while inactive, oxidized protein or any contaminating products will flow through the column in the mobile phase. Right: Once 100 mM soluble lactose is added to the column, it will compete with the lactose covalently linked to the beads for galectin's CRD. This will move active galectin to the mobile phase. By fractionating the solution flowing off the column in both the pre-elution and elution phases, oxidized and reduced galectin can be separated.

Alkylation of Galectin

Lactose and 2-mercaptoethanol (βME) are removed from the protein sample by running the sample over a PBS-equilibrated PD-10 column. This size-exclusion column will

separate the larger protein from smaller ligand molecules (lactose) and βME which help stored galectin remain active and reduced. Positive protein fractions as determined by spectrophotometry are pooled and incubated in 18 mg/mL iodoacetamide and 36 mg/mL lactose for 15 hours at 4ºC. The reaction is covered in aluminum foil as iodoacetamide will spontaneously hydrolyze in light. Following completion of the reaction (Figure 4), excess iodoacetamide is removed by again running the protein solution over a PBS-equilibrated PD-10 column. Positive protein fractions are then aliquoted for use in other assays.

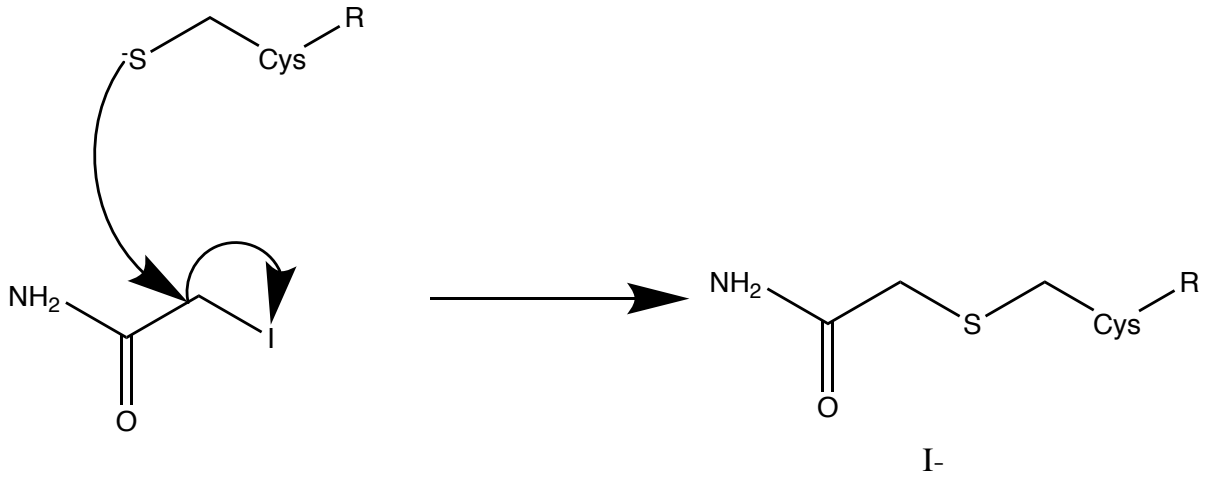


Figure 4: Cysteine residue stabilization by iodoacetamide

Protein cysteine residues can be stabilized from oxidation by alkylating them with iodoacetamide. As cysteine residues interact with iodoacetamide molecules, a lone pair on a cysteine sulfur atom acts as a Lewis base and participates in SN2 reaction with the alpha carbon on iodoacetamide. This results in the release of an iodide anion with an amide linked to the cysteine residue. The relatively stable new bond prevents further chemistry at the cysteine residue including oxidative disulfide bond formation.

Galectin-Bacteria Killing Assay

A strain of bacteria is grown to an OD of 0.1 (A_{600}). In culture tubes, 1-part bacteria and 1-part PBS or galectin (at varying concentrations) are mixed (Figure 5). The culture tubes are incubated in a shaker at 37 ºC for 1.5 hours. 1:10 serial dilutions are performed for each condition in a 96-well plate. 20 μL of the 10^{-3} and 10^{-4} dilutions are plated onto LB agar plates. Each condition and dilution is plated at least twice, such that there are at least two replicates. Plates are left in a 37 ºC incubator for 15 hours and then counted.

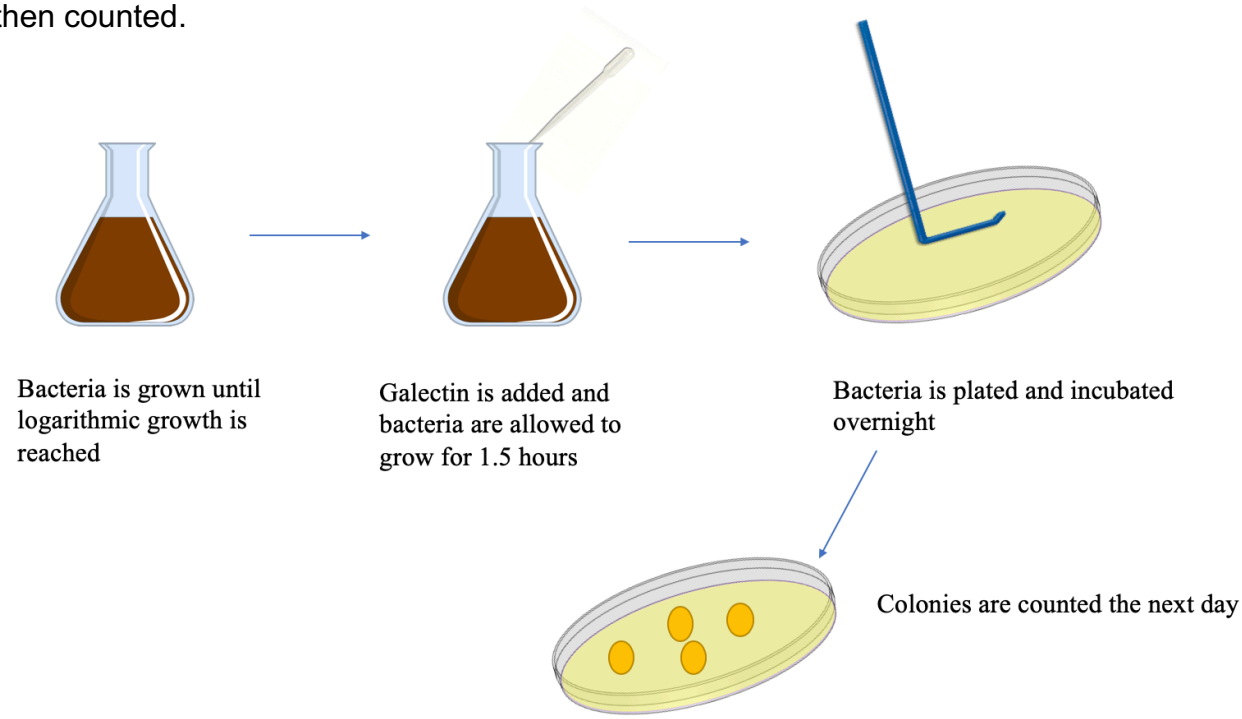


Figure 5: Schematic of the galectin killing assay protocol

Binding Assay

6 ug of biotinylated Gal-1, 3 uL of Streptavidin conjugated to FITC (1 g/mL), and 300 uL of PBS are incubated on ice for 15 minutes. PAO5 bacteria is resuspended in PBS at an OD_{600} of 0.1. 45 uL of PAO5 is added to the protein solution and incubated for 15 minutes on ice in darkness. The protein-bacteria solution is then washed twice with PBS to remove unbound Gal-1. The protein-bacteria solution is then suspended in 200 uL of PBS and run on 4-color BD FACSCaliber flow cytometer. Samples were analyzed in FlowJo Software.

HL-60 Cell Culture

HL-60 (ATCC CCL-240™) cells are cultured in RPMI-1640 media supplemented with 15% Fetal Bovine Serum, 100 U/mL Penicillin, and 100 U/mL Streptomycin. Cells are expanded in T-75 sterile tissue culture flasks and split once they reach 70% confluence.

Oxidation of Gal-1 by Hydrogen Peroxide

Hydrogen peroxide of varying concentration is added to samples of galectin in 15 mL conical tubes. Once oxidation reaction time is reached, protein samples are run over a PBS size exclusion PD-10 column to separate unreacted hydrogen peroxide from the protein. Alternatively, bovine catalase can be added to the reaction to scavenge any excess hydrogen peroxide. Protein positive fractions, as measured through spectrophotometry at 280 nm, are pooled and assayed for carbohydrate binding capacity.

Gel Electrophoresis to Determine Aggregation

Protein concentrations are determined through spectrophotometry at 280 nm. Protein concentrations are then normalized such that each well contains the same mass of protein. Samples are combined with 6x protein gel loading dye absent of 2-mercaptoethanol to sustain disulfide bonds and heated for 10 minutes at 70° C. Samples are loaded into an SDS-PAGE gradient gel. Samples are run for 1.5 hours at 100V. The gel is then probed for protein with Coomassie Brilliant Blue by staining overnight. After staining the gel is washed with double-distilled water to remove dye unbound to protein.

Hemagglutination Activity Assay

10% sheep red blood cells are diluted in a 96-well plate at a 1:4 dilution with PBS containing Gal-1 of known concentration. Once a PBS control well containing no galectin settles such that the red blood cells in the well form a small circle at the bottom of the well, the plate is photographed. Wells in which the red blood cells disperse into a flat lattice contain sufficient active galectin protein to agglutinate the red blood cells via surface carbohydrate structures. By comparing samples for the maximum dilution in which agglutination is observed, relative levels of activity and therefore oxidation can be observed.

Extinction Coefficient Calculation

Sequence information is determined from PubMed Protein (www.ncbi.nlm.nih.gov

/protein). The sequence is then pasted into ExPASy ProtParam tool (https://web.expasy.org/protparam/) which calculates a molar extinction coefficient based on known absorbances of amino acid residues. This molar extinction coefficient represents ε in the Lambert-Beer Law ($A_{280}=\varepsilon cl$). This equation with known extinction coefficient can then be used to calculate protein concentrations from 280 nm absorbances.

MALDI-TOF/TOF Mass Spectrometry

Protein matrix solution is made by combining 10 mg/mL 3,5-dimethoxy-4 hydroxycinnamic acid in a solution of 50% acetonitrile and 0.1% trifluoroacetic acid. 1 uL of sample and 1 uL of matrix are spotted and mixed by pipetting on the MALDI plate. Sample data is acquired in Emory Comprehensive Glycomics Core Bruker UltraFlexII MALDI-TOF/TOF instrument. Mass spectrometry data is then analyzed using FlexAnalysis Software.

HL-60 Agglutination Assay

In a 48-well tissue culture plate, 20,000 HL-60 cells in 200 uL are loaded into each well. Galectin samples are then loaded into each well and pipetted up and down to mix. At different time points the cells are observed under a light microscope for agglutination. Each well is scored on a scale from 0 to +4 for agglutination strength. A guide for how scores were assigned can be found in Figure 8.

Data Analysis and Presentation

Graphs were created in GraphPad Prism. Data analysis was done in GraphPad Prism and one-way ANOVA with Tukey multiple comparison test was used to analyze data with three or more groups.

Results

Alkylation and protein oxidation

We first wanted to confirm our method of alkylation would successfully alter the cysteine residues of the Gal-1. As alkylation with iodoacetamide leads to the addition of an amide to the cysteine residues, one would expect that the molecular weight of the protein would change following alkylation. In order to observe this shift in molecular weight, we used MALDI-TOF/TOF mass spectrometry of Gal-1 samples, both with and without alkylation. Alkylation resulted in an increase of 319 Da to the molecular weight of the homodimeric protein (Figure 6). This increase confirms that the alkylation successfully altered the molecule and did not alter its ability to dimerize in solution.

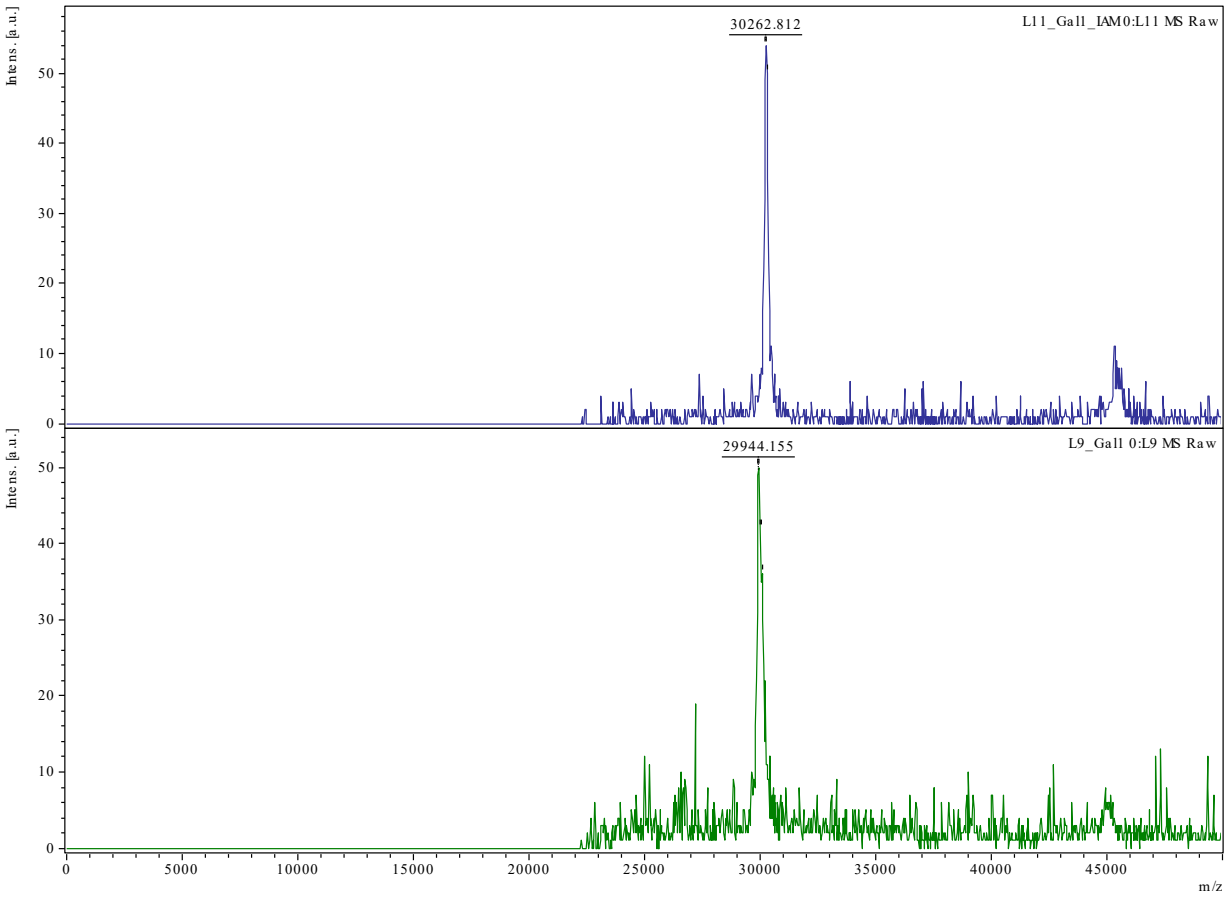


Figure 6: MALDI-TOF/TOF Mass Spectrometry Confirmation of Alkylation

Gal-1-IAM (Top) and Gal-1 (Bottom) were sampled through mass spectrometry to assess for mass increase indicative of alkylation.

To understand how the stabilization of Gal-1 through alkylation affected the protein, we sought to characterize the protein under oxidative stress. After alkylating the protein and confirming its activity through affinity chromatography, 34 uM samples of Gal-1 and alkylated Gal-1 (Gal-1 IAM) were chemically oxidized with 10 mM hydrogen peroxide for 2 hours. Following this oxidative stress, Gal-1 and Gal-1 IAM were tested for carbohydrate binding activity to determine which fraction of protein had lost its ability to bind lactose, a defining feature of galectin oxidation. A much greater fraction of Gal-1 lost carbohydrate binding potential compared with Gal-1 IAM which is indicative of oxidation (Figure 7). The unstabilized Gal-1 has a large peak of protein flow off the column before elution with lactose while stabilized Gal-1 IAM has a relatively small peak of inactive protein flow off the column before elution with lactose. Similarly, the Gal-1 IAM post-elution peak is much larger than the Gal-1 peak, demonstrating that more of the Gal-1 IAM retained its carbohydrate binding lectin properties and was more resistant to chemical oxidation by hydrogen peroxide. These data show that alkylation of Gal-1 with iodoacetamide successfully inhibits oxidation and prolongs Gal-1's ability to bind carbohydrate ligands.

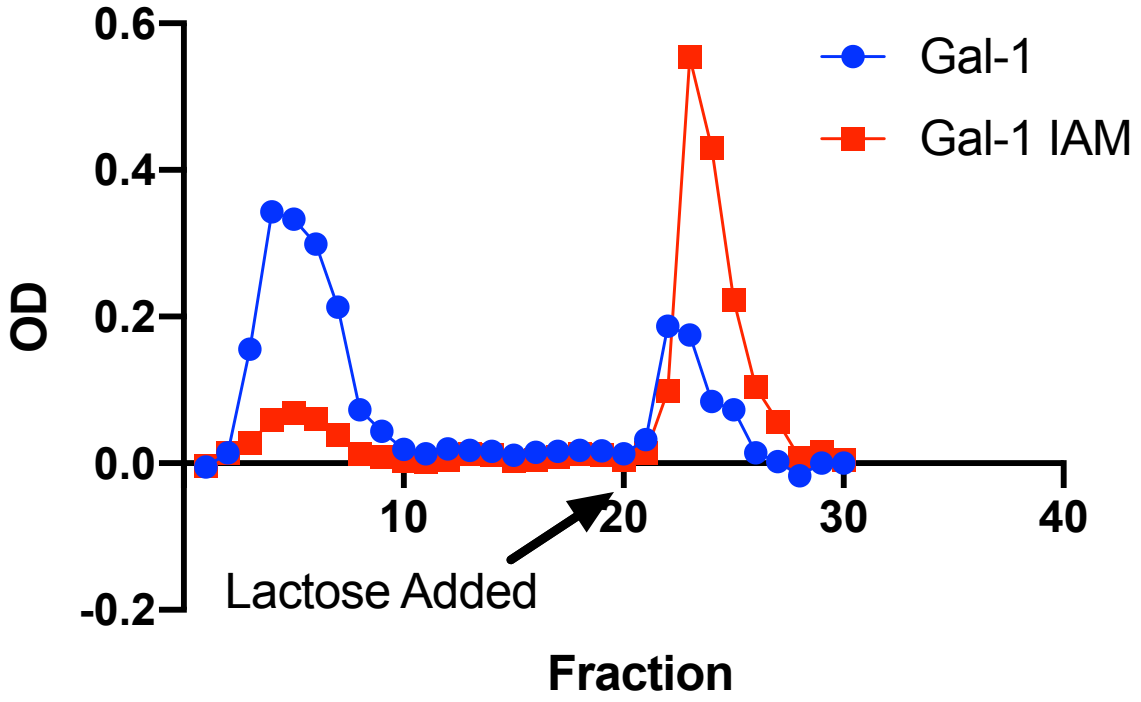


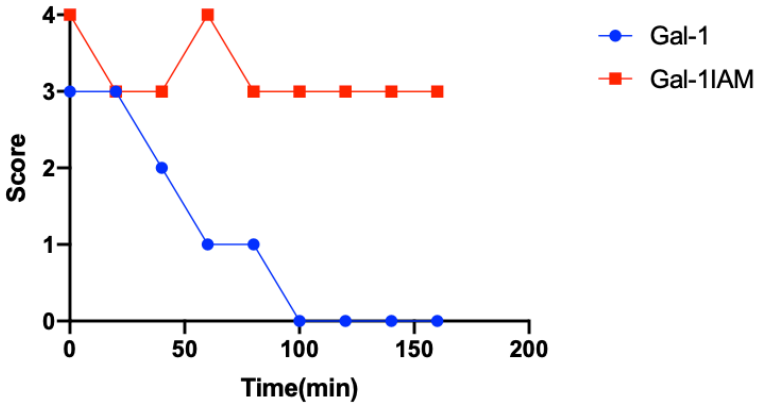
Figure 7: Alkylation of Gal-1 prevents oxidation as measured by column binding affinity

34 uM Gal-1 and Gal-1 IAM were oxidized with 10 mM hydrogen peroxide for 2 hours. Each sample was then run over a lactosyl-Sepharose column to test for binding activity to lactose. Gal-1 loses most of its activity while Gal-1 IAM is mostly protected from oxidation.

To further characterize how alkylation impacted Gal-1 oxidation, Gal-1 agglutination of HL-60 cells was examined. HL-60 cells possess surface carbohydrate ligands to which Gal-1 can bind. When homodimeric Gal-1 molecules bind to these carbohydrate antigens, the cells agglutinate and clump in culture. As carbohydrate binding potential is

lost, Gal-1 can no longer link the cells in solution and they disperse without agglutination. By scoring HL-60 cell agglutination, one can determine the relative activity Gal-1 samples. Additionally, Gal-1 activity can be tracked over time as the protein oxidizes. This is a key advantage to monitoring Gal-1 activity by HL-60 cell agglutination rather than affinity chromatography as the same information would require a large number of columns that would need to be run in a relatively short succession. Furthermore, in the HL-60 agglutination assay the protein is being oxidized in culture with cells. This more accurately resembles how Gal-1 would oxidize in a living system as it is exposed to extracellular oxidative metabolites and molecules. Addition of 5 uM Gal-1 and Gal-1 IAM to HL-60 cells resulted in both samples agglutinating at 0 and 20 minutes. However, by 60 minutes most of the Gal-1 sample had oxidized while Gal-1 IAM retained very strong agglutination activity. By 100 minutes, Gal-1 had lost all lectin activity while Gal-1 IAM retained relatively strong agglutination capacity throughout the end of the experiment to the 160 minute timepoint (Figure 8). These data further show that alkylation of Gal-1 with iodoacetamide protects the protein from oxidative inactivation and significantly prolongs its activity under oxidative stress. These data also suggest that carbohydrate binding functions of Gal-1 may be amplified by stabilizing Gal-1 from oxidation to extend the protein's functional time.

A.



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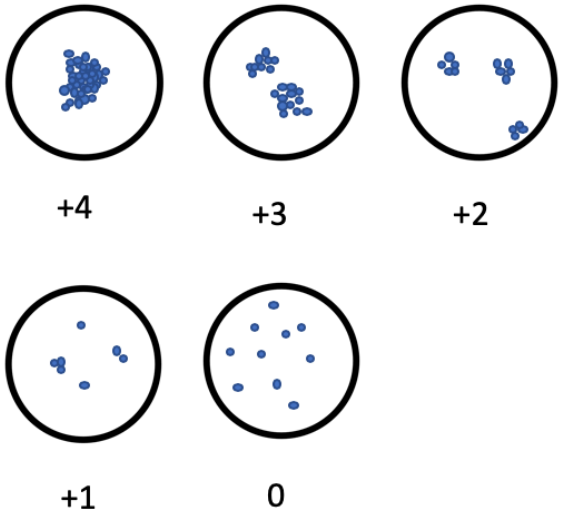


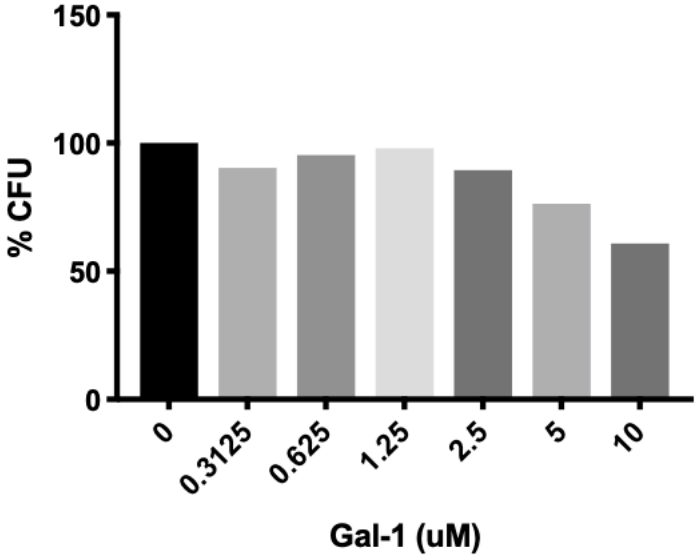
Figure 8: Alkylation protects Gal-1 from oxidation as measured by HL-60 agglutination

A: 5 uM Gal-1 and Gal-1 IAM were added to HL-60 cells in RPMI complete media (No hydrogen peroxide). Samples were observed every 20 minutes by light microscope and scored for agglutination. B: Guide used to score agglutination of cells.

Killing of Bacteria by Gal-1

We first investigated a strain of *Klebsiella pneumoniae* (KPO1) that expressed carbohydrate surface structures that were potential ligands for Gal-1. Previously unpublished data from our lab showed that Gal-1 did in fact bind KPO1; however, its killing capacity remained unknown. KPO1 was first challenged with unstabilized Gal-1 for 1.5 hours (Figure 9A). Following plating and colony enumeration, there did not appear to be significant killing of KPO1 by Gal-1 at a concentration of 10 uM Gal-1. Nevertheless, we repeated the killing assay with alkylated Gal-1 IAM to see if potential Gal-1 killing activity may be hidden do to oxidation which resulted in Gal-1 inactivation (Figure 9B). However again, there was no notable bacteria killing observed by colony enumeration. While these results did not support the notion that Gal-1 possessed antimicrobial properties, the results could derive from an assay sensitivity issue or that Gal-1 does not possess the ability to kill this specific strain of bacteria despite binding its surface.

A.



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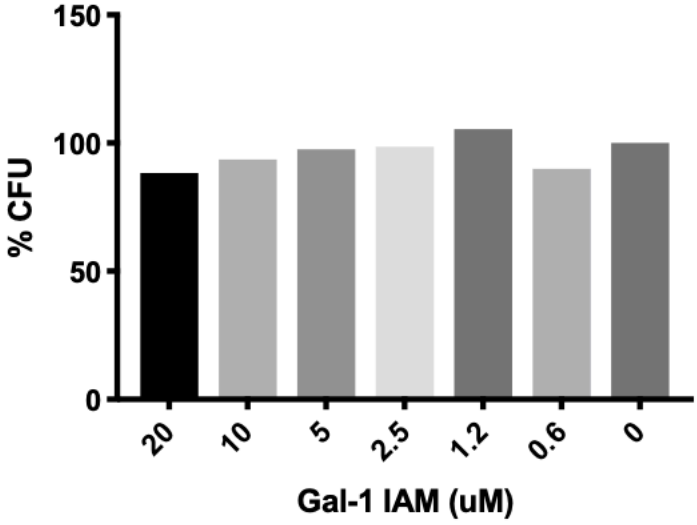


Figure 9: Killing Assay of KPO1 with Gal-1 and Gal-1 IAM

KPO1 bacteria was treated with various concentrations of Gal-1 (A) and Gal-1 IAM (B) to assess for killing ability. 2 replicate plates from each group were counted and %CFU of PBS control was plotted

We proceeded to investigate another bacteria strain, *Providencia alcalifaciens* O5 (PAO5) that also expressed potential O-glycan carbohydrate ligands for Gal-1. Our lab had previous unpublished data that demonstrated Gal-3, the chimeric galectin, could kill PAO5. Therefore, Gal-3 could be used as a positive control for PAO5 killing to ensure both assay sensitivity and that our PAO5 strain did not evolve resistance or become contaminated throughout experiments. Gal-3 killed PAO5 in a dose-dependent manner with concentrations above 3 uM killing nearly all PAO5 cells in culture (Figure 10).

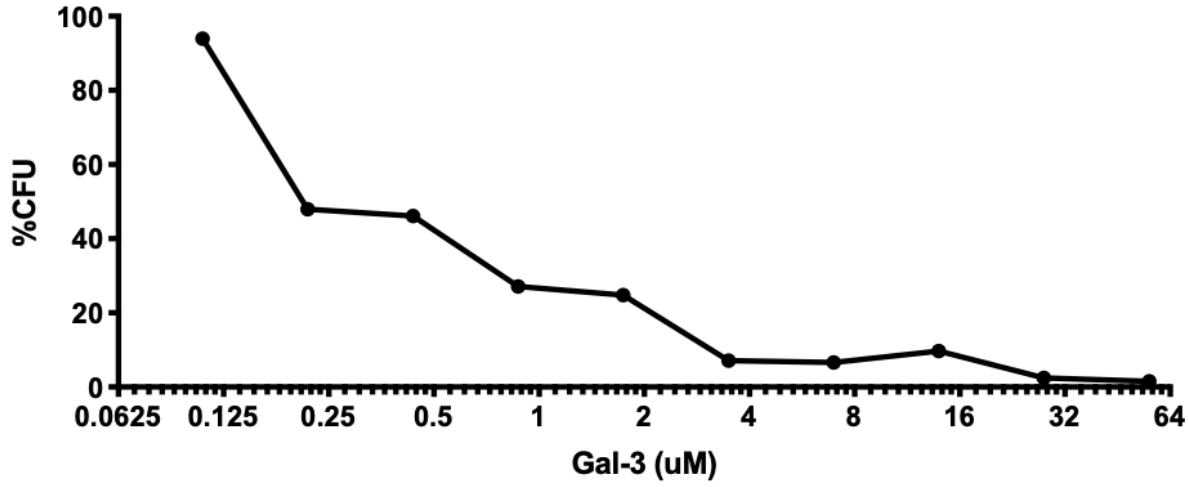


Figure 10: Dose-dependent killing of PAO5 by Gal-3

PAO5 bacteria was treated with different concentrations of Gal-3. Average %CFU of PBS control was plotted with 2 replicates in each concentration group.

With Gal-3 killing activity confirmed, we then tested PAO5 bacteria growth against Gal-1, Gal-1 IAM, and Gal-3. 35 uM of each protein was added to PAO5 undergoing logarithmic growth. After 1 hour of protein treatment, the cultures were plated and enumerated following overnight growth. As seen previously, the Gal-3 treated culture had nearly complete killing. However, with PAO5 in contrast to KPO1, Gal-1 killing could now be observed (Figure 11). Notably, this is the first instance of prototypical galectin exhibiting antimicrobial properties. Furthermore, alkylated Gal-1 IAM exhibited stronger killing than Gal-1 suggesting that oxidative inactivation may regulate Gal-1 killing by preventing Gal-1:bacteria surface carbohydrate interactions.

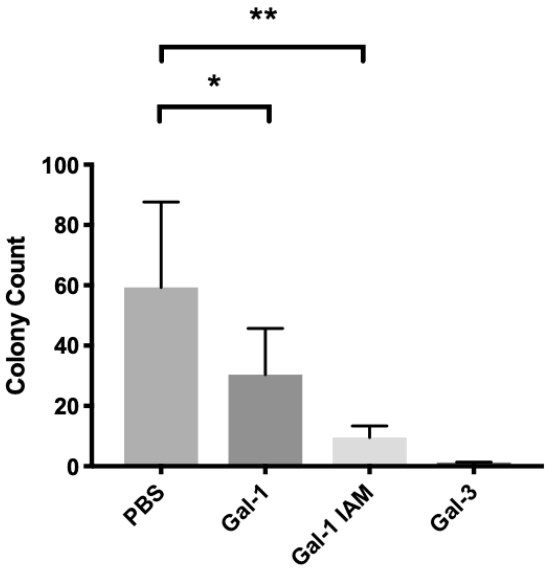
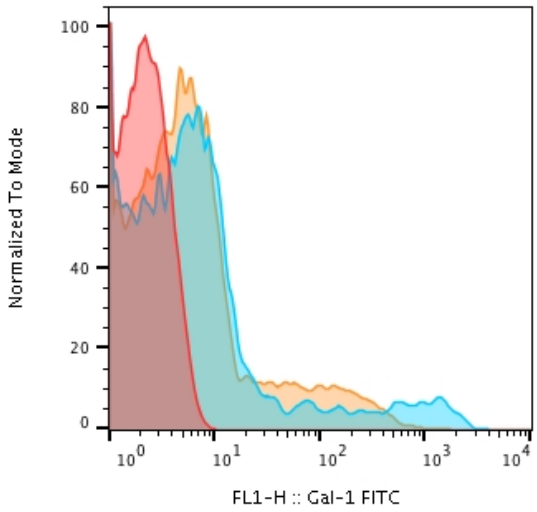


Figure 11: PAO5 Killing by Gal-1 and Gal-1 IAM

PAO5 bacteria was cultured with 35 uM Gal-1, Gal-1 IAM, and Gal-3 (n=3-4). Average colony counts from each group were plotted. One-way ANOVA with Tukey multiple comparison test was used to analyze data. Error bars represent standard deviation.

To ensure that stabilization of Gal-1 through alkylation did not fundamentally affect the protein's binding properties with PAO5, Gal-1 and Gal-1 IAM were tested for binding with PAO5. Gal-1 and Gal-1 IAM were both biotinylated using Sulfo-NHS-Biotin. The protein was then incubated with PAO5 bacteria and stained with streptavidin conjugated to fluorescein isothiocyanate (FITC). Using flow cytometry, PAO5 cells were tested for FITC positivity. In both the Gal-1 and Gal-1 IAM groups, there were FITC positive bacteria cells compared with untreated control bacteria (Figure 12). This suggests that alkylation does not fundamentally alter Gal-1's ability to bind PAO5.



	Sample Name	Subset Name	Count
	20190311_PAO5.001	Bacteria	13566
	20190311_PAO5.002	Bacteria	9790
	20190311_PAO5.003	Bacteria	14523

Figure 12: Flow cytometry based binding assay of PAO5 with Gal-1 and Gal-1 IAM

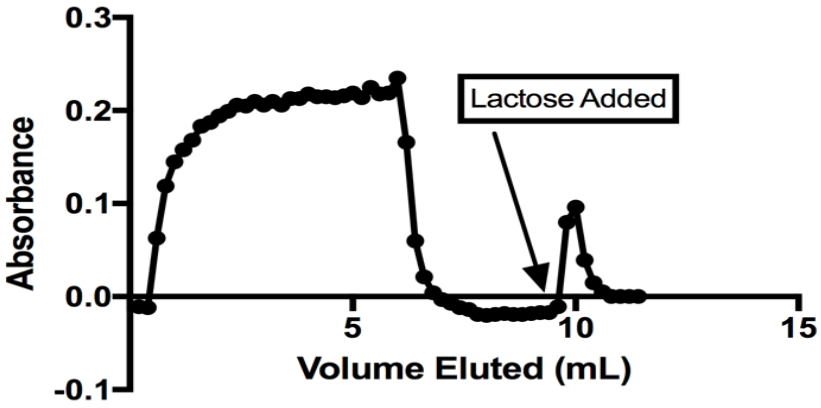
PAO5 bacteria was incubated with biotinylated Gal-1 (Blue) and Gal-1 IAM (Orange). The protein was stained with streptavidin conjugated to FITC. Samples are compared with bacteria only control (red). PAO5 bacteria was similarly FITC positive in both Gal-1 and Gal-1 IAM treated samples indicating binding by both unstabilized and stabilized Gal-1.

Optimizing Gal-1 Oxidation for Crystal Structure

As part of understanding how oxidation of Gal-1 regulates function with respect to antimicrobial properties, we sought to better characterize the structural changes Gal-1 undergoes during oxidation. To do this, we wanted to crystallize monomeric oxidized Gal-1 to examine changes in the CRD that prevent carbohydrate binding and understand post-oxidation functions of Gal-1. As protein structure and function are inextricably linked, crystallizing oxidized Gal-1 may also suggest additional roles for Gal-1 following oxidation. In order to form crystals, highly-pure oxidized Gal-1 was required.[27]

One approach to oxidizing the protein was to use hydrogen peroxide as an oxidizing agent. The first conditions tested were 40 uM Gal-1 with 15 mM hydrogen peroxide incubated for 2 hrs. When the protein was run over a lactosyl-Sepharose affinity column, nearly all the protein was oxidized (Figure 13A). However, as there are 6 cysteine residues in each monomer of Gal-1, there is the potential for intermolecular disulfide linkages that cause oligomerization. To test whether these conditions resulted in oligomers, the oxidized and reduced protein fractions were run on a non-reducing SDS-PAGE gel. After development with Coomassie Brilliant Blue, a large streak of protein was observed in the oxidized lane meaning that the conditions were potentially too harsh and resulted in intermolecular oxidation (Figure 13B).

A



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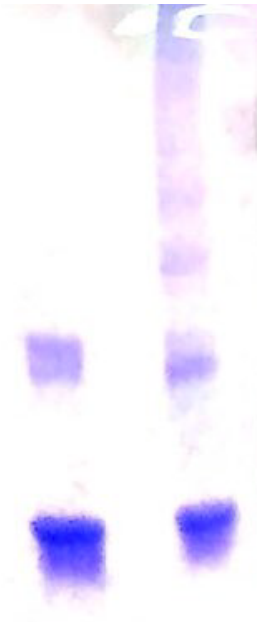


Figure 13: Gal-1 oxidation with 15 mM hydrogen peroxide

40 uM Gal-1 was oxidized by 15 mM hydrogen peroxide for 2 hours. A: Following the reaction, the protein sample was run over an affinity lactosyl-Sepharose column. Protein collected in fractions prior to lactose elution was considered oxidized while protein following lactose elution was considered reduced. B: Reduced (Left Lane) and oxidized (Right Lane) protein from column in A was run on an SDS-PAGE non-reducing gel and stained with Coomassie Brilliant Blue. The oxidized sample contained oligomers as indicated by the protein streaking on the gel.

Because we believed that chemical oxidation by hydrogen peroxide was too harsh, we sought other ways to oxidize the protein such as heating the protein or leaving solutions of protein on the benchtop for extended periods of time. Unfortunately, the solution would turn turbid as Gal-1 formed aggregates despite the gentler conditions.

Empirically derived oxidation conditions

Because 20 mM hydrogen peroxide for 2 hours was too harsh of an oxidative condition, we tried to optimize the conditions such that the protein will oxidize but not aggregate. To find the optimal conditions, we switched to another technique to assess galectin activity. Galectin will bind carbohydrates on sheep red blood cells (SRBCs) and cause the cells to form a lattice in a 96-well plate. Therefore, if there is enough active, reduced protein, the cells will appear as a wide sheet in the plate; while if there is not enough active protein or it is oxidized, the cells will settle to a point on the bottom of the plate. From this assay, we observed that 5 mM hydrogen peroxide did not oxidize Gal-1 at 1 hour but did oxidize Gal-1 at 2 hours (Figure 14). Therefore, we concluded 5 mM hydrogen peroxide for 2 hours is a relatively gentle treatment to oxidize Gal-1.

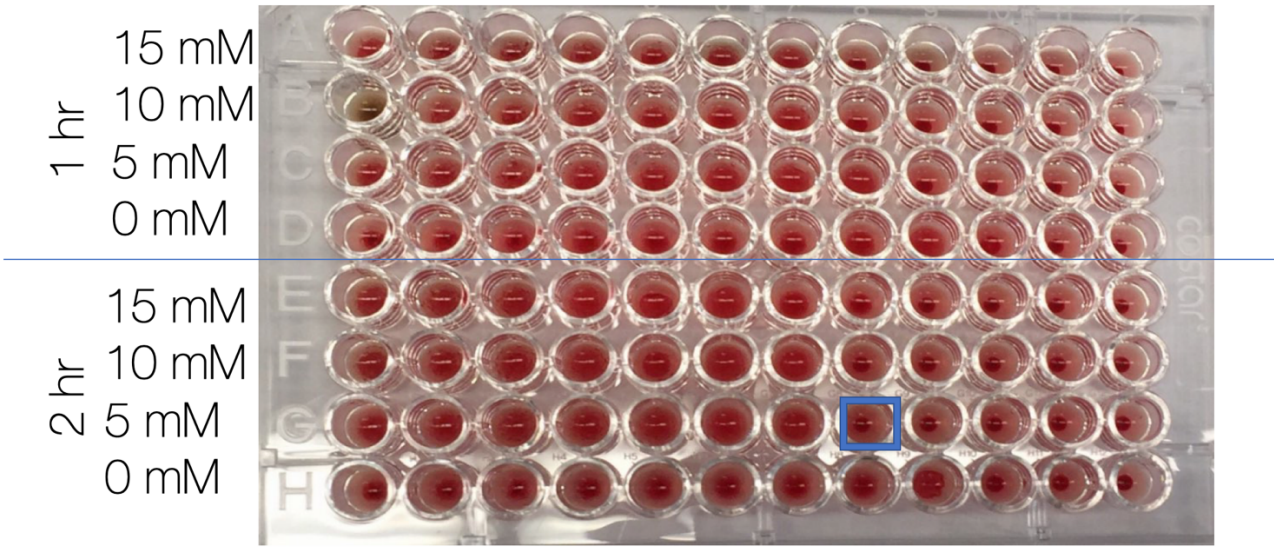


Figure 14: Hemagglutination activity assay of Gal-1 to optimize oxidation condition

180 uM Gal-1 was treated with 0-15 mM hydrogen peroxide for 1 or 2 hours. These samples were then serial diluted 1:2 from left to right across the plate. 2.5% SRBCs were added and the plate was photographed 50 minutes later. The blue square represents a well that was reduced at 1 hour but oxidized at 2 hours. The conditions that led to this oxidation were 5 mM hydrogen peroxide for 2 hours.

With the oxidizing conditions determined, Gal-1 oxidation was confirmed by affinity chromatography. 4.5 uM Gal-1 was oxidized by 5 mM hydrogen peroxide for 2 hrs. Approximately 50 % of the sample was oxidized as analyzed by taking the area under the elution curve (Figure 15A). Similar to before, the oxidized and reduced fractions were run on a non-reducing SDS-PAGE gel (Figure 15B). This time, however, there was no visible higher order aggregation and a relatively large amount of oxidized monomer.

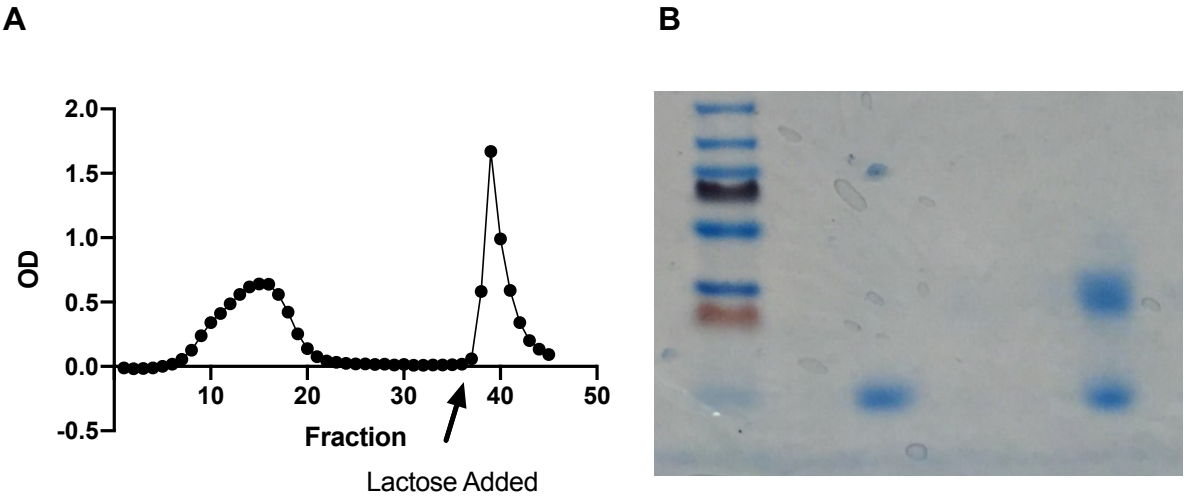


Figure 15: 5mM Hydrogen Peroxide Oxidation for 2 hours

4.5 uM Gal-1 was oxidized by 5 mM hydrogen peroxide for 2 hours. A: Affinity chromatography of Gal-1 after reaction. B: Reduced (Left Lane) and oxidized (Right Lane) protein fractions from A were run on an SDS-PAGE gel with very few aggregates in the oxidized lane.

We were then skeptical whether all of the protein determined to be oxidized was truly oxidized. To confirm that the protein was inactive, we recycled the pre-elution fractions over the same affinity column. Surprisingly, over 70% of the protein that had been labeled inactive was able to bind the column (Figure 16A). We realized that out of standard practice with the lactosyl-Sepharose column, there was βME in the wash and elution buffers. βME is a strong reducing agent, which reversed the oxidation of the protein. When we recycled the inactive peak from our second column again, this time without any βME, all of the protein remained inactive (Figure 16B). This shows that Gal-1 oxidation is reversible by βME as carbohydrate-binding ability is recovered when oxidized Gal-1 is exposed to a sufficiently strong reducing agent.

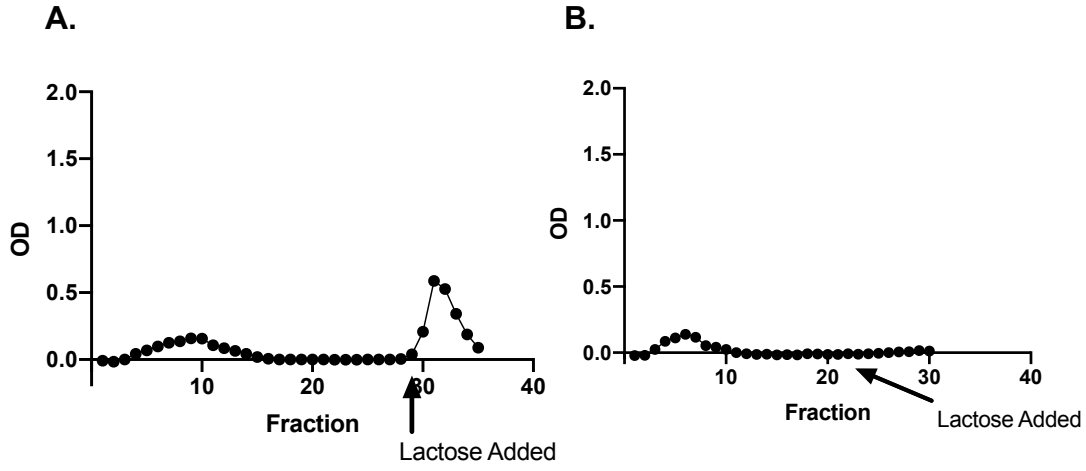
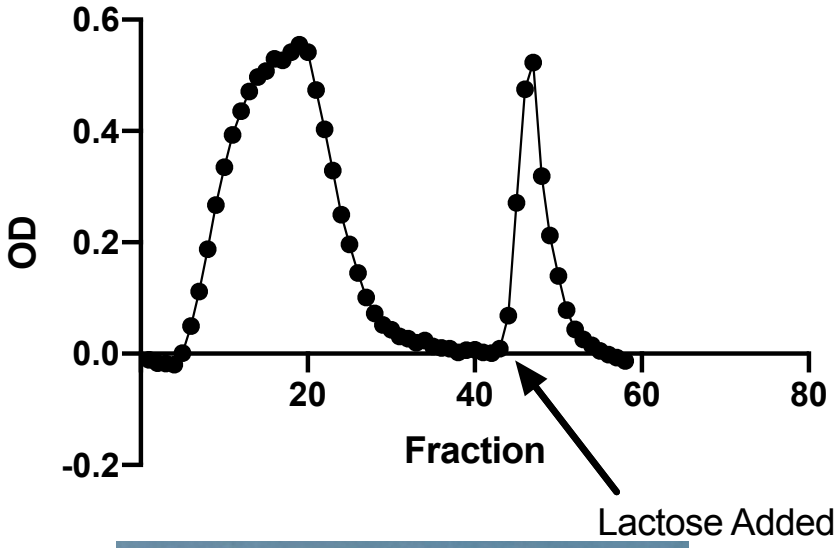


Figure 16: Oxidized protein reduced by βME in column wash buffer

A. Oxidized protein fractions from Figure 15A were rerun over a lactosyl-Sepharose column without βME. Lectin activity was recovered in the protein showing the reversibility of Gal-1 oxidation. B. Oxidized protein fractions from Figure 16A were rerun over lactosyl-Sepharose column without βME. All protein remained oxidized.

Keeping the reversibility of Gal-1 oxidation in mind, we repeated our oxidation conditions without any βME in the buffers. 60 uM Gal-1 was exposed to 5 mM hydrogen peroxide for 2 hours. The protein sample was then assayed for activity by affinity chromatography and over 75% of the protein was oxidized (Figure 17A). Again, the oxidized and reduced fractions were run on an SDS-PAGE gel. Nearly all of the oxidized protein remained monomeric with very little protein forming dimers (Figure 17B). There is not much concern for the protein that remains active from the oxidation as this protein purifies itself from the solution with the lactosyl-Sepharose column. Additionally, the small amount of oxidized dimer can be easily separated by size-exclusion HPLC.

A



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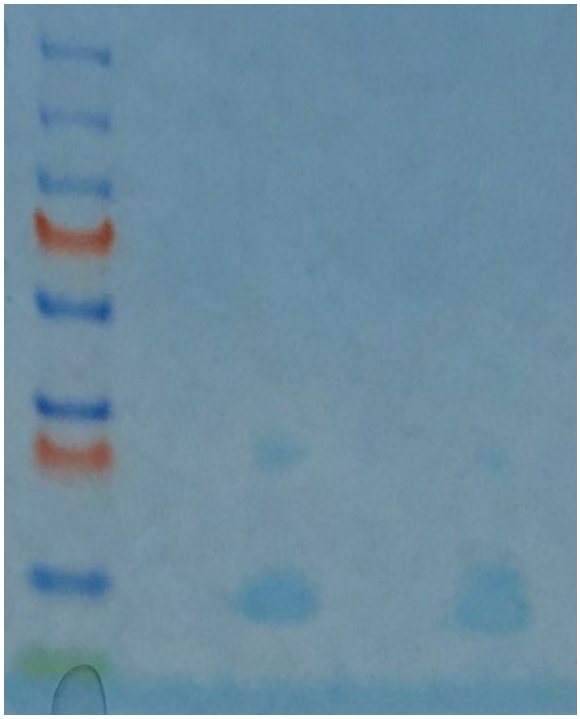
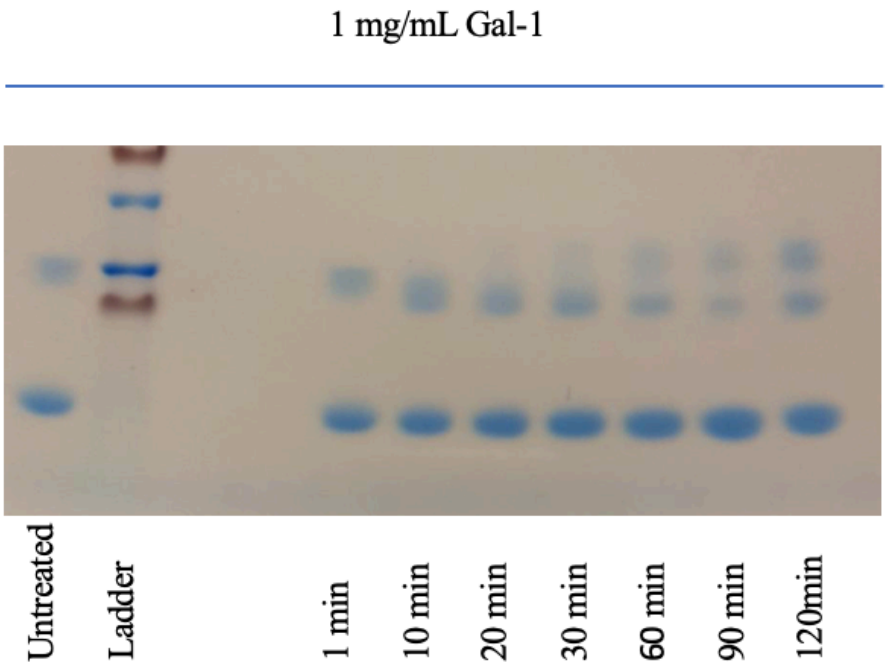


Figure 17: 60 uM Gal-1 treated with 5 mM hydrogen peroxide for 2 hours results in highly monomeric oxidation

A. Lactosyl-Sepharose affinity chromatography binding of Gal-1 oxidation reaction. B. Reduced (Left Lane) and oxidized (Right Lane) fractions from A were run on an SDS-PAGE non-reducing gel.

We also strove to characterize the timing of oligomerization during Gal-1 oxidation. Gal-1 at concentrations of 1 mg/mL and 3 mg/mL was oxidized by 5 mM hydrogen peroxide. At 1 minute, 10 minute, 20 minute, 30 minute, 60 minute, 90 minute, and 120 minute timepoints, oxidation was stopped by quenching unreacted hydrogen peroxide with bovine catalase. These samples were then run on an SDS-PAGE gel to observe oligomerization over time. The 3 mg/mL sample of Gal-1 that was oxidized for 90 minutes had nearly 100% monomer (Figure 18). Although the redox state of these samples is unknown as they were not assayed for activity, the 90 minute timepoint fits previous data showing Gal-1 oxidizing between 1 and 2 hours with 5 mM hydrogen peroxide. The 90 minute oxidation preserves more monomer than the 120 minute oxidation which potentially makes it a more efficient and less wasteful condition to produce oxidized Gal-1.

A



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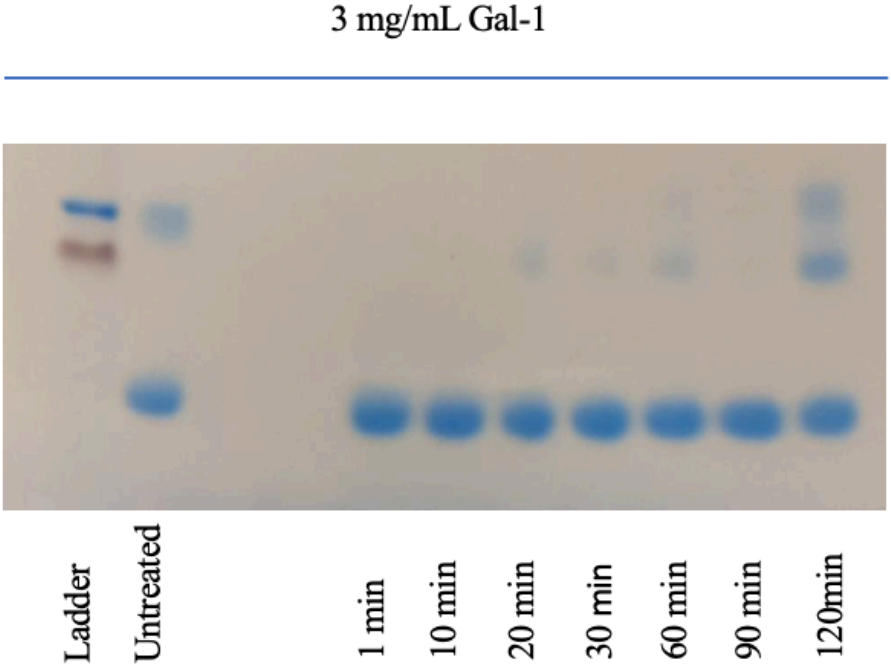

Figure 18: Time-dependent oligomerization of Gal-1 oxidation

A.1 mg/mL(30 uM) Gal-1 and B. 3 mg/mL (90 uM) Gal-1 were treated with 5 mM hydrogen peroxide. Reactions were stopped at the listed times by bovine catalase and run on an SDS-PAGE non-reducing gel.

Discussion

Many studies have investigated galectins as antimicrobial peptides against pathogenic bacteria and fungi; however, of the three galectin classes only tandem-repeat and chimeric galectins have been shown to possess antimicrobial properties. Prior to this work, no literature existed describing prototypical galectins as antimicrobial peptides. We have demonstrated, however, that Gal-1 has the ability to directly target and kill *Providencia alcalifaciens* O5. The O-glycosylation on the bacterial surface provides a carbohydrate ligand through which Gal-1 can bind and kill the bacteria. Though the molecular mechanism of this killing has yet to be described, studies suggest that galectins can penetrate lipid bilayers and this membrane permeability may reduce the viability of bacterial cells.[28]

Previously, redox state has been shown to be an important factor in protein function regulation.[29] This is especially seen in redox shifting environments such as the mitochondria where proteins are exposed to both reductive and oxidative agents associated with glycolysis. Galectins are secreted proteins but do not follow canonical secretory pathways through the endoplasmic reticulum, Golgi apparatus, and secretory vesicles.[30] This means the protein traverses the cellular membrane and is therefore exposed to both the reductive intracellular environment and the oxidative extracellular environment. Galectins also have a high propensity for oxidative inactivation. Following oxidation, galectins lose lectin activity. As galectins are exposed to both reductive and oxidative environments, redox state may act as a functional regulator for galectin. In this work, we demonstrate that Gal-1 carbohydrate binding activity relies on the protein's

oxidative state. We demonstrate this concept in three separate measures for Gal-1 activity: column binding affinity; agglutination of sheep red blood cells; and HL-60 cell agglutination. Furthermore, we show that Gal-1 cysteine residues can be alkylated with iodoacetamide, decreasing Gal-1's propensity to oxidize. By comparing stabilized and unstabilized Gal-1, we created a model to understand how oxidation acts as a functional regulator of Gal-1 carbohydrate dependent functions.

Although unstabilized Gal-1 can kill PAO5 bacteria, alkylated Gal-1 appeared to exhibit stronger killing than unstabilized Gal-1. This suggests that oxidation may act as a regulatory switch to deactivate Gal-1 as it diffuses from its secreting cell. This relatively quick oxidation gives plasticity to the system as Gal-1 activity can quickly be shut down following secretion. Should more active Gal-1 be necessary for defense, the secreting cell can produce more active protein. While altering Gal-1 cysteine residues with iodoacetamide is not a physiologically relevant method to stabilize the protein, nitrosylation cysteine stabilization of protein is found in nature. Interestingly, Gal-2, another prototypical galectin, can be nitrosylated which stabilizes it from oxidation.[31] We have not yet optimized the Gal-1 nitrosylation to observe potential stabilizing effects but doing so may increase the physiological relevance Gal-1 oxidative stabilization.

Providencia alcalifaciens has been shown to infect host cells intracellularly.[25] While our study of Gal-1 bacterial killing is done in culture with oxidizing molecules in solution, Gal-1 could potentially kill bacteria that are intracellular to infected cells. Cytotoxic T lymphocytes (CTLs) are a major cellular defender against intracellular bacterial

infections.[9] When CTLs encounter an infected cell, they will degranulate wherein the cell exocytoses cytotoxic granules that enter the infected cell. These granules contain important antimicrobial peptides including perforin, granzyme A, and granzyme B. Intriguingly these granules also contain Gal-1. Gal-1 is believed to be important in the actual mechanism of degranulation by modulating Fas-Fas ligand interactions,[32] but these cytotoxic granules provide an avenue that Gal-1 could employ to kill intracellular bacteria without traversing the oxidative extracellular environment (Figure 19).

For further study of Gal-1 antimicrobial properties, it would be interesting to identify more strains of bacteria which Gal-1 can kill. As we have demonstrated that Gal-1 alkylation enhances Gal-1 bactericidal effects, screening should utilize Gal-1 IAM to amplify the power of subtle Gal-1 killing. Additionally, all experiments that were performed in this study utilized exogenous Gal-1 that was expressed in *E. coli*. Chinese hamster ovary (CHO) cells are a cell line that produces and secretes Gal-1. To examine endogenous Gal-1 killing of bacteria CHO cells could be cocultured with target bacteria to observe potential antimicrobial properties. This would better demonstrate Gal-1 killing in a cellular context.

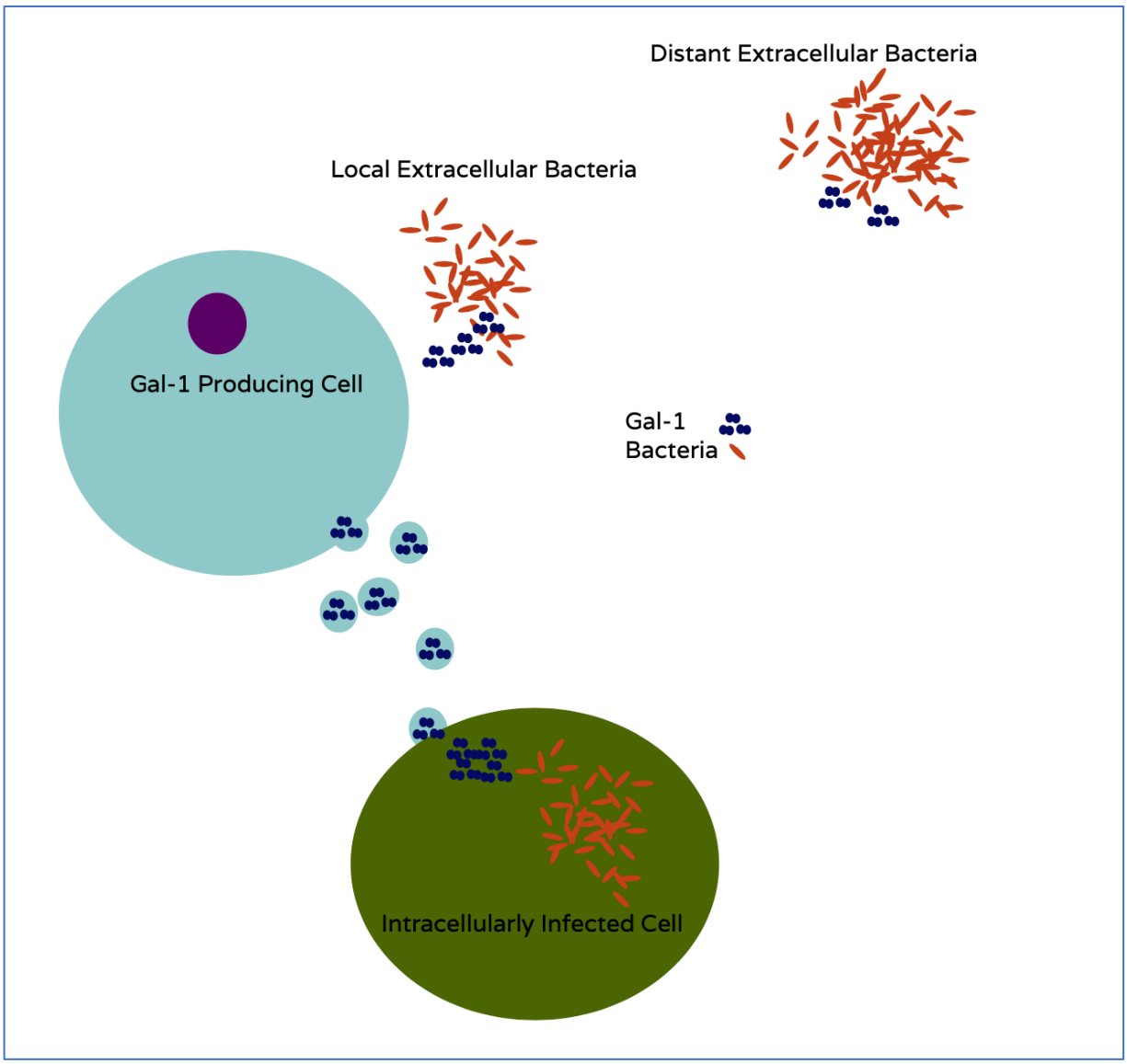

Figure 19: Potential methods of Gal-1 killing intracellular and/or extracellular bacteria

Gal-1 may be non-canonically secreted by a Gal-1 producing cell in to kill local extracellular bacteria before oxidizing. If Gal-1 can be stabilized *in vivo* it may extend its killing capacity to distant extracellular bacteria. Gal-1 may also kill intracellular bacteria by traveling in exocytosed cytotoxic granules with other antimicrobial peptides.

This project also examined the oxidation of Gal-1 and successfully optimized the protocol to produce pure monomeric oxidized Gal-1 for structural analysis. In optimizing the conditions, we had to balance oxidation methods that were alternately too harsh and resulted in aggregation and precipitation of protein with oxidation methods that were too gentle and either took too long to oxidize large amounts of protein or incompletely and heterogeneously oxidized the sample. Through empirical condition testing, we determined that 5 mM hydrogen peroxide for 2 hours was the most efficient reaction condition. We demonstrated that Gal-1 treated with 5 mM hydrogen peroxide for 2 hours led to a loss of lactose binding ability. Additionally, this oxidized protein remained mostly monomeric which is necessary for achieving pure crystals.

These conditions can be used to further study post-oxidation Gal-1 functions. Though oxidized Gal-1 has currently only been shown to promote axonal regeneration in damaged neurons,[19] there may be other post-oxidative functions in which having homogenous soluble oxidized Gal-1 will prove invaluable for elucidation. Finally, crystal analysis of oxidized Gal-1 will provide atomic level insight into Gal-1's post-oxidation functions. Furthermore, it may hint at novel functions through potential binding partners. In order to determine the crystal structure of oxidized Gal-1 we are using HPLC to separate the monomeric oxidized protein. Once we have sufficient quantities, will crystallize the protein and determine the atomic scale changes between reduced and oxidized Gal-1.

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