

Case study of Glycomimetics against Multi-Drug Resistant Pathogens

Rafael Bermeo

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ABSTRACT

Multi-drug resistant (MDR) pathogens such as Burkholderia cenocepacia have become a hazard in the context of healthcare-associated infections, especially for patients admitted with compromising or aggravating conditions. Like other opportunistic Gram-negative bacteria, this pathogen establishes virulence and biofilms through lectin-mediated adhesion. Glycans and glycomimetics have become devices of choice to antagonize or disrupt such interactions. We provide an overview of this topic, highlighting anti-microbial resistance (AMR), anti-adhesion therapy (AAT), carbohydrate-lectin interactions, and glycomimetics as therapeutic agents. Furthermore, we focus on the case of MDR lung pathogens, in particular B. cenocepacia. We describe its position amongst other pathogens and highlight the *Burkholderia cenocepacia* lectin (BC2L) family and the potential of targeting this pathogen with glycomimetics directed to the superlectin BC2L-C.

INTRODUCTION

Many barriers have been met and overcome across the history of the human race and its progress. One of such, and particularly significant, is humans' fight against pathogenic microorganisms. Very relevant to current times, pathogenic viruses can rise to become global threats, but so can bacteria. Be it the bubonic plague, tuberculosis, cholera, or others. These names still resonate, echoes of times in which the battle against pathogens was lost. In such times, a unicellular organism could singlehandedly decimate a percentage of the human population: for example, tuberculosis peaked in the XIXth century and is estimated to, now, have killed 14% of humanity (all humans that had ever lived to that point), making it the deadliest bacterial infection in history, so far. In 2019, it still managed to infect 10 million and kill 1.4 million people. (WHO, 2020) Adding to this, bacteria have acted in conjunction with pathogenic viruses, for example, during the early XXth century, when the influenza pandemic later called 'the Spanish Flu' left millions vulnerable to opportunistic bacterial pneumonia. This pandemic decimated 5% of the world's population at the time.

The aforementioned dark times came to an end in relatively recent times: as the XIXth century gave its way to the XXth, the rapid development and introduction of many vaccines gave a prophylactic means to fight infectious diseases. More importantly, Alexander Flemming's chance encounter with penicillin in 1928 paved the way for the direct fight against bacterial infections with antibiotics. Penicillin's widespread use started in the 1940s during World War 2 and was followed by the 'Golden Age' of antibiotics (1950-70s), humanity's highest point in the fight against microbes. However, by 1955, antimicrobial resistance (AMR) to penicillin was a fact only twelve years after the start of its extensive use, as Flemming himself had predicted. Thus, AMR loomed large over modern medicine and scientists, who kept finding new antibiotics, hoping to stay ahead in the race between humans and AMR pathogens (see Table 1). (Bushak, 2016)

At the beginning of the XXIst century, there is no denying it : we are losing the antibiotics race. As seen in Table 1, the most recently discovered antibiotics (Daptomycin in 2003 and Ceftazidime-avibactam in 2015) lasted only one year before resistance appeared and was documented. (US-CDC, 2019) Names such as MRSA (Methicillin-resistant *Staphylococcus aureus*) have reached the general public, and terms such as 'superbugs' have been coined for MDR and PDR (Multidrug- and Pandrug-resistant) bacteria.

Cases of patients infected with superbugs resistant to 'last-resort' antibiotics such as colistin already surfaced in 2016. (McGann et al., 2016) These pathogens, resistant to most existing therapies, are especially threatening to hospitalized patients who present risk factors. Risk factors include medical conditions such as cancer, diabetes and immunosuppression, for example, due to chemotherapy. Additionally, immunodeficiency due to either physiological stress (for example, skin damage or malnutrition) or old age can render a patient prey to these pathogens, in what is called an 'opportunistic' infection. (Appelgren et al., 2001 ; Ozer et al., 2010 ; van Duin et al., 2016) It is evident that the mere presence of these pathogens in medical environments could quickly turn into a worst-case scenario: fragile patients threatened by untreatable bacterial infections. Indeed. MDR microorganisms already represent the leading cause of death from hospital-acquired infection (HAI).(Cassini et al., 2019; van Duin et al., 2016)

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Development		Resistance		
Antibiotic	Year Released	Year Identified	Resistant Strain	
Ponicillin	10/11	1942	Penicillin-resistant	
Penicilin	1941	1942	Staphylococcus aureus	
Vancomycin	1958	1988	Plasmid-mediated vancomycin-resistant	
Vancomychi	1550	1500	Enterococcus faecium	
Amphotericin B	1959	2016	Amphotericin B-resistant	
			Candida auris	
Methicillin	1960	1960	Methicillin-resistant	
Fisher de deservations	1000		Staphylococcus aureus (MRSA)	
Extended-spectrum	1980	1983	Extended-spectrum beta-lactamase-producing	
cephalosporins	(Cerotaxime)		Azithromucin-registant	
Azithromycin	1980	2011	Noissaria goporrhoga	
			Klebsiella pneumonige carbapenemase	
Imipenem	1985	1996	(KPC)-producing	
			Klebsiella pneumoniae	
c: () .	4007	2007	Ciprofloxacin-resistant	
Ciprofloxacin	1987	2007	Neisseria gonorrhoeae	
Elucopozolo	1099	1099	Fluconazole-resistant	
Fluconazole	1900	1900	Candida	
Caspofungin	2001	2004	Caspofungin-resistant	
casporangin	2001	2004	Candida	
Daptomycin	2003	2004	Daptomycin-resistant methicillin-resistant	
buptomyem	2005	2004	Staphylococcus aureus	
Ceftazidime-			Ceftazidime-avibactam-resistant	
avibactam	2015	2015	KPC-producing	
			Klebsiella pneumoniae	

 Table 1. The race between antibiotic development and AMR. Adapted from the U.S. Centers for Disease Control and Prevention.(US-CDC, 2019)

Undeniably, HAIs by resistant pathogens can grow into a bigger problem, to the point of reversing years of advances in modern medicine. This issue is illustrated by the situation of patients afflicted with cystic fibrosis (CF). CF is a well-studied genetic disease caused by a cvstic fibrosis transmembrane conductance regulator (CFTR) mutation. This dysfunction results in thick mucus accumulating in different organs. Its chief consequence is progressive respiratory problems and increased susceptibility to lung inflammation and infections. Although no definitive cure exists, regular advances in modern medicine have enabled specialized treatment and care for CF patients, improving their quality of life. In terms of life expectancy, children born with CF in 2021 are expected to live 20 years more than the previous generation of patients.(O'Sullivan et al., 2009). Despite this, the leading cause for morbidity and mortality (at least 80%) in this population are bacterial respiratory infections. Indeed, the thick mucus characteristic of CF translates into a reduced capacity for airway cleansing, making the lungs an ideal breeding ground for opportunistic pathogens.(Ciofu et al., 2013) Due to this, CF is considered a high-risk factor in HAIs. It is responsible for mortality among genetic diseases in the Caucasian population. Similar to antibiotics, CF patients are losing the battle against MDR pathogens.

Proportional to what is becoming one of the main challenges of the XXIst century, a coordinated response against AMR has been erected at the highest levels: the World Health Organization (WHO), the European Commission, and the United States CDC (Centers for Disease Control and Prevention) all have action plans to implement against the rise of MDR pathogens.(EU-Comission, 2017; US-CDC, 2019; WHO, 2015) These plans provide solid advice on reducing resistance by better handling of antibiotics and highlight the necessity for alternatives in this fight. Therapies involving vaccines, antibodies and bacteriophages are some of the alternatives presented. Another alternative to antibiotics, less conventional but more relevant to this communication, is anti-adhesion therapy.

ANTI-ADHESION THERAPY (AAT), A POSSIBLE SOLUTION

To act efficiently, infective pathogens need to interact with their environment. First and foremost, a virus or a bacterium needs to recognize the cells of its host to start the infective process. At this point, it becomes necessary for the pathogen to remain in close vicinity to its host cells. In this vicinity, pathogens thrive: enhanced access to nutrients, shelter from cleansing mechanisms such as airflow or liquid flow, cover from immune factors all converge to facilitate infection. Consequently, host-adhesion is a determinant factor in the infective process.

The concept of anti-adhesion therapy surfaced in the 90s and consisted of using monoclonal antibodies to disrupt adhesive interactions between leukocytes and endothelial cells. Disrupting those interactions was therapeutically beneficial in models of inflammation or immune response. (Carlos et al., 1994) Currently, AAT aims to disrupt adhesive interactions but has broadened its scope considerably. One of its main applications is relevant to our study: to disrupt the interactions between invasive pathogens and their hosts (see Figure 1).



Figure 1. Schematic representation of bacterial adhesion to epithelial cells and the anti-adhesion strategy. A detail of the workings of anti-adhesion therapy is presented in Figure 5.

Considering the increasingly complex challenges to antibiotic therapy and the emergence of drug-resistant fungal pathogens, anti-adhesion has gained momentum as a complementary type of therapy. AAT can be deemed complementary to antibiotic therapy because of its lack of selective evolutionary pressure: only the drug-resistant mutants survive and constitute the next generation. Conversely, AAT does not result in the elimination of pathogens. By merely obstructing the infective action of the pathogens, this type of therapy does not induce selective pressure in such a direct way. Nevertheless, it is arguable that mutant organisms that evade AAT and proceed to successful infection will gain an evolutionary advantage: enhanced access to nutrients and capacity to multiply, especially for viruses. Alternatively, these 'favoured' strains will have to compete with normal strains, instead of being the sole survivors of their generation, instead of the unhindered growth of antibiotic-resistant strains. The result is that the resistance to AAT is possible but on a different scale than the dramatic race observed for antibiotics. (Cozens et al., 2012) Furthermore, the prospects of AAT will undoubtedly benefit from the lessons learned from antibiotics, such as the need to limit over-prescription and encourage combination therapies, among others.

Indeed, combination therapies may be instrumental in curbing otherwise unsurmountable MDR pathogens. Recently, modern computational tools became available to model and predict outcomes of combination therapies on simple disease models. Encouraging results showed that antibiotics and anti-adhesives combine synergistically, generating better outcomes than the isolated treatments. Furthermore, the study optimized the treatment to arrive at a predicted 'best-case' outcome, in which the minimum antibiotic dose was lower, reducing the chances of resistance to develop.(Roberts et al., 2019) Thus, it would seem that AAT coupled with the gathered knowledge and the newest technologies can turn the tide in the fight against pathogens.

Among the different AAT approaches against infections, some highlights include the disruption of the biosynthesis of adhesion factors of either pathogen or host, the use of antibodies targeting adhesion factors, the immunization of patients against the adhesion and the competition against binding epitopes by tailored therapeutic agents. (Krachler et al., 2013) We will develop this last example : the design of AAT agents intended to mimic and compete against epitopes that are usually targeted during the adhesion process in the context of early infection.

As mentioned earlier, adhesion is a staple of infection, meaning that adhesion machinery has evolved throughout time and become increasingly effective and varied. This machinery has also gained specificity in its variety : many different virulence factors specifically target their corresponding epitopes in the host/pathogen interface. Consequently, an understanding of these virulence factors, their targets and the host/pathogen interface is necessary to attempt AAT. One key element of this same interface is the so-called glycocalyx : a carbohydrate-populated matrix that encapsulates different cells, including epithelial and bacterial cells.

THE ROLE OF LECTINS AND CARBOHYDRATES IN INFECTION

Human epithelial cells are found at the forefront of human anatomy separate the body and its cavities from the exterior environment. Their glycocalyx nanolayer comprises glycoconjugates: glycoproteins and glycolipids, which present their carbohydrate portion to the extracellular environment. The role of the glycocalyx and its actors is to sense and communicate with their environment in different ways. For example, epithelial cells are the gatekeepers of the body compartments and, as such, need to communicate to establish a stable cellular tissue. This endothelial tissue assembly is ensured by glycocalyx-mediated communication. (Reitsma et al., 2007) Another example of this communication is how glycoconjugates mediate immune self-recognition, allowing the immune system to discern between own and foreign cells and act accordingly. Finally, the glycocalyx can be a biomarker of diseased states like cancer.(Mereiter et al., 2019; Pinho et al., 2015) Theoretically, the structural versatility of glycans allows them to hold an unfathomably large quantity of information. In reality, this information is filtered through physical and biological constraints, resulting in the glycan structures observed in living organisms. The resulting information held by these glycan structures remains vast: the 'sugar code' is considered the third

alphabet of life, employing monosaccharides as letters in parallel to nucleobases and amino acids.(Gabius, 2018; Kaltner et al., 2019; Solís et al., 2015)



Figure 2. Left : Electron microscopy picture of the endothelial glycocalyx. Right : Schematic depiction of the glycocalyx and some of its roles. Glycoconjugates and oligosaccharide epitopes are schematized at the surface of an epithelial cell. Adapted from Zausig and co-workers (2013). (Zausig et al., 2013)

Naturally, for every glycan presented by the glycocalyx as a 'message' to its environment, another biomolecule plays the complementary role of 'reader'. Lectins are ubiquitous carbohydrate-binding proteins, key recognition agents for intercellular interactions at the extracellular matrix. Lectins have been studied extensively, owing to their role and potential for deciphering the sugar code and providing valuable knowledge over its significance on biological processes.(Sharon et al., 2004; Solís et al., 2015)



Figure 3. Schematic representation of different types of symmetry observed in lectins. The symmetry family and symmetry axis are noted for each schematic representation. Adapted from Notova and co-workers (2020). (Notova et al., 2020)

Generally having a weak millimolar affinity for the monosaccharide version of their ligand, lectins compensate by establishing multivalent interactions mediated by the presence of several binding sites. Indeed, lectins often present elements of structural symmetry: β -propellers, β -trefoils and β -sandwiches in homo-multimeric assemblies are not uncommon.

As lectins typically rely on multivalent interactions, they present their binding sites on the same face of the carbohydrate recognition domain (CRD). The prototypical lectin presents many equivalent or quasi-equivalent binding sites on one of its faces, around a symmetry axis, as seen in Figure 3.

Although this seems to imply that lectins have low structural diversity, the opposite is true: lectins hold the structural diversity to match the sugar code. Indeed, the richness of specificity and topology observed in lectin scaffolds have made them exciting tools for generating engineered 'neolectins' with applications in diagnostics, therapy and material science, among others.(Notova et al., 2020; Ribeiro et al., 2018) Developed in recent years, UniLectin3D is a valuable database for exploring and comparing lectins and scaffolds: it curates lectins by structural features and carbohydrate specificity and even species, highlighting that lectins are ubiguitous. (Bonnardel et al., 2019)

Although lectins do not exclusively mediate intercellular communication, these proteins are notably represented in the interactions between humans and microbes. As mentioned earlier, a prerequisite to attempting AAT is a thorough understanding of microbial virulence factors and their targets in the host/pathogen interface. Carbohydrate-binding molecules (lectins, toxins, adhesins) from bacteria, viruses and even parasites are famously known virulence factors. On the one hand, adhesins are found atop bacterial extracellular organelles – fimbriae and mediate the adhesion of the whole bacterium to any surface that exposes the corresponding carbohydrate epitope.

For example, FimH is an extensively studied adhesin that allows Escherichia coli's fimbriae to adhere to mannosylated residues on human epithelial cells, thus facilitating urinary tract infection (UTI). Recently, mechanical studies performed by atom force microscopy (AFM) have characterized the interactions of FimH and other adhesins as 'catch bonds' : interactions that get stronger under mechanical tension. (Viela et al., 2020) The mechanical strength observed supplements another characteristic of these virulent interactions : whereas animal and plant lectins usually have low affinity for their targets, microbial lectins and adhesins present sub-micromolar or stronger affinities. (Imberty et al., 2005)

On the other hand, toxins and lectins are, contrary to adhesins, soluble. Toxins are proteins that usually feature different sub-units. The pathogen releases them to recognize epitopes on the surface of target cells, which is mediated by a first sub-unit. Upon binding, toxins are internalized, and their second sub-unit enacts a toxic effect, often leading to cell death. A classic example of such toxins is seen in Figure 4: the AB5 toxin family. AB5 toxins featured in organisms such as E. coli and Bordella pertussis present a cytotoxic ADPribosyltransferase (A) domain linked to five (B5) lectin subunits with the capacity to recognize endothelial surfaces.(Imberty et al., 2005; Merritt et al., 1995) Finally, many soluble lectins do not fill the role of either adhesin or toxin. These agents often present specificity to epitopes located at the glycocalyx but are not reduced to these targets. Lectins are versatile and can fill complex roles related to guorum sensing, biofilm formation and even cooperativity across different species of pathogens.



Figure 4. Strategies used by pathogens for host recognition and adhesion. Adapted from Imberty and co-workers (2008). (Imberty et al., 2008)

The list of pathogens using lectins for adhesion, infection and toxicity is long: E. coli, Staphylococcus aureus, Pseudomonas aeruginosa, Vibrio cholera, Clostridium tetani, Influenza viruses, etc. (Imberty et al., 2008). As transpires from Figure 4, many illnesses and pathologies rely on lectins in their initial stages, cementing the idea that AAT would be beneficial to counter MDR pathogens. (Poole et al., 2018) Moreover, lectins have a role in establishing and holding biofilms together, thus boosting resistance to antibiotics. Biofilms occur when the bacterial and fungal cells adhere to a surface and each other to form an extracellular matrix. For pathogenic bacteria, the advantages of forming biofilm are many::stability for growth, change into an infection-adapted phenotype, elasticity against physical forces and, more importantly, resilience against host immune factors and antibiotics. (Hall-Stoodley et al., 2004) Interestingly for AAT, the knock-out or inhibition of biofilm-mediating lectins disrupts the biofilm integrity.(Diggle et al., 2006; Inhulsen et al., 2012; Tielker et al., 2005)

As a result, bacterial lectins are twice-verified targets for AAT : antagonizing all pathogenic lectins would certainly be therapeutically advantageous in the context of early infection. However, every project targeting lectins must be unique: most carbohydrate/lectin interactions are specific. Indeed, lectins are as diverse as carbohydrate structures are. Nevertheless, trends do exist in the context of microbial virulence factors and infections.

Among the typical targets for lectins, the role of histo-blood group oligosaccharides in microbial infections is undeniable. (Heggelund et al., 2017) Human oligosaccharides are tightly bound to infection, to the point that evolutionary strategies have developed around them. A clear example of this can be drawn from the staple of mammalian biology: breastfeeding. High concentrations of oligosaccharides occur in the milk of humans and other mammals: these are HMOs (human milk oligosaccharides).

Interestingly, these HMOs present the same epitopes usually recognized by virulence factors. Studies analyzing the influence of HMOs in pathogenicity showed better outcomes for breastfed infants. (Sharon, 2006) It means that mammals confer a true anti-adhesion therapy to the next generation by the mere action of breastfeeding. Regarding the epithelial glycocalyx, the histo-blood group oligosaccharides present large yet well-defined epitopes for lectins to recognize, which explains the high diversity of microbial lectins and the high specificity for their targets

Some pathogens take the unorthodox approach and display carbohydrates that human lectins can recognize as an alternative to microbial lectins. By high-jacking human bio-machinery, they can infect and enter the human cell in question in the case of viruses. Among the pathogens using this strategy is the well-known HIV: it targets Langerin and the Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN). This receptor belongs to the immune system and can recognize mannosylated glycans characteristic of invasive pathogens such as Ebola, Hepatitis C and HIV. Dendritic cells can travel to lymph nodes and elicit immune responses by binding to these viruses. However, HIV uses this dynamic to propagate and find its way to lymph nodes. Another family of pathogens that have become relevant in recent times uses a similar process: coronaviruses. Indeed, recent studies have confirmed the ability of SARS-CoV-2 to use its spike glycoprotein to target human lectin DC-SIGN and others. (Chiodo et al., 2020) Thanks to its spike protein and human angiotensin-converting enzyme (ACE2), the virus enters human cells. (Letko et al., 2020) It remains to be seen whether the carbohydrate/lectin interactions discovered are also relevant for adhesion and infection.

Returning to AAT, we mentioned the concept of designing therapeutic agents to mimic and compete against epitopes targeted by virulence factors. Applied to virulent lectins, this translates into designing carbohydrate ligands that can compete against the human oligosaccharides by efficiently binding to the lectins, effectively impeding microbial adhesion, as schematized in Figure 5.



Figure 5. Schematic depiction of the carbohydrate/lectin interactions involving epitopes presented by the glycocalyx and the bacterial cell surface leading to adhesion. AAT disrupts the interactions by replacing these epitopes with therapeutic molecules.

GLYCANS AND GLYCOMIMETICS AS THERAPEUTIC AGENTS

On paper, the concept of using naturally occurring carbohydrates in AAT can be considered a revamping of the successful story involving breastfeeding and HMOs. Unsurprisingly, it was implemented as early as the late 70s and was successful to an extent: in vitro and in vivo experiments repeatedly prevented infections in models featuring common pathogens such as *E. coli*.(Aronson et al., 1979; Sharon, 2006; Sharon et al., 2000) In animals, successful administration of soluble carbohydrates led to the protection of diverse environments: gastrointestinal and urinary tracts, eyes and lungs. Nevertheless, these successes were pushed only to a certain extent, as mono and oligosaccharides showed shortcomings on the prospect of their use for widespread therapy.(Parente et al., 2003; Sharon, 2006; Ukkonen et al., 2000)

Indeed, sugars, by their nature, are not viable therapeutic molecules. The leading problem is that naturally occurring sugars are 'accounted for' by human biology, meaning that metabolization machinery does a quick job of degrading them into smaller building blocks for recycling. Indeed, sugars are part of the 'building-blocks-of-life', meaning that a cohort of enzymes exists with the sole purpose of assembling, modi-fying and dismantling carbohydrate structures. Naturally, this would deplete the effective concentration of any sugar, lowering their therapeutic effect and calling for higher dosage to reach the desired outcome. On a related note, carbohydrate epitopes are found in the glycocalyx and elsewhere, with roles to fill in human biology: communication, immunity or others. It means that overloading the human body with saccharides can have undesired and potentially harmful off-site side effects.

Another issue with sugars is their large polar surface area (PSA). On the one hand, polar molecules are easily dissolved in aqueous solutions, allowing easy administration to epithelial interfaces of infection. On the other hand, the design of therapeutic molecules avoids large PSA values: strongly polar molecules cannot permeate membranes, meaning that some compartments are out of their reach. An excellent example of this issue is biofilms: if the bacterial targets hide behind a lipophilic matrix, polar anti-adhesives are as useless as antibiotics. The PSA of a monosaccharide is already high on the scale of drug design: 120 Å2 for glucose, nearing the 140 Å2 upper limits recommended. It means that oligosaccharides are too polar on this scale.

The last argument separating sugars from drug-like molecules is their low stability: carbohydrates present reactive chemical functions that react with the biological matrix, either facilitated or not by metabolic enzymes. Furthermore, monosaccharides maintain a dynamic equilibrium between different forms (cyclic forms and open chains). It might not be a problem for their biological role, but chemical stability is necessary for a therapeutic entity.

Recapitulating: on the one hand, carbohydrates have proven their anti-adhesive potential both in nature and in the laboratory. On the other hand, they fail to align with what modern medicine considers a 'viable' drug. Synthetic organic chemistry has provided a solution to this predicament: glycomimetics. As their name indicates, this relatively new class of therapeutic agents aims to mimic carbohydrates in terms of shape and effect. Their parallel objective is to present an optimized pharmacokinetic profile. The resulting therapeutic molecules, or glycodrugs, boast increased metabolic and chemical stability, specificity for their targets, and the ability to be adjusted and redesigned through organic synthesis to adapt them to new needs continuously.



Figure 6. Examples of monovalent glycomimetics. Adapted from Tamburrini and co-workers (2020). (Tamburrini et al., 2020)

Indeed, glycomimetics are molecules tailored to their target: rather than merely copying the original carbohydrate-mediated interaction, they draw on it and try to perfect it. For example, a representative glycomimetic could be modelled after a monosaccharide but be functionalized with lipophilic moieties that complement the lectin's binding site to boost affinity and specificity for its target. Under increased lipophilicity, the now moderate polarity of the molecule would grant it access to spaces normally barred for monosaccharides. Finally, the molecule could be synthesized from scratch to replace the ring oxygen with a carbon atom, making it a 'carbasugar' as seen in Figure 6. This modification would further reduce the PSA, grant it metabolic stability, and secure the cyclic form from opening. Compared to its monosaccharide equivalent, this hypothetical glycomimetic is already far ahead down the roads of drug-likeness and therapeutic effect.

It's not necessary to go very far to find a real-life glycomimetic success story: carbohydrate-based oseltamivir/Tamiflu is a widespread antiviral drug that prevents and treats influenza A and B. Oseltamivir was designed to mimic the transition state generated when the viruses' neuraminidase cleaves the terminal sialic acid of its substrate glycocompounds. Synthetic strategies were applied to boost potency and remove structural weak points detrimental to stability or affinity, starting from a slightly modified monosaccharide. (Ernst et al., 2009; Kim et al., 1997) In addition to installing a hydrophobic moiety to match an apolar pocket of the binding site, the chemical modification also allowed to generate a prodrug derivative, leading to an orally bioavailable glycodrug. Onwards from this early example, the great potential of glycomimetics has sparked a growing number of projects for a range of targets. Some obvious targets for glycomimetics are sugar-metabolizing enzymes: for example, glycodrugs voglibose and miglitol target glycosidases to achieve glycemic control in the context of diabetes. (Campbell et al., 2000 ; Chen et al., 2006 ; Ernst et al., 2009) These molecules, an iminosugar and an N-glycoside are examples of how replacing oxygen atoms with nitrogens can lead to viable glycodrugs. New drugs such as these are always welcome, especially in the case of diabetes: the ever-growing pathology of the modern-day and the eighth leading cause of death in 2012. (WHO, 2016)

A second significant pathology in which glycomimetics have their role to play is cancer: abnormal cancerous cells exhibit unusual modifications in their glycocalyx, opening an avenue for studying and using cancer-related carbohydrates. Indeed, selectins and galectins are lectin families that have shown involvement with cancer and aberrant oligosaccharides.(Natoni et al., 2016; Takenaka et al., 2002) Many glycomimetic antagonists to selectin and galectin are being developed for cancer combination therapy and are currently undergoing clinical trials.(Festuccia et al., 2019; Wdowiak et al., 2018) Apart from targeting these lectin families, glycomimetics have found their way into cancer therapy in other ways. For example, Gemcitabine is a nucleoside analogue that features a fluorinated ribose mimetic used in chemotherapy for decades. (Noble et al., 1997) The list of glycomimetics developed against these and other pathologies is long. Therefore, the synthetic methodologies leading to glycomimetics are ever-growing, as has been recently documented. (Hevey, 2019; Tamburrini et al., 2020)

Similarly, and closer to our interest, glycomimetics have met success as anti-adhesives. Among many successfully drugged targets, we encounter HIV-related DC-SIGN: based on the oligosaccharide epitopes bound by the lectin, new synthetic glycomimetics have been designed and synthesized throughout the years. They can be separated into the two families recognized by DC-SIGN: mannosides mimicking the epitope Man9, and fucosides, mimics of Lewis oligosaccharides. Among the many types of glycomimetics designed, high-affinity monovalent structures were created, mirroring the oligosaccharide assembly, yet replacing each sugar with a glycomimetic counterpart.(Medve et al., 2019; Sattin et al., 2016)

An exciting avenue that synthetic chemistry opens for glycomimetics is covalent inhibition: absent in natural structures, reactive groups can be synthetically added to glycomimetics to tether them to their targets. This strategy applies to carbohydrate-modifying enzymes by taking advantage of their machinery in mechanism-based design. (Ren et al., 2018) Closer to our interest, anti-adhesive covalent compounds that inhibit lectins persistently have shown promising potential to impede the virulence of the corresponding organism. (Wagner et al., 2017)

Nevertheless, the avenue of covalent inhibition entails particular considerations, such as the possibility of unspecific binding and unforeseen side effects. It follows that, for covalent glycomimetic design, ensuring selectivity for the target becomes equally or more important than ensuring high affinity. Incidentally, improving the selectivity and affinity of monovalent ligands is a practical step to take before taking glycomimetics to the next level : multivalency.(Martínez-Ávila et al., 2009; Varga et al., 2014)

Enabled by synthetic chemistry and its tools, the multivalent assembly of glycan ligands has opened the gate to otherwise inaccessible rewards. Ever-increasing numbers of scaffolds and coupling procedures allow straightforward construction of macromolecules bearing repeated units of monovalent ligands. The relevance of multivalent glycocompounds is quite clear: by presenting several copies of the ligand, the monovalent affinities increase to deliver multivalent affinities several orders of magnitude higher. However, this is not new: multivalent glycocompounds aim to emulate nature, which usually handles carbohydrate/lectin interactions with multivalency. Indeed, lectins present many equivalent binding sites simultaneously to compensate for low-affinity monovalent interactions. Furthermore, carbohydrates destined for molecular recognition usually occur in clusters of epitopes yielding the so-called 'Cluster Glycoside Effect'. This effect, multivalency, and its implications for therapy have been studied and discussed for decades. (Bernardi et al., 2013 ; Lee et al., 1995 ; Lundquist et al., 2002 ; Pieters, 2009) Some important lessons to retain from the use of multivalent glycocompounds relate to their design and their mechanisms of function.

Regarding design, multivalency has infinite possibilities: glycans have been attached to increasingly large frames, and the valency of these structures has exploded accordingly. Some multivalent designs have entirely left behind the idea of drug-likeness to produce therapeutic agents at an entirely different scale; carbohydrates supported by nanoparticles, guantum dots, vesicles, micelles, proteins, polymers, and dendrimers have been successfully implemented as tools or therapeutic agents in various projects.(Ashree et al., 2020; Budhadev et al., 2020; Kim et al., 2005; Prost et al., 2012; Schaeffer et al., 2013 ; Soria-Martinez et al., 2020 ; Zubkova et al., 2018) Pushing design to the limit, virus-like structures bearing over a thousand carbohydrates have been generated, bringing the level of mimicry to a new height (Figure 7), (Ribeiro-Viana et al., 2012) Nonetheless, this infinite potential can be regulated by some metrics: the structure's geometry can be defined by the relative orientation of units and the distance between them. Other factors that have a proven influence are the rigidity of the construction and, naturally, the

number of epitopes presented. Particularly in the case of lectins, it has been established that tailoring the multivalent agent to its target ('lectin-based design') increases its effectiveness dramatically.(Bernardi et al., 2013; Cecioni et al., 2015; Kane, 2010; Ordanini et al., 2015; Pieters, 2009)



Figure 7. Schematic depiction of a multivalent compound's nested assembly on a virus-like scaffold. The resulting glycodendrinanoparticles are used to compete against the Ebola virus in an infection model. Adapted from Ribeiro-Viana and co-workers (2012). (Ribeiro-Viana et al., 2012)

The second lesson to be learned from multivalent glycocompounds relates to their mechanisms of function : more than one effect takes place at the same time when these ligands are confronted with their target. Firstly, it is essential to understand what makes an effective multivalent ligand : comparing it to the monovalent unit is useful to assess its affinity and applicability for practical purposes. This 'functional' affinity is called avidity since it results from many equivalent interactions, each with its affinity. Characterizing the efficacy of a multivalent design requires correcting from the multivalent avidity and relating it to a single unit. Comparing this value to the affinity of a monovalent ligand leads to what could be called a relative potency per sugar or epitope. The increase in relative potency observed when sugars are presented multivalently is the true meaning of the 'multivalent effect'. With this distinction in mind, it is easier to study the different effects leading to increased affinities and relative potencies, as schematized in Figure 8. The most intuitive effect is chelation, which describes the ability of a molecule to engage two or more binding sites of a target simultaneously. Once a first binding event has established the availability of a multivalent ligand, the affinity of the subsequent interactions is increased compared to the initial binding event. Facilitated binding is one of the drivers of the multivalent effect.



Figure 8. Binding of (1) monovalent or (2-4) multivalent glycomimetic mannosides to a hexameric lectin. Multivalent effects include chelation (2), receptor clustering (3), and statistical rebinding (4).

long

Multivalent design can envision perfectly tailored compounds that fit their lectin targets as a lid fits a pot to push the chelation effect to the limit. Nevertheless, it is a difficult task: any design mistake or fluctuation in the ligand/target dynamics can dramatically affect the affinity measured. Parallel to chelation, a second effect called statistical rebinding describes the increased likelihood of a second interaction happening on the same site where a first binding event has taken place. This effect works synergistically with chelation and drives the chelation effect even further. Importantly, statistical rebinding can also occur in the absence of chelation: a single site may be consecutively engaged by the multiple copies of the ligand presented in a multivalent structure. Therefore, the off-rate of the ligands is reduced, and the affinity is increased. Finally, other effects exist, such as when a compound simultaneously engages two lectins if the three participants' steric bulk allows it. In this case, the 'recruitment' of targets by a multivalent ligand can be called receptor clustering and elicit signalling cascades. (Kiessling et al., 2006) Cross-linking is possible in the case of considerable/long participants with high valencies and can lead to reticulation and even aggregation and precipitation of masses of protein. (Lundquist et al., 2002) This aggregation can be beneficial if the aim is to disable the target, like in AAT. It follows that multivalent compounds can also be designed to encompass various targets at once instead of the 'lid and pot' approach. Today, the multivalent glycomimetics design retains a heavy empirical factor, as every target is and behaves differently.

A final word to be said about multivalent glycocompounds is that, although they work well by presenting basic unmodified sugar units, they can benefit from preceding glycomimetic optimization. Indeed, the increased affinity of a monovalent ligand works synergistically: implementing an optimized glycomimetic in a multivalent design can improve its affinity by additional orders of magnitude, as the gain of affinities multiply themselves, rather than adding to each other.(Cecioni et al., 2015; Sattin et al., 2016; Varga et al., 2014)

OPPORTUNISTIC LUNG PATHOGENS:

BURKHOLDERIA CENOCEPACIA AND COMPANY

As previously mentioned, multidrug-resistant (MDR) pathogens are a constant threat to hospitalized patients, especially those with risk factors such as cancer, diabetes, immunodeficiency, etc. Indeed, opportunistic pathogens take advantage of their weakened organisms for infecting and spreading among patients, leading to outbreaks of hospital-acquired infections (HAIs). Among MDR opportunistic pathogens, lung pathogens are especially notorious: lower respiratory infections have been the fourth leading cause of death for the last 20 years. (WHO, 2020) Among the victims of lower respiratory infections by an MDR lung pathogen can easily translate into a death sentence.

Indeed, lung pathogens thrive in the conditions created by CF: thick mucus hinders the action of immune factors and therapeutic agents and reduces the ability for airway cleansing through mucociliary clearance or expectoration. Infected patients often carry infections by one or multiple pathogens throughout the years. Even the most invasive interventions such as lung transplantation do not guarantee recovery. What is more: re-infection of the lungs after transplant is not uncommon, meaning that the confirmed presence of lung pathogens can be a decisive factor leading to a denial of this life-saving procedure.(Ciofu et al., 2013; O'Sullivan et al., 2009) The list of infection pathogens associated with chronic lung is long : Pseudomonas aeruginosa, Staphylococcus aure-Rafael Bermeo

us, Haemophilus influenza, Achromobacter xylosoxidans, Stenotrophomonas maltophilia, and members of the genus Burkholderia, with more being discovered over time. (Ciofu et al., 2013) Two notorious specimens are *P. aeruginosa and B. cenocepacia,* albeit for different reasons (Figure 9).



Figure 9. Scanning electron microscopy (SEM) images of P. aeruginosa PAO1 and B. cenocepacia K56-2 cells. Top left: P. aeruginosa biofilm on a granite pebble, scale: 10μ m. Top middle : Magnification on P. aeruginosa cells, scale : 1μ m. Top right: Magnification on B. cenocepacia cells. Bottom: B. cenocepacia adhesion to human bronchial epithelial cells (cell line 16HBE14o-). Adapted from Whiteley and co-workers (2001) and Pimenta

P. aeruginosa forms part of the ESKAPE pathogens: high-profile threats to human healthcare. In 2017, it was set as a priority for developing new antibiotics by the WHO.(WHO, 2017) P. aeruginosa is a Gram-negative bacterium that displays intrinsic drug resistance and guickly develops MDR in clinical settings. It is not an inherently easy target to treat, yet P. aeruginosa lung infections rarely affect healthy individuals. It is due to its opportunistic behaviour: in humans, infections by this pathogen occur in conjunction with ailments such as eye injuries, burns, immunodeficiency (AIDS, cancer), and, above all, inflammatory airway diseases (CF, asthma, chronic obstructive pulmonary disease). (Diggle et al., 2020; Garcia-Clemente et al., 2020) P. aeruginosa is responsible for many HAIs (10 % worldwide) and is mainly responsible for mortality in CF populations. Two factors are responsible for its high impact; its ubiquitous presence and its capacity to form biofilms. Firstly, this bacterium finds its way to hospitals by virtually every possible path: newly admitted visitors and patients, unsterilized medical equipment, aerators, water supply, and even healthcare personnel. (van Duin et al., 2016) Secondly, once it establishes itself in a host organism, this pathogen can deploy biofilm and even change its phenotype from 'non-mucoid' to 'mucoid', meaning that it becomes increasingly persistent in antibiotic treatment and deploys factors to boost its virulence. (Diggle et al., 2020; Garcia-Clemente et al., 2020) This adaptive plasticity is a testimony of how difficult it is to eradicate infections by P. aeruginosa permanently.

Among its many virulence factors, *P. aeruginosa* produces two widely studied lectins: LecA and LecB, formerly known as PA-IL and PA-IL. (Gilboa-Garber, 1982) Regulated by quorum sensing, these lectins are released into the extracellular matrix and are known to be essential to biofilm formation, meaning they play a vital role in the infection bio-machinery.(Diggle et al., 2006; Passos da Silva et al., 2019; Tielker et al., 2005; Winzer et al., 2000) Moreover, both have shown Glycomimetics against Multi-Drug Resistance Pathogens

similar roles in pathogenicity either by mediating cell adhesion, blocking epithelial ciliary beating or having a cytotoxic effect on lung cells.(Adam et al., 1997; Bajolet-Laudinat et al., 1994; Chemani et al., 2009)

As such, both lectins have become targets for AAT: in vitro and in vivo studies demonstrated the usefulness of using the corresponding sugars (galactose, mannose, fucose) for inhibiting the effects of LecA and LecB.(Bucior et al., 2013; Chemani et al., 2009; Diggle et al., 2006) A small pilot study explored the treatment of CF patients by inhalation of monosaccharides: the treatment was well-tolerated and led to promising results, but the size of the study limited any claims. (Hauber et al., 2008) Further down the line, mono- and multivalent glycomimetics were developed, with ever-improving affinities and inhibition effects.(Boukerb et al., 2014 ; Gustke et al., 2012 ; Sommer et al., 2019 ; Sommer et al., 2018) Moreover, as prototypical targets for AAT, these lectins have been used to test innovative strategies such glycomimetic-mediated antibiotic delivery and the first case of covalent lectin inhibition. (Meiers et al., 2020; Wagner et al., 2017) These recent advancements on the glycomimetic avenue and advances in many other fields hold promise in treating P. aeruginosa infections. Indeed, decades of study and efforts may remove P. aeruginosa from its place among the most threatening lung pathogens in the not-so-distant future.

The information thus presented concerning P. aeruginosa can also be related to the main lung pathogen described in this text: Burkholderia cenocepacia. In many aspects, B. cenocepacia bears a close resemblance to P. aeruginosa: it is opportunistic, multidrug-resistant, ubiquitous in the environment, and has led to HAIs in the same way P. aeruginosa has. (Tavares et al., 2020) Furthermore. B. cenocepacia mediates its infection through auorum sensing, adhesion and virulence vectors.(Loutet et al., 2010; Mil-Homens et al., 2012; Pimenta et al., 2021; Scoffone et al., 2017) Lastly, it has shown the ability to modulate its phenotype during chronic infection and form biofilms, even in cooperation with P. aeruginosa.(Bragonzi et al., 2012; Inhulsen et al., 2012; Mira et al., 2011) On the other hand, key differences exist between these highprofile pathogens: for instance. B. cenocepacia also infects patients suffering from a chronic granulomatous disease (CGD). (Speert et al., 1994) More importantly, its drug-resistance profile is broader, and its lung infections are much more likely to spread than P. aeruginosa.(Campana et al., 2005; Mahenthiralingam et al., 2005; van Duin et al., 2016) Adding to this, although B. cenocepacia affects fewer patients than P. aeruginosa, its pathogenicity is much more severe and associated with worse patient outcomes. Its infections are usually considered more concerning than those of *P. aeruginosa*. (Tavares et al., 2020)

Indeed, B. cenocepacia, along with more than 20 strains of the Burkholderia genus, have been compiled in the BCC : Burkholderia cepacia complex. (Vandamme et al., 1997) The BCC was introduced in 1997 by the 'International Burkholderia cepacia Working Group' - IBCWG, which was assembled to discuss the emerging threat to public health. (IBCWG; Vandamme et al., 1997) Among the BCC species, a handful is defined by their role as opportunistic pathogens in lung infection : infection outcomes range from asymptomatic carriage to chronic infection and, in the worst cases, deadly 'cepacia syndrome'. Cepacia syndrome defines a rapid exacerbation of the pulmonary infection: necrotizing pneumonia and septicaemia lead to systemic infection and, if left untreated, death.(Mahenthiralingam et al., 2005) Although sometimes cured,

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this syndrome is considered almost untreatable. Due to this, CF patients infected with *B. cenocepacia* and other BCC members are often segregated to protect other susceptible patients. (Jones et al., 2004)

In recent times BCC bacteria have sparked severe predicaments: year-spanning outbreaks of *B. stabilis* in Swiss hospitals were studied in 2019, tracing the origin of the contamination to commercially available gloves. (Seth-Smith et al., 2019) Similarly, in 2019, the French ANSM (National Agency for Medicines and Health Products Safety) had to swiftly release an alert recalling batches of contaminated disinfectant agents. (2019; ANSM, 2019) It happened after the manufacturer Anios, a European market leader in terms of hospital-related disinfection, reported two of their products were contaminated by bacteria: *B. cepacia* and *Pseudomonas oryzihabitans*. (Carlotti, 2020; Duthoit, 2019) In this case, the origin of the bacteria was traced to the water supply, highlighting the fact that these ubiquitous bacteria represent a pervasive threat.

Among the species in the BCC, *B. cenocepacia* is multidrug-resistant and is the species most commonly transmitted among BCC-infected populations and often accounts for half or more of the total BCCinfections among the studied CF populations.(Campana et al., 2005; Drevinek et al., 2010; Scoffone et al., 2017) Lastly, *B. cenocepacia* is responsible for cepacia syndrome, making it the deadliest species from its genus. It is undoubtedly related to its prevalence but is also a testament to the particularly aggressive infections that B. cenocepacia elicits compared to other members of the BCC. (Jones et al., 2004) In conjunction, these facts explain why infection with *B. cenocepacia* is considered most critical and has been studied the most.

Before its reclassification to 'cenocepacia' in 2003, the pathogenic traits of this species were observed early on as *B. cepacia*'s genomovar III. (Vandamme et al., 2003; Vandamme et al., 1997) Extensive study of this bacterium has continued today, from its genome sequencing in 2009 to recent studies dissecting virulence, pathogenicity, existing treatments, and new possible therapies. (Holden et al., 2009; Loutet et al., 2010; Pimenta et al., 2021; Regan et al., 2019; Scoffone et al., 2017) The current stance in terms of treatment remains antibiotic combination therapy: early aggressive treatment may prevent chronic BCC infections. Nevertheless, in the particular case of *B. cenocepacia*, infections become chronic in over 90% of the cases. (Regan et al., 2019) Because no consensus on a standardized protocol for treatment exists, the recurring conclusion is that better tools are needed to understand and treat infections by B. cenocepacia.

On a different note, pioneering work on gene editing has shown that *B. cenocepacia* and one of its toxins may hold the key to accomplishing mitochondrial gene editing. (Mok et al., 2020) This discovery highlights the importance of exploring a pathogenic target through all the available avenues, which may uncover therapeutic potential or other unexpected applications. As stated previously, recent review articles have explored *B. cenocepacia* and its machinery in terms of determinants for biofilm formation and quorum sensing, adhesins, toxins, etc. Nevertheless, those studies seem to have overlooked the existence of soluble lectins in the proteome of *B. cenocepacia*.

LECTINS OF B. CENOCEPACIA: THE BC2L FAMILY

As mentioned earlier, lectins are key actors in cell adhesion leading to infection and have proven to be interesting anti-adhesion and combination therapy targets. A prime example of these notions is how inhibiting the soluble lectins of *P. aeruginosa* with drug-like glycomimetics has led to biofilm disruption and enhanced susceptibility to antibiotics. (Sommer et al., 2018)

Connecting the dots between *B. cenocepacia* and *P. aeruginosa* is simple: both hold the same characteristics as MDR opportunistic pathogens, target the same populations, are considered critical lung pathogens, and have been extensively studied through the lens of CF-related research. Moreover, both have similar bio-machinery to establish chronic infection: they rely on quorum sensing, adhesion, phenotypic adaptation, biofilm formation and resistance to therapy. Therefore, screening the genome of B. cenocepacia and other BBC strains for putative lectins using P. aeruginosa's heavily studied lectins as a template can be considered a reasonable venture. The search thus conducted identified four homologs of lecB on B. cenocepacia strain J2315. (Lameignere et al., 2008) The homologs were called BC2L(-A, -B, -C and -D). There are three genes in chromosome 2, coding for putative lectins A to C, and a final gene on chromosome 3, coding for putative lectin BC2L-D. Although a frameshift invalidated the gene coding for BC2L-D, it was valid in other strains.



Figure 1.1. Structural similarity between LecB from P. aeruginosa, BC2L-A, and the C-terminal domain of BC2L-C, from B. cenocepacia. **A:** Homotetramer, the ligand is L-fucose (from PDB entry 1GZT). **B:** Homodimer, ligand is αMe-D-Mannoside (from PDB entry 2VNV). **C:** Homodimer, no ligand (from PDB entry 2XR4). Ligands depicted as sticks, ions as spheres: sulfate (SO₄²⁻, red and yellow), calcium (Ca²⁺, green).(Berman et al., 2000; Lameignere et al., 2008; Mitchell et al., 2002; RCSB; Sulak et al., 2011)

The study of these lecB-like lectin families started by BC2L-A, whose original name 'BclA' was aptly changed to avoid redundancy with other protein names such as 'BclA' from *Bacillus anthracis* and the heavily studied 'Bcl-2' family of apoptosis regulators involved in cancer research. (Cory et al., 2002) Leading to BC2L-A, the lecB-like gene bclA occurred on other six *Burkholderia* strains, well-conserved and maintaining 32% similarity with lecB. (Lameignere et al., 2008) It coded for 129 residues, longer than LecB, mainly through insertion in a non-functional region and an elongated N-terminus. Nevertheless, BC2L-A was successfully expressed in native form from *B. cenocepacia* strain J2315 and later cloned in E. coli and produced in recombinant form, showing the expected LecB-like calcium-mediated specificity for mannoside saccharides.

Indeed, LecB is a fucose-binding lectin that binds mannosides and requires two calcium Ca2+ ions for carbohydrate binding. Interesting-

ly, BC2L-A shows exclusive specificity for mannosides. It is due to a difference in their sequence: a specificity loop formed by residues 22-24 in LecB features two serine residues (22 & 23), which are replaced by alanine (29 & 30) in BC2L-A, thus allowing rationalization for the specificity. Nevertheless, both lectins have an unusually strong affinity for their respective ligands compared to usual monosaccharide/lectin interactions.(Lameignere et al., 2010; Sabin et al., 2006) The structural and functional study of BC2L-A went on to provide crystal structures, extensive probing against mannosides and even successful inhibition with mannoside glycomimetics.(Beshr et al., 2016; Csavas et al., 2017; Lameignere et al., 2008; Lameignere et al., 2010; Pifferi et al., 2017) In these studies, the structural similarities and differences between LecB and BC2L-A were detailed: the homotetrameric form of LecB is inaccessible to BC2L-A, which instead remains a homodimer (see Figure 10). Additionally, the potential of glycomimetics as antagonists of BC2L-A was proven through structural and biophysical evaluation, and BC2L-A proved to be a valuable model to optimize a multivalent glycomimetic design. (Reynolds et al., 2013)

A report of utmost relevance described interactions of BC2L-A with epitopes obtained from bacterial lipopolysaccharides (LPS). (Marchetti et al., 2012) LPS are structural staples of the Gram-negative outer membrane and cover most of the bacterium's surface. (Bernardi et al., 2013) This could mean that the likely biological function of BC2L-A is to mediate cell-cell adhesion between bacterial cells. Finally, fluorescent-tagged BC2L-A was used for imaging experiments: *E. coli and B. cenocepacia* cells were incubated with the lectin, which accumulated exclusively at the surface of *B. cenocepacia* and within its biofilm. This study confirmed the ability of this soluble lectin to interact not only with the host mannosylated glycoproteins but also with bacterial cells participating in the biofilm matrix.

As the study of BC2L-A advanced, so did the interest in the other orthologs of lecB: bclB, bclC and bclD. Indeed, these putative proteins were longer than BC2L-A, featuring N-terminal domains with no relation to LecB. Their role was considered in *B. cenocepacia's* virulence studies thanks to their identification as soluble lectins. One study evaluated genomic expression and chronic infection evolution on a single patient who suffered from cepacia syndrome. (Mira et al., 2011) The transcriptomic analysis surveyed which genes were up-or down-regulated in 3 years of chronic infection. Among others, genes coding for BC2L-B and -C were up-regulated, whereas the corresponding gene for BC2L-A was down-regulated. This difference of outcomes pointed towards the possibility of secondary roles for the N-termini of these lectins.

Other studies detailed the genes regulated by quorum sensing in *B. cenocepacia*.(Inhulsen et al., 2012; Schmid et al., 2012) The influence of the lectins -A, -B and -C on biofilm formation was assessed in these. After proving that the operon bcIACB coding for the three lectins was regulated by quorum sensing, it was also uncovered that it plays a role in maintaining biofilm structure. Indeed, gene knockout strategies confirmed that biofilm was slower to grow when the lectins were absent and structurally flawed compared to wild-type biofilm: it presented cavities and alterations in thickness and biomass. More importantly, lectin-specific knockouts revealed that all three lectins were necessary, and the lack of any one of them led to defective biofilm. This discovery hints at specific roles of each lectin, again pointing to the role of the N-termini of BC2L-B and -C. Compared to *P. aeruginosa*, blocking lectin action on *B. cenocepacia* produced a mitigated effect (malfunction instead of disruption). Nevertheless,

this information remains encouraging if these lectins become targets for AAT.

Considering the growing body of data, it was clear that the discovery of LecB-like proteins featuring additional N-terminal domains was not trivial. The study of these domains could uncover information related to the role of the BC2L family in virulence and the adhesion mechanisms of B. cenocepacia. It was so that the lectin BC2L-C and its N-terminal came under close scrutiny. (Sulak et al., 2010) Their study would reveal a superlectin. (Sulak et al., 2011)

THE SUPERLECTIN BC2L-C: STATE OF THE ART

Like BC2L-A, the protein BC2L-C is a "Lec-B" like lectin: its Cterminal domain is 116 residues long and shares 43% identity with LecB. (Sulak et al., 2011) Much like BC2L-A, this domain assembles itself as a homodimeric lectin, featuring two calcium-dependent binding sites. Continuing the similarities, the specificity loop in the binding site bears alanine residues, ensuring specific affinity for mannosides and mannosylated structures in the low micromolar range. Nevertheless, beyond the 116 residues of its C-terminal domain, BC2L-C departs from BC2L-A and becomes a unique lectin.

BC2L-C was initially identified from *B. cenocepacia* strain J2315: its gene bclC (NCBI-GI 206562055) codes for 272 amino acids and has been consistently found in other B. cenocepacia strains. (Lameignere et al., 2008; Sulak et al., 2010) The C-terminal Lec-B like lectin that led to its discovery accounts for 116 residues. The following 26 amino acids form a serine- and glycine-rich flexible region considered a linker; the remaining amino acids of BC2L-C form its 130 residues-long N-terminal domain. As previously mentioned, lectins presenting many domains are not uncommon: multivalency occurs by repeating the same lectin unit. Nevertheless, the N-terminal domain of BC2L-C (BC2L-C-Nter) was found to be a lectin domain structurally different to BC2L-C-Cter, with well-defined carbohydrate specificity for fuco-sides. (Sulak et al., 2010) This dual carbohydrate specificity is exceptional and defines the chimeric BC2L-C as a superlectin. (Sulak et al., 2011)

Two seminal studies characterized BC2L-C. In 2010, Šulák and coworkers expressed the 28 kDa native protein, then designed a gene coding for the 156 N-terminal residues of the protein and cloned it in E. coli for recombinant production. (Sulak et al., 2010) Indeed, this initial design included the linker region, which was only characterized as such in retrospective. The protein construct thus obtained was labelled BC2L-C-nt and was 187 residues-long due to the uncleavable 31 residues-long C-terminal histidine tag (HisTag) that was engineered for purification. Size exclusion chromatography revealed the first structural feature of this domain: its elution size corresponded to a 58 kDa protein rather than the expected 19 kDa, signifying a homotrimeric assembly. Assuming it to be a lectin, Šulák and coworkers characterized the new construct by probing it against different monosaccharides. BC2L-C-nt showed specific millimolar affinity towards L-fucose by surface plasmon resonance (SPR). The construct was probed against a glycan array to define specificity further, resulting in a marked preference for fucosylated histo-blood group epitopes. Indeed, the N-terminal of the BC2L-C superlectin is specific for a well-known lectin target for adhesion, present in the glycocalyx of human epithelial cells. Isothermal titration calorimetry (ITC) allowed further characterization of affinity against human oligosaccharides, returning micromolar values detailed in Table 1.2. The best affinity for BC2L-C-nt occurred for Lewis y (Ley: aFuc1-2βGal1 $4[\alpha$ Fuc1-3] β GlcNAc), with a KD of 54 μ M. The data collected confirmed one binding site per monomer, meaning three per trimer.

Ligand	Terminal Epitope	Affinity (μM)	Reference		
BC2L-C-C _{ter}					
D-Mannose	Man	37.4			
αMeMan	Man	27.6	Šulák and		
Trimannose	Manα1-3(Manα1-6)Man 28.8		co-workers (2011)		
αMeHept	L,D-manHep				
Diheptose	L,D-manHepa1-3L,D-manHep	88.1			
BC2L-C-N _{ter}					
αMe-L-Fuc	Fuc	2700			
H-type 2	Fucα1-2Galβ1-4GlcNAc	1236			
Lewis b	$Fuc\alpha 1-2Gal\beta 1-3[\alpha Fuc 1-4]GlcNAc$	213	Šulák and co-workers		
Lewis x	Galβ1-4[αFuc1-3]GlcNAc	196			
Lewis a	Galβ1-3[αFuc1-4]GlcNAc 132		(2010)		
H-type 1	Fucα1-2Galβ1-3GlcNAc	77.2			
Lewis y	$Fuc\alpha 1-2Gal\beta 1-4[\alpha Fuc 1-3]GlcNAc$	53.9			
BC2L-C					
D-Mannose	Man	21.8	Šulák and		
αMeMan	Man	18.3	co-workers		
Lewis y	$Fuc\alpha 1\text{-}2Gal\beta 1\text{-}4[\alphaFuc1\text{-}3]GlcNAc$	47.5	(2011)		

Table 2. Affinities measured by ITC for different ligands of the two domains of BC2L-C. Standard deviations are below 5%. Adapted from Šulák and co-workers (2010 and 2011). (Sulak et al., 2010; Sulak et al., 2011)

In the same study, the first crystal structure of this domain (PDB-ID: 2WQ4) was solved at 1.42 Å, as seen in Figure 11. It revealed a trimeric structure presenting β sheets in a jellyroll – Greek-key architecture, which was unprecedented for lectins. It closely resembled the structure of the human tumor necrosis factor (TNF), heavily studied for its role in signalling and immunity, despite no sequence identity. It is worth mentioning at this point that the sequence coding for BC2L-C-Nter did not match any other known sequence, except for a putative protein from the unrelated *Photorhabdus luminescens*, an insect pathogen. Three fucoside-populated binding sites occurred at the protomeric interfaces, presented on the same structure face, facilitating interactions with surface-bound epitopes, as observed in many lectins.

The study of the binding interaction provided a rationalization of the observed L-fucose selectivity. Arginine residues Arg111 and Arg85 belong to the two adjacent protomers, encase the monosaccharide from the side and below. They establish hydrogen bonds (H-bonds) with oxygen atoms O2, O3, and O4, O6, respectively. Other noteworthy interactions involve a water molecule buried between ligand and protein, establishing H-bonds with the ligand's O3 atom and residues Tyr75 (carbonyl) and Ser82 (side chain). The remaining interactions appear in Figure 11. They define a novel fucose binding mode previously unseen in other lectins. The selectivity for L-fucosides and related L-galacto-configured structures can be condensed to the substituent at the C4 position: residue Arg85 allows a downward axial substituent, whereas an equatorial substituent would generate steric conflict with the side chain of Ser82.

Although the crystal structure was solved at high resolution, the residues corresponding to the linker region were not visible due to disorder and high mobility. This study highlights the main limitations of this study of BC2L-C-Nter: the unaccounted flexible tail of the construct was detrimental for stability, with precipitation being a common problem. (Houser et al., 2021) Similarly, attempts to co

crystallize the protein with larger ligands were unsuccessful since accessibility to the binding side. Thus, structural information of the carbohydrate/ligand interaction with human oligosaccharides could not be obtained, although their affinity for the lectin is several orders of magnitude stronger than the monosaccharide. Molecular modelling led to predicted binding modes of oligosaccharides such as Ley and H-type 1, to be verified by future studies.



Figure 11. Crystal structure of the N-terminal domain of BC2L-C. A and B : Side view of the homotrimer, ligand is aMe-Seleno-L-Fucoside. C : Details of the binding site of BC2L-C-Nter and interactions with the ligand. Water molecules are depicted as red spheres, ligands as spheres or sticks, H-bonds as yellow dashed lines. Adapted from Šulák and co-workers (2010). (Sulak et al., 2010)

In 2011, a subsequent study from Šulák and co-workers uncovered the superlectin as a whole: recombinant versions of the C-terminal domain and the entire protein were produced and probed. The similarities mentioned earlier with BC2L-A were defined, and the affinity for different mannoside and manno-configured heptose ligands were guantified (see Table 2). (Sulak et al., 2011) A crystal structure of the recombinant BC2L-C-Cter dimeric lectin domain was obtained (PDB: 2XR4, Figure 10) and was used for successful computational docking of mannosides as ligands.(2011) Apart from the characterization of the LecB-like domain, the entire protein was characterized in terms of affinity and structure. Glycan array, SPR and ITC technologies were reprised to confirm the dual-specificity, revealing no overlaps between the binding abilities of both domains. Structural analysis by small-angle X-ray scattering (SAXS) and electron microscopy (EM) revealed a flexible hexameric structure, which accounted for the (three) dimeric and (two) trimeric C- and N-terminal domains (see Figure 12).

 a
 This arrangement supported the working theory that the superlectin acts as a 'cellular bridge' or cross-linker of human and bacterial cells

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by simultaneously engaging with the surface-bound epitopes recognized specifically by each terminal. Indeed, the hypothesis is further supported by three additional findings. Firstly, BC2L-C and -A and -B are secreted by the bacterial cell into the extracellular medium by a yet unknown mechanism. Secondly, these lectins can be found at the bacterial surface, later confirmed for BC2L-A and -B in separate studies. (Inhulsen et al., 2012; Marchetti et al., 2012) Lastly, BC2L-C was released into the extracellular matrix only upon incubation of the cells with mannose, hinting heavily at its regulation by quorum sensing and involvement in virulence. (Sulak et al., 2011)



Figure 12. Left: Likely hexameric arrangement of BC2L-C from the SAXS and EM reconstructions. Right: Schematic depiction of the 'cellular bridge' hypothesis: BC2L-C cross-links B. cenocepacia and human epithelial cells by binding their LPS mannoside and histo-blood fucoside epitopes, respectively. Adapted from Šulák and co-workers (2011). (Sulak et al., 2011)

This study evaluated the recently discovered structural relation of BC2L-C-Nter to inflammatory elicitor TNF on a different note. In particular, the study assessed whether exposing epithelial cells to the superlectin would trigger an immune response. A marked increase in interleukin 8 (IL-8) secretion upon treatment with the whole protein or its N-terminus proved the hypothesis. Nevertheless, the inflammatory pathway remained obscured because the obvious candidate, TNF receptor 1 (TNFR1), was not engaged by BC2L-C-Nter. Alternatively, it was demonstrated that the inflammatory response was not linked to carbohydrate binding. The capacity of a virulence factor from B. cenocepacia to elicit inflammation through a cytokine-like structure can be tied to the heavy inflammation seen in patients with cepacia syndrome. Demanding proof of the relationship between BC2L-C, inflammation and cepacia syndrome remains to be obtained by further study.

In the decade since its initial characterization as superlectin, BC2L-C has been studied and implemented by many. Across many works, Tateno, Ito and co-workers have established the lectin affinity for fucosylated oligosaccharide epitopes on human pluripotent stem cells. Their new construct of BC2L-C-Nter, called rBC2LC-N, was produced and implemented for fluorescence-based techniques to detect induced pluripotent stem cells and embryonic stem cells against differentiated stem cells (iPSCs, ESCs, etc. SCs, respectively). (Onuma et al., 2013 ; Tateno et al., 2020 ; Tateno et al., 2011) The glycoprotein Podocalyxin was identified as a cell-surface ligand of rBC2LC-N through its H-type three epitopes. (Tateno et al., 2013) This discovery allowed the development of a method for detecting and eliminating tumorigenic pluripotent cells, with a direct use for safety in stem cell therapy.(Tateno et al., 2014; Tateno et al., 2015) In recent years, their efforts have developed chimeric proteins featuring rBC2LC-N and various toxins: lectin-drug conjugates (LDC),

aimed at varied cell targets recognized explicitly by the lectin domain.(Shimomura et al., 2018; Tateno et al., 2015) Finally, they have highlighted the usefulness of BC2L-C-Nter¬¬ to detect specific populations of cancer cells.(Mawaribuchi et al., 2020; Mawaribuchi et al., 2019) Among the many discoveries from this line of research, more support for the 'cellular bridge' hypothesis can be found: BC2L-C-Nter¬¬ probes bind to human cells via the histo-blood groups in their glycocalyx with antibody-level sensitivity, and they seem to bind to cell lines with epithelial characteristics specifically.(Breiman et al., 2016; Mawaribuchi et al., 2019; Onuma et al., 2013; Shimomura et al., 2018)

Apart from the work of this group on stem and cancer cells, others have benefitted from BC2L-C as a tool for varied endeavours: detection of histo-blood epitopes for cell characterization, development of protein stability screening kits, validation of microbe-oriented glycan arrays, and validation of Fragment Molecular Orbital (FMO) tools for the analysis of protein/ligand interactions.(Geissner et al., 2019; Houser et al., 2021; Sugahara et al., 2017; Tokiwa et al., 2019; Ziganshina et al., 2020) Finally, some groups have challenged antagonizing the superlectin with an early array of fucoside ligands, reaching some degree of success thanks to multivalency.(Kasakova et al., 2018; Thai Le et al., 2019) From these campaigns, the best synthetic ligand for BC2L-C-Nter was a calix[4]arene-based tetravalent fucoside, which showed a 256-fold increase of potency compared to L-fucose for inhibition of hemagglutination of red blood cells. A cross-linking test confirmed the capacity of this inhibitor to aggregate B. cenocepacia cells by engaging the surface-bound lectin. Bearing mostly unmodified C-fucosides, this compound proves a successful multivalent effect : the potency per sugar corresponds to a 64-fold increase. With multivalency validated as a viable strategy to inhibit this virulence factor, S-fucoside glycomimetics were put forward in an attempt to develop glycomimetic monovalent inhibitors. Nevertheless, this optimization was hindered by a lack of biophysical techniques to assess affinity constants and structural data to rationalize the relative potency observed in hemagglutination assays. (Thai Le et al., 2019)

CONCLUSION

In conclusion, two undeniable truths must be considered: antimicrobial resistance is becoming a critical problem for modern medicine, and the use of glycomimetics to fight against drug-resistant infections holds great potential. Indeed, the roles played by lectins and carbohydrates during the infective process makes them excellent targets for disrupting and preventing infections. Moreover, scientific and technological advances facilitate this task: novel synthetic methodologies allow new glycomimetic structures. The thorough study of carbohydrate-lectin interactions allows for better ligand design. Even computational methods are being developed to maximize the potential of glycomimetics by implementing them into combination therapies.

A relevant group of targets within this effort are lung pathogens: they are tough to manage with antibiotics alone and lay great economic and human burdens on society. A particular case we studied is which of multi-drug resistant *Burkholderia cenocepacia* and its family of lectins. By delving into a deeper understanding of the lectins' involvement in the infective process, the design of tailored glycomimetic antagonists can start, paving the way to a realistic anti-adhesive therapy against this pathogen.

Glycomimetics against Multi-Drug Resistance Pathogens

THE AUTHOR



Born and raised in Ecuador, Rafael Bermeo moved to France in 2011 to attend the Lyon Superior School of Chemistry, Physics and Electronics (CPE Lyon), where he obtained his Bachelor, then his Chemical Engineering Diploma in 2018. During his diploma. Rafael spent one year as an exchange graduate student at Oregon State University (Corvallis, OR, USA), specialising in Organic Synthesis and Chemical Biology. In 2018, he started his PhD as a Marie Skłodowska-Curie fellow of the European network PhD4GlycoDrug. Enrolled at the Grenoble and Milan universities under Dr Annabelle Varrot and Prof. Anna Bernardi, he worked towards the design of inhibitors for the superlectin BC2L-C. He was recently granted a doctoral degree by both universities and will continue his career within the Novartis Innovation postdoctoral program (Basel, Switzerland), Rafael's main interest revolves around using organic synthesis, medicinal chemistry and chemical biology to tackle current pathologies in new, efficient ways.

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