GLYCAN DIVERSITY IN FUNGI, BACTERIA AND SEA ORGANISMS

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GLYCAN DIVERSITY IN FUNGI, BACTERIA AND SEA ORGANISMS

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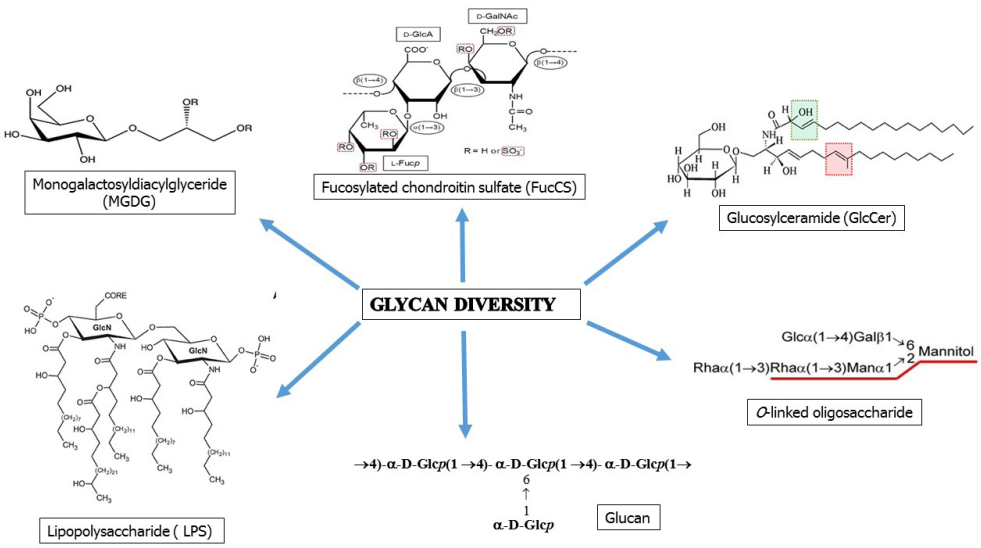

Diversity of glycan structures in fungi, bacteria and sea organisms.
Figure by Eliana Barreto-Bergter

The cell surface of fungi, bacteria and sea organisms is highly glycosylated. These glycans are oligo- or polysaccharide molecules that can be secreted or attached to protein or lipids forming glycoconjugates. They present extraordinary structural diversity that could explain their involvement in many fundamental cellular processes, including growth, differentiation and morphogenesis. Considerable advances have been made on the structural elucidation of these glycans. Their primary structures were determined based on a combination of mass spectrometry and NMR spectroscopy techniques. The combination of these sensitive and powerful techniques has allowed us to increase our structural knowledge of a wide variety of glycans expressed by different fungi, bacteria and sea organisms.

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EDITORIAL
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Editorial: Glycan diversity in fungi, bacteria, and sea organisms

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Keywords: fungal glyconjugates, marine invertebrates, glycosaminoglycans, lipopolysaccharide, biological activity

The cell surface of fungi, bacteria, and sea organisms is highly glycosylated. These glycans are oligo- or polysaccharide molecules that can be secreted or attached to protein or lipids forming glycoconjugates. They present extraordinary structural diversity that could explain their involvement in many fundamental cellular processes, including growth, differentiation, and morphogenesis. Considerable advances have been made on the structural elucidation of these glycans. Their primary structures were determined based on a combination of mass spectrometry and NMR spectroscopy techniques. The combination of these sensitive and powerful techniques has allowed us to increase our structural knowledge of a wide variety of glycans expressed by different fungi, bacteria and sea organisms.

The Research topic "Glycan diversity in fungi, bacteria, and sea organisms" covered important aspects related to polysaccharides, glycoproteins and glycolipids from different organisms and their biological functions.

Some contributions to this Research topic have highlighted the importance of glycosaminoglycans analogs with unique structures from different marine invertebrates. Many sulfated fucans (SFs), sulfated galactans (SGs), and glycosaminoglycans (GAGs) of new structures have been characterized and described (Vieira and Mourão, 1988; Mourão, 2004; Pomin and Mourão, 2008). Pomin and Mourão (2014) have made clear the relevance of certain structural combinations of sulfation and glycosylation to the anticoagulant activity of the marine carbohydrates of well-defined chemical structures.

Pomin (2014) has described the most important marine carbohydrates with therapeutic actions, as well as their main structural and medical properties.

Pavão (2014) emphasized that whereas these marine organisms will be a source of new heparin analogs with significant therapeutic effect in thrombosis, inflammation and cancer in the future will depend on the economic pressure of the pharmaceutical industry and the increasing demand for new natural drugs with less undesired side effects to treat specific diseases.

Plouguerné et al. (2014) reviewed the literature on the glycolipids from seaweeds and their potential biotechnological applications. The most reported biological activities for glycolipids from seaweeds were antibacterial, antitumor, and antiviral activities, enhancing the pharmacological potential of these compounds. Antifouling, and antiherbivory activities were already reported for glycolipids from *Sargassum muticum* and *Fucus vesiculosus*, respectively (Deal et al., 2003; Plouguerné et al., 2010).

The diversity of the composition of the fungal cell surface and important aspects related to structure and function of fungal glycans has also been reviewed in this Research topic. These contributions highlighted the importance of surface molecules of fungal cells for the fungal pathogenesis, physiology, and immune recognition.

Guimarães et al. (2014) focused on glycan structures carried on sphingolipids of pathogenic/opportunistic fungi, and aspects of their biological significance have been discussed.

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A review from Barreto-Bergter and Figueiredo (2014) showed that the variety of carbohydrate structures present in the different fungal pathogens offers exceptional targets for the innate immune recognition which has evolved to recognize specific fungal glycans through a plethora of different receptors.

Burjack et al. (2014) demonstrated a structural diversity of the polysacharides from *Fonsecae monophora* isolated from clinical and environmental origins.

Mannoproteins with different molecular masses were identified and characterized as *Cryptococcus neoformans* immunoreactive antigens by Teixeira et al. (2014) with potential cryptococcosis vaccine candidates.

In addition to glycans from sea organisms and fungal cells, this Research topic included a mini review on the lipopolysaccharide from bacteria. Serrato (2014) has described the structure of diazotrophic bacteria and highlights the importance of these glycolipids in the microbe-plant interaction.

All these articles strongly indicate that knowledge on structure and functions of glycans from fungi, bacteria and sea organisms may open new perpectives allowing to identify specific targets for new generation of antifungal drugs, development of new classes of immunomodulators, antigens, and adjuvants and also marine carbohydrate-based drug development.

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Marine medicinal glycomics

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Glycomics is an international initiative aimed to understand the structure and function of the glycans from a given type of cell, tissue, organism, kingdom or even environment, as found under certain conditions. Glycomics is one of the latest areas of intense biological research. Glycans of marine sources are unique in terms of structure and function. They differ considerably from those of terrestrial origin. This review discusses the most known marine glycans of potential therapeutic properties. They are chitin, chitosan, and sulfated polysaccharides named glycosaminoglycans, sulfated fucans, and sulfated galactans. Their medical actions are very broad. When certain structural requirements are found, these glycans can exhibit beneficial effects in inflammation, coagulation, thrombosis, cancer growth/metastasis, and vascular biology. Both structure and therapeutic mechanisms of action of these marine glycans are discussed here in straight context with the current glycomic age through a project suggestively named marine medicinal glycomics.

Keywords: carbohydrate-based drug development, chitin, chitosan, glycosaminoglycans, sulfated fucans, sulfated galactans

INTRODUCTION

After the launch of many international biological *ome* initiatives, the glycome has now emerged as a source of great information (Hart and Copeland, 2010). Glycome is the project and glycomics is the studies concerned with the science of carbohydrates or glycobiology. Glycomics aims to describe systematically and comparatively the specific or general properties of the carbohydrates. These carbohydrates may be within a repertoire of a given type of cell, tissue, organism, kingdom, or a certain environment as found under specific conditions. Glycomics is focused on the studies and description of the structural and biological functions of carbohydrates. The particular underlying mechanisms of sugar biosynthesis, catabolism, and the nature of molecular interactions with functional proteins involved in health and pathology are also relevant topics of study in glycomics.

Glycomics has brought more challenges than other *ome* projects. The reason is that carbohydrates are the utmost complex biomolecules in terms of structure. High dynamic behavior, conformational fluctuations, diversity of monomers, glycosidic linkages, enantiomers, anomericity, extensive and inhomogeneous post-polymerization modifications are all relevant contributors to greatly enhance structural complexity in glycobiology. Moreover, the number of carbohydrate classes is very high. They include *N*-linked or *O*-linked oligosaccharides in glycoproteins, glycosaminoglycans (GAGs) in proteoglycans, sulfated fucans (SFs), sulfated galactans (SGs) and many others. Because of this, glycomics is a sum of many individual subprojects rather than a single and unique project. This helps to decrease the complexity of the system. Based on this natural division new terminologies are being created to describe the subprojects. Some examples are sialome (for sialic acid-containing glycans) (Cohen and Varki, 2010), glycosaminoglycanome (for GAGs) (Gesslbauer and Kungl, 2006), heparanome (for heparan sulfate) (Lamanna et al., 2007), proteoglycanome (for proteoglycans) (Gesslbauer et al., 2007), fucanome (for SFs) (Pomin, 2012a,b), and galactanome (for SGs) (Pomin, 2012a,b).

The most medically relevant functions of carbohydrates are those related with clinical treatment (therapy) or prevention (prophylaxis). These areas of glycobiology are boosted not only to develop new health care products but due to the efforts of multinational pharmaceutical companies to design and manufacture novel carbohydrate-based drugs. Although several glycans have therapeutic properties those of marine origin have a special position. This is particularly due to the unique structural features that are not found in naturally occurring terrestrial sources. The medicinal mechanisms of action of the marine glycans are also quite distinct (Pomin and Mourão, 2008; Pomin, 2009). Research using structurally well-defined glycans from marine organisms helps to achieve accurate structure-function relationships (Pomin, 2012b,c). Marine sources are rich in glycans of

Abbreviations: AMCase, acidic mammalian chitinase; aPTT, activated partial thromboplastin time; AT, antithrombin; bFGF, basic fibroblast growth factor; BCT, blood coagulation time; FGFR, fibroblast growth factor receptor; DS, dermatan sulfate; Fuc*p*, L-fucopyranose; FucCS, fucosylated chondroitin sulfate; GAGs, glycosaminoglycans; GalNAc, N-acetyl D-galactosamine; Gal*p*, galactopyranose; GlcA, D-glucuronic acid; GlcN, D-glucosamine; GlcNAc, N-acetyl D-glucosamine; GnT-V, N-acetylglucosaminyltransferase-V; HCII, heparin cofactor II; HMWC, high molecular weight chitosans; ICAM, intercellular cellular adhesion molecule; IdoA, L-iduronic acid; LMWC, low molecular weight chitosans; Man, mannose; MMWC, medium molecular weight chitosans; MSPs, marine sulfated polysaccharides; MW, molecular weight; PA, platelet aggregation; PRP, platelet-rich plasma; PDGF-AB, platelet derived growth factor-AB; PSGL-1, P-selectin glycoprotein ligand-1; RANTES, regulated on activation normal T-cell expressed and secreted; SGs, sulfated galactans; SFs, sulfated fucans; SPs, sulfated polysaccharides; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor; IIa, thrombin; Xa, factor X activated; XIIa, factor XII activated.

well-defined chemical structures that can be used to achieve these accurate relationships, as discussed further. These accurate correlations between structure and medical function are extremely important for drug discovery and development, especially when novel glycans are under investigation.

This document aims to describe, in a systematic way, the main structural and medical properties of the most well known glycans from the sea. These glycans are chitin, chitosan, and sulfated polysaccharides (SPs), named GAGs, SFs, and SGs. When certain structural features are present, these glycans can exhibit beneficial activities in inflammation, coagulation, thrombosis, cancer, and vascular biology. The underlying mechanism of actions for their medical effects will be described here individually for each class of marine polysaccharide. All the background provided herein will be discussed in direct connection with glycomics. In fact, this set of information strongly supports the incorporation and development of a new subproject in glycomics, which is suggestively named *marine medicinal glycomics*. The objective of this subproject in the currently ongoing glycomic era is not limited to dissemination of knowledge regarding therapeutic marine carbohydrates but meant to assist research programs focused on marine carbohydrate-based drug discovery and development.

CHITIN AND CHITOSAN

Chitin is the second most abundant polysaccharide on earth after cellulose. Cellulose is mostly terrestrial while chitin is marine and terrestrial. In the marine environment, chitin is certainly the most abundant biopolymer. Chitin is structurally composed of 2-acetamino-D-glucose, also named *N*-acetyl D-glucosamine (GlcNAc), and 2-amino-D-glucose also known as D-glucosamine (GlcN) units. These units are linked by $\beta(1 \rightarrow 4)$ glycosidic bonds (**Figure 1A**). In chitin the GlcNAc content is above 70% of the total monosaccharide. This implies that this polysaccharide is highly *N*-acetylated. This in turn significantly decreases its hydrosolubility property. Low hydrosolubility levels give rise to the main natural function of chitin, which is to create a protective surface in invertebrate and fungal organisms. The major examples are exoskeletons in arthropods, especially insects and arachnids, shells in crustaceans and mollusks and cell walls in fungi.

The unique structure and particular physicochemical properties of chitin make this glycan very useful to industries of several kinds. Chitin, its derivatives, and enzymes involved in their processing are all globally explored by manufacturers of cosmetics and food products. Chitin is also used by agricultural, pharmaceutical, and biomedical companies. However, the interest and application in medicine clearly surpasses any other area (Sugano et al., 1980; Suzuki et al., 1982; Nishimura et al., 1986; Bourbouze et al., 1991; Fukada et al., 1991; Ikeda et al., 1993; Maezaki et al., 1993; Deuchi et al., 1995; Bleau et al., 1999; Shibata et al., 1997, 2000; Cho et al., 1998; Khor, 2001; Barone et al., 2003; Okamoto et al., 2003; Qian and Glanville, 2005; Di Rosa et al., 2005; Malaguarnera et al., 2005; Owens et al., 2006; Zhou et al., 2006; Harish Prashanth and Tharanathan, 2007; Jayakumar et al., 2007; Bonferoni et al., 2008; Liu et al., 2008; Wu et al., 2008; Yang et al., 2008; Muzzarelli, 2009; Paolicelli et al., 2009; Perioli et al., 2009; Tan et al., 2009).

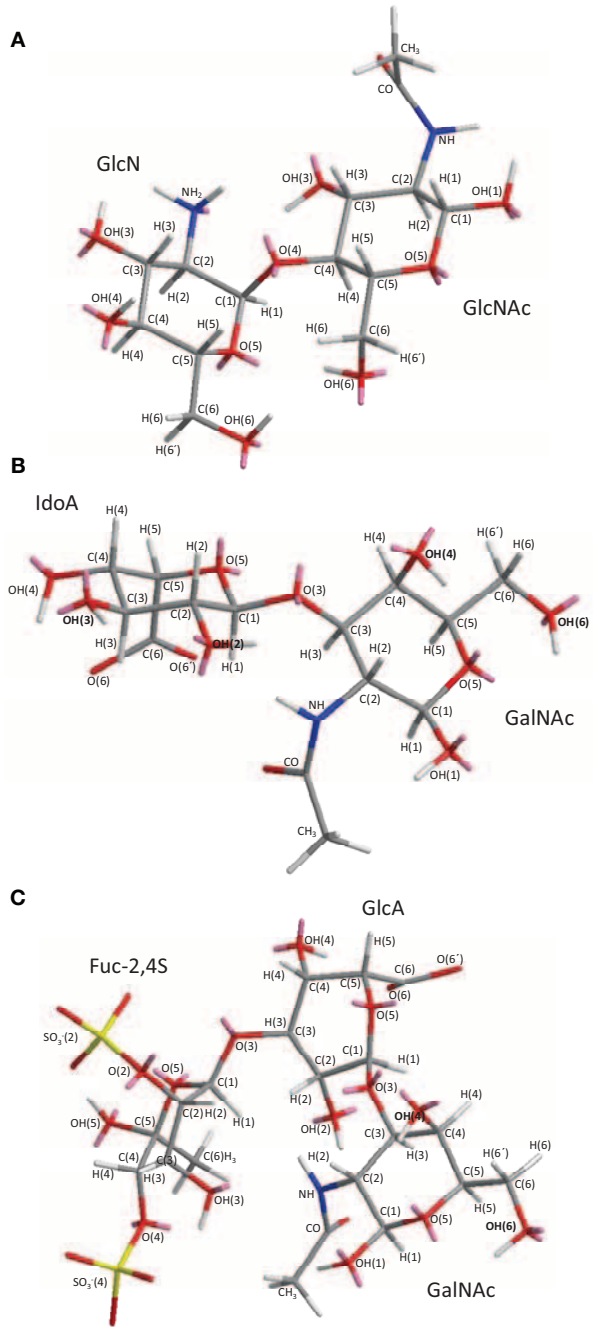

FIGURE 1 | 3D structural representation of the marine glycans (A) chitin and chitosan, (B) ascidian dermatan sulfates (DSs), and (C) sea-cucumber fucosylated chondroitin sulfate (FucCS). These pictures represent the lowest-energy conformations obtained by computational simulation on Chem3D Ultra 8.0 software using 10,000 step intervals of 2.0 fentosecond each, at 298 K and heating/cooling rate of 1000 Kcal/atom/ps. **(A)** Chitin and chitosan are composed of β-(1–4)-linked D-glucosamine (GlcN) and *N*-acetyl D-glucosamine (GlcNAc) units with different amounts. Chitin has ≥70% GlcNAc units while chitosan is composed of ≤30% of this same unit. **(B)** The DS from ascidian *Styela plicata, Halocynthia pyriformis*, and *Phallusia nigra* are composed of $[\rightarrow 4)$-α-L-IdoA-$(2R^1,3R^2)$-$(1\rightarrow 3)$-β-D-GalNAc-$(4R^3, 6R^4)$-$(1\rightarrow]_n$ with different

(Continued)

FIGURE 1 | Continued
sulfation patterns (Pavão et al., 1995, 1998). *S. plicata* DS has R^1, R^2, R^3, and R^4 at 66, <5, 94, 6%, respectively. *H. pyriformis* DS has R^1, R^2, R^3, and R^4 at 70, <5, 99, 1%, respectively. *P nigra* DS has R^1, R^2, R^3, and R^4 at 80, <5, <5, and 100%, respectively. **(C)** The FucCS from *Ludwigothurea grisea* composed of {→4)-β-D-GlcA-3[→1)-α-L-Fuc*p*-2,4-di(OSO_3^-)]-(1→3)-β-D-GalNAc-(1→]$_n$ (Vieira and Mourão, 1988; Vieira et al., 1991; Mourão et al., 1996; Fonseca et al., 2010). IdoA, GalNAc, GlcA, and Fuc*p* stand for iduronic acid, *N*-acetyl galactosamine, glucuronic acid, and fucopyranosyl units. Carbon (C), oxygen (O), hydrogen (H), sulfur (S) and nitrogen (N) atoms are represented in gray, red, white, yellow, and blue, and indicated by numbers within their positions in the sugar rings. The unpaired electrons of oxygens and nitrogens are shown in pink. The OH groups in **(B)** DS and **(C)** FucCS molecules that can be substituted by sulfate ester groups are highlighted in bold for rapid visualization.

The structure of chitin polymers can be found at three forms, α, β, and γ. The α-chitin is known to have a parallel-sheet conformation and is the most abundant form in nature. This form can be found in the shells of crabs and shrimps. The β-chitin is found in the spines of diatoms, squid pens, and pogonophoran tubes. The β-chitin polymers are made of anti-parallel sheets. The γ-chitin, which occurs in fungi and yeast, is comprised of both α and β forms, thus, having a mixture of both anti-parallel and parallel sheets.

Chitin, which has a compact conformation made of highly acetylated regions and sheet-rich 3D-structures, is poorly water-soluble. These properties make industrial and commercial exploration of this structure difficult. To enhance hydrosolubility, chemically modified or hydrolyzed derivatives are usually generated. For example, alkaline hydrolysis removes the acetyl groups and leaves just the amino groups allowing the polymer to be converted from a poorly water-soluble molecule into a highly water-soluble one.

Chitosan is a cationic polysaccharide made up of the same units and glycosidic linkage of chitin (**Figure 1A**). However, low amounts of GlcNAc are found in chitosan, usually less than 30%. Physicochemical characteristics like hydrophobicity and inter-chain interactions depend on the amount and distribution of acetyl groups. Another physicochemical characteristic that varies naturally among different chitosan samples is the molecular weight (MW). Based on this characteristic, three categories of chitosan exist. These categories are named accordingly to their different MWs: high molecular weight chitosans (HMWC), medium molecular weight chitosans (MMWC), and low molecular weight chitosans (LMWC). The MW ranges between 10–100 kDa for LMWC, 100–300 kDa for MMWC, and over 300 kDa for HMWC. In aqueous solution the HMWC sample are more viscous than those prepared with LMWC or MMWC polymers. Although LMWC can be obtained by size exclusion chromatography of unmodified chitosans, enzymatic methods can be additionally employed to produce LMWC derivatives. Although chitosan and its derivatives are all cationic by nature, structural differences among them account for differences in their biological activities and physicochemical properties (Zhang et al., 2009; Ozhan Aytekin et al., 2012).

Chitin and chitosan are widely explored as dietary supplements. Some pharmaceutical functions of chitin and chitosan occur due to their unique physicochemical properties as naturally occurring molecules. They are non-toxic, renewable, and biodegradable. Depending on structures, they exert antitumor, immunoenhancement, antimicrobial, and hypocholesterolemic properties. These properties and activities make these polymers very promising therapeutic candidates (Ilium, 1998). Other therapeutic applications of chitin and chitosan are also under current investigation. Examples are their multiple effects in drug delivery and gene therapy. These activities include ocular, nasal, and vaginal delivery as well as targeted delivery into tumor sites, colon, and wound dressing (bandages). These two marine carbohydrates have also the capability in interacting with receptors on macrophage surfaces to stimulate an immune response in cells (Muzzarelli, 2009) as detailed further. Other clinical effects are also discussed below.

EFFECTS ON IMMUNE RESPONSE

It has been shown that chitin microparticles are effective in clinical treatments including tumor cases, bacterial and viral infections (Suzuki et al., 1982; Nishimura et al., 1986; Shibata et al., 1997). Administration of these particles through the vascular system enhances the generation and release of cytokines by macrophages. The action of chemokines responsible to activate leukocytes in immunological events is mediated by various surface receptors. These receptors act as agents that help internalization of chitin microparticles. Because of the stimulatory action on macrophages, it is believed that chitin plays a pivotal role in depressing allergen-induced type 2 inflammatory responses. This belief is supported by the fact that cytokines are involved in the regulation of allergic immune responses (Shibata et al., 2000). Moreover, it is also known that chitin is a T helper cell type 1 (Th1) adjuvant agent. It has the ability to up-regulate Th1 immunity at the same time it down-regulates T helper cell type 2 (Th2) immunity. The principal type of chitin with this property is the shrimp α-chitin. Microparticles made of shrimp α-chitin have the ability to convert an allergic response mediated by Th2 immunity into an inflammatory response mediated by Th1 immunity (Muzzarelli, 2009).

Studies in mammals have shown that in cases of infection, chitinase enzymes can increase immunity (Bleau et al., 1999). This finding was supported by trials on allergic and asthmatic patients in which macrophages have shown increased expression of acidic mammalian chitinase (AMCase) (Barone et al., 2003; Di Rosa et al., 2005; Malaguarnera et al., 2005). Although some researches of chitin-related enzymes has clearly pointed toward beneficial properties in immunologic system, the specific roles of such enzymes in host defense mechanisms as possible therapeutic agents are yet to be uncovered.

IN FORMULATIONS FOR DRUG DELIVERY

In the recent years of the glycomics age, researches about drug delivery and development has placed a great deal on chitosan due to its capacity of addressing drugs to target tissues. This can be done efficiently by different administration routes such as nasal, oral, intra-peritoneal, and intravenous. Some outcomes provided by these different routes of administration or targeted treatments using chitosan molecules are shown in **Table 1**.

Table 1 | Successful applications of chitin and chitosan in drug delivery.

Delivery systems	Application	References
Ocular delivery	Ocular nanomedicines to be used in clinical practices from chitosan-based nanosystems	Zhang et al., 2009
Nasal delivery	Insulin transportation due to mucoadhesive, cationic and biodegradable properties of PEG-g-chitosan nanoparticles	Paolicelli et al., 2009
Targeted delivery to tumors	Reduction of systematic cytotoxicity, inhibition of cancer cell growth, induction of apoptosis of bladder tumor cells	Tan et al., 2009
Vaginal delivery	Mucoadhesion, enhanced penetration, peptidase inhibition by chitosan containing tablets	Perioli et al., 2009
Wound dressing	Healing of wounded soft tissue, bone, nerve, cartilage by chitin and chitosan based materials	Bonferoni et al., 2008

HYPOCHOLESTEROLEMIC AND HYPOLIPIDEMIC PROPERTIES

As hypocholesterolemic and hypolipidemic agents, chitosan molecules can lower the total cholesterol, plasma and liver triacylglycerol levels quite effectively (Sugano et al., 1980; Fukada et al., 1991; Ikeda et al., 1993; Maezaki et al., 1993; Cho et al., 1998). These activities have been reported with little or no drastic side effects. Chitosans of different MW exhibit distinct effects (Maezaki et al., 1993). The varying activity was demonstrated by *in vitro* studies using LMWC derivatives of different MW ranges. Results have indicated that LMWC derivatives of different MWs have different fat-binding and bile-salt-binding capacities (Zhou et al., 2006; Liu et al., 2008). Another influencing factor in binding properties of chitosan fibers is the particle size of LMWC derivatives. Powdered forms of chitosan have shown to have higher binding capacities when compared to flake forms. The hypocholesterolemic activity of LMWC derivatives may be explained by electrostatic attraction and absorption mechanisms with bile-salts and fatty acids. In the stomach, LMWC derivatives entrap fat droplets when chitosan fibers and fat are consumed together. This entrapment mechanism leads to precipitation of the fat molecules together with LMWC derivatives, which leads to formation of clusters at neutral pH in the small intestine. This prevents fat digestion (Deuchi et al., 1995; Zhou et al., 2006). This is a procedure widely explored by pharmaceutical industries to develop dietary and health care chitosan-based products, mainly used for weight control or reduction. Nevertheless, the ability to reduce fat-absorption by LMWC fibers is likely to be significantly lower or nonexistent if very acidic conditions are found in the stomach.

EFFECTS ON HEMOSTASIS

Pure chitin/chitosan fibers have wound healing and blood coagulating properties. They can be used either as internal hemostatic dressing or as hemostatic bandages (Qian and Glanville, 2005; Harish Prashanth and Tharanathan, 2007; Jayakumar et al., 2007; Khor, 2001). Purity levels of this marine glycan are influential for these activities. This molecule is mostly obtained from shells of marine organisms and, during isolation procedures, other naturally occurring molecules can be co-extracted as contaminants. Studies have demonstrated that depending on the dose and purity, both chitin and chitosan are significantly effective on decreasing the blood coagulation time (BCT) (Okamoto et al., 2003). In this work, the effects of both chitin and chitosan on blood coagulation and platelet aggregation (PA) were evaluated using canine blood in *in vitro* experiments. Whole blood was mixed with chitin and chitosan suspensions (0.0001–1.0 mg/ml), and then the BCT was measured. Chitin and chitosan have been proven to reduce BCT in a dose-dependent manner. Platelet-rich plasma (PRP) was mixed with chitin- and chitosan-suspensions, and then PA was measured in a dual aggregometer. The PA level induced by chitin was the strongest of all samples tested including chitosan, cellulose and latex used as comparative standards. When washed platelets were used, the PA level induced by chitin was similar to that of chitosan, while the rate of coagulation was lower than that of PRP. Chitin and chitosan have shown the ability to enhance the release of platelet derived growth factor-AB (PDGF-AB) and transforming growth factor-β (TGF-β) from platelets (Okamoto et al., 2003).

The hemostatic effect of chitosan as an internal dressing agent against bleeding of liver, aorta, lung, kidney, and cardiac ventricle wounds have been tested and certified by *in vivo* experiments (Owens et al., 2006). Hemostatic property of chitosan may benefit patients with coagulopathies since this therapeutic property is independent of coagulation (co)factors (Yang et al., 2008; Zhang et al., 2009). The beneficial activity of chitosan depends almost entirely on platelets, as supported previously (Okamoto et al., 2003; Wu et al., 2008). *In vitro* experiments have proven that the hemostatic activity of chitosan can contribute effectively to PA and adhesion (Zhang et al., 2009). Therefore, serpin-dependent and -independent anticoagulant and antithrombotic pathways are not involved in the effect of chitosan.

EFFECTS AGAINST CANCER

Enzymes that are involved in chitin/chitosan synthesis and degradation are generally named glycosyltransferases and glycosidases, respectively. They are highly specific in terms of reaction. In biosyntheses, for instance, the presence and amounts of the correct substrate, sugar donors, and enzyme dictate whether the reaction will occur or not. These enzymes have been noted to be expressed in different levels accordingly to healthy or pathological conditions. The over- or down-expression of these enzymes will result in significant changes of the structures of the cellular glycans. Therefore, the structural integrity of the surface glycans at the surface of healthy cells is intimately controlled by the activities of glycosyltransferases and glycosidades. A small change in the balance of the activities of these two enzymes can lead to diseases (Ohtsubo and Marth, 2006). Studies have demonstrated that changed expressions of these enzymes are in fact indicators

of carcinogenesis. For example, the β(1 → 6) branch levels of *N*-linked glycans, found between mannose (Man) and GlcNAc units are seen to be increased in tumor cases. Interestingly, these units are products from digestions of chitin and chitosan polysaccharides. More specifically, the structure GlcNAc-β(1 → 6)-Man-α(1 → 6)Man-β results from a combination of available substrate (the digested chitin/chitosan) and the specific glycosyltransferase, N-acetylglucosaminyltransferase-V (GnT-V) (Humphries et al., 1986; Fernandes et al., 1991; Handerson and Pawelek, 2003; Dube and Bertozzi, 2005; Wattenberg, 2006). *In vivo* studies have shown that β(1 → 6) GlcNAc branching, catalyzed by GnT-V activity, is intimately related with carcinogenesis (Wattenberg, 2006). In terms of therapy, the regulation of the up-take levels of chitin and chitosan and the control of the enzyme activities related with the degradation of these polymers, by gene and/or enzymatic therapy, are effective clinical routes to decrease availability of substrates used to build up glycans involved in tumor development.

In addition to what has been mentioned above, chitin synthase and chitinase that work on synthesis and degradation of chitin, respectively, have also shown to play a key role in invasion by many pathogens, including tumor cells. Hence, inhibitors of chitin synthases might have therapeutic uses in cancer. In fact, several reports using *in vitro* and *in vivo* experiments have pointed out that plant and bacterial chitinases are indeed effective agents in cancer regressions (Pan et al., 2005; Sotgiu et al., 2008; Xu et al., 2008).

SULFATED POLYSACCHARIDES

Marine GAGs have different structures than those present in common mammal GAGs. For example, dermatan sulfate (DS) isolated from the ascidian species *Phallusia nigra* is composed of [→4)-α-L-IdoA-(2R^1,3R^2)-(1→3)-β-D-GalNAc-(4R^3,6R^4)-(1→]$_n$, where IdoA is iduronic acid, GalNAc is *N*-acetyl galactosamine, R^1, R^2, R^3, and R^4 are sulfate groups at 80, <5, <5, and 100 percent, respectively, (**Figure 1B**) (Pavão et al., 1995). Conversely, the commonest mammalian DS is mostly composed of 2-sulfated IdoA units together with occasional C4 sulfation at GalNAc units. Another different GAG from marine invertebrates is fucosylated chondroitin sulfate (FucCS) isolated from the sea-cucumber *Ludwigothurea grisea*, which is composed of [→4)-β-D-GlcA-3[→1)-α-L-Fuc*p*-2,4-di(OSO_3^-)]-(1→3)-β-D-GalNAc-(1→]$_n$, in which GlcA is glucuronic acid, and Fuc*p* is a fucopyranosyl residue (**Figure 1C**) (Vieira and Mourão, 1988). Conversely, the commonest chondroitin sulfate (CS) in mammals is composed of [→4)-β-D-GlcA-(1→3)-β-D-GalNAc-(1→]$_n$ where its GalNAc units can be either mostly 4-sulfated (CS-A) or predominantly 6-sulfated (CS-C) (Pomin et al., 2012).

As opposed to ascidian DS and sea-cucumber FucCS which are heterogeneous marine sulfated polysaccharides (MSPs) in terms of monosaccharide composition, the SFs and SGs are very homogeneous given that they are composed of only Fuc*p* or galactopyranose (Gal*p*) units distributed in a quite regular and repeating backbone (**Table 2**). The major differences between species from SFs or SGs are either the sulfation pattern or the glycosidic linkage type (**Figure 2** and **Table 2**). From comparative studies using the SFs and SGs shown in **Table 2**, their biomedical responses can be understood based on some structural requirements (Pereira et al., 2002). This analytical procedure helps to uncover the underlying mechanisms of action of their biomedical effects through a very accurate and efficient way. Some of the results in these advanced structure-function relationship studies are detailed below.

Besides the unique structures of the MSPs, they also show differential medical properties (Cumashi et al., 2007). This is especially evident when compared to the common mammalian SPs, GAGs. The medical properties of MSPs are directly related to some of their unique structural features, which are not found

Table 2 | Oligosaccharide repetitive units of SFs and SGs from echinoderms sea-urchins (Echinoidea), and sea-cucumber (Holothuroidea), red algae (Rhodophyta), and ascidians or tunicates (Ascidiacea).

Species (class)	Structure
Ludwigothuria grisea (holothurioidea)	[→3)-α-L-Fucp-2,4(OSO_3^-)-(1→3)-α-L-Fuc*p*-(1→3)-α-L-Fuc*p*-2(OSO_3^-)-(1→3)-α-L-Fuc*p*-2(OSO_3^-)-(1→]$_n$
Strongylocentrotus purpuratus II (echinoidea)	[→3)-α-L-Fuc*p*-2,4di(OSO_3^-)-(1→3)-α-L-Fuc*p*-4(OSO_3^-)-(1→3)-α-L-Fuc*p*-4(OSO_3^-)-(1→]$_n$
Strongylocentrotus purpuratus I (echinoidea)	80% [→3)-α-L-Fuc*p*-2,4di(OSO_3^-)-(1→]$_n$ and 20% [→3)-α-L-Fuc*p*-2(OSO_3^-)-(1→]$_n$
Strongylocentrotus franciscanus (echinoidea)	[3)-α-L-Fuc*p*-2(OSO_3^-)-(1→]$_n$
Strongylocentrotus droebachiensis (echinoidea)	[→4)-α-L-Fuc*p*-2(OSO_3^-)-(1→]$_n$
Strongylocentrotus pallidus (echinoidea)	[→3)-α-L-Fuc*p*-2(OSO_3^-)-(1→3)-α-L-Fuc*p*-2(OSO_3^-)-(1→3)-α-L-Fuc*p*-4(OSO_3^-)-(1→3)-α-L-Fuc*p*-4(OSO_3^-)-(1→]$_n$
Lytechinus variegatus (echinoidea)	[→3)-α-L-Fuc*p*-2(OSO_3^-)-(1→3)-α-L-Fuc*p*-2(OSO_3^-)-(1→3)-α-L-Fuc*p*-4(OSO_3^-)-(1→3)-α-L-Fuc*p*-2,4di(OSO_3^-)-(1→]$_n$
Arbacia lixula (echinoidea)	[→4)-α-L-Fuc*p*-2(OSO_3^-)-(1→4)-α-L-Fuc*p*-2(OSO_3^-)-(1→4)-α-L-Fuc*p*-(1→4)-α-L-Fuc*p*-(1→]$_n$
Echinometra lucunter (echinoidea)	[→3)-α-L-Gal*p*-2(OSO_3^-)-(1→]$_n$
Glyptosidaris crenularis (echinoidea)	[→3)-β-D-Gal*p*-2(OSO_3^-)-(1→3)-β-D-Gal*p*-(1→]$_n$
Botryocladia occidentalis (rodophyta)	[→3)-β-D-Gal*p*-2R_1-3R_2-(1→4)-α-D-Gal*p*-2R_3-3R_4-(1→]$_n$, where $R_\#$ = OSO_3^- or OH, R_1 and R_2= OSO_3^- in ∼66 and 33%, respectively
Gelidium crinale (rodophyta)	[→3)-β-D-Gal*p*-2R_1-4R_2-(1→4)-α-D-Gal*p*-2R_3-3R_4-(1→]$_n$, where $R_\#$ = OSO_3^- or OH, R_1 and R_2= OSO_3^- in ∼60 and 15%, respectively
Styela plicata (ascidiacea)	{→4)-α-L-Gal*p*-2[→1)-α-L-Gal*p*]-3(OSO_3^-)-(1→}$_n$
Hedmania monus (ascidiacea)	[→4)-α-L-Gal*p*-3(OSO_3^-)-(1→]$_n$

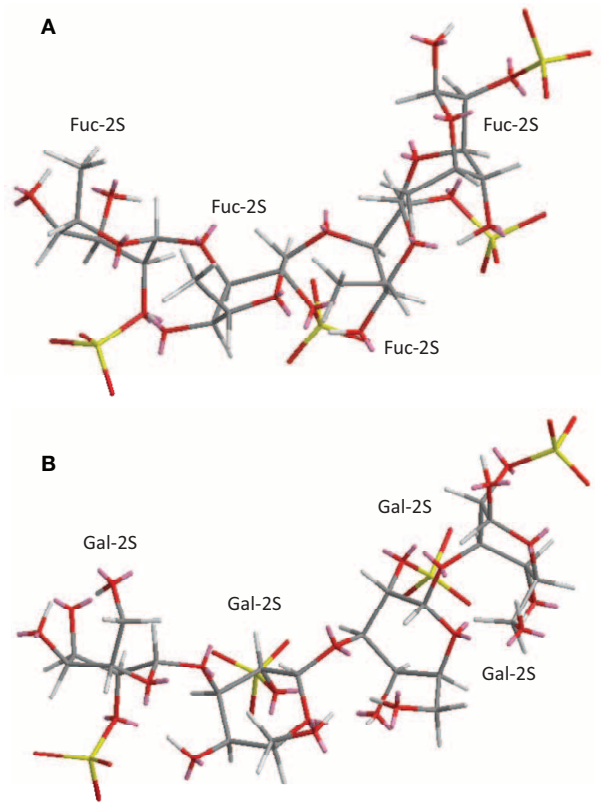

FIGURE 2 | 3D structural representation of the sea-urchin 3-linked 2-sulfated glycans: (A) sulfated fucan (SF) from *Strongylocentrotus franciscanus,* and (B) sulfated galactan (SG) from *Echinometra lucunter* both shown at their tetrasaccharide models. These pictures represent the lowest-energy conformations obtained by computational simulation on Chem3D Ultra 8.0 software using 10,000 step intervals of 2.0 fentosecond each, at 298 K and heating/cooling rate of 1000 Kcal/atom/ps. The polymers are made of the following structures **(A)** [→3)-α-L-Fuc*p*-2(OSO_3^-)-(1→]$_n$ (Alves et al., 1997) for sea-urchin *S. franciscanus*, and **(B)** [3)-α-L-Gal*p*-2(OSO_3^-)-(1→]$_n$ (Vilela-Silva et al., 1999) for sea-urchin *E. lucunter*. Gal*p* and Fuc*p* stand for galactopyranosyl and fucopyranosyl units, respectively. Carbon (C), oxygen (O), hydrogen (H), and sulfur (S) atoms are represented in gray, red, white, and yellow. They have not been indicated because of the big conformational overlap. The unpaired electrons of oxygens are shown in pink.

in mammalian counterparts. For example, while the mammalian CS, which lacks a fucosyl branch, is a non-anticoagulant polysaccharide, the marine FucCS is anticoagulant since it naturally bears the fucosyl branch (Mourão et al., 1996). If this branch is removed in the MSP, for example, by mild acid hydrolysis, it becomes inactive as anticoagulant (Mourão et al., 1996). Below, some of these unique structural requirements necessary to achieve a good response in the medical actions of the MSPs will be described. This will be made through a systematic discussion about the structure-function relationship in the medical activities of the ascidian DS, sea-cucumber FucCS, sea-urchin and red algal SFs and SGs whose mechanisms of action have been elucidated. The events in which these mechanisms of action have been elucidated are inflammation, coagulation, thrombosis, cancer, and angiogenesis.

ANTI-INFLAMMATORY EFFECTS

When some structural requirements are present, the MSPs (ascidian DS, sea-cucumber FucCS and sea-urchin or algal SFs and SGs) may exhibit anti-inflammatory activities, as observed by *in vitro* and *in vivo* experiments (Borsig et al., 2007; Cumashi et al., 2007; Melo-Filho et al., 2010; Belmiro et al., 2011; Kozlowski et al., 2011; Pomin, 2012b,c). The anti-inflammatory action of these MSPs essentially resides in abrogating the P- and L-selectin-mediated leukocyte trafficking, and recruitment and the chemokine-related leukocyte activation during inflammatory events. Hypotheses that the MSPs can also sequester chemokines also exist (Pomin, 2012b). Hence, the MSPs may exhibit anti-inflammatory activities via both cellular and molecular mechanisms of inflammation. A detailed description of the mechanisms of action is illustrated in **Figure 3** for SFs and SGs used as examples. It seems that the same mechanisms of action also occur for the ascidian DS and the sea-cucumber FucCS (Borsig et al., 2007; Melo-Filho et al., 2010; Belmiro et al., 2011; Kozlowski et al., 2011).

As seen in most steroidal anti-inflammatory drugs, such as the glucocorticoids, downside immunosuppressive effects for the above-mentioned anti-inflammatory mechanisms of the MSPs can exist. Since the extravasation of leukocytes to the sites of infection are impaired by the use of MSPs in optimal anti-inflammatory doses, the lower levels of leukocytes at the infected or injured sites are somewhat disrupted. This can decrease the ability of patients to fight infections.

The work of Melo-Filho and coworkers has shown that the sea-cucumber FucCS can greatly attenuate progression of renal fibrosis. This was observed using animals submitted to unilateral ureteral obstruction. The anti-fibrotic mechanism occurs through the stoppage of the P-selectin-driven cell migrations (Melo-Filho et al., 2010). In this work essentially based on *in vivo* experiments, mice were given 4 mg/kg body weight of FucCS intraperitoneally, once a day. After 14 days of injection, their kidneys were examined by histological, immune-histochemical, and biochemical methods. Compared with control mice, collagen deposition decreased in the course of renal fibrosis in the mice receiving FucCS as revealed by Sirius red staining and hydroxyproline content. The cellularity related to myofibroblasts and macrophages was also clearly reduced, as was the production of TGF-β. Fibrosis induced by unilateral ureteral obstruction was observed markedly decreased in P-selectin-deficient mice, which was also proved insensitive to the invertebrate GAG. In this reference, the authors have clearly demonstrated the attenuation ability of FucCS in renal fibrosis using the ureteral obstruction model in mice. As conclusion, the anti-inflammatory mechanism in which FucCS works is mostly driven by P-selectin-mediated cell migration (Melo-Filho et al., 2010).

The phenomenon of P-selection blocking activity by FucCS was demonstrated again in the work of Borsig and co-authors (Borsig et al., 2007). In this work, the authors have shown by *in vitro* experiments that not only heparin can block P- and L-selection, but also the sea-cucumber FucCS. The blocking action of these GAGs impairs the binding of selectins with sialyl Lewis(x). This blocking action disrupts the rolling and migration of the leukocytes on the vessel surfaces close to the

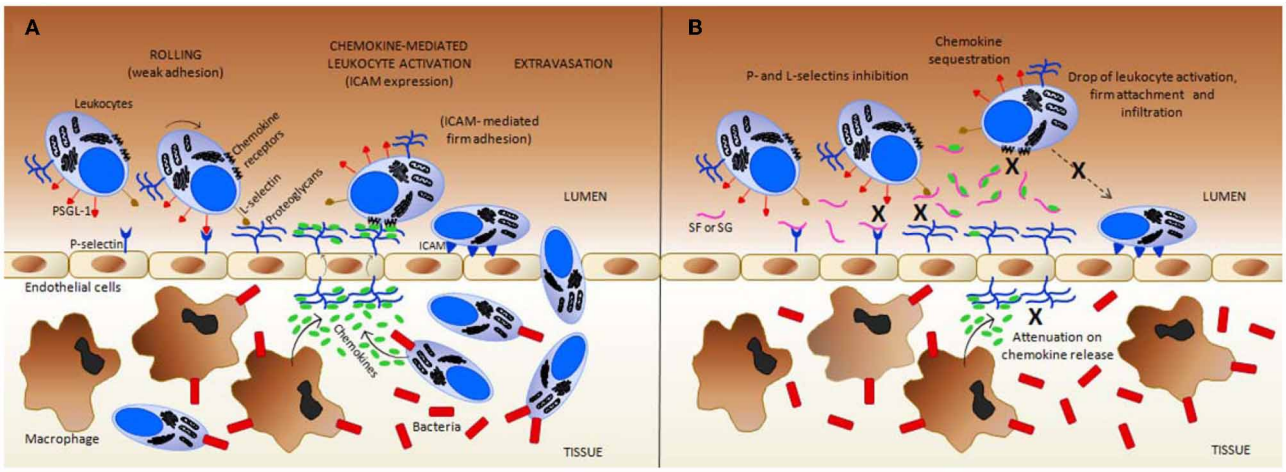

FIGURE 3 | Simplified scheme regarding the inflammation mechanisms in (A) normal (untreated) vs. (B) the treated condition with exogenous sulfated fucans (SFs) and sulfated galactans (SGs). These glycans can target multiple points during the inflammatory process. **(A)** In response to an inflammatory stimuli, such as a bacterial infection, resident macrophages in inner tissues produce both chemokines that attract more leukocytes into these inflamed tissues, and cytokines (such as tumor necrosis factor, TNF) that trigger, at the early stages, the display of pre-formed P-selectins on the luminal surface of endothelial cells (the cytokine-induced P-selectin exposure is not shown at the panels). Cytokines can also induce the expression of E-selectin by endothelial cells (mechanism not shown). GAGs at endothelial proteoglycans play an important role in L-selectin binding, in chemokine presentation to chemokine receptors on neutrophils, and in the transportation of chemokines produced by tissue macrophages and further infiltrated leukocytes. Intercellular adhesion molecule (ICAM), and P-selectin glycoprotein ligand-1 (PSGL) are important leukocyte cell-membrane proteins involved in rolling and firm adhesion, respectively. **(B)** In the presence of SFs, and likely SGs, by direct contact, both P- and L-selectins are blocked to interact further with PSGL-1, and GAGs, respectively, thus, causing a reduction on the leukocyte recruitment. In addition, at certain concentrations, SFs and SGs sequestrate the chemokines responsible to drive and to activate the leukocytes. This is another anti-inflammatory action of these marine glycans. This sequestration occurs most likely because of the presence of conserved heparin-binding sites (BBXB motifs, where B and X are basic and neutral amino acids) in some pro-inflammatory chemokines such as CCL5/RANTES. Due to chemokine sequestration, the numbers of activated defense cells, their firm attachment to the endothelial surface and further infiltration become all consequently reduced in treatment cases. Besides those actions, the number of released chemokine as a pro-inflammatory feedback process from inner tissues is also attenuated due to the decreased number of infiltrated cells. This latter event enhances the anti-inflammatory activity of the MSPs. All mechanisms marked by X in **(B)** collaborate in conjunction to the resultant anti-inflammatory action of SFs and SGs. Figure reproduced with permission from (Pomin, 2012b).

inflamed sites. The sea-cucumber FucCS was proven to be a potent inhibitor of P- and L-selectin binding to immobilized sialyl Lewis(x), and of LS180 carcinoma cell attachment to immobilized P- and L-selectins. Inhibitions have been shown to occur in a concentration-dependent manner. Interestingly, FucCS was 4–8-fold more potent than heparin in the inhibition of P- and L-selectin-sialyl Lewis(x) interactions. No inhibition of E-selectin was observed. This was expected based on similar studies undertaken by Cumashi and coworkers on the anti-inflammatory activity of some brown algal SFs (Cumashi et al., 2007). In the work of Borsig et al. (2007), FucCS demonstrated to have inhibitory properties on lung colonization of adenocarcinoma MC-38 cells in an experimental metastasis using mice. This inhibitory activity was also observed in neutrophil recruitment in two *in vivo* models of inflammation (thioglycollate-induced peritonitis and lipopolysaccharide-induced lung inflammation). Inhibition occurred at a dose that produces no significant change in plasma activated partial thromboplastin time (aPTT). Removal of the sulfated fucose branches in the FucCS (**Figure 1C**) abolished its inhibitory effect as observed by both *in vitro* and *in vivo* experiments. This proves the importance for the fucosyl branch for this activity. The results from this reference suggest that invertebrate FucCS may be a potential alternative to heparin for blocking metastasis and inflammation without the undesirable anticoagulant side effects seen in heparin.

Another beneficial aspect of MSPs was shown in studies of the anti-inflammatory potential of ascidian DS with different structures (**Figure 1B**) (Belmiro et al., 2011; Kozlowski et al., 2011). Subcutaneous administration of ascidian DS has shown therapeutic effects against colon inflammation in rats by reducing macrophage and T-cell recruitment and activation. These activities are in perfect coherence with the mechanisms described in **Figure 3**. The work of Belmiro also showed the capacity of DS as an anti-inflammatory agent in decreasing the myofibroblast population in fibrosis-induced mice submitted to unilateral ureteral obstruction. The *in vivo* experiment used was similar to that used in the work of Melo-Filho et al. (2010). In the work of Kozlowski, the investigators showed *in vivo* anti-inflammatory action of two ascidian DSs. The conclusion was based on the ascidian DS capacity to block infiltration of defense cells in a thioglycollate-induced peritonitis mouse experiment (Kozlowski et al., 2011).

Cumashi and coworkers have shown anti-inflammatory effects of some brown algal SFs using *in vitro* assays to test the binding properties of the MSPs with selectins. Curiously, the brown algal heterogenous SFs (also known as fucoidans) were able to clear inhibit P- and L-selectins but not E-selectin (Cumashi et al., 2007).

ANTICOAGULATION AND ANTITHROMBOSIS: THE SERPIN-INDEPENDENT MECHANISM

The effects of MSPs on hemostasis are the mostly studied medical activities of these compounds. A detailed scheme describing their major mechanism of action, as possible anticoagulants and antithrombotics, is provided at **Figure 4**, in which SFs and SGs are used as examples. The mechanisms of action reside on the inhibition of some coagulation proteases like thrombin (IIa) and factor Xa, via their physiological inhibitors, named serpins (serine-protease inhibitors). The most common serpins of this system are antithrombin (AT) and heparin cofactor II (HCII). Although at different degrees of response, the majority of the MSPs described herein: the ascidian DS (**Figure 1B**) (Vicente et al., 2004; Kozlowski et al., 2011), the sea-cucumber FucCS (**Figure 1C**) (Mourão et al., 1996; Mourão, 2004), the algal SFs and SGs (**Table 2**) (Pereira et al., 1999; Farias et al., 2000; Mourão, 2004; Pomin and Mourão, 2012) and the invertebrate SFs or SGs (**Figure 2** and **Table 2**) (Pereira et al., 1999; Farias et al.,

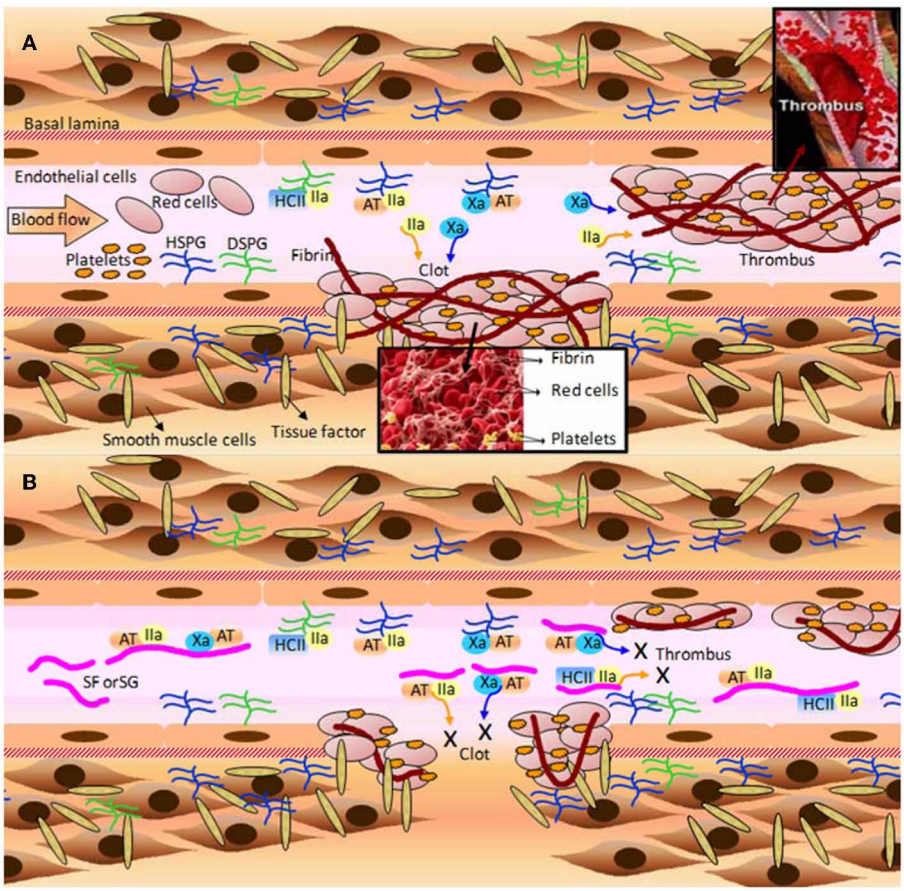

FIGURE 4 | A few of the molecular and cellular players in (A) blood coagulation, clot and thrombus formation; and (B) the anticoagulant and antithrombotic mechanisms of the marine sulfated fucans (SFs) and sulfated galactans (SGs). (A) When the blood vessel wall is disrupted by an injury (atherosclerotic plaque or a physical rupture, for example) tissue factors normally expressed and localized below the basal lamina become exposed to the blood stream. Blood factor XII is recognized by tissue factor, and after making complex with it, becomes factor XII activated (XIIa) (complex not shown). XIIa initiates the blood coagulation cascade leading to the expressive formation of thrombin (IIa) and factor X activated (Xa). These factors feed the formation of more blood coagulation factors which will result in clot or thrombus formation. Thrombin acts directly on fibrinogen in order to form fibrin fibers, which stabilizes the clots and thrombus through cross-linked fibers. Platelets play an important role to this stabilization as well. The natural inhibitors of the two proteases (Xa and IIa) are the serpins antithrombin (AT), and heparin cofactor II (HCII). AT is able to act directly on either Xa or IIa, whereas HCII acts only on IIa. Upon interaction with heparan sulfates and dermatan sulfates of proteoglycans distributed throughout the endothelial surface of blood vessels, AT and HCII become activated for inhibiting actions. This leads to sequestration of the plasma soluble Xa and IIa factors. It is worth to mention that AT is a heparin-binding protein with the BBXB motif of high-affinity to SPs. HSPG and DSPG stand for heparan sulfate and dermatan sulfate proteoglycans, respectively. **(B)** The inhibitory mechanisms provoked by MSPs are analogous to the natural inhibitory mechanisms caused by the proteoglycans at surfaces of the vessels. However, due to the large plasmatic amounts of SFs and SGs in treatment conditions, the cofactors AT and HCII would have their natural inhibitory actions enhanced by certain orders of magnitude, consequently lowering the plasmatic concentration of active factors IIa and Xa. The decreased amounts of these blood factors abrogate the clotting and thrombus formation, as a consequent result. Fibrinolytic activity is responsible to undertake metabolic process on formed clots and thrombus after significant inactivation of the proteases Xa and IIa. All the mechanisms marked by X in **(B)** lead to the anticoagulant and antithrombotic actions of SFs and SGs. Figure reproduced with permission from (Pomin, 2012b).

2000; Pomin, 2012b), have all effects in this serpin-dependent mechanism (**Figure 4**). The anticoagulant effects of the MSPs are intimately dependent on some of their structural features. For example, the SF from *Strongylocentrotus franciscanus* (**Figure 2A** and **Table 2**) is not an anticoagulant polysaccharide while the SG from *Echinometra lucunter* (**Figure 2B** and **Table 2**) is anticoagulant (Pereira et al., 2002). The only difference between these two compounds is the monosaccharide type. The other features C3-glycosydic linkage, 2-sulfation, L-enatiomericity, and α-anomericity are equal (**Figure 2**). This single structural difference is enough to make either an active or an inactive compound.

Besides the common serpin-dependent anticoagulant activity of the FucCS from the sea-cucumber *L. grisea* (**Figure 1C**), and the SG from the red alga *Botryocaldia occidentalis* (**Table 2**), these glycans have also shown serpin-independent anticoagulant actions (Glauser et al., 2008, 2009). Initially, their anticoagulant actions were essentially attributed by their capacity in potentiate factors Xa and IIa inhibition via AT and HCII, as summarized in **Figure 4**. Currently, the sea-cucumber FucCS and the red algal SG are also known to inhibit the generation of factor Xa and IIa by interfering in the formation of the blood cofactor complexes at the surface of the cells. Factor Xa is activated mainly by the intrinsic tenase complex, while IIa is converted from II by the prothrombinase complex. FucCS and SG were shown the ability to inhibit the activation of these tenase and prothrombinase complexes (Glauser et al., 2008, 2009). The formation of these complexes is a key step for the generation and amplification of the coagulation cofactors. Besides this serpin-independent mechanism, another novel mechanism is the inhibition of thrombosis by targeting tissue factor in combination with factor Xa, as reported by the recent work of Zhao and coworkers using the sea-cucumber FucCS (Zhao et al., 2013).

THERAPEUTIC EFFECTS AGAINST CANCER GROWTH AND METASTASIS

The effects of MSPs against cancer growth seem to be related to the blocking of tumor angiogenesis that feeds the growth of tumor cells (Pomin, 2012b), as illustrated in **Figure 5**. Like some mammal GAGs, such as heparin, MSPs have shown the capacity to bind growth factors such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). This binding will impair, respectively, the differentiation of mesodermal cells into angioblasts and angioblasts into endothelial cells (**Figure 5**). These cellular differentiations are important to the neovascularization process (**Figure 5**). Several articles have demonstrated the capacity of MSPs in binding with these growth factors (Tapon-Bretaudière et al., 2000, 2002; Cumashi et al., 2007).

Besides interfering in tumor neovascularization, the MSPs have also the capacity to inhibit, to some extent, the metastasis of tumor cells. This action is driven by blocking the adhesion capacity of the tumor cell onto the surface of the blood vessels (**Figure 5**) (Croci et al., 2001; Borsig et al., 2007; Kozlowski et al., 2011). This step is crucial for proper migration and invasion of the primary and mature cancer cells toward new spots of growth (metastasis). The mechanism of action of this tumor adhesion inhibition by MSPs seems to be related to the blocking of P- and L-selectins. This inhibitory mechanism is similar to that described for the anti-inflammatory activities described above (Borsig et al., 2007; Kozlowski et al., 2011). In the recent work of Zhao and coworkers, the investigators have additionally demonstrated that the sea-cucumber FucCS also inhibits metastasis by targeting the nuclear factor-κB pathway in melanoma B16F10 cells (Zhao et al., 2013).

OTHER ACTIVITIES

Besides coagulation, inflammation, and tumor angiogenesis, the MSPs can also show therapeutic actions in other systems. They can act like wound healing (O'Leary et al., 2004), oxidative-stress (Dore et al., 2013), nociception (Rodrigues et al., 2012), and viral infections (Ponce et al., 2003). In wound healing a combination of chitosan-fucoidan hydrogels were created for therapeutic purposes (Sezer et al., 2008). Nevertheless, the mechanisms of action of MSPs in these systems are yet unclear. However, it is strongly believed that for antiviral activity the MSPs might be impairing the adhesion of the virus particle onto host cells since many virus need the negatively charged polysaccharides on host cell surfaces for attachment and invasion. The clinical systems just described here comprise new research areas for MSPs in terms of studying their underlying mechanisms of action and the structural features necessary for the effectiveness.

REMARKED CONCLUSIONS

Here, we have made clear the clinical significance of MSPs. Chitin and chitosan (**Figure 1A**) are likely the mostly abundant polysaccharides from the marine environment. They can show beneficial effects in immune response, against cancer, in hemostasis, as hypocholesteromic and hypolipidemic agents besides exhibiting capacity to enhance drug delivery. Even though they exhibit an impressive range of therapeutic actions, chitin/chitosan fibers have mostly been used in the pharmaceutical market as merely dietary supplements for weight control. GAGs from marine organisms are really distinct in terms of function and structure. Two main examples are the ascidian DS with different patterns of sulfation (**Figure 1B**) and the sea-cucumber FucCS (**Figure 1C**). The latter differs considerably from the common CS due to the presence of fucosyl branches. This branch is a structural requirement for the biomedical properties since when it is removed the sea-cucumber SP losses its medical properties. As opposed to CS, FucCS can be used as a potential anti-inflammatory and anticoagulant agent. Both ascidian DS and FucCS have not been employed in researches of clinical trials. They have been used only in *in vitro* and *in vivo* studies. The *in vivo* experiments have mostly used laboratory wild and mutant mice models. SFs and SGs are other important classes of SPs found in the sea. In invertebrates and in some red algae, these compounds may exist with well-defined chemical structures (**Table 2**). The use of these structurally well-defined glycans has helped the development of drug discovery by achieving accurate structure-function relationships. These unique glycans has also helped to understand the underlying mechanisms of action involved in some clinical effects of the MSPs. The clinical events with mechanisms of action mostly elucidated so far are anti-inflammation, anticoagulation, antithrombosis, and anti-tumor angiogenesis. Although brown algae SFs, widely known as fucoidans, do not have well-defined

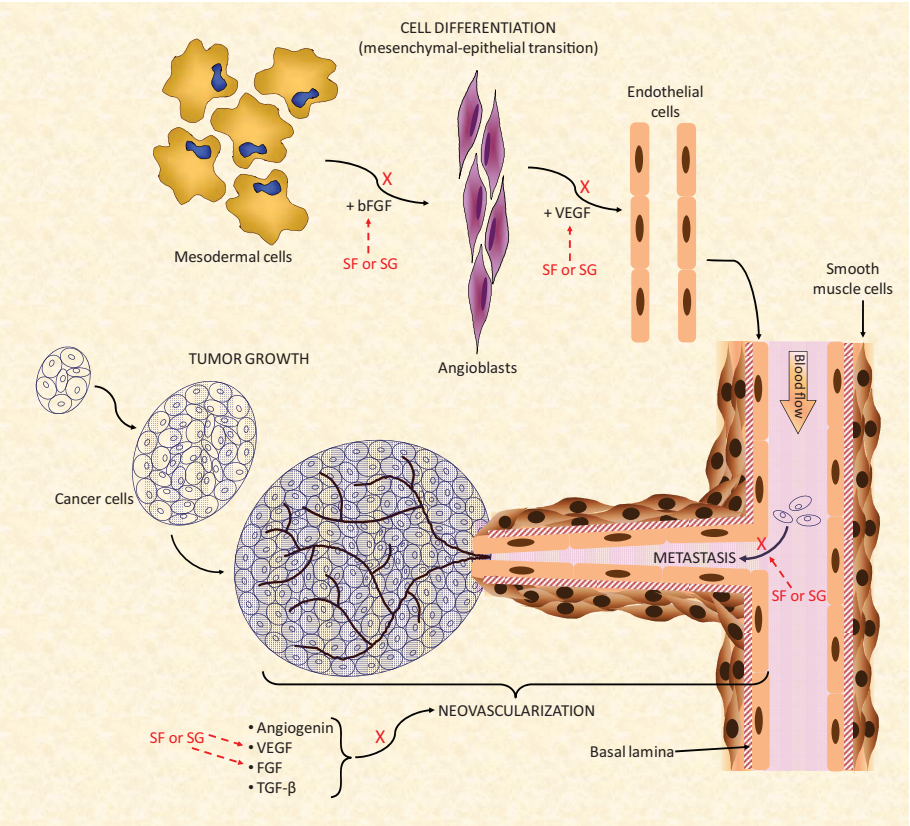

FIGURE 5 | A simplified scheme of the major biochemical mechanisms involved in tumor angiogenesis. Multiple points of action are targeted by the SFs and SGs. For a new blood vessel to be formed and to grow properly there should be a feeding of stimulatory angiogenic factors such as angiogenin, VEGF, FGF, and TGF-β for formation of the new vessels. The mesenchymal–epithelial transition must also occur concomitantly to provide newly formed endothelial cell to help the construction of the new blood ducts. In this event, modulated also by FGF molecules, mesodermal cells undergo transition until angioblasts which is the precursors of mature endothelial cells. Under the influence of VEGF, newly formed endothelial cells will be used for building the novel vessels (Lamalice et al., 2007). Neovascularization is a fundamental process for cancer growth in primary tumors as well as to feed the tumor growth at new metastatic spots. SFs and SGs can inhibit the action of FGF and VEGF molecules either at the endothelial cell differentiation as well as during the feeding of the angiogenesis development. Interactions of SFs and SGs with these factors as well as with their respective receptors have been observed. Besides this neovascularization inhibitory function, SFs and SGs were also reported to synergically reduced tumor spreading by decreasing their cell-adhesion capacity (Croci et al., 2001) during the tumor proliferation stage. All the mechanisms marked by X collaborate in conjunction to the anti-angiogenic and/or antitumoral effects of SFs and SGs. Figure reproduced with permission from (Pomin, 2012b).

chemical structures, they are the mostly used MSPs in research. Like chitin and chitosan fibers, the brown algal SFs have been used as dietary supplement products in the market. Clinical trials in animals are likely to be unknown for the majority of the MSPs discussed here. The clinical tests available so far are just those found in the referential works cited through this document.

MARINE MEDICIAL GLYCOMICS

This document has as its main objective the description of the most important marine carbohydrates with therapeutic actions, as well as their main structural and medical properties. These glycans are really unique, and this uniqueness seems to be related to the marine source. Glycomics, as an area of research, has grown significantly over the last few years. Based on the discoveries made with respect to therapeutic properties of marine glycans, as discussed here, we want to propose to the major international scientific societies involved with drug development, glycobiology, and marine biology, a glycomics subproject named *marine medicinal glycomics*. The subproject *marine medicinal glycomics* would be very useful to push forward the research programs involved with marine carbohydrate-based drug development. Since clinical tests using the marine glycans here discussed, especially those of **Table 2**, are virtually inexistent, the implementation of this subproject would support research programs of licensed clinical trials using these sugars. The implementation of this subproject would also enhance the medical contribution of carbohydrates in the currently ongoing glycomic age. Not only chitin/chitosan, invertebrate GAGs, SFs, and SGs would benefit from this subproject, but actually, any marine carbohydrate possessed of medical properties. Certainly the number of marine carbohydrate-based drugs would increase significantly with the implementation of such subproject.

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Specific sulfation and glycosylation—a structural combination for the anticoagulation of marine carbohydrates

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Based on considered achievements of the last 25 years, specific combinations of sulfation patterns and glycosylation types have been proved to be key structural players for the anticoagulant activity of certain marine glycans. These conclusions were obtained from comparative and systematic analyses on the structure-anticoagulation relationships of chemically well-defined sulfated polysaccharides of marine invertebrates and red algae. These sulfated polysaccharides are known as sulfated fucans (SFs), sulfated galactans (SGs) and glycosaminoglycans (GAGs). The structural combinations necessary for the anticoagulant activities are the 2-sulfation in α-L-SGs, the 2,4-di-sulfation in α-L-fucopyranosyl units found as composing units of certain sea-urchin and sea-cucumber linear SFs, or as branching units of the fucosylated chondroitin sulfate, a unique GAG from sea-cucumbers. Another unique GAG type from marine organisms is the dermatan sulfate isolated from ascidians. The high levels of 4-sulfation at the galactosamine units combined with certain levels of 2-sulfation at the iduronic acid units is the anticoagulant structural requirements of these GAGs. When the backbones of red algal SGs are homogeneous, the anticoagulation is proportionally dependent of their sulfation content. Finally, 4-sulfation was observed to be the structural motif required to enhance the inhibition of thrombin via heparin cofactor-II by invertebrate SFs.

Keywords: algae, carbohydrate-based drug development, fucosylated chondroitin sulfate, sea cucumber, sea urchin, sulfated galactan, sulfated fucan

INTRODUCTION

Marine organisms represent a very special source of potential therapeutic molecules with unique structures. Among innumerous of these compounds, the sulfated polysaccharides (SPs) have awakened great interest in the scientific community. This happens mostly because of the fact that these compounds hold the characteristic of being naturally polyanionic. This feature makes the SPs suitable to interact with important functional proteins, especially those involved in the balance of health and disease. The nature and quality of the SP-protein interactions control and regulate the activity of these functional proteins in the body. Although the affinity of these molecular complexes are mostly driven by electrostatic interactions in which sulfation content of the SPs play a crucial role, the overall structural features of the SPs are still more influential in the process (Pomin, 2009). The structural features of the SPs involved into the quality of these molecular interactions are sulfation patterns and glycosylation. This latter includes anomeric and enantiomeric configurations, glycosidic linkage position, monosaccharide type and composition.

In the last 25 years, our group has made great efforts in scientific researches related with marine and medicinal glycobiology. As a consequence, many sulfated fucans (SFs), sulfated galactans (SGs) and glycosaminoglycans (GAGs) of new structures have been characterized and described (Vieira and Mourão, 1988; Mourão, 2004; Pomin and Mourão, 2008). We believe that we have fully characterized over 20 new structures of these marine sulfated polysaccharides (MSPs) in our studies (Vieira and Mourão, 1988; Mourão, 2004; Pomin and Mourão, 2008; Pomin, 2009, 2012a,b, 2014a,b). We have also submitted most of these new MSPs to *in vitro* experiments to assess their possible anticoagulant effects (Mourão, 2004; Pomin and Mourão, 2008; Pomin, 2009). Curiously, we noticed that even though bearing significant levels of sulfation, some of these MSPs have insignificant effects toward the coagulation system; while other MSPs, even carrying lower sulfation content, can show surprising levels of anticoagulant activity (Mourão, 2004; Pomin, 2009). Moreover, some SFs and SGs, even though exhibiting nearly equal sulfation levels, but within different sulfation patterns, have completely different anticoagulant effects. This observation has clearly proved the concept that sulfation and thus electronegative-charge density in marine carbohydrates are not the solely structural determinants for the resultant anticoagulant activities of these molecules.

Abbreviations: aPTT, activated partial thromboplastin time; AT, antithrombin; DS, dermatan sulfate; Fuc*p*, L-fucopyranose; FucCS, fucosylated chondroitin sulfate; GAGs, glycosaminoglycans; GalNAc, N-acetyl D-galactosamine; Gal*p*, galactopyranose; GlcA, D-glucuronic acid; HCII, heparin cofactor II; IdoA, L-iduronic acid; MSPs, marine sulfated polysaccharides; SGs, sulfated galactans; SFs, sulfated fucans; SPs, sulfated polysaccharides; UFH, unfractionated heparin; IIa, thrombin; XIIa, factor XII activated.

Since we have characterized and used many different structures in our anticoagulant tests across the last years, we should be able to state by now some of the structural features of the MSPs necessary for their differential anticoagulant properties. Based on comparative and systematic analyses on the structure-anticoagulation relationships of certain MSPs, we noticed that to achieve a good anticoagulant response, certain structural combinations of sulfation and glycosylation are indeed required. In these analyses, we have particularly given more preference to examine SFs, SGs and GAGs from marine invertebrates or red algae, since these organisms can provide molecules of well-defined chemical structures (Mourão, 2004; Pomin, 2009, 2012c; Pomin and Mourão, 2012). This kind of structural regularity has facilitated interpretation and this in turn enables us to establish advanced structure-anticoagulation relationships (Pereira et al., 2002; Pomin, 2009, 2012c). Here, we describe based on data of some of our previous works, the major structural combinations of the invertebrate and red algal MSPs that have been proved to be necessary to make a satisfactory anticoagulant effect.

THE ROLE OF SFs AND SGs OF WELL-DEFINED STRUCTURES: ALGAL vs. INVERTEBRATE MOLECULES

SFs are a class of SPs composed mostly of α-L-fucopyranosyl (Fuc*p*) units. SFs can be extracted mostly from marine organisms, such as sea-urchins (echinoidea), sea-cucumbers (holothuroidea) and brown algae (phaeophyta). These molecules are also known as fucoidans when isolated from brown algae. Besides Fuc*p*, the fucoidans are also composed of other sugars, such as xylose and uronic acids. This heterogeneous monosaccharide composition, associated with the lack or an unclear structural pattern of regularity and the presence of branching residues, makes the establishment of the structure-function relationships for algal molecules very hard (Pereira et al., 1999). On the other hand, the SFs isolated from invertebrates can reveal structures quite regular (**Figures 1A–I**). This type of structural pattern helps to achieve advanced structure-function correlations about their anticoagulant properties. Using the invertebrate SFs, we are able to understand which structural features are important for the anticoagulant activity of these molecules (Pereira et al., 1999; Pomin, 2009, 2012b,c). Below, some of these influential structural features on coagulation of the MSPs of well-defined chemical structures will be described.

SGs are a class of SPs composed of α-L-, α-D-, or β-D-galactopyranosyl (Gal*p*) units. SGs can be found in green algae (clorophyta), red algae (rodophyta), sea-urchins and ascidians which are also known as tunicates (ascidiacea). Like SFs, algal SGs have structures more complex than the regular and simpler invertebrate counterparts. In green algae, for example, the sulfation pattern is usually complex, with additional substitutions like pyruvates, and the possibility of branches (Farias et al., 2008). In red algae, although the SGs show heterogeneous sulfation patterns, they are usually composed of disaccharide repeating units of 3-linked β-D-Gal*p* and 4-linked α-Gal units in their backbones. Sometimes, the latter unit can be seen forming an extra carbon ring which results in an anhydro-sugar (Quinderé et al., 2014). This is an additional heterogeneity that enhances structural complexity in the red algal molecule. However, some red algal species can show very simple structures whose sulfation patterns vary accordingly to the species of extraction. Two examples of these structures are shown at panels N and O of **Figure 1**. Advanced structure-function correlations can be reached when these red algal SGs are used, as opposed to the more heterogeneous SGs from red or green algae. Conversely, the invertebrate SGs are very often composed of well-defined chemical structures (**Figures 1J–M**), which allow accurate structure-function correlations.

The information in the two previous paragraphs have made clear the advantages of the structures of the invertebrate SFs and SGs over the algal molecules, except few cases of red algal molecules (**Figures 1N,O**). These advantages come from the fact that the invertebrate molecules and the regular red algal SGs (**Figure 1**), which are composed of well-defined chemical structures, can be successfully used in structure-function relationship studies (**Table 1**). These studies allow prediction of the most influential structural combinations of the MSPs to achieve desirable anticoagulant responses. This knowledge is relevant to the development of these molecules as future therapeutic candidates.

MARINE GAGs HAVE UNIQUE STRUCTURES

Marine GAGs have different structures than those present in common mammal GAGs. For example, dermatan sulfate (DS) isolated from the ascidian species *Styela plicata* is composed of $[\rightarrow 4)$-α-L-IdoA-$(2R^1,3R^2)$-$(1\rightarrow 3)$-β-D-GalNAc-$(4R^3,6R^4)$-$(1\rightarrow]_n$, where IdoA is iduronic acid, GalNAc is *N*-acetyl galactosamine. The R^1, R^2, R^3, and R^4 are sulfate groups at 66, < 5, 94, and 6 percent, respectively (Pavão et al., 1998). The *S. plicata* is mostly 2-sulfated at the IdoA unit but largely 4-sulfated at the GalNAc unit. Conversely, the commonest mammalian DS is mostly composed of 2-sulfated IdoA units together with occasional C4 sulfation at GalNAc units. Another different GAG from marine invertebrates is the fucosylated chondroitin sulfate (FucCS) isolated from sea-cucumbers. These molecules are composed of the following structure $\{\rightarrow 3)$-β-D-GalNAc-$(1\rightarrow 4)$-[α-L-Fuc*p*-$(1\rightarrow 3)$]-β-D-GlcA-$(1\rightarrow\}_n$ (**Figure 2**) (Pomin, 2014a). The branching Fuc*p* unit can be sulfated at the 2, and/or 3 and/or 4-positions within different percentages according to the species of occurrence (**Table 2**). Conversely, the commonest chondroitin sulfates (CSs) of mammals are made of the following structure $[\rightarrow 4)$-β-D-GlcA-$(1\rightarrow 3)$-β-D-GalNAc-$(1\rightarrow]_n$, in which the GalNAc units can be either mostly 4-sulfated (CS-A) or predominantly 6-sulfated (CS-C) (Pomin et al., 2012).

ANTICOAGULANT MECHANISMS OF ACTION OF THE MSPs

The effects of MSPs on hemostasis are the mostly studied medical activities of these compounds. The mechanisms of action in this particular clinical activity reside basically on the inhibition of some coagulation proteases like thrombin citation(IIa) and factor Xa, via their natural inhibitors, named serpins citation(serine-protease inhibitors). The most common serpins of this system are antithrombin citation(AT) and heparin cofactor II citation(HCII). Although at different degrees of response, the majority of the MSPs described in this review, the ascidian DS (Pavão et al., 1998; Vicente et al., 2004; Kozlowski et al., 2011), the sea-cucumber FucCS (Mourão et al., 1996; Mourão, 2004), the algal

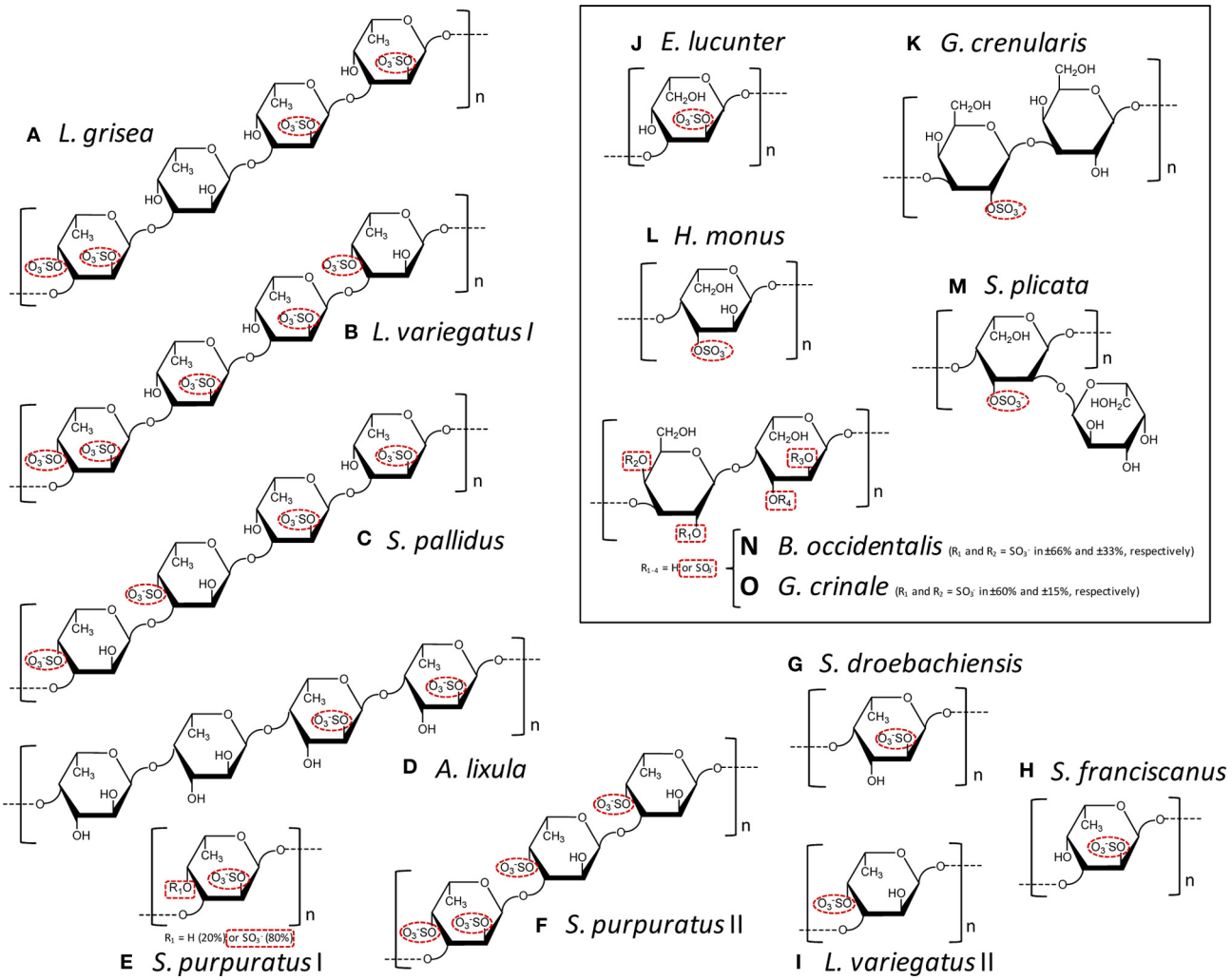

FIGURE 1 | Structural representation of the repetitive oligomeric units of the SFs (A–I) and SGs (J–O, inside the box) of well-defined chemical structures isolated from cell wall of sea cucumbers (A), from egg jelly coat of sea urchins (B–K), from tunic of ascidians (L,M), and from cell wall of red algae (N,O). Modified with permission from Pomin (2012b). These MSP are composed of α-L-fucopyranoses (α-l-Fuc*p*) **(A–I)**, α-L-galactopyranoses (α-L-Gal*p*) (I, M-O), or β-D-galactopyranosyl units (β-D-Gal*p*) **(K,N,O)**. The species-specific structures vary in sulfation patterns (exclusively 2-*O*- and/or 4-*O*-, or 3-*O*-positions), in glycosidic linkages: α(1→3) **(A–C,E,F,H–K)**, α(1→4) **(D,G,L,M)**, β(1→3) **(K)**, or alternating β(1→4) and α(1→3) **(N,O)**; and in number of composing residues of the repetitive units: tetrasaccharides **(A–D)**, trisaccharides **(F)**, disaccharides **(K,N,O)**, or monosaccharides **(E,G–J,L)**, all in linear chains, except **(M)**. The sulfation groups are highlighted by red dashed ellipses or rectangle when just percentage can be estimated. The structures are the following: **(A)** *Ludwgothurea grisea* [→3)-α-L-Fuc*p*-2,4(OSO_3^-)-(1→3)-α-L-Fuc*p*-(1→3)-α-L-Fuc*p*-2(OSO_3^-)-(1→3)-α-L-Fuc*p*-2(OSO_3^-)-(1→]$_n$ (Mulloy et al., 1994); **(B)** *Lytechinus variegates* I [→3)-α-L-Fuc*p*-2,4(OSO_3^-)-(1→3)-α-L-Fuc*p*-2(OSO_3^-)-(1→3)-α-L-Fuc*p*-2(OSO_3^-)-(1→3)-α-L-Fuc*p*-4(OSO_3^-)-(1→]$_n$ (Mulloy et al., 1994); **(C)** *Strongylocentrotus pallidus* [→3)-α-L-Fuc*p*-4(OSO_3^-)-(1→3)-α-L-Fuc*p*-4(OSO_3^-)-(1→3)-α-L-Fuc*p*-2(OSO_3^-)-(1→3)-α-L-Fuc*p*-2(OSO_3^-)-(1→]$_n$ (Vilela-Silva et al., 2002); **(D)** *Arbacia lixula* [→4)-α-L-Fuc*p*-2(OSO_3^-)-(1→4)-α-L-Fuc*p*-2(OSO_3^-)-(1→4)-α-L-Fuc*p*-(1→4)-α-L-Fuc*p*-(1→]$_n$ (Alves et al., 1997); **(E)** *Strongylocentrotus purpuratus* I ~80% [→3)-α-L-Fuc*p*-2,4(OSO_3^-)-(1→]$_n$and ~20% [→3)-α-L-Fuc*p*-2(OSO_3^-)-(1→]$_n$(Alves, Mulloy, Moy, Vacquier and Mourão, 1998) and **(F)** *S. purpuratus* II [→3)-α-L-Fuc*p*-2,4(OSO_3^-)-(1→3)-α-L-Fuc*p*-4(OSO_3^-)-(1→3)-α-L-Fuc*p*-4(OSO_3^-)-(1→]$_n$ (Alves, Mulloy, Moy, Vacquier and Mourão, 1998); **(G)** *Strongylocentrotus droebachiensis* [→4)-α-L-Fuc*p*-2(OSO_3^-)-(1→]$_n$ (Vilela-Silva et al., 2002); **(H)** *Strongylocentrotus franciscanus* [3)-α-L-Fuc*p*-2(OSO_3^-)-(1→]$_n$ (Vilela-Silva et al., 1999); **(I)** *L. varieagtus* II [3)-α-L-Fuc*p*-4(OSO_3^-)-(1→]$_n$ (Cinelli et al., 2007); **(J)** *Echinometra lucunter* [→3-α-L-Gal*p*-2(OSO_3^-)-1→]$_n$ (Alves et al., 1997); **(K)** *Glyptocidaris crenularis* [→3-β-L-Gal*p*-2(OSO_3^-)-1→3-β-L-Gal*p*-1→]$_n$ (Castro et al., 2009); **(L)** *Herdmania monus* [→4)-α-L-Gal*p*-3(SO_3^-)-(1→]$_n$ (Santos et al., 1992); **(M)** *Styela plicata* {→4)-α-L-Gal*p*-2[→1)-α-L-Gal*p*-3(OSO_3^-)]-3(OSO_3^-)-(1→}$_n$ (Mourão and Perlin, 1987); **(N,O)** both *Botriocladia occidentalis* and *Gelidium crinale* express structures of [3-β-D-Gal*p*-1→4-α-Gal-1→]$_n$with different sulfation contents (Farias et al., 2000; Pereira et al., 2005b).

Table 1 | Anticoagulant activities of MSPs of well-defined structures (Figure 1) measured by aPTT[a] and by IC_{50} for thrombin citation(IIa) and factor Xa inhibition in the presence of antithrombin citation(AT) or heparin cofactor II (HCII).

Polysaccharide	Source	Structure (Figure)	APTTcitation(IU/mg) [a]	IC_{50} citation(μg/mL)		
				IIa/AT	IIa/HCII	Xa/AT
3-linked sulfated α-L-fucans	*S. purpuratus* I	1E	76	0.3	0.3	2
	S. purpuratus II	1F	10	0.9	2	Nd[b]
	S. pallidus	1C	18	>500	>500	>500
	L. variegatus I	1B	3	>500	>500	>500
	L. variegatus II	1I	Nd	Nd	Nd	Nd
	S. franciscanus	1H	~2	>500	>500	250
	L. grisea	1A	<1	>500	>500	>500
4-linked sulfated α-L-fucans	*S. droebachiensis*	1G	<1	Nd	Nd	Nd
	A. lixula	1D	~2	150	150	>500
sulfated α-L-galactans	*E. lucunter*	1J	20	3	6	20
	G. crenularis	1K	Nd	Nd	Nd	Nd
	H. monus	1L	~2	>500	>500	>500
	S. plicata	1M	<1	>500	>500	>500
algal SGs	*B. occidentalis*	1N	93	0.02	1.1	2.5
	G. crinale	1O	65	0.02	25	1.5

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[a] *The activity is expressed as international units/mg using a parallel standard curve based on the International Heparin Standard citation(193 units/mg).*

[b] *Not determined.*

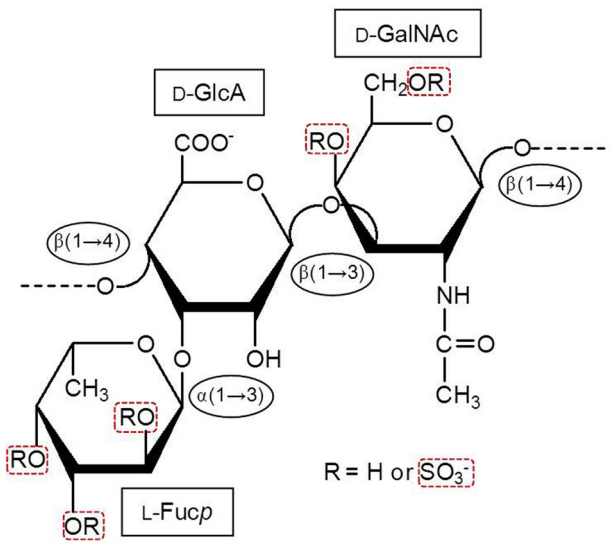

FIGURE 2 | Structural representation of the holothurian FucCS. The monosaccharides are indicated by rectangles. They are α-l-fucopyranose (l-Fuc*p*), β-d-glucuronic acid (d-GlcA), and *N*-acetyl β-d-galactosamine (d-GalNAc). The glycosidic linkage types are indicated in ellipses. Modified with permission from Pomin (2014a).

SFs and SGs (Pereira et al., 1999; Farias et al., 2000; Mourão, 2004) and the invertebrate SFs and SGs (Pereira et al., 1999, 2002; Pomin, 2012c) have either negligible or impressive effects in this serpin-dependent mechanism. The resultant anticoagulant activities of these MSPs (**Tables 1**, **2**) are intimately and ultimately dependent on their structural features, as described below.

Besides the common serpin-dependent anticoagulant mechanism, the FucCS from the sea-cucumber *Ludwigothurea grisea,* which its fucosyl unit is mostly 2,4-di-sulfated (**Figure 2**; **Table 2**), and the SG from the red alga *Botryocladia occidentalis* (**Figure 1N**) have also shown serpin-independent anticoagulant mechanism (Glauser et al., 2008, 2009). Initially, their anticoagulant actions were primarily attributed by their capacity in potentiate the inhibition of factors Xa and IIa, via either AT or HCII. However, the sea-cucumber FucCS and the red algal SG are also known to inhibit the generation of factor Xa and IIa by interfering in the formation of the blood cofactor complexes at the surface of the cells. Factor Xa is activated mainly by the intrinsic tenase complex, while IIa is converted from II by the prothrombinase complex. FucCS and SG were shown the ability to inhibit the activation of these tenase and prothrombinase complexes (Glauser et al., 2008, 2009). The formation of these complexes is a key step for the generation and amplification of the coagulation cofactors. This serpin-independent mechanism has also been reported for other types of SGs (Quinderé et al., 2014). Unfortunately, advanced structure-function correlations are yet to be established for this novel mechanism. Therefore, the structural requirements necessary to achieve the anticoagulant activity via the serpin-independent mechanism are still unknown. For this reason, we will keep this new anticoagulant mechanism out of discussion here.

THE ANTICOAGULANT STRUCTURAL COMBINATIONS

The anticoagulant structural features responsible for the anticoagulant activity of the MSPs can be determined from systematic analyses using the MSPs of well-defined chemical structures

Table 2 | Sulfation patterns (proportions of the branching sulfated fucose units) and the anticoagulant potential (measured by aPTT) of FucCS from 12 sea cucumber species analyzed so far.

Species	Fuc0S	Fuc3S	Fuc4S	Fuc2S4S	Fuc3S4S	aPTT	References
Ludwigothurea grisea[a]	0	-	∼49	∼20	∼17	55[b]	Mourão et al., 1996; Fonseca et al., 2009
Pearsonothuria graeffei	-	-	81.6	18.4	-	35[c]	Chen et al., 2011
Holothuria vagabunda	25.6	-	50.2	15.8	8.4	42[c]	Chen et al., 2011
Stichopus tremulus	-	-	24.8	22.4	52.8	135[c]	Chen et al., 2011
Isostichopus badionotus	-	-	4.1	95.9	-	183[c]	Chen et al., 2011
Thelenata ananas	0	∼25	∼22	∼53	0	348[d]	Wu et al., 2010, 2012
Stichopus japonicus[e]	0	Nd[f]	11.1	55.6	33.3	Ns[g]	Yoshida and Minami, 1992
Holothuria edulis[h]	-	-	Nd	18	Nd	89[i]	Luo et al., 2013
Apostichopus japonicas[h]	-	-	Nd	45	Nd	116[i]	Luo et al., 2013
Holothuria nobilis[j]	-	Nd	Nd	-	Nd	59[i]	Luo et al., 2013
Acaudina molpadioidea[k]	-	-	-	-	-	Nc[l]	Ye et al., 2012
Athyonidium chilensis[k]	-	-	-	-	-	Nc[l]	Matsuhiro et al., 2012

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[a] The CS backbone of FucCS from L. grisea has been extensively characterized. It is composed of GalNAc units with the following substitution percentages: 12% 4,6-di-sulfated, 53% 6-mono-sulfated, 4% 4-mono-sulfated, and 31% non-sulfated (Fonseca et al., 2009).

[b] aPTT values expressed as international units/mg citation(IU/mg) using a parallel standard curve based on the International Heparin Standard (UFH) whose activity is 229 units/mg (Fonseca et al., 2009).

[c] aPTT values expressed as international units/mg citation(IU/mg) using a parallel standard curve based on the International Heparin Standard citation(UFH) whose activity is 150 units/mg (Chen et al., 2011).

[d] aPTT values expressed as international units/mg citation(IU/mg) using a parallel standard curve based on the International Heparin Standard citation(UFH) whose activity is 204 units/mg (Wu et al., 2012).

[e] The CS backbone of this FucCS was mostly characterized as CS-E (Nagase et al., 1995), which is predominantly composed of 4,6-O-di-sulfated GalNAc units.

[f] Not determined.

[g] Not studied.

[h] Although the mono-4S and di-3S4S fucosyl units have been assigned in the FucCS of H. edulis and A. japonicas in Luo et al. (2013), the amounts of these units were not provided therein.

[i] aPTT values expressed as international units/mg citation(IU/mg) using a parallel standard curve based on the International Heparin Standard citation(UFH) whose activity is 212 units/mg (Luo et al., 2013).

[j] The FucCS from H. nobilis was studied by NMR but the anomeric signals belonging to the fucose residues were rather to weak and broad to allow integration and further quantitation of their proportions. However, mono-3S, mono-4S, and di-3S4S fucosyl units were clearly observed (Luo et al., 2013).

[k] Structures studied by Fourier transformed-infrared spectroscopy. Just a few structural features were raised. The sulfation patterns of FucCS from these two holothurian species are still an unknown.

[l] Not clear. Although the aPTT assay was undertaken and values were measured for different FucCSs concentration, the final values in IU/mg in comparison with a standard UFH curve were not provided.

(**Figure 1**). On this way, comparing all the structures in **Figure 1**, one can discern structural similarities and differences in these molecules. For example, both the SF from *Strongylocentrotus franciscanus* (**Figure 1H**), and the SG from *Echinometra lucunter* (**Figure 1J**) present the same sulfation pattern (exclusive and entirely 2-sulfated), the same anomeric configuration (α-form), the same glycosidic linkage (1→3), and based on previous works (Pereira et al., 2005a), the same molecular mass (∼100 kDa). Their single difference is the sugar type (Fuc*p* or Gal*p*). Interestingly, this single structural difference is itself enough to promote great changes in the anticoagulant outcomes of these homopolysaccharides. The 2-sulfated α-galactan from *E. lucunter* exhibits a significant anticoagulant activity monitored by the activated partial thromboplastin time (aPTT) method (Pomin, 2014b). The anticoagulant potential of this SG was determined to be 20 IU mg, although almost 10-fold less than unfractionated heparin (UFH) (**Table 1**). The specific anticoagulant assay with the purified proteases revealed that this SG enhances both IIa and factor Xa inhibition by either AT or HCII (**Table 1**). Conversely, the anticoagulant effect of 2-sulfated α-fucan from *S. franciscanus* is exclusively based on catalysis of AT inhibition over factor Xa, although it is 12.5-fold less active than the α-SG. This single effect on the Xa/AT system explains the much lower activity of the compound from *S. franciscanus* (aPTT of ∼2 IU mg^{-1}, 100-fold less active than UFH) since the anti-Xa activity has a relatively minor influence on the aPTT. This is an illustrative and typical example of a sugar-type-dependent biological effect of polysaccharides. The structural combination of 2-sulfation with 3-linked α-Gal*p* units results therefore in the anticoagulant response.

Based on this same systematic and comparative analysis, the SGs from the red algae *Botryocladia occidentalis* and *Gelidium crinale* were seen to exhibit identical backbones, and the same chain sizes (Farias et al., 2000; Pereira et al., 2005b). However, there are slight differences in their sulfation patterns

(**Figures 1M,O**). As summarized in **Table 1**, these structural differences account for the 30% difference in anticoagulant activity as observed by the aPTT values of these algal macromolecules and the even greater difference in catalytic effect of the SP from *B. occidentalis* on the HCII-mediated anti-IIA activity, which was approximately 25-fold more than the catalytic effect of the SP from *G. crinale*. When the backbones of the red algal SGs are identical but bearing sulfation as their main difference, the anticoagulant activity seems to be proportionally dependent of the sulfation content (**Table 1**).

The structural requirements for the interaction of the MSP of well-defined chemical structures with the coagulation cofactors and their target proteases and inhibitors were determined to be very stereospecific (Mourão, 2004; Pomin and Mourão, 2008; Pomin, 2009). The site of sulfation has a major impact on the activity. This can be illustrated by the fact that 2,4-di-sulfated units have an amplifying effect on the AT-mediated anticoagulant activity in the series of 3-linked α-L-fucans (**Figure 1**; **Table 1**). Specific sulfation sites are required for the interaction with plasma serine-protease inhibitors. Note the occurrence of the 4-sulfated unit content in the 3-linked α-L-fucans vs. their anticoagulant activities (**Table 1**). *L. variegatus* (a single 4-sulfated unit/tetrasaccharide, **Figure 1B**, has 3 IU/mg of activity), *S. pallidus* (a double 4-sulfated unit/tetrasaccharide, **Figure 1C**, has 18 IU/mg of activity), and *S. purpuratus* isotype II (a double 4-sulfated unit/trisaccharide, **Figure 1F**, has 76 IU/mg of activity). This 4-sulfation is the structural motif required to enhance the inhibition of IIa by HCII. In contrast, the presence of 2-sulfated residues seems to have a deleterious effect on HCII-mediated anti-IIA activity of the polysaccharides (Mourão, 2004).

In the studies using the ascidian DS, Pavão and co-authors demonstrated that 4-sulfation at GalNAc units together with some 2-sulfation at the IdoA units is a structural motif of anticoagulant properties in these molecules (Pavão et al., 1998). This was observed based on the fact that DS from the species *Styela plicata* and *Halocynthia pyriformis* have large amounts of 4-*O*-sulfated GalNac units (94 and 99%, respectively), together with the reasonable amounts of 66 and 70% of 2-sulfation at the IdoA units. Their anticoagulant actions were measured by aPTT and shown to be 11 and 8 IU/mg, respectively; while the activity of the DS from bovine mucosa was shown to be only 2 units/mg (Pavão et al., 1998). Unlike the ascidian DSs, the mammalian DS is largely 2-sulfated at the IdoA unit and much less sulfated at the 4-position of the GalNAc units, as above-mentioned.

The sulfated fucosyl branches in the sea-cucumber FucCS were shown to be essential to their anticoagulant activities. This statement is based on the fact that when these branching units are removed, for example, by mild acid hydrolysis, or desulfated, by desulfation reactions, their anticoagulant effects disappears (Pomin, 2014a). Besides the necessary existence of these branching units, their sulfation patterns are also influential to the levels of anticoagulant activity. This can be seen from the structures and aPTT values of **Table 2**. The 2,4-di-sulfation in the fucosyl branching units appears to be the best sulfation pattern to the anticoagulant activity of this class of SPs. The importance of the 2,4-di-sulfation in Fuc*p* units was already pointed out in the work of Fonseca et al. (2009), as above for the AT-mediated anticoagulant activities of the series of 3-linked α-L-fucans (**Figure 1**, **Table 1**). In the work of Fonseca et al. (2009), the 2,4-di-sulfated Fuc*p* units were reported to be crucial to the anticoagulant activity of both sea-cucumber FucCS molecules and the linear SFs from invertebrates (**Figure 1**, **Table 1**).

MAJOR CONCLUSIONS

Here, we have made clear the relevance of certain structural combinations of sulfation and glycosylation to the anticoagulant activity of the marine carbohydrates of well-defined chemical structures. For example, 2-sulfation together with 3-linked α-L-Gal*p* units, as found in the SG from the sea-urchin *E. lucunter* (**Figure 1J**), represents one of these structural combinations. Another combination is the 2,4-di-sulfation with α-L-Fuc*p* units. This combination can be found either in linear SFs of sea-urchins and sea-cucumbers (**Figures 1A,B,F**), or in the branched FucCS molecules (**Figure 2**, **Table 2**). The FucCS is a unique GAG found exclusively in sea-cucumbers. Another unique GAG from marine sources are the ascidian DS. For a good anticoagulant response of these GAGs, the 4-sulfation in GalNAc units combined with occasional 2-sulfation in IdoA units seems to be essential. When SGs from red algae exhibit simple backbones in which sulfation comprises the single modification and difference, sulfation content regardless the pattern seems to be relevant for the anticoagulant activity. In this case, the anticoagulant activity increases proportionally with the sulfation content. Finally, in invertebrate SFs, the 4-sulfation was observed to be a structural motif required to enhance the HCII-mediated inhibition of IIa. All these conclusions are summarized at **Table 3** for a straightforward representation. Although we have clearly revised the relevance of sulfation and glycosylation to the anticoagulant activity of MSPs, molecular weights are also known to be influential to the biological activity (Melo et al., 2004; Quinderé et al., 2014). To ensure that our interpretation on the anticoagulant effects of MSPs have been based solely on sulfation and glycosylation patterns, our comparative analyses using the different MSP structures were

Table 3 | Summary of the structural requirements and effects in anticoagulation of the MSPs.

Structural requirement	Outcome
2-sulfated 3-linked α-L-Gal*p*[a]	Enhance serpin (HCII and AT) inhibitory activity over the coagulation proteases (IIa and Xa)
2,4-disulfation in Fuc*p*[b]	
4-sulfated GalNAc + 2-sulfated IdoA in ascidian DS[c]	
Sulfation content in red algal homogeneous SGs[d]	
4-sulfation in invertebrate 3-linked SFs[e]	Enhance HCII-dependent IIa inhibition

[a] *(Pereira et al., 2005a).*
[b] *(Fonseca et al., 2009).*
[c] *(Pavão et al., 1998).*
[d] *(Pereira et al., 2005b).*
[e] *(Pereira et al., 2002).*

performed on samples of similar molecular weights. This procedure disregards the contribution from differential molecular weights.

One of the major goals of research programs involved with drug development nowadays, especially those related with potential carbohydrate-based drug candidates, is to understand the structural requirements of the new compounds in their specific therapeutic functions. Here, we have presented in a clear and straightforward way some of these structural requirements of the invertebrate and red algal SPs for their anticoagulant properties.

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Mannoprotein MP84 mediates the adhesion of *Cryptococcus neoformans* to epithelial lung cells

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The capsule is the most important virulence factor of the fungal pathogen *Cryptococcus neoformans*. This structure consists of highly hydrated polysaccharides, including glucuronoxylomannan (GXM), and galactoxylomannan (GalXM). It is also composed of mannoproteins (MPs) which corresponds to less than 1% of the capsular weight. Despite MPs being the minority and least studied components, four of these molecules with molecular masses of 115, 98, 88, and 84 kDa were identified and characterized as *C. neoformans* immunoreactive antigens involved in the pathogenesis, and are potential cryptococcosis vaccine candidates. With the aim to describe the adhesive property of MPs, we cloned and expressed the MP84, a mannoprotein with molecular weight of 84 kDa, on *Pichia pastoris* yeast, and performed interaction assays of *C. neoformans* with epithelial lung cells, in the presence or absence of capsule components. Two fungal strains, the wild type, NE-241, and a mutant, CAP67, deficient in GXM production, were used throughout this study. The adhesion assays were completed using epithelial lung cells, A549, and human prostate cancer cells, PC3, as a control. We observed that capsulated wild type (NE-241), and acapsular (CAP67) strains adhered significantly to A549 cells, compared with PC3 cells ($p < 0.05$). GXM inhibits the NE-241 adhesion, but not the CAP67. In contrast, CAP67 adhesion was only inhibited in the presence of MP84. These results demonstrate the involvement of MP in the adhesion of *C. neoformans* to epithelial lung cells. We conclude that this interaction possibly involves an adhesion-like interaction between MP on the fungal surface and the complementary receptor molecules on the epithelial cells.

Keywords: *Cryptococcus neoformans*, mannoprotein MP84, cell adhesion, A549 epithelial lung cell

INTRODUCTION

The opportunistic fungus *Cryptococcus neoformans* is the etiological agent of cryptococcosis, a disease that kills about 630,000 people per year globally (Park et al., 2009). This infection is most probably acquired by inhalation of desiccated cells, which are present in the environment as basidiospores or poorly encapsulated yeasts (Rodrigues et al., 1999). This disease can manifest into different clinical forms, with the most severe being cryptococcal meningitis that affects mainly immunocompromised patients, such as individuals with HIV/AIDS. Although the incidence of HIV-associated cryptococcosis has decreased in developed countries since the introduction of antiretroviral therapy against AIDS, this disease continues to cause significant morbidity and mortality in the less developed world (La Hoz and Pappas, 2013).

C. neoformans has certain attributes that provide their survival in specific ecological niches. Some attributes include the ability to grow at 37°C and melanin production. These are indispensable for the adaptation into the mammalian host environment. However, the cryptococcal capsule, which surrounds the cell body, is considered the major determinant of virulence of this pathogen, with potent anti-phagocytic properties (Zaragoza et al., 2009). Many microbes possess capsules that play important roles, for example, in resistance to stressful conditions (such as dehydration), and in the interaction with the environment (Zaragoza et al., 2009). The *C. neoformans* capsular network consists of a highly hydrated polysaccharide gel, composed of high-molecular weight polysaccharide polymers, such as glucuronoxylomannan (GXM), which represents almost 90% of the total capsule; the remainder being galactoxylomanna (GalXM) (Kumar et al., 2011). GXM is composed of a large backbone of 6-O-acetylated α-1,3-mannose residues with β-D-xylopyranosyl, β-D-glucuronosyl monosubstituted side chains (Cherniak and Sundstrom, 1994). Recently, Heiss et al. (2009) re-examined the structure of *C. neoformans* GalXM by Nuclear Magnetic Resonance (NMR) spectroscopy and Gas-liquid Chromatography-Mass Spectrometry (GC–MS), and proposed GalXM to be termed glucuronoxylomannogalactan (GXMGal).

The complexity of the cell surface architecture of *C. neoformans* is increased by the presence of mannoproteins (MPs), that comprises less than 1% of the capsule mass (Rodrigues and Nimrichter, 2012). These proteins are highly mannosylated antigens, usually containing 80–90% of mannose, and found in a wide range of fungal species (Zhang and Ballou, 1981; Murphy, 1988; Cao et al., 1998; Nguyen et al., 1998; Nisini et al., 2001; Frieman et al., 2002; Mansour and Levitz, 2003; Chong et al., 2004). The

C. neoformans MPs were first identified in culture filtrates (Reiss et al., 1985; Murphy, 1988) and were defined as MPs by their ability to adhere to a Concanavalin A (Con A) affinity column (Murphy et al., 1988).

Investigation on the location of MPs on cryptococcal cells concluded that they are mainly found in the inner capsule, near the cell wall, and not associated with GXM or GXMGal (Vartivarian et al., 1989; Jesus et al., 2010).

The role of MPs in capsule architecture has never been established. However, their effects on the host cells have long been studied. It is well known that *C. neoformans* MPs are highly immunogenic. Some authors consider MPs as a key inflammatory mediator that induces a protective immune response against *C. neoformans* infection, promoting them as a vaccine candidate against fungi (Pietrella et al., 2001, 2005; Levitz and Specht, 2006).

Several *C. neoformans* MPs have been isolated and new MP roles have been identified, besides the immunogenic function. In 2002, a specific MP involved in T-cell activation was identified. This protein had an apparent molecular weight of 88 kDa, and was therefore named MP88 (Huang et al., 2002). One year earlier, a 98 kDa MP involved in the stimulation of T-cell responses was identified and named MP98. Analysis of the predicted amino acid sequence of MP98 reveals a domain with chitin deacetylase activity (Levitz et al., 2001). In 2005, new immunogenic MPs have been identified, with molecular weights of 250, 125, 115, and 84 kDa. The genes encoding MP115 and MP84 were cloned and had homology with carboxylesterases and chitin deacetylases, respectively (Biondo et al., 2005). Recently, the MP Cig1 was described to be a mediator of iron uptake, functioning as a hemophore at the cell surface, and contributing to virulence in a mouse model of cryptococcosis (Cadieux et al., 2013).

Inhalation of *C. neoformans* is the main pathway for infection. An effective interaction of this fungus with epithelial alveolar cells is crucial for disease establishment. Once adhered to the pulmonary epithelia, *C. neoformans* can proliferate and induce primary lesions in the lung (Kawakami, 2004). A previous study suggested that *C. neoformans* can use GXM for attachment to epithelial lung cells (Barbosa et al., 2006). Since the alveolar epithelium is the first host site to be challenged by *C. neoformans* to establish a successful infection, we aimed to clone and express the MP84 on *Pichia pastoris* yeast, and investigate the role of this MP in the interaction of *C. neoformans* with epithelial lung cells. We found that these cells apparently express MPs binding sites responsible for the attachment of *C. neoformans* to A549 monolayers.

MATERIALS AND METHODS

MICROORGANISM AND CULTURE CONDITIONS

Two *C. neoformans* strains were used throughout this study, a wild type strain, NE-241, and a mutant strain, deficient in GXM production, CAP67. They were kindly provided by Professor Tamara Doering (Department of Molecular Microbiology, Washington University School of Medicine, St Louis, MO, USA) and Professor Robert Cherniak (Georgia State University, Atlanta, GA, USA), respectively. The strains were grown on YPD agar, for 72 h at 37°C and stored at 4°C as stock cultures. To obtain free cells, NE-241 and CAP67 were cultivated at 37°C under constant shaking (150 rpm) for 120 h, in a chemically defined CDCB 2550 medium containing (g/L) dextrose, 20; urea, 1.29; $KH_2PO_4.H_2O$, 1.36; $MgSO_4.7H_2O$, 0.3; sodium glutamate, 1.0; thiamine-HCl, 2×10^{-3}; biotin, 1×10^{-5}. Yeast cells were obtained by centrifugation and washed twice in 0.01 M phosphate buffered saline (PBS), pH 7.2. Cell growth was estimated by counting the number of yeasts in a Neubauer chamber. Capsule expression was determined by India ink staining.

A549 EPITHELIAL LUNG CELLS

A549 is a human type II alveolar epithelial-like lineage, which is derived from lung carcinomatous tissue of a human patient, and is widely used as a model for infection of respiratory pathogens (Hahn, 1997). The cultures were maintained and grown to confluence in 25 cm^2 culture flasks containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), at 37°C in a 5% CO_2 atmosphere. For interaction experiments with *C. neoformans* or ELISA with recombinant MP84, A549 cells were cultivated on 96 or 24-well plates, respectively.

ISOLATION AND PURIFICATION OF CAPSULAR POLYSACCHARIDES

The capsular components GXM, GXMGal and MPs were obtained from the NE-241 strain, which was grown in a chemically defined medium (CDCB 2550). These capsular polysaccharides were isolated and purified as previously described by our group (Villena et al., 2008). Briefly, the culture supernatant was separated from cells by centrifugation (6000 *g*, for 30 min at 4°C) and precipitated by addition of three volumes of cold ethanol. The precipitate was collected by centrifugation, dissolved and dialyzed against distilled water and lyophilized. The lyophilized material was solubilized in distilled water and precipitated with cetyltrimethylammonium bromide (CTAB). The mixture was maintained for 18 h at room temperature, for precipitation. The precipitated material was suspended in 10% ethanol, centrifuged and the pellet was solubilized in 1M NaCl. This solution was precipitated with three volumes of ethanol; the pellet was solubilized in distilled water, dialyzed and lyophilized, resulting in the GXM fraction. The supernatant was concentrated and precipitated in three volumes of ethanol. The pellet was solubilized in distilled water, dialyzed and lyophilized, resulting in the GXMGal+MP fraction.

C. NEOFORMANS RNA EXTRACTION AND cDNA OBTAINMENT

C. neoformans RNA was extracted from CAP67 strain using Brazol reagent (LGC Biotecnologia). Briefly, 0.75 ml of Brazil was added to 5×10^6 CAP67 yeast cells and incubated under room temperature for 5 min; 0.2 mL of chloroform was then added and incubated at room temperature for 3 min. The suspension was centrifuged (12,000 *g*) for 15 min at 4°C, and 0.5 ml of 100% isopropanol was added to the aqueous phase. After incubation at room temperature for 10 min, the tube was centrifuged (12,000 *g*) for 10 min at 4°C. The pellet was washed with 75% ethanol and resuspended in *Rnase free* MilliQ water. Any DNA genomic contamination was removed by treatment of 5 µg of the total RNA with 1 U RQ1 RNase-free DNas I (Promega) for 15 min at 37°C.

The cDNA was obtained from the extracted RNA (1 μg), using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas Life Science), as described in the manufacturer's manual.

MP84 GENE AMPLIFICATION AND CLONING

The gene encoding the 84 KDa MP was amplified by Polymerase Chain Reaction (PCR), using *Taq* DNA polymerase (Promega) with oligonucleotides 5′ATA*CTGCAG*GGCACGAGTCATGGCT TCAGCC 3′ (sense) and 5′ATA*TCTAGA*CCACTACCGCGT GGCACCAGTTGGGAGCTGGCAGCAGAGG 3′ (antisense) (the underlined regions are restriction sites of *Pst*I and *Xba*I enzymes, respectively). This consisted of an initial denaturation step of 5 min at 95°C; 25 cycles of 45 s at 95°C, 45 s at 55°C, and 60 s at 72°C; and a final extension step for 7 min at 72°C. The amplified fragments were purified from agarose gels using Gel Extraction Kit (Qiagen).

To clone the MP84 gene, pPICZαB plasmid (Invitrogen) and purified amplified fragment were digested with *Pst*I and *Xba*I restriction enzymes (New England Biolabs), and the ligation was performed with T_4 DNA ligase enzyme (Promega). After transformation in *Escherichia coli* DH5α, some clones were selected to performance the Mini-prep using Gel Extraction Kit (Qiagen) and differential digestion assays were done to confirm the cloning. The positive clones were sequenced in an API-3100 (Applied Biosystems) automated sequencer and sequences were analyzed by DNASTAR Lasergene software (Version 7.2).

PICHIA PASTORIS TRANSFORMATION

P. pastoris X-33 strain was used to express and secrete recombinant MP84 in culture medium. After sequence analysis, the pPICZαB plasmid with MP84 gene was digested by *Bgl*II restriction enzyme (New England Biolabs), for 1 h at 37°C, to linearize the plasmid. The transformation occurred by the addition of 5 μL of linearized plasmid (10 μg/μL) to the competent *P. pastoris* cells, suspended in 1 M cold Sorbitol, and incubated for 5 min on ice before electroporation. The electroporator (BioRad) was set at 2.43 kV, 25 μF, and 400 Ω, under pulse time of 9.6. An aliquot of 600 μL 1 M cold Sorbitol was added to the electroporated cell suspension, followed by incubation at 30°C for 30 min. YPD medium was added to the cell suspension and incubated for an additional 30 min at 30°C. The cells were then plated on YPD agar with 100 μg/mL zeocin (Invitrogen) and cultivated at 28°C for 48 h to select the transformants.

EXPRESSION TEST

With the aim to select the colony that expressed and secreted recombinant MP84 the most, 5 colonies were randomly chosen and cultivated overnight at 28°C on BMGY (buffered complex glycerol medium). After centrifugation, the cells were transferred to BMMY (buffered complex methanol medium) for induction of recombinant MP84 expression. 100% methanol was added for a final concentration of 0.5% for each colony at 24 h of growth. Culture medium samples were collected on each colony at 24, 36, 48, 60, 72, 84, and 96 h. These samples were analyzed by Western Blot, as described in detail elsewhere (Lam et al., 2005), using anti-histidine antibody (Pierce) and mouse anti-IgG HRP conjugated and revealed with SuperSignal West Pico Chemiluminescent Substrate (Pierce), to determinate recombinant MP84 expression levels and the duration of the secretion of this protein for each *P. pastoris* colony. A colony with pPICZαB without MP84 gene was used as negative control.

PURIFICATION OF RECOMBINANT MP84

Recombinant MP84 was purified by an immobilized metal affinity chromatography (IMAC) using precharged Ni Sepharose™ High Performance (His Trap HP, Amersham Biosciences), as described by manufacturer's manual. The selected *P. pastoris* colony was grown on 2 L of BMMY medium with addition of 0.5% methanol every day at 28°C, for the time selected. After centrifugation, the culture supernatant was applied in a nickel affinity column (His Trap HP column), which recognizes the histidine tag added to the recombinant MP84 during molecular biology steps. Elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4) was used to elute the recombinant MP84. This protein was then dialyzed against 20 mM sodium phosphate, 0.5 M NaCl to remove imidazole. Before storing at −80°C, the eluate was analyzed by SDS-PAGE, and stained with comassie blue to verify the purity of the sample.

INTERACTION OF *C. NEOFORMANS* WITH EPITHELIAL LUNG CELLS

A549 epithelial lung cells and PC3 prostate human epithelial cells were cultivated on 24-well plates on DMEM supplemented with 10% fetal bovine serum (FBS), at 37°C in a 5% CO_2 atmosphere, for about 36 h until the monolayer formation. Each well was then inoculated with 10^6 *C. neoformans* yeast cells suspended on DMEM medium to a final volume of 500 μL per well, and incubated at 37°C for 1 h. For inhibition assays, A549 cells were pre-incubated with GXM, GXMGal+MP or MP84, at 37°C for 1 h. After three washings with DMEM medium to remove non-adhered cells, the A549 cells were lysed with sterile cold water. The cell lysate was recovered and plated onto YPD agar and incubated at 37°C. After 48 h, the number of colony-forming units (CFU) was determined. The experiment was performed in triplicate and statistically analyzed by using Student's *t*-test. $p < 0.05$ was considered statistically significant.

BINDING OF MP84 TO EPITHELIAL LUNG CELLS

The binding between recombinant MP84 and A549 cells was evaluated by ELISA assays. A549 cells were suspended in DMEM and placed into the wells of a 96-well plate and cultivated at 37°C until the cells were confluent. Then, the lung cells were incubated with recombinant MP84 in DMEM, for 1 h at 37°C. After three washings with PBS containing 0.05% Tween 20 (PBS-T), the A549 cells were fixed with 4% of paraformoldehyde and blocked with 0.5% casein in PBS-T for 1 h at 37°C. After washing with PBS-T, cells were incubated with 1:3000 anti-histidine antibody (GE Healthcare) in PBS-T for 1 h at room temperature, washed, and incubated with mouse anti-IgG HRP conjugated (Cell Signaling) diluted 1:2000 under the same conditions. After washing, the reaction was revealed by TMB substrate solution (eBioscience). The absorbance was determined on the ELISA reader (Beckman Counter) at 450 nm. Negative control was performed by replacing the recombinant MP84 with PBS. The experiment was performed

in triplicate and statistically analyzed by using Student's *t*-test. $p < 0.05$ was considered statistically significant.

RESULTS

C. NEOFORMANS RNA EXTRACTION AND MP84 GENE AMPLIFICATION

With the aim to extract the *C. neoformans* total RNA, the poorly capsulated strain, CAP67, was grown for 18 h. The RNA was extracted with Brazol reagent as described in the Materials and Methods. The integrity and DNA free appearance were observed by agarose gel electrophoresis (**Figure 1A**). The extracted RNA was quantified and used to synthesize the *C. neoformans* cDNA, which was utilized as a template for the obtainment of the MP84 gene. The amplification of this gene was performed by PCR using high fidelity enzymes. Oligonucleotides driven to the coding region of MP84 resulted in the amplification of a single fragment of 1230 bp (**Figure 1B**).

CLONING MP84 IN pPICZαB PLASMID

Competent *E. coli* DH5α bacteria were transformed with insert-vector, and were grown in culture medium containing zeocin antibiotic to screen for transformants. Some colonies had their plasmid extracted, linearized and analyzed on an electrophoresis gel to select the positive clones (data not shown).

To confirm the positive clones of MP84 gene in pPICZαB plasmid, the DNA of some clones was digested with *Pst*I and *Xba*I restriction enzymes, which produced two fragments of approximately 3600 and 1200 bp of molecular weight, corresponding to the correct size of pPICZαB and MP84, respectively (**Figure 2B**, lane 2). When the same insert-vector was digested with *Pst*I, *Eco*RI, and *Xba*I restriction enzymes, we observed three fragments with at approximately 3600, 700, and 500 bp, corresponding to the vector and two insert parts (**Figure 2B**, lane 3). The presence of fragments with molecular weight corresponding to the expression vector, the insert and the insert cut, suggests that the insertion of the MP84 gene in the pPICZαB plasmid was successful.

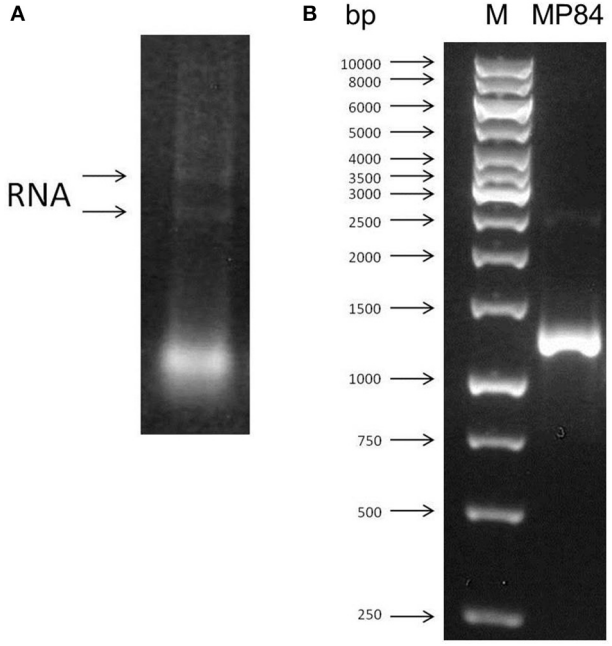

FIGURE 1 | RNA extraction and amplification of MP84 gene. (A) Ethidium bromide-stained 1% agarose gel of total RNA (0.5 μg) extracted from *C. neoformans* CAP67. **(B)** PCR fragment amplified using specific oligonucletides for MP84. (M) Markers (BioTools).

The sequences analyzed in DNASTAR software showed that the insert without mutation was in frame with the α-factor signal sequence as well as with the C-terminal c-myc and HIS_6 (data not shown).

P. PASTORIS TRANSFORMATION AND PURIFICATION OF RECOMBINANT MP84

After the screening of transformants with zeocin antibiotic, expression tests were performed on five selected colonies, which verified the duration and expression levels of MP84 recombinant protein for each colony. The duration and expression levels were evaluated by Western Blot assays (**Figure 3A**), using anti-histidine as primary antibody. The five clones were grown in culture medium containing methanol for 24, 36, 48, 60, 72, 84, and 96 h. The Western Blot analyses demonstrated that all colonies tested were able to express and secrete recombinant MP84 to the culture medium. However, colony 2 was chosen for large-scale purification due to its capacity to secrete a large amount of integrated MP earlier (72 h) than the other clones (**Figure 3A**).

Large-scale purification of recombinant MP84 protein secreted from methanol-induced yeast cells was performed by concentration of cell-free growth medium, followed by affinity purification by an immobilized metal affinity chromatography (IMAC) using precharged Ni Sepharose™ High Performance (His Trap HP, Amersham Biosciences), as described in the Methods. The eluate was analyzed by SDS-PAGE, and the purity of the recombinant MP84 sample was attested (**Figure 3B**).

INTERACTION OF *C. NEOFORMANS* WITH A549 EPITHELIAL LUNG CELLS

Adhesion is the first step to colonization and dissemination into the host. This observation leads us to study the binding between *C. neoformans* strains and host cells. Capsulated (NE-241) and non-capsulated (CAP67) strains were incubated for 1 h with epithelial lung cells or with prostate human epithelial cells (PC3). After culture and CFU counting, we observed that both strains adhered significantly to A549 cells, compared with PC3 cells ($p < 0.05$), used as a negative control (**Figure 4A**). We also observed that capsule size did not interfere in the interaction between the fungal cells and the epithelial lung cells, due to the capacity of both strains to bind to A549 cells. Additionally, no significant differences were observed in the adhesion levels between the two strains ($P > 0.05$) (**Figure 4A**). This result corroborates previous study, from Barbosa et al. (2006), which showed that *C. neoformans* yeast cells were able to specifically bind to A549 epithelial lung cells by capsular envelope components, such as GXM, however, disagrees with Merkel and Scofield (1997) who observed that the non-capsulated strain was the most adherent strain under the tested conditions.

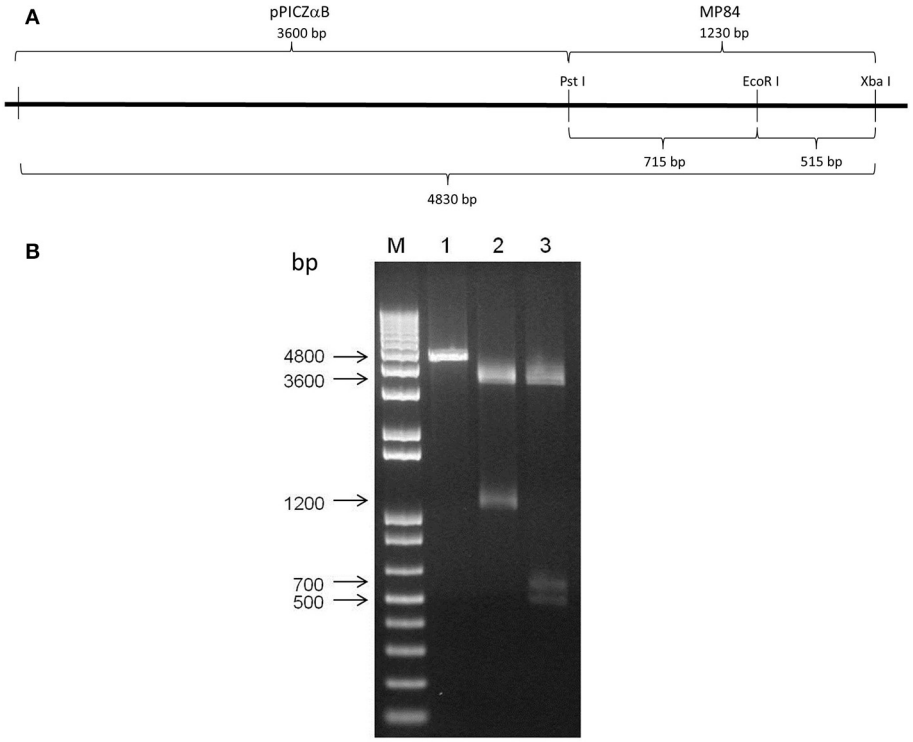

FIGURE 2 | Cloning of MP84 mannoprotein in pPICZαB plasmid. The amplified gene of MP84 by PCR was ligated in expression vector pPICZαB and then transformed into *E. coli* DH5α. After the selection of the positive clones, the plasmids were extracted and the differential digestion was performed. All positives clones were sequenced to confirm the correct sequence. **(A)** Schematic representation of clone pPICZαB-MP84 and the restriction enzymes map. **(B)** pPICZαB-MP84 (lane 1) digested with restriction enzymes *Pst*I and *Xba*I (lane 2) and with *Pst*I, *Eco*RI and *Xba*I (lane 3).

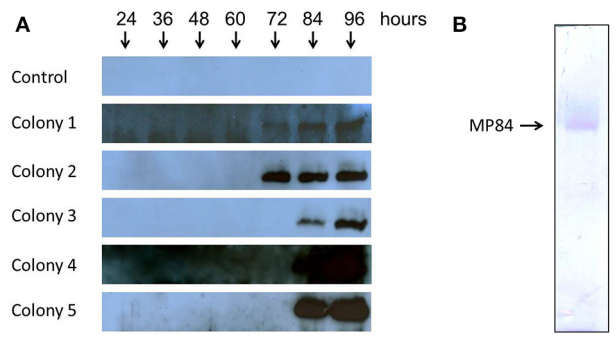

FIGURE 3 | Expression of MP84 mannoprotein in *P. pastoris*. *P. pastoris* was transformed with pPICZαB-MP84 plasmid and positive colonies were selected in the presence of the drug. Expression tests were performed to choose colonies with high levels of expression. Colony 2 was selected with a high amount of protein secreted. MP84 was then purified from the culture supernatant by affinity chromatography with nickel columns. **(A)** Western Blot analysis of the recombinant MP84 expression of the indicated colonies with 24, 36, 48, 60, 72, 84, and 96 hours of growth. A colony with pPICZαB without MP84 gene was used as negative control (Control). **(B)** SDS-PAGE of *P. pastoris* colony 2 culture supernatant after purification by affinity chromatography with nickel columns.

Furthermore, we aimed to investigate the molecules involved in this interaction. To test the adhesion inhibition by capsule components, GXM, GXMGal+MP and recombinant MP84 were pre-incubated with A549 cells before the addition of fungal cells. We observed that GXM inhibits 85% of the adhesion of the capsulated strain to A549 cells, contrarily; this polysaccharide did not affect the interaction between the mutant strain and the host cells (**Figure 4B**). In addition, 90% of the adhesion of the non-capsulated strain (CAP67) was inhibited by GXMGal+MP fraction or recombinant MP84, which was not observed with the capsulated strain (NE-241) (**Figure 4B**).

BINDING OF RECOMBINANT MP84 TO A549 EPITHELIAL LUNG CELLS

The results from the experiments of interactions between fungal and host cells suggest that when GXM, GXMGal or MP84 putative receptors are blocked, *C. neoformans* cells become less adhesive to alveolar cells, leading us to investigate whether A549 cells are able to recognize MP84.

Epithelial lung cells, cultivated in 96-well plate, were incubated with purified recombinant MP84 and analyzed by ELISA with anti-histidine antibody, as demonstrated in **Figure 5**. After 1 h of interaction with recombinant MP84, the A549 cells were strongly recognized by the anti-histidine antibody, suggesting the existence of MP binding sites in the host cells.

DISCUSSION

C. neoformans infection begins in the lung, by the inhalation of desiccated basidiospores or yeasts living in the environment, mainly in pigeon guano. In some cases, this fungus can surpass the local host defense and reach the central nervous system, causing fungal meningoencephalitis (Rodrigues et al., 1999).

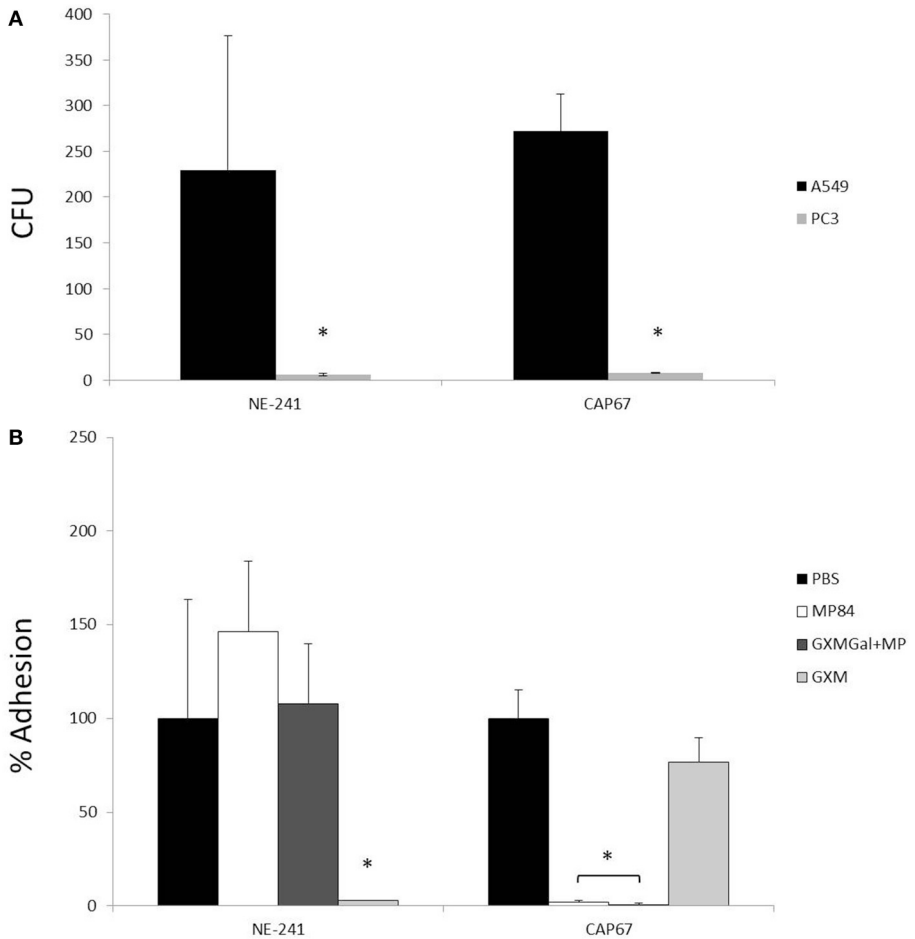

FIGURE 4 | Interaction of *C. neoformans* with epithelial lung cells. Capsulated (NE-241) and non-capsulated (CAP67) *C. neoformans* strains were interacted with prostate human epithelial cells (PC3) or with epithelial lung cells (A549), with or without the presence of capsule constituents (GXM, GXMGal+MP or MP84). The interaction of *C. neoformans* with host cells was determined after counting colony forming units (CFUs) of viable yeasts recovered after 1 h of incubation. **(A)** CFUs of capsulated (NE-241) and non-capsulated (CAP67) strains incubated with A549 and PC3 cells. *C. neoformans* strains adhere significantly more to A549 than to PC3 cells. No significant difference was observed between the adhesion levels of the two strains. **(B)** Percentage of adhesion of *C. neoformans* strains to A549 cells in the presence or not of capsule constituents. Pretreatment of host cells with GXM inhibited NE-241 adhesion, while the incubation of lung cells with GXMGal+MP or MP84 inhibited the adhesion of CAP67. $*p < 0.05$.

This observation implies that for a successful infectious process, *C. neoformans* must interact with different host tissues, such as the lung epithelia, endothelial cells and the blood brain barrier (Goldman et al., 1994; Chen et al., 2003; Chang et al., 2004).

After inhalation, *C. neoformans* cells are deposited into the alveolar space, and interact with types 1 and 2 epithelial cells, macrophages and lung surfactant proteins. In this regard, the ability of *C. neoformans* to interact with alveolar macrophages (Tucker and Casadevall, 2002) and to bind surfactant proteins (Van de Wetering et al., 2004) has been well studied; however, the mechanisms involved in the fungal interaction with epithelial cells remain largely unknown. Few studies reported the ability of *C. neoformans* yeasts to interact with human epithelial lung cells. In 1997, Merkel and Scofield demonstrated that capsulated and non-capsulated strains were able to adhere to A549 cells. They also observed that simple carbohydrates, such as sucrose, inositol, *N*-acetylglucosamine and *N*-acetylgalactosamine, as well as a monoclonal antibody produced against whole cryptococcal cells, inhibited adherence of *C. neoformans* to host cells, suggesting a specific host-pathogen interaction mediated by yeast adhesins (Merkel and Scofield, 1997). Later, Barbosa and co-authors demonstrated that the interaction between capsulated or acapsular *C. neoformans* yeasts and alveolar epithelia was mediated by GXM, allowing the fungus to reach the intracellular environment and damage host cells (Barbosa et al., 2006). The inhibitory activity of mannose or MPs was not evaluated in both studies.

Here, we observed that capsule size did not interfere in the interaction between the *C. neoformans* and human A549 human cells, since no significant differences ($P > 0.05$) were observed in the adhesion levels between the strains NE-241 (capsulated) and CAP67 (acapsular) with epithelial cells (**Figure 4A**). This suggests that in the absence of GXM, *C. neoformans* can utilize other capsule components as adhesins to interact with host cells. As

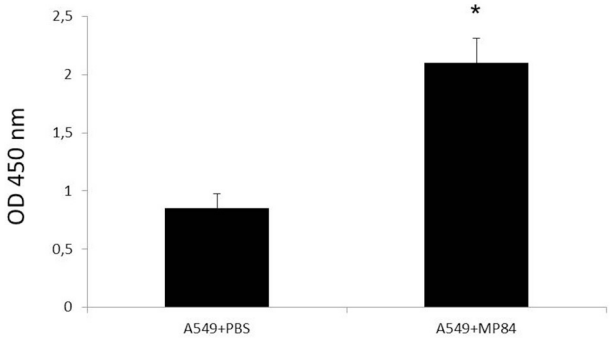

FIGURE 5 | Binding of recombinant MP84 to A549 cells was determinated by ELISA. Incubation of epithelial lung cells with recombinant MP84 for 1 h, for further reaction with the anti-histidine antibody and a mouse anti-IgG HRP conjugated, shows that A549 cells are able to bind recombinant MP84. Control preparations were submitted to the same steps, but the recombinant MP84 was replaced by PBS. $^*p < 0.05$.

MPs are internal capsule molecules that become most exposed in the absence of GXM, we aimed to investigate whether MPs could function as *C. neoformans* adhesin and mediate the interaction between the fungus and the host cells. For this, the first step was to obtain a good volume of MPs. This was achieved by the obtainment of a GXMGal+MP fraction, purified from the culture supernatant, but mainly, by the clone and expression of MP84 in *P. pastoris* yeasts.

MP84 was first identified in 2005, by Biondo and co-workers, together with MP115. N-terminal amino acid sequences of both proteins were used to search the *C. neoformans* nucleotide databases, and homologous genomic sequences were used to synthesize DNA probes and isolate cDNA clones containing the full-length genes.

Analysis of the gene sequence showed the presence of a serine/threonine-rich region, a potential site for heavy N-glycosylation, which was confirmed by marked shifts in the molecular mass of MP84 (23 kDa) after peptide-*N*-glycosidase F treatment (Biondo et al., 2005). Moreover, homology with chitin deacetylases from other organisms was found during analysis of the deduced amino acids sequence. Chitin deacetylases are enzymes that convert chitin to chitosan, the deacetylated form of chitin. Chitosan is indispensable for fungal cell architecture, by maintaining cell integrity, normal capsule and bud separation (Levitz et al., 2001; Biondo et al., 2002). Additionally, MP84 contains a putative GPI anchor motif in the C-terminal portion. Since GPI anchors are used to link proteins to the cell wall or to the cell membrane, the presence of a putative GPI anchor site suggests a cell wall or capsule inner layer MP84 localization (Biondo et al., 2005).

In the present study, MP84 was, for the first time, expressed using the *P. pastoris* yeast model, with the aim to obtain a good amount of glycosylated recombinant protein, which is not possible using *E. coli* model. Mannosylation seems to be strongly required for MP functions. Unglycosylated MP, for example, had a greatly impaired capacity to stimulate antigen-specific IL-2 production from $CD4^+$ T cells, compared with the mannosylated protein (Specht et al., 2007). In addition, the polysaccharide GXM was reported as mediator of host-pathogen interaction (Barbosa et al., 2006). These observations reinforced the necessity to use the *P. pastoris* model to express MP84. For this, two sense primers were constructed based on the MP84 gene sequence accessed in EMBL Nucleotide Sequence Database with AJ938050 accession number. These primers were used to amplify the MP84 gene from cDNA obtained from *C. neoformans* RNA (**Figure 1**). The MP84 amplified gene had to be bound to the expression vector (pPICZαB) and transformed into *E. coli* for large scale amplification of the insert-vector before being transformed into *P. pastoris*. Differential digestion assays confirmed the correct binding between the MP84 gene and pPICZαB (**Figure 2**), and the analysis of the insert-vector sequence confirmed that the insert was in frame with the α-factor signal sequence as well as with the C-terminal c-myc and HIS_6 epitopes encoded by the vector (data not shown). Finally, the pPICZαB+MP84 gene was transformed into *P. pastoris* yeasts and expression tests were performed to verify the colonies expression levels and select the most secretory colony. Colony 2 was chosen to express the recombinant MP84 in large-scale, and this protein was purified by affinity chromatography in a nickel column (**Figure 3**).

Recombinant MP84, GXM or GXMGal+MP fraction was incubated with epithelial lung cells to test their capacity to inhibit the adhesion between fungal and host cells. Capsulated (NE-241) and non-capsulated (CAP67) *C. neoformans* strains were interacted with host cells. We observed that the fractions containing MP, as recombinant MP84 and GXMGal+MP, only inhibited the non-capsulated strain adhesion, whereas GXM only inhibited the capsulated strain adhesion (**Figure 4B**). These results led us verify the existence of putative binding sites that could recognize MPs on the surface of epithelial lung cells. Thus, we incubated recombinant MP84 with A549 cells, and after 1 h, we observed a specific interaction between this host cell and that MP (**Figure 5**).

Adhesion is one of the most important steps for the establishment of an infection. The outer layer molecules of microorganisms are the first antigens to interact with the host substrate, functioning as adhesins. *C. neoformans* yeasts are typically highly capsulated, and as GXM is abundant in this fungus capsule (more than 90%), this polysaccharide can mediate the interaction with host cells, as described elsewhere (Barbosa et al., 2006). However, not all *C. neoformans* wild yeasts are highly capsulated; this fungus exhibits striking variations in cellular structure and size, which have important consequences during infection. The morphological variations in *C. neoformans* can be divided into three classes, as reviewed by Zaragoza in 2011: (1) changes in capsule structure, (2) changes in capsule size, and (3) changes in the total size of the cell. These variations have profound consequences on the interaction with the host, involving survival, phagocytosis escape and immune evasion and dissemination.

Changes in cell size can be achieved by the formation of cryptococcal giant/titan cells or microforms. Giant/titan cells, which are most common during chronic infection, help *C. neoformans* evade the host immune system, since they avoid macrophage phagocytosis (Okagaki et al., 2010; Okagaki and Nielsen, 2012). In contrast, in recent review, Zaragoza hypothesized that micro cells could have a particular ability to disseminate and cross biological

barriers, such as endothelia and the brain–blood barrier, and in consequence, contribute to the development of cryptococcal meningitis (Zaragoza, 2011). Changes in capsular structures mainly occur during the crossing of the brain–blood barrier, suggesting that capsular variations are required for dissemination and organ colonization (Garcia-Hermoso et al., 2004; Charlier et al., 2005). The last variation in cell morphology described by Zaragoza (2011) is the change in capsule size. It is well known that the capsule is an important *C. neoformans* virulence factor, indispensable for the establishment of cryptococcosis, however, in the environment, before inhalation, the fungus capsule is still small, but significantly increases in size after a few hours of post-infection (Feldmesser et al., 2001). This observation led us to hypothesize that in the first moments of the interaction between *C. neoformans* and alveolar epithelia, just after the inhalation of poorly encapsulated yeasts, the fungus can utilize other molecules besides GXM to adhere to host cells, due to the low amount of this polysaccharide in the thin capsule. Since there are big variations in morphology of *C. neoformans* cells, both highly capsulated and poorly capsulated cells have the ability to interact with host cells during infection. Here, we demonstrated, for the first time, the involvement of MPs in the interaction of *C. neoformans* to epithelial lung cells.

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Lipopolysaccharides in diazotrophic bacteria

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Biological nitrogen fixation (BNF) is a process in which the atmospheric nitrogen (N_2) is transformed into ammonia (NH_3) by a select group of nitrogen-fixing organisms, or diazotrophic bacteria. In order to furnish the biologically useful nitrogen to plants, these bacteria must be in constant molecular communication with their host plants. Some of these molecular plant-microbe interactions are very specific, resulting in a symbiotic relationship between the diazotroph and the host. Others are found between associative diazotrophs and plants, resulting in plant infection and colonization of internal tissues. Independent of the type of ecological interaction, glycans, and glycoconjugates produced by these bacteria play an important role in the molecular communication prior and during colonization. Even though exopolysaccharides (EPS) and lipochitooligosaccharides (LCO) produced by diazotrophic bacteria and released onto the environment have their importance in the microbe-plant interaction, it is the lipopolysaccharides (LPS), anchored on the external membrane of these bacteria, that mediates the direct contact of the diazotroph with the host cells. These molecules are extremely variable among the several species of nitrogen fixing-bacteria, and there are evidences of the mechanisms of infection being closely related to their structure.

Keywords: lipopolysaccharide, plant-bacterium interaction, nitrogen-fixation, associative diazotrophs, nodulating diazotrophs

INTRODUCTION

With the exception of water, nitrogen is the most limiting compound for plant growth and production. Despite being found in abundance in the Earth's atmosphere as molecular dinitrogen (N_2), it is unavailable to plants which can only use reduced forms of this element, such as ammonia (NH_3). A very specialized group of prokaryotes, named diazotrophs, are able to carry out the conversion of gaseous N_2 into ammonia in a process known as biological nitrogen fixation (BNF), discovered by Martinus Beijerinck in 1901. The BNF process had a major breakthrough in the early 1970's during the oil crisis, when the price of petroleum rose vertiginously, thus affecting the prices of production and transportation of chemical fertilizers. With the aid of BNF plants can readily assimilate NH_3 to produce important biomolecules such as proteins, nucleic acids, ATP, chlorophyll, among others. Diazotrophic microorganisms include aquatic cyanobacteria and free-living bacteria in soil, but a variety of these prokaryotes form associative relationships with plants, and most interestingly, a few have developed an interdependent symbiosis with their hosts, especially legumes, in which specialized structures (nodules) where BNF takes place are formed in the roots. The infection process in which soil bacteria interact with their plant hosts is very complex and yet not fully understood. In the case of nodulating diazotrophs, it is known that exopolysaccharides (EPS) and lipochitooligosaccharides (LCO) that are released in the surrounding microbe environment have paramount importance in all different stages of infection, as well as on the stimulation of cell division in the plant causing the nodule to form in legumes. In this mini-review, however, the focus is on the role of lipopolysaccharides (LPS) during the diazotroph-plant interaction, since these glycoconjugates are present on the outer membrane of these microorganisms and create an intimate "face-to-face" interaction between plant root-cells and nitrogen-fixing bacteria. Knowledge gained in the understanding of the molecular basis for these interactions may lead to improving the yield of economically important crops, as well as diminish the impact of chemical fertilizers on the environment by using nitrogen provided by BNF.

GENERAL STRUCTURE OF LIPOPOLYSACCHARIDES

Most Gram-negative bacteria possess LPS as the major component of the outer membrane. Typically, LPS consist of an oligo- or polysaccharide portion, respectively the core and the O-antigen moiety, anchored in the outer leaflet of the bacterium external membrane by a hydrophobic moiety named lipid-A. The latter is structurally conserved among different classes of bacteria, being formed by two units of 2-amino-2-deoxy-D-glucose (GlcN) linked by a β-(1→6) glycosidic bond and phosphorylated at positions 1 and 4′ (Zähringer et al., 1999; Trent, 2004; Wang and Quinn, 2010). Long-chain acyl groups are found either esterifying free hydroxyl groups or N-linked as amide-type substitutions on C2 of both GlcN units (Trent et al., 2006; Raetz et al., 2007). The oligosaccharide core is usually bound to the lipid-A by a Kdo unit (3-deoxy-D-manno-octulosonic acid) linked at C6 of one of the GlcN units (Raetz and Whitfield, 2002). The core varies in monosaccharide composition, but the presence of Kdo (or its derivative, Ko—D-glycero-D-talo-octulosonic acid) is almost mandatory. Some species contain D-glycero-D-mannoheptose

(D,D-Hep) alone, or in combination with the most commonly found L,D-Hep, while others may have a diversity of hexopyranoses and aminosugars (Zähringer et al., 1999; Holst, 2011). Within a genus or family the structure of the inner core tends to be conserved, and the fact that distantly related bacteria share structural features is a reflection of the importance of the core in outer membrane integrity (Raetz and Whitfield, 2002). The outermost part of the LPS, the polysaccharide chain or O-antigen, lies in the interface between the bacterium and its surrounding environment, and is where the most structural heterogeneity is found. The enormous structural diversity of O-antigens lies on monosaccharide composition, glycosidic linkage position, size of repeating unit, and chain length, as well as on non-carbohydrate substitutions that may occur (Lerouge and Vanderleyden, 2001; Raetz and Whitfield, 2002). O-antigen modifications seem to play and important role at several stages of the infection process during plant-microbe interactions, including adherence, bypassing or overcoming host defenses, and establishing and maintaining intercellular communication (Knirel, 2011). **Figure 1** shows a schematic model of the general structure found for LPS in Gram-negative bacteria. The complexity of LPS reflects the difficulties encountered to determine their fine structures. In many cases, only the structure of the predominant polysaccharide backbone is known. LPS extraction from bacterial cultures may also be affected by culture age and growth condition. In the case of plant-associated bacteria, culture conditions may be inadequate in order to observe the LPS present during interaction.

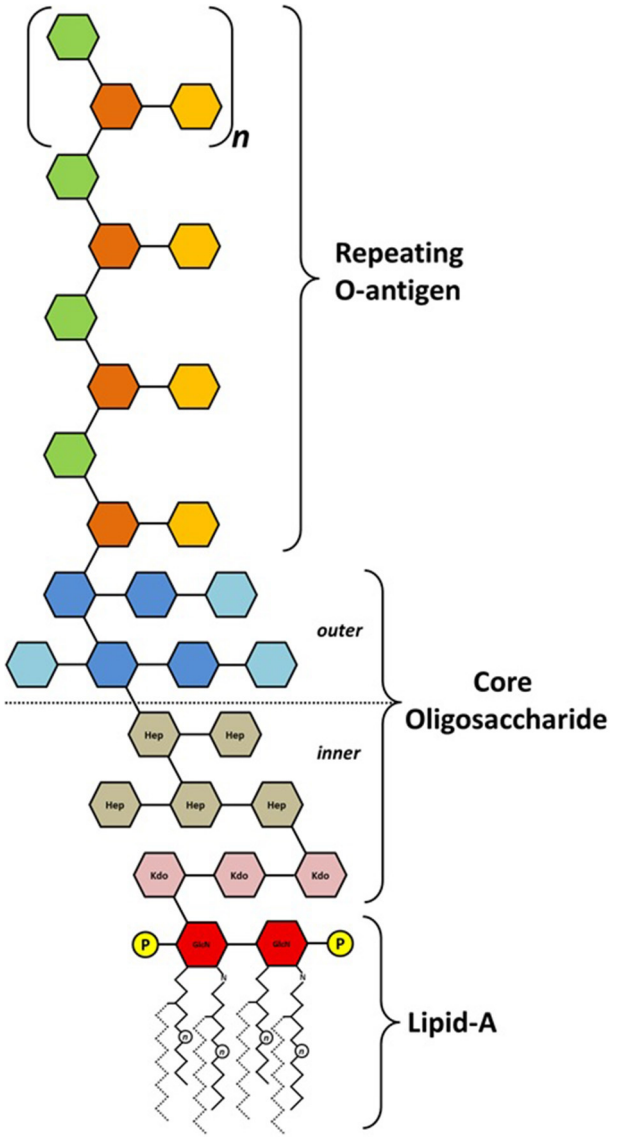

FIGURE 1 | Schematic representation of the general structure found on lipopolysaccharides. Fatty acid chain length (*n*) and position may vary greatly among different species (secondary fatty acids shown as dotted lines). Phosphate substitutions (P) are commonly found at C1 and C4′ of both GlcN (2-amino-2-deoxy-D-glucose) units that form the lipid-A moiety. Phosphate substitutions may also be found attached to core or O-antigen units. Kdo (3-deoxy-D-manno-octulosonic acid) and Hep (D-glycero-D-mannoheptose) are most commonly found on the inner core structure, but other monosaccharides may occur.

LIPOPOLYSACCHARIDES IN RHIZOBIACEAE

Among all diazotrophic bacteria, those belonging to the family Rhizobiaceae have certainly the greater number of species studied in regards to their LPS. Extensive work has been done on structural characterization, biosynthesis and involvement of LPS during Rhizobia-legume interaction (Carlson et al., 1999, 2010; Price, 1999; Noel and Duelli, 2000; Fraysse et al., 2003; Kesawat et al., 2009). Lipid and monosaccharide composition in LPS found for Rhizobiaceae vary considerably, but the basic architecture for this molecule is conserved (Kannenberg et al., 1998). The LPS produced by *Rhizobium etli*, strain CE3, and *R. leguminosarum* have the same basic lipid-A backbone. Instead of the typical GlcN disaccharide, both structures are formed by a trisaccharide containing GlcN, GalA, and GlcNate (gluconate) (1:1:1) (Carlson et al., 1999) (**Figure 2A**). In this case, the phosphate in position 4′ is replaced by a galacturonic acid unit, and both GlcN and GlcNate are N-acylated at C2 and O-acylated at C3 by β-hydroxy-fatty acids of different chain length (Bhat et al., 1994). Most lipid-A structures found in Rhizobiaceae, including *R. etli*, have very-long-chain fatty acids such as 27-hydroxyoctacosanoic acid (27-OH-$C_{28:0}$) (Hollingsworth and Carlson, 1989; Kannenberg et al., 1998) (**Figure 2B**). The inner core of *R. etli* CE3 is formed by a complex highly-branched octasaccharide containing Kdo, Gal, GalA, and Man, while the outer core that binds the O-antigen has Fuc, Man, and QuiNAc (N-acetyl-quinovosamine) (Forsberg and Carlson, 1998). Despite the structural variations found in the O-antigen, the presence of deoxy-hexoses, methylated hexoses, 6-deoxy-amino-sugars, and N-methyl-6-deoxysugars is common together with the presence of acetyl substituents in the structure (Schnaitman and Klena, 1993). The O-antigen of the LPS described for *R. etli* CE3 has a trisaccharide repeating unit on its terminal portion formed by GlcA*p*, Fuc*p*, and 3Me-6dTal*p* (3-methyl-6-deoxy-talose). A cap unit of 2,3,4-tri-O-metyl-fucose is also found as non-reducing terminal (Bhat and Carlson, 1992; Forsberg and Carlson, 1998; Forsberg et al., 2000).

The nodulation process during symbiosis of rhizobia with legumes seems to be affected by the presence of truncated LPS or by the complete lack of these molecules (Carlson et al., 1995).

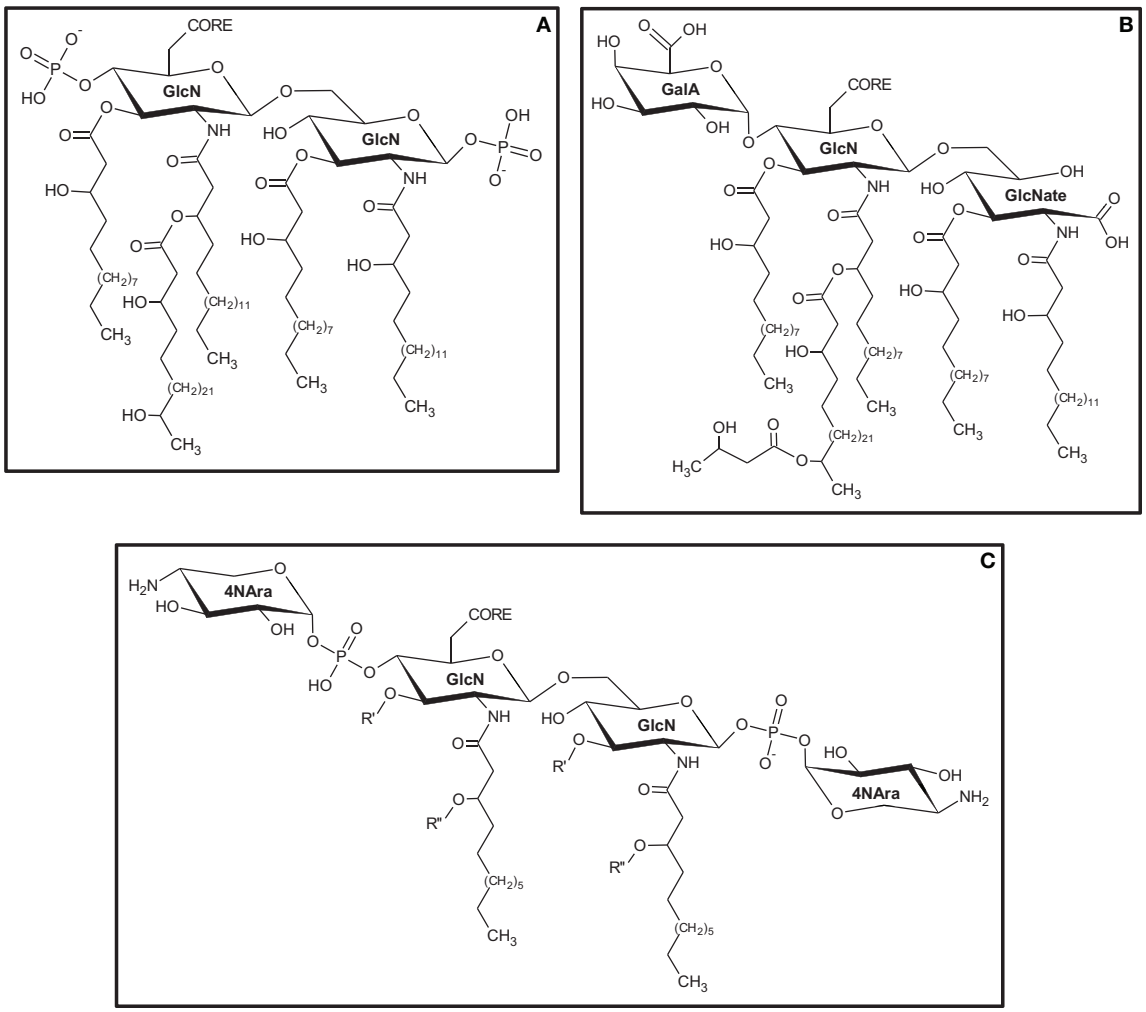

FIGURE 2 | Structural variations found on the lipid-A portion of LPS produced by diazotrophic bacteria. (A) *Sinorhizobium meliloti*; **(B)** *Rhizobium etli* CE3; **(C)** *Herbaspirillum seropedicae* SMR1. Primary (R′) and secondary (R″) ester-linked fatty acids were not determined for *H. seropedicae* SMR1. GlcN, 2-amino-2-deoxy-D-glucose; GalA, Galacturonic Acid; GlcNate, N-acetyl-Gluconate; 4NAra, 4-deoxy-4-amino-Arabinose.

Genes related to LPS expression and biosynthesis are modulated during symbiosis, and LPS structures are modified during the transition of free-living cells to nodule bacteroids (Broughton et al., 2006). These changes may be induced by plant extracts, and especially by flavonoids (Duelli and Noel, 1997). Mutants of rhizobia deficient in LPS biosynthesis remain on the infection thread during the early stages of nodulogenesis and are unable to complete cellular differentiation into mature nitrogen-fixing bacteroids (Noel et al., 1986; Campbell et al., 2002; Broughton et al., 2006). Mutants of *R. etli* that produce truncated LPS structures have promoted the growth of deformed nodules without the ability to fix nitrogen (Noel and Duelli, 2000). It has been proposed that LPS in rhizobia are not involved in the early stages of symbiosis (attachment, root hair curling and infection thread development), but have a central role in maintaining viable differentiated cells once de nodules are formed (Kannenberg et al., 1998; Noel et al., 2000). Furthermore, bacteroids of *Rhizobium leguminosarum* found inside the nodules of some legumes show drastic alterations in their LPS structures in comparison to the structures found for the non-differentiated cells (Goosen-Deroo et al., 1991; Kannenberg and Brewin, 1994). Bacteroids of *R. etli* and *Sinorhizobium meliloti* found in nodules of their respective plant hosts have structural differences in the O-antigen of their LPS structures similar to those found when these bacteria are cultivated in low levels of oxygen and low pH, indicating that changes in LPS structure may be due to physiological conditions to which they are exposed (Tao et al., 1992; Kannenberg et al., 1998; Reuhs et al., 1999). These data indicate that the degree of structural alterations on rhizobial LPS influence the chances of bacteroid survival and guarantees the development of an adequate nitrogen-fixing nodule on the plant host (Carlson et al., 1995).

LIPOPOLYSACCHARIDES IN ASSOCIATIVE AND ENDOPHYTIC DIAZOTROPHS

Other than nodulating rhizobia, diazotrophic bacteria are also found associated with roots and rizosphere, and even inside plant

tissues. This now well-known class of nitrogen-fixing bacteria, capable of establishing endophytic associations with economically important cereals and forage grasses, such as wheat, rice, sugarcane, and maize, has been investigated in recent years with regards to their LPS structures and function during the infection process. To what concerns the structure of LPS during plant-bacterium interactions, some reports have shown that different portions of these molecules may be involved in different stages of the infection process. In *Pseudomonas syringae*, the loss or alteration of the O-antigen structure is related to an impaired virulence (Smith et al., 1994). Some works have reported the role of LPS in the adhesion process of *Agrobacterium tumefaciens* to their host cells (Pueppke, 1984; Matthysse, 1986). Mutants of this bacterium that produce LPS with an altered core structure but that maintain a non-defective o-antigen are still able to attach normally to carrot root cells (Metts et al., 1991), showing that the total structure of the LPS is not necessary to the process. The LPS produced by several strains of *Herbaspirillum* was analyzed by Serrato and coworkers (Serrato et al., 2010) showing that the LPS produced by *H. seropedicae* SmR1 was different in monosaccharide and fatty acid composition when compared to other strains. Later, the structure of the lipid-A portion of the LPS isolated from strain SmR1 was determined as having a typical β-(1→6)-linked GlcN disaccharide backbone, both units phosphorylated and decorated with units of 4-deoxy-4-amino-arabinose (4NAra) (Serrato et al., 2012) (**Figure 2C**). Alterations in the structure of the LPS where observed when genes related to the biosynthesis of rhamnose where knocked out in *H. seropedicae*. The LPS of both *rfbB*$^{-}$ and *rfbC*$^{-}$ mutants lack the entire o-antigen portion and both 4NAra units in the lipid-A. The main effect observed for both mutants is the impaired ability to colonize internal tissues of maize root (Balsanelli et al., 2010). More recently, it has been proposed that N-acetyl-glucosamine (GlcNAc) units found in the o-antigen of *H. seropedicae* LPS structure are responsible for mediating the interaction with lectins found in the root cells (Balsanelli et al., 2013).

A number of other associative nitrogen-fixing bacteria have been studied with regards to their LPS. In *Azospirillum brasilense*, the structure of the o-antigen is linear rhamnan where every unit is found as D-Rha (Fedonenko et al., 2002). Immunochemical and structural characteristics of the LPS of *A. brasilense* are also reported (Konnova et al., 2006). Another species, *A. lipoferum*, has an o-antigen backbone of α-L-Rha with a branching β-D-Glc unit (Fedonenko et al., 2008), while strain Sp59b presents a very distinct structure formed by a backbone of α/β-D-Gal*p*, branched by a tetrasaccharide containing α-L-Rha*p* and β-D-Man*p* (3:1) (Fedonenko et al., 2005). The lipid-A portion of *A. lipoferum* was described to have two β-(1→6)-linked D-GlcN units but lacks phosphate residues. Moreover, the reducing end of the backbone is found α-linked with a D-galacturonic acid unit (Choma and Komaiecka, 2008). Recent findings on the structure of o-antigen from strain SR80 of *A. brasilense* have shown that two distinct oligosaccharide repeating units are found, a trisaccharide containing D-Rha, L-Fuc, and D-Xyl (1:1:1 molar ratio respectively), and a tetrasaccharide containing D-GalNAc, L-Fuc and D-Gal (1:1:2) (Sigida et al., 2013a). Structural variations for the LPS of other strains of *A. brasilense* include the presence of 3-O-methyl-D-rhamnose units (strain Jm6B2) (Boyko et al., 2012) and 2-O-methyl-D-rhamnose (Strain Sp7) (Sigida et al., 2013b). The importance of LPS in the *Azospirillum*-plant association has been reported (Skvortsov and Ignatov, 1998; Bashan et al., 2004), but the actual role of LPS in molecular communication is yet to be understood.

A comparative analysis performed in six different strains of *Gluconacetobacter diazotrophicus* has shown a great structural variability within this species (Fontaine et al., 1995). However, the structure described for the O-antigens of *G. diazotrophicus* is similar to that previously described for some other alpha-proteobacteria, except for the presence of 2-O-substituted ribofuranose units (Previato et al., 1997). Diazotrophs of the beta-proteobacterium class have shown very distinct structures, in many cases rare and uncommon monosaccharide units are found. The presence of 3,6-dideoxy-4-C-(4′-hydroxyethyl)-D-xyloheptose, or yersiniose (YerA), has been described in the structure of the EPS produced by *Burkholderia brasiliensis* (Mattos et al., 2005). The O-antigen of *Ralstonia picketti* has shown to have units of BacNAc (4-acetamide-2-amino-2,4,6-trideoxy-D-glucose) in its structure (Vinogradov et al., 2004).

CONCLUSIONS

BNF performed by diazotrophic bacteria has been extensively studied over the past decades, as have the symbiotic and associative processes that allow these microorganisms to invade plant tissues and deliver ammonia together with other growth-promoting substances. Even though the role of glycans and glycoconjugates, such as LPS, have been determined for some species during the infection and colonization process with their plant hosts, there are several gaps in the process that are poorly understood and require more investigation. The recent availability of numerous nitrogen-fixing bacteria genome sequences, allied to the chemical, and structural characterization of LPS, offer the tools to determine the functional aspects that these molecules play during the plant-diazotroph molecular interaction.

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Glycosaminoglycans analogs from marine invertebrates: structure, biological effects, and potential as new therapeutics

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In this review, several glycosaminoglycan analogs obtained from different marine invertebrate are reported. The structure, biological activity and mechanism of action of these unique molecules are detailed reviewed and exemplified by experiments *in vitro* and *in vivo*. Among the glycans studied are low-sulfated heparin-like polymers from ascidians, containing significant anticoagulant activity and no bleeding effect; dermatan sulfates with significant neurite outgrowth promoting activity and anti-P-selectin from ascidians, and a unique fucosylated chondroitin sulfate from sea cucumbers, possessing anticoagulant activity after oral administration and high anti P- and L-selectin activities. The therapeutic value and safety of these invertebrate glycans have been extensively proved by several experimental animal models of diseases, including thrombosis, inflammation and metastasis. These invertebrate glycans can be obtained in high concentrations from marine organisms that have been used as a food source for decades, and usually obtained from marine farms in sufficient quantities to be used as starting material for new therapeutics.

Keywords: glycosaminoglycans, heparin, dermatan sulfate, selectins, marine invertebrates

INTRODUCTION

Heparin is a highly sulfated glycosaminoglycan composed by disaccharide units containing a hexuronic acid (α-L-iduronic acid or β-D-glucuronic acid) linked 1,4 to α-D-glucosamine. The heparin molecules consist of a heterogeneous mixture of polymers with a similar backbone, which results from variations of sulfation on the D-glucosamine (N-acethylated, N-sulfated, O-sulfated at C6 and/or C3) and on the uronic acid residue (O-sulfated at C2) (Lindahl et al., 1989).

Because of its unique binding to antithrombin, involving a specific pentasaccharide sequence containing a 3-O-sulfated glucosamine, heparin is endowed of a potent anticoagulant activity (Lindahl et al., 1980, 1989). In fact, based on its ability to inhibit fluid phase coagulation, unfractionated heparin (UF) isolated from porcine and bovine intestinal mucosa has been used clinically for decades. However, the therapeutic use of UH is limited mostly by its potent hemorrhagic effect, implying that patients under heparin therapy have to be closely monitored (Hirsh, 1984). UH also has poor bioavailability, requires multiple daily dosing, and has side effects such as heparin-induced thrombocytopenia (Hirsh and Raschke, 2004; Hirsh et al., 2004). To circumvent these problems, different low-molecular-weight heparins (LMW-Hep) have been produced by degrading UF, using a variety of methods, including chemical depolymerization and enzymatic digestion (Hirsh and Raschke, 2004).

In addition to its anticoagulant effect, mammalian UH has also anti-inflammatory effect in several animal models of inflammation, which is possibly mediated by P- and L-selectins. The recruitment of leukocytes from blood and lymphatic systems into tissues facilitates the response to tissue injury. Adhesion molecules of the selectin family (E, P, and L) mediate the initial events that direct the movement of leukocytes across the endothelium in inflamed tissues by interacting with sialylated, fucosylated carbohydrate antigens related to sialyl Lewis^x [Sle^x, Neu5Acα2,3Galβ1,4(Fucα1,3)GlcNAcβ-] found at the cell surface (Lasky, 1995; McEver, 1995; Nelson et al., 1995; Butcher and Picker, 1996). It has been reported (Stevenson et al., 2005) that the dose of UH required for the inhibitory effect on the selectins is higher than that required for the anticoagulant action, which increases the hemorrhagic risk and makes the clinical use of UH impractical to treat inflammation. Similarly, the use of LMW-Hep, which has a much lower hemorrhagic effect, is not

Abbreviations: HexA, hexuronic acid; GlcA, glucuronic acid; IdoA, iduronic acid; GalNAc, N-acetyl galactosamine; GlcNAc, N-acetyl glucosamine; GlcA2S, 2-O-sulfated glucuronic acid; GlcA3S, 3-O-sulfated glucuronic acid; IdoA2S, 2-O-sulfated iduronic acid; GalNAc4S, 4-O-sulfated N-acetyl galactosamine; GalNAc6S, 6-O-sulfated N-acetyl galactosamine; GalNAc4S,6S, 4-O- and 6-Osulfated N-acetyl galactosamine; GlcNS, N-sulfated glucosamine; GlcNS,6S, N- and 6-sulfated glucosamine; GlcNS,3S,6S, N, 3-O- and 6-Osulfated glucosamine.

a good alternative for UH, since it is a poor inhibitor of selectins (Stevenson et al., 2005).

UF and heparin-like oligosaccharides inhibit L- and P-selectin binding to Sle^x and has been shown to dramatically reduce leukocyte infiltration in thioglycollate-induced peritoneal inflammation in mice (Burg et al., 1997; Wang et al., 2002). In addition, UH has also been used successfully as a therapeutic agent in different animal models of nephropathy. Subcutaneous injection of non-anticoagulant UH reduces glomerulosclerosis in rats (Burg et al., 1997), and ameliorates the progression of renal disease in rats with subtotal renal ablation (Purkerson et al., 1988). In addition, it has also been demonstrated that heparin inhibits macrophage infiltration and TGF-β synthesis in puromycin glomerulosclerosis (Ceol et al., 2003).

The risk of contamination with pathogens is an important aspect to take into account in the therapeutic use of natural products from mammalian origin. For example, the association of mammalian prionic proteins with transmissible spongiform encephalopathy has restricted the use of bovine heparin in Europe, USA and Japan. In these countries, commercial heparin is obtained exclusively from porcine tissues and the risk of contamination with a prionic protein or even a virus is still present.

Therefore, as we consider the molecular mechanism of the anti-inflammatory effect of UF, its side effects, and the possibility of pathogen contamination, it becomes extremely relevant the search for alternative heparin-like compounds, obtained from non-mammalian sources, possessing similar biological activities, but devoid of the undesired side effects.

UNIQUE INVERTEBRATE GLYCOSAMINOGLYCANS

HEPARIN-LIKE GLYCANS

Heparin with a structure similar to that of vertebrate heparin but with lower anticoagulant activity has been identified in the tissues of the ascidian *Styela plicata* (Chordata-Tunicata) (Cavalcante et al., 2000). An extensive structural analysis of the polymer indicated that the invertebrate heparin is composed mainly of the disaccharide α-L-IdoA(2SO_4)-1→4β-D-GlcN(SO_4)(6SO_4)-1, with a minor contribution (∼25%) of the disaccharide α-L-IdoA-1→4β-D-GlcN(SO_4)(6SO_4)-1. Activated partial thromboplastin time (APTT) assays of the ascidian heparin showed that the polymer has lower anticoagulant activity than mammalian heparin. In addition, it is about 20 times less potent than mammalian heparin in stimulating the inhibition of thrombin by purified ATIII. However, *S. plicata* heparin activates HCII with approximately the same potency as vertebrate heparin, as indicated by comparison of the IC_{50} values for inhibition of thrombin by purified HCII. To compare the hemorrhagic effect of the ascidian and mammalian heparin, we used a rat cut-tail bleeding model. *S. plicata* heparin at a dose of 4 mg/kg body weight, which is above the dose required to prevent thrombus on an animal experimental model did not increase the amount of blood loss in comparison with the saline control. In contrast, mammalian heparin, compared to the control, increased blood loss almost 2-fold (Cardilo-Reis et al., 2006).

In a TNBS-induced colitis model in rats, *S. plicata* heparin drastically reduced inflammation after subcutaneous administration during a 7-day period (Belmiro et al., 2009), as observed by the normalization of the macroscopic and histological characteristics of the colon. At molecular level, TNF-alpha, TGF-beta, and VEGF were reduced to normal values. Lymphocyte and macrophage recruitment and epithelial cell apoptosis were also decreased after the treatment. A drastic reduction in collagen-mediated fibrosis was also observed. No hemorrhagic events were observed after glycan treatment (Belmiro et al., 2009). These results strongly indicate a potential therapeutic use of the ascidian heparin in the treatment of colonic inflammation with a lower risk of hemorrhage, when compared with mammalian heparin.

The bivalve mollusk *Nodipecten nodosus*, currently cultivated in different parts of the world with an annual production of about 75,000 tons (http://www.fao.org/fishery/topic/14884/en), has been shown to contain high amounts of a heparan sulfate-like glycosaminoglycan. 1D ^1H, 2D COZY and HSQC nuclear magnetic resonance revealed characteristic signals of non-sulfated, 3- or 2-sulfated glucuronic acid, as well as *N*-sulfated and/or 6-sulfated glucosamine (Gomes et al., 2010). The mollusk glycan possesses an anticoagulant activity of 36 IU mg^{-1}, 5-fold lower than bovine heparin (180 IU mg^{-1}). It inhibits factor Xa (IC_{50} = 0.835 μg mL^{-1}) and thrombin (IC_{50} = 9.3 μg mL^{-1}) in the presence of antithrombin. Experiments *in vivo*, demonstrated that, the mollusk HS inhibited thrombus growth in photochemicaly injured arteries, at the dose of 1 mg Kg^{-1}. No bleeding effect, factor XIIa-mediated kallikrein activity or toxic effect on fibroblast cells were induced by the invertebrate HS at the antithrombotic dose (Gomes et al., 2010).

OVERSULFATED DERMATANS

The ascidians *S. plicata* and *Halocyntia pyriformis*, contain dermatan sulfates formed by [α-L-IdoA(2SO_4)-1→3β-D-GalNAc(4SO_4)] disaccharide units. These oversulfated dermatans have high heparin cofactor II-mediated anticoagulant activity (Pavao et al., 1998). Due to a higher concentration of [α-L-IdoA(2SO_4)-1→3β-D-GalNAc(4SO_4)]-containing sequences, that bind to the glycosaminoglycan binding site in the inhibitor, their heparin cofactor II activities are at least 10 times higher than that of the mammalian counterpart.

The occurrence of a well-defined relationship between sulfate position on the disaccharides and biological activity can be observed studying dermatan sulfates from different species of ascidians. In the ascidian *Phallusia nigra*, the dermatan sulfate has the same degree of sulfation of that from *S. plicata*, but is composed mainly by [α-L-IdoA(2SO_4)-1→3β-D-GalNAc(6SO_4)] disaccharide units (Pavao et al., 1995). As a result of the different sulfation on the N-acetylgalactosamine (6-sulfated instead of 4-sulfated), *P. nigra* dermatan sulfate has very low heparin cofactor II-mediated anticoagulant activity. Overall, these results strongly suggest that binding of oversulfated dermatan sulfate polymers to heparin cofactor II requires a specific sulfation pattern on the glycans, composed by [α-L-IdoA(2SO_4)-1→3β-D-GalNAc(4SO_4)]-enriched sequences.

These unique oversulfated dermatan sulfates from ascidians, with different heparin cofactor II activities, allowed the study of the relationship between heparin cofactor II activity and antithrombotic effect. Thus, after intravascular administration *S. plicata* dermatan sulfate, with high heparin cofactor II activity,

prevents thrombus formation in veins (Vicente et al., 2001). On the other hand, *P. nigra* dermatan sulfate, with a discernible heparin cofactor II activity, has no antithrombotic effect in the same venous thrombotic model and dose (Vicente et al., 2001). These results indicate that a heparin cofactor II-mediated mechanism is associated with the antithrombotic effect of dermatan sulfate polymers.

The oversulfated dermatan sulfates from the ascidian *P. nigra* and *S. plicata*, composed by [α-L-IdoA($2SO_4$)-1→3β-D-GalNAc($6SO_4$)] and [α-L-IdoA($2SO_4$)-1→3β-D-GalNAc($4SO_4$)] disaccharide unities were used in the study of mouse hippocampal neurons behavior. *P. nigra* dermatan possesses significant neurite outgrowth-promoting activity, which resulted specific morphological features. The ascidian dermatan sulfate induced a flattened neuronal cell soma and dendrite-like multiple neurites (Hikino et al., 2003). *S. plicata* dermatan sulfate, composed by [α-L-IdoA($2SO_4$)-1→3β-D-GalNAc($4SO_4$)], on the other hand, exhibited only a modest neurite outgrowth-promoting activity (Hikino et al., 2003; Bao et al., 2005).

Heparin has been shown to exhibit P- and/or L-selectin-mediated antimetastatic and antiinflammatory activities. P-selectin-mediated platelet-tumor cell and tumor-cell endothelium interactions facilitate the initial steps of hematogeneous metastasis (Borsig et al., 2001, 2002). The dermatan sulfates from *S. plicata* and *P. nigra*, that contain the same disaccharide core structure [α-L-IdoA($2SO_4$)-1→3β-D-GalNAc]$_n$, but sulfated at carbon 4 or 6 of the GalNAc residues, respectively, and opposed HCII activities are potent inhibitors of P-selectin (Kozlowski and Pavao, 2011; Kozlowski et al., 2011). Both ascidian dermatan sulfates regardless of the position of sulfation on the N-acetylgalactosamine drastically attenuate metastasis of both MC-38 colon carcinoma and B16-BL6 melanoma cells, and the infiltration of inflammatory cells in a thioglycollate peritonitis mouse model (Kozlowski and Pavao, 2011; Kozlowski et al., 2011). Additionally, both ascidians glycosaminoglycans reduced thrombus size in a $FeCl_3$-induced arterial thrombosis model, irrespective of their HCII activities. Interestingly, the arterial thrombi demonstrated a markedly reduced platelet deposition after dermatan sulfate treatment (Kozlowski and Pavao, 2011; Kozlowski et al., 2011), suggesting that the ascidians glycosaminoglycan inhibited P-selectin and thereby the binding of activated platelets during thrombus formation. These results provide evidence that inhibition of P-selectin is a potential therapeutic target in thrombosis, inflammation and metastasis, and ascidian dermatan sulfates may serve as anti-selectin agents.

FUCOSYLATED CHONDROTITIN SULFATE

A unique natural chondroitin sulfate analog, containing sulfated fucose branches has been identified in several species of sea cucumbers (Vieira and Mourao, 1988; Vieira et al., 1991; Kariya et al., 1997). The *Ludwigothurea grisea* glycan has a core like that of mammalian chondroitin sulfate but substituted at the 3-position of the glucuronic acid residues with fucose-2,4 disulfated branches (Vieira and Mourao, 1988; Vieira et al., 1991). The fucosylated chondroitin sulfate has high anticoagulant and antithrombotic activities that disappear after removal of the sulfated fucose branches by mild acid hydrolysis (Mourao et al., 1996). Two anticoagulant mechanism have been proposed: activation of thrombin inhibition by heparin cofactor II, and inhibition of factor-Xa and thrombin generation by the tenase and prothrombinase complexes, respectively (Glauser et al., 2008; Buyue and Sheehan, 2009).

Interestingly, thrombosis inhibition in artery by the fucosylated chondroitin sulfate occurs at low doses, and does not modify the plasma anticoagulant activity. On the contrary, in venous thrombosis the antithrombotic activity of the fucosylated chondroitin requires high doses and occurs with an increase in the plasma anticoagulant activity (Zancan and Mourao, 2004). Additionally, daily oral doses of this glycosaminoglycan showed a decrease in thrombus weight on experimental models of venous and arterial thrombosis in experimental animals (Fonseca and Mourao, 2006).

The fucosylated chondroitin sulfate from *H. grisea* inhibited P- and L-selectin binding to immobilized sialyl Lewis^x, a component of leukocyte surface glycoproteins, which is also overexpressed in several tumor cells (Borsig et al., 2007). The glycan also inhibited LS180 carcinoma cell attachment to immobilized P- and L-selectins (Borsig et al., 2007). As a result of its anti-selectin effect, the intact sea cucumber glycan attenuates lung colonization by adenocarcinoma MC-38 cells in an experimental metastasis model in mice, as well as neutrophil recruitment in thioglycollate-induced peritonitis. Removal of the sulfated fucose branches abolishes the inhibitory effect *in vitro* and *in vivo* (Borsig et al., 2007). These results suggest that this glycan may be a potential therapeutic drug for blocking metastasis and inflammatory reactions.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Glycosaminoglycans analogs with unique structures and pharmacological activities have been described in different marine invertebrates (**Table 1**). The structure, biological activity and mechanism of action have been extensively studied and the glycans evaluated in pre-clinical experiments in rodent animals with promising results. Heparin-like polymers with low anticoagulant activity, significant antithrombotic and anti-inflammatory activities, but devoid of bleeding effects occur in different species of ascidians and mollusks. Similarly, oversulfated dermatan sulfates containing the same disaccharide core structure of [α-L-IdoA($2SO_4$)-1→3β-D-GalNAc]$_n$, but differing in the position of sulfation (4-sulfate or 6-sulfate) on the N-acetylgalactosamine, occur in high amounts in ascidians. These polymers have high anti-selectin activity, which results in attenuation of metastasis and leukocyte recruitment. The 2,6-sulfated dermatan sulfate from *P. nigra* has very low heparin cofactor II activity and antithrombotic effect, different from its 2,4-sulfated high anticoagulant counterpart from *S. plicata*. The anti-P-selectin activity of these disulfated glycans is involved in low platelet arterial thrombus formation. Moreover a significant neurite outgrowth promoting activity is associated with the di-sulfated ascidian dermatan sulfates. The fucosylated chondroitin sulfate from *L.grisea* has significant therapeutic effect after oral administration, attenuating metastasis and inflammation due to the high anti-selectin activity of the sulfated fucose branches. So far, no significant toxic effect has been associated with the use of these marine

Table 1 | Effect of invertebrate glycans on coagulation, inflammation, and metastasis.

Glycan	Disaccharide composition	Marine invertebrate source	Anticoagulant properties					Anti P-selectin-mediated events		
			aPTT (U/mg)	IC_{50} for IIa inhibition (μg/ ml)		Effect on thrombosis	Bleeding effect (rat tail)	Inhibition of tumor cell-selectin adhesion (μg/ml)	Inhibition of neutrophil recruit-ment on Tioglycolate-induced peritonitis (%)	Inhibition of carcinoma cell metastasis to the lungs (%)
				Protease inhibitor		Dose for 100% inhibition (mg/kg)				
		Ascidian		**AT**	**HCII**					
Dermatan sulfate	IdoA**2**S-GalNAc**4**S	*S. plicata*	8	ND	0.31	4	Low	13.51***	>90**	>95***
Dermatan sulfate	IdoA**2**S-GalNAc**6**S	*P. nigra*	<0.5	ND	320	>400	Low	12.19***	>90**	>95***
		Bivalve mollusk								
Heparan sulfate-like	**GlcA-GlcNAc** (GlcA**2**S/GlcA**3**S) (Glc**N**S/GlcNAc**6**S)	*N. nodosus*	38.3	0.012	4	1	Low	0.07****	>90*****	~50****
		Sea cucumber								
Fucosylated chondroitin sulfate	(Fuc**2**/**3**S)-GlcA-GalNAc**6**S	*L. grisea*	40	~9[∫]	~1[∫]	3	Low	10.4***	~80*	>95***

*Dose of 0.5 mg/mouse, see Borsig et al. (2007).

**Dose of 0.1 mg/mouse, see Kozlowski et al. (2011).

***Carcinoma MC-38 cell, see Kozlowski et al. (2011).

****Carcinoma LS 180 cell, unpublished results.

*****Dose of 0.5 mg/mouse, unpublished results.

[∫] See reference Mourao et al. (1996).

compounds. The marine invertebrates that are the primary source of these potential therapeutic compounds abound in different parts of the world, mainly in western seas, where they are cultivated in large scale. In general, the marine invertebrate glycans occur in higher concentration in the tissue (about 0.5% of the dry weight, comparing to 0.022% from pig intestinal mucosa) and can be easily isolated by procedures similar to those already employed in the preparation of pharmaceutical heparin. Whereas these marine organisms will be a source of new heparin analogs with significant therapeutic effect in thrombosis, inflammation and cancer in the future will depend on the economic pressure of the pharmaceutical industry and the increasing demand for new natural drugs with less undesired side effects to treat specific diseases.

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Structural diversity and biological significance of glycosphingolipids in pathogenic and opportunistic fungi

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Glycosphingolipids (GSLs) are ubiquitous membrane components and have key roles in biological systems, acting as second messengers or modulators of signal transduction by affecting several events, ranging from cell adhesion, cell growth, cell motility, regulation of apoptosis and cell cycle. Over the last 20 years our laboratory and other research groups determined the glycan and ceramide structures of more than 20 GSLs from several pathogenic/opportunistic fungi, using a combination of gas chromatography, mass spectrometry, nuclear magnetic resonance as well as other immunochemical and biochemical techniques. Fungal GSLs can be divided in two major classes: neutral GSLs, galactosyl- and glucosylceramide (GlcCer), and acidic GSLs, the glycosylinositol-phosphorylceramides (GIPCs). Glycosyl structures in fungal GIPCs exhibited significant structural diversity and distinct composition when compared to mammalian GSLs, e.g., the expression of inositol-mannose and inositol-glucosamine cores and the terminal residue of β-D-galactofuranose which are absent in mammalian cells. Studies performed by our group demonstrated that GIPC (Gal*f*β6[Manα3]Manα2InsPCer) elicited in patients with paracoccidioidomycosis an immune response with production of antibodies directed to the terminal residue of β-D-galactofuranose. Further studies also showed that inhibition of GlcCer biosynthetic pathways affects fungal colony formation, spore germination and hyphal growth, indicating that enzymes involved in GlcCer biosynthesis may represent promising targets for the therapy of fungal infections. Recently, it was shown that GlcCer and GIPCs are preferentially localized in membrane microdomains and monoclonal antibodies directed to these GSLs interfere in several fungal biological processes such as growth and morphological transition. This review focuses on glycan structures carried on sphingolipids of pathogenic/opportunistic fungi, and aspects of their biological significance are discussed.

Keywords: fungal glycosphingolipids, glucosylceramide, glycosylinositol phosphorylceramides, glycosphingolipid synthesis inhibitors, fungal membrane microdomains

INTRODUCTION

Glycosphingolipids (GSLs) are ubiquitous membrane components present mostly in the outer leaflet of the plasma membrane with their carbohydrate head groups exposed to the extracellular space, and mainly organized in microdomains by association with sterols and specific proteins.

The glycosphingolipid biosynthesis starts by the action of serine palmitoyltransferase which condensates palmitoyl-CoA with serine forming 3-keto-sphinganine, the keto group is reduced to hydroxyl group thus forming the dihydrosphingosine (also termed sphinganine), at this point, as shown in **Figure 1**, the dihydrosphingosine may be hydroxylated on the C4 to form the phytosphingosine (4-hydroxysphinganine). Specific acyl transferases act upon dihydrosphingosine and phytosphingosine forming dihydroceramide or phytoceramide, respectively. In dihydroceramides, a desaturase inserts a double bound at C4 converting it in ceramide (Rhome et al., 2007). In fungi Glc or Gal residues are transferred to ceramide in the assembly of GlcCer or GalCer whereas the more complex glycan moieties of GIPCs are built up on phytoceramide (Takahashi et al., 2009). After glycosylation, GSLs are transported to the outer leaflet of the cell membrane forming membrane microdomains, also as described by Longo et al. (2013), GlcCer may also be found in lipid vesicles in the cell wall, probably being part of the exocytosis transport system.

Studies analyzing the roles of glycosphingolipids (GSLs) in different biological systems have demonstrated their association with: (i) oncogenic transformation; (ii) embryonic development; (iii) control of cell division; (iv) cell adhesion and motility; (v) signal transduction pathways by glycosinapses, e.g., carbohydrate-carbohydrate interactions; and (vi) control of cell phenotype (Hakomori, 2004, 2008). Since the first description of GSLs in fungi (Steiner et al., 1969), numerous studies focused in structural elucidation and biological significance of these GSLs

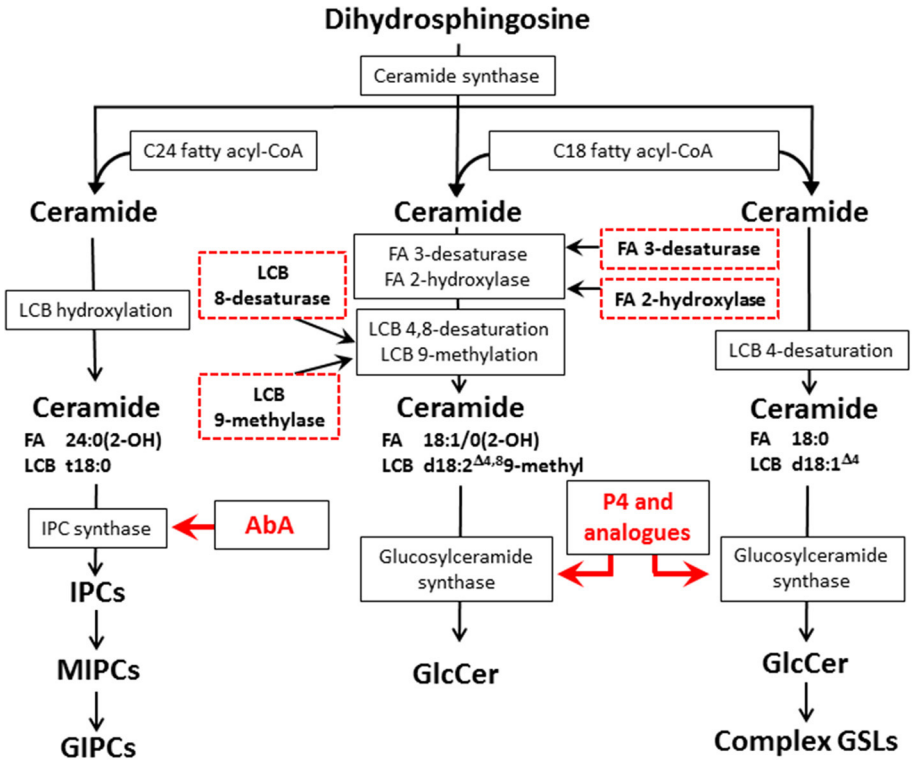

FIGURE 1 | Fungal and mammal GSLs biosynthetic pathways and potential targets for new antifungal therapies. The scheme displays the biosynthetic pathways of fungal GIPCs (left), fungal GlcCer (center), and mammalian GSLs (right). The red arrows indicates the biosynthetic step where the inhibition by AbA and P4/analogs occurs, the red dashed boxes in the glycosphingolipid biosynthetic pathway indicate potential targets for the development of new antifungal therapies. LCB, long chain base; FA, fatty acid; IPC, inositol phosphorylceramide; MIPC, mannosylinositol phosphorylceramide; GIPC, glycosylinositol phosphorylceramide; AbA, Aureobasidin A; P4, D-threo-1-phenyl-2-palmitoyl-3-pyrrolidinopropanol.

revealed the importance of these sphingolipids in normal fungal morphogenesis and infectivity. In the last 25 years our lab has made efforts to isolate and analyze the structure of more than 20 new fungal GSLs. In this review we focus on glycan structures carried on sphingolipids of pathogenic/opportunistic fungi and multiple aspects of their biological significance.

STRUCTURAL ELUCIDATION OF GSLs ISOLATED FROM PATHOGENIC FUNGI

Our laboratory and other research groups have characterized a number of neutral GSLs, glucosyl- and galactosylceramide (GlcCer and GalCer), as well as acidic GSLs—the glycosylinositol-phosphorylceramides (GIPCs) from pathogenic/opportunistic fungi, using a combination of high performance thin layer chromatography (HPTLC), gas chromatography/mass spectrometry (GC/MS), single quadrupole mass spectrometry, $^{1}H/^{13}C$ nuclear magnetic resonance—Correlation Spectroscopy (COSY), Total Correlation Spectroscopy (TOCSY), Nuclear Overhauser Effect Spectroscopy (NOESY) as well as other immunochemical and biochemical techniques.

As shown in **Figure 2** contrasting to mammalian monohexosilceramides, fungal GlcCer and GalCer displays interesting structural peculiarities in their ceramide moiety, such as the presence of (E)-Δ^8-unsaturation and a methyl group linked to sphingosine, i.e., (4E,8E)-9-methyl-4,8-sphingadienine as long chain

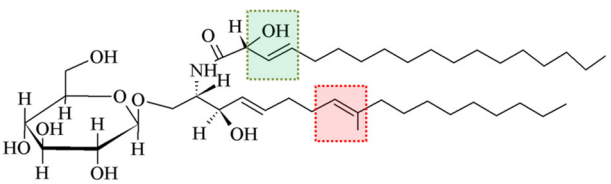

FIGURE 2 | A typical glucosylceramide from fungi. Differently from mammalian GlcCer, fungal GlcCer displays structural peculiarities in their ceramide, such as presence of (E)-Δ^8-unsaturation and a methyl group linked to sphingosine (highlighted by red box). Another feature that is particular to fungal GlcCer is the (E)-Δ^3-unsaturation and the presence of a hydroxyl group linked to C2 of the fatty acid moiety (highlighted by green box) (Toledo et al., 1999).

base (d19:2), In *Cryptococcus neoformans*, the methylation in C9 on the sphingosine backbone of GlcCer is associated with fungi virulence, since the mutation of the sphingolipid C9 methyltransferase gene (SMT1), resulted in loss of more than 80% of its virulence, when compared to the wild-type and/or the reconstituted strains (Singh et al., 2012).

Another feature that is particular to fungal GlcCer/GalCer is the (E)-Δ^3-unsaturation and the presence of a hydroxyl group linked to C2 of the fatty acid moiety, having either N-2′-hydroxyoctadecanoate (h18:0) or the unsaturated form

N-2′-hydroxy-(E)-3′-octadecenoate (h18:1) [(E)-13-unsaturated fatty acid] (Takahashi et al., 2009). GlcCer from mycelium forms of *Paracoccidioides brasiliensis* and *Histoplasma capsulatum* present a higher percentage of unsaturated fatty acids, indicating that the temperature change which induces the transition of mycelium to yeast forms possibly activates a fatty acid desaturase (Toledo et al., 1999, 2001).

Additionally, for yeast forms of *Sporothrix schenckii* it was observed that the expression of both GlcCer and GalCer was approximately equimolar, while mycelial forms displayed only GlcCer. These differences in neutral GSLs expression suggest that the activation of GalCer synthase may accompanies the mycelium to yeast transition, or, conversely, the suppression of this activity may accompany the yeast to mycelium transition in this fungus (Toledo et al., 2000). Concurrently in two non-dimorphic fungi *Aspergillus fumigatus* and *Aspergillus niger*, it was reported the expression of both GlcCer and GalCer, with GalCer bearing a high percentage of unsaturated fatty acid when compared to GlcCer (Villas Boas et al., 1994; Toledo et al., 1999; Levery et al., 2000).

On the other hand, inositol phosphorylceramides (IPCs) and their glycosylated derivatives (GIPCs) are widely distributed among fungal species of the two phyla Ascomycota and Basidiomycota. Fungal GIPCs, display a significant glycosyl structural variation (Barr and Lester, 1984; Barr et al., 1984; Levery et al., 1996, 1998; Loureiro y Penha et al., 2001; Toledo et al., 2007; Suzuki et al., 2008). The glycan structures of fungal GIPCs are built-up on three different "cores": (i) GlcNα1-2Ins; (ii)Manα1-6Ins; and (iii) Manα1-2Ins. The glycosylation of IPCs seems to occur upon a ceramide mainly composed by t18:0 4-hydroxysphinganine (phytosphingosine) and h24:0 fatty acid (Takahashi et al., 2009). It is worth mentioning that the lipid moiety of these GIPCs when compared to glucosyl- and galactosylceramide displayed different sphingosines, with 4,8-diene-9-methyl-sphingo base as precursor for GlcCer/GalCer synthesis, and phytosphingosine for IPC synthesis, suggesting a dichotomy in the biosynthetic pathway of fungal neutral and acidic GSLs (Leipelt et al., 2001). The expression of GIPCs in fungi is summarized in **Figure 3**.

From an evolutionary perspective, an analysis of GSLs in basal lineages of fungi, such as zygomycetes (James et al., 2006; McLaughlin et al., 2009), revealed that this phylum expresses only neutral GSLs, no inositol-containing sphingolipids were detected (Aoki et al., 2004), differently from higher fungi representatives such as Ascomycota and Basidiomycota where both neutral GSLs and GIPCs are expressed.

Thus, considering the structural diversity of fungal GSLs, studies aiming to investigate the structure-function relationship of these glycoconjugates and their phylogenetic distribution in fungi kingdom may open new perspectives allowing to identify specific targets for new generation of antifungal drugs.

INHIBITION OF GSL BIOSYNTHETIC PATHWAYS AS TARGETS FOR NEW ANTIFUNGAL THERAPIES

Hence, in order to better understand the importance/biological role of GSLs in different fungi, combined with studies searching for new alternatives for antifungal therapies, a series of studies were conducted in our laboratory to analyze the inhibition effect of key enzymes involved in biosynthetic pathways of fungal GlcCer and GIPCs.

Studies performed with inhibitors of GlcCer synthase, D-threo-1-phenyl-2-palmitoyl-3-pyrrolidinopropanol (P4) and D-threo-3P,4P-ethylenedioxy-P4 (EDO-P4), showed a strong inhibition of germination and hyphal growth, affecting also fungal colony formation of *A. fumigatus* and *A. nidulans* (Levery et al., 2002). Similar results were observed when *P. brasiliensis*, *H. capsulatum*, *S. schenckii*, and *C. neoformans* were cultivated in the presence of P4 (Takahashi et al., 2009). It is worth mentioning that antimicrobial peptides, such as the plant defensin RsAFP2, also display antifungal activity against *Candida* isolates by interaction with fungal GlcCer (Tavares et al., 2008; Thevissen et al., 2012; Silva et al., 2014). Further improvement of existing GlcCer synthase inhibitors, based on the active site of the fungal enzyme may confer higher selectivity for these compounds, a key step for a more efficient therapy of fungal infections, with fewer side effects on the patients.

Other approaches may also lead to interesting results in studies regarding GlcCer and its influence in host/pathogen interactions, which consists in the use of GlcCer-deficient mutants (Δgcs1) of pathogenic fungi. As shown by Rittershaus et al. (2006) *C. neoformans* mutant strain lacking GlcCer was unable to grow *in vitro* at a neutral/alkaline pH in the presence of 5% CO_2, a condition that mimics the host extracellular environment, such as in alveolar spaces or in the bloodstream. However, growth of these mutants was similar to wild type at acidic pH, which mimics the host intracellular environment, such as macrophage-phagolysosome. Furthermore, when these GlcCer defective mutants were incubated with J774.16 macrophage-like cells, no differences in intracellular growth of mutant cells were observed in comparison to the wild-type, suggesting that GlcCer does not have a relevant role in *C. neoformans* intracellular development. Considering the fact that in *Cryptococcus* infections they are predominantly in the extracellular environment, GlcCer may represent a highly relevant molecule associated with virulence of *C. neoformans*.

In the last few years, RNAi technology has been used in silencing genes in *Saccharomyces* spp and *Candida albicans* yeasts (Drinnenberg et al., 2009; Moazeni et al., 2012). Also, some specific features of fungal GlcCer may represent potential targets for therapy, e.g., methylation at C9 and desaturation at C8 of sphingosine, hydroxylation at C2 and desaturation at C3 of the fatty acid (**Figure 1**). Using a similar approach, the expression of fungal glucosylceramide synthase (GCS) as well as other enzymes related to this biosynthetic pathway could be reduced. As pointed out in a recent review by Del Poeta et al. (2014) GlcCer may be considered a key molecule in fungal infectivity, therefore, this approach may help to develop new therapeutic strategies based on silencing specific target sequences not present in mammals.

Concerning the other biosynthetic route of GSLs in fungi, the IPC and GIPCs synthesis, the first step is catalyzed by the transfer of a phosphoinositol group from a phosphatidylinositol to a ceramide (or phytoceramide) (Nagiec et al., 1997), which also represents potential target for the development of new antifungal drugs. In cultures of *Saccharomyces cerevisiae*, the inhibition of IPC synthase by Aureobasidin A (AbA), a highly specific pharmacological inhibitor of IPC synthase (Takesako et al., 1993)

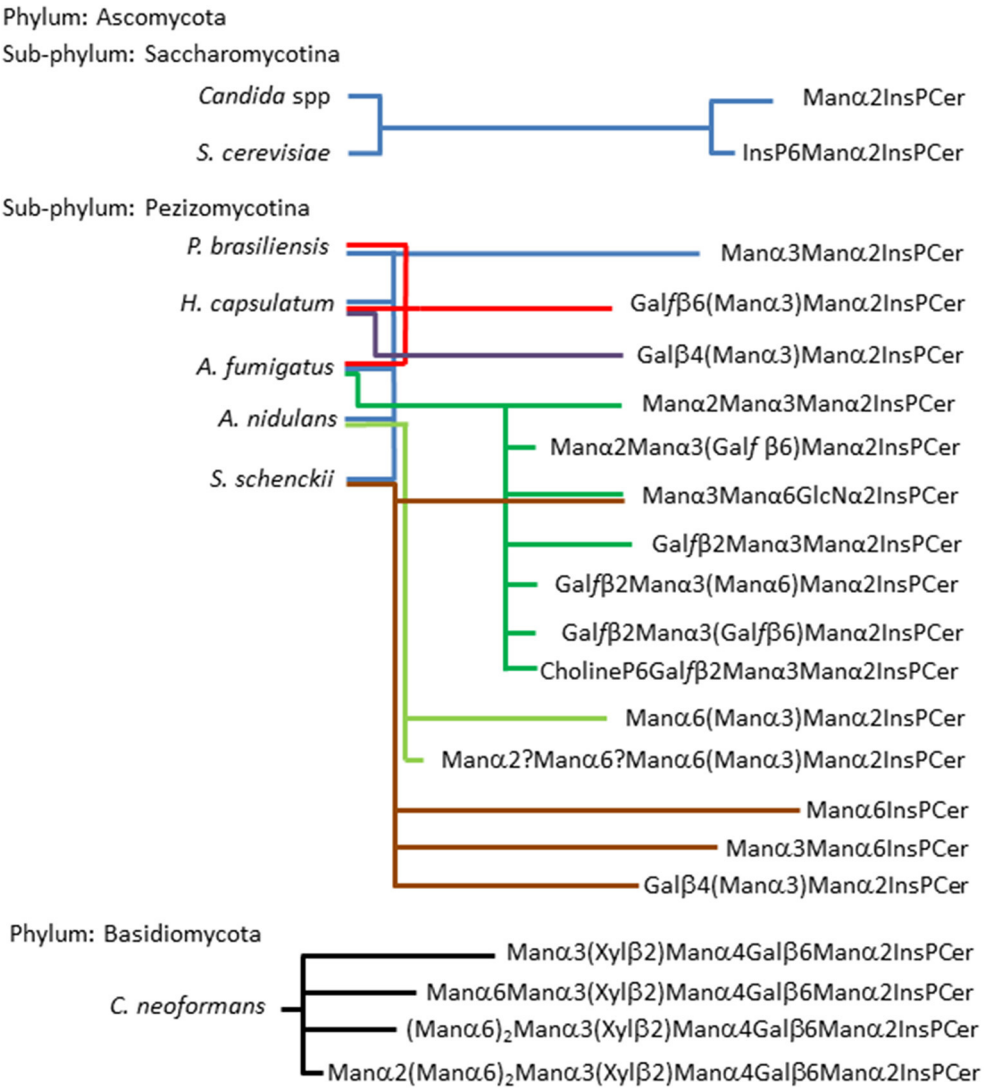

FIGURE 3 | Acidic glycosphingolipids found in fungi according to fungal phylogenetic relationships. The scheme shows two sub-phylum from the Phylum Ascomycota: sub-phylum Saccharomycotina and sub-phylum Pezizomycotina. The former is represented by two of the most studies yeast, *Candida spp* and *S. cerevisiae*, their GIPCs were described by Steiner (Steiner et al., 1969), as two basic structures (Man2IPC and IP6Man2IPC). Five fungi are representing the sub-phylum Pezizomycotina, as can be observed they present the most variable repertoire of glycan structures in three possible core: Manα2IPC, which is present in all five fungi; GlcNα2IPC, which is present in *A. fumigatus* and *S. schenckii*; and Manα6IPC which is present only in *S. schenckii* (Barr and Lester, 1984; Barr et al., 1984; Toledo et al., 1995, 2000, 2007; Levery et al., 1996, 1998; Loureiro y Penha et al., 2001; Aoki et al., 2004; Suzuki et al., 2008). Phylum Basidiomycota is represented by *C. neoformans* which presents GIPCs with up to eight residues of carbohydrates expressing xylose residues. It is noteworthy that its capsule is rich in xylose (Heise et al., 2002).

(**Figure 1**), led to the abnormal budding and fungal death (Endo et al., 1997). Takesako et al. (1993) also showed that AbA oral treatment in mice with systemic candidiasis was effective and showed low toxicity for the host. In a more recent experimental approach Tan and Tay (2013) tested the *in vitro* susceptibility of 92 clinical isolates of various *Candida* species to AbA. These authors described that planktonic Candida yeasts were more susceptible to AbA than *Candida* forms present in biofilm (MIC_{50} of 1.0 vs. 8.0 $\mu g.mL^{-1}$, respectively). In this study it was also demonstrated that AbA inhibited filamentation and lead to short hyphae formation which may have disabled the biofilm development by *C. albicans*, though biofilm formation and development is a highly complex process which still remain to be fully understood (Shopova et al., 2013; Guimarães and Takahashi, 2014).

Blocking the synthesis of GIPCs with inhibitors also may confer the additional advantage of microbial selectivity considering the fact that this class of GSL is absent in mammalian cells. However, fungi that lack GIPC biosynthetic pathway, such as zygomycetes presented resistance to AbA (Aoki et al., 2004) and they will probably be resistant to others inhibitors of IPC synthase. Therefore, the use of enzymatic inhibitors for one or both GSL biosynthetic pathways must vary according to the fungi,

allowing the combination of both therapies for most efficient antifungal therapies by blocking GSC and IPC synthase without affecting the biosynthetic pathways of the host.

FUNGAL GSLs AS MODULATORS OF HOST IMMUNE RESPONSE

Besides the importance of GSLs for normal fungal development, studies performed by our group and other investigators have shown that some fungi elicit immune responses in the infected host. More specifically, it was demonstrated that GIPC Pb-3 (Gal*f*β6[Manα3]Manα2InsPCer) elicited in patients with paracoccidioidomycosis (PCM) an immune response with production of antibodies directed to the terminal residue β-D-galactofuranose. Also, several GIPCs from *H. capsulatum* and *A. fumigatus* bearing a terminal residue of βGal*f* presented cross-reactivity with sera of PCM (Barr and Lester, 1984; Toledo et al., 1995, 2007; Bertini et al., 2007). The primary immune response of patients with PCM was associated with IgM production and further switched to IgG1. IgG1 titers decreased after 5 months of antifungal therapy with sulfamethoxazole-trimethoprim accompanied with the decline of the symptoms (Bertini et al., 2007).

Passive immunization with mouse monoclonal antibody directed to *Cryptococcus* GlcCer was reported to prolong survival of mice infected with *C. neoformans* (Rodrigues et al., 2007). Conversely, data from our laboratory using a specific monoclonal antibody directed to fungal GlcCer did not show any significant inhibitory effect on *P. brasiliensis*, *H. capsulatum,* and *S. schenckii* colony formation units and fungal growth rate (Toledo et al., 2010). Although promising, the effect of anti-GlcCer antibodies on humans should be carefully assessed in order to fully understand the mechanisms of the modulatory response to anti-GlcCer antibodies as well as to determine their effectiveness as a therapy for different mycosis.

Concerning the cellular immune response against GSLs, experiments were carried out with concanavalin A activated BALB/c lymphonode cells and showed that purified preparations of GIPCs from *P. brasiliensis* (Manα3Manα2IPC and Gal*f*β6[Manα3]Manα2IPC), *A. fumigatus* (Manα2Manα3[Gal*f*β6]Manα2IPC) and *S. schenckii* (Manα3Manα6GlcNα2IPC), as well as, GlcCer and GalCer from these three fungi were able to inhibit T lymphocyte proliferation *in vitro* in a dose-dependent manner. It was observed an $IC_{50} \leq 5\,\mu M$ for GIPCs, whereas an IC_{50} of 20 μM was observed for GlcCer and GalCer (Takahashi et al., 2009). Studies performed with GlcCer from *A. fumigatus* revealed that this GSL was able to activate *in vitro* mouse and human natural killer T cells (iNKT cells), and to induce airway hyperreactivity in mice (Albacker et al., 2013).

The data above indicate that fungal GSLs, presenting unique monosaccharide sequences and ceramide moieties may influence both humoral and cellular responses and potentially may open new vistas in this field.

FUNGAL GSLs IN MEMBRANE DOMAINS

Recently it was shown by membrane microdomain isolation protocols that GlcCer and GIPCs are preferentially localized in these membrane domains. Studies performed by our group demonstrated that∼40% of ergosterol from membranes of *H. capsulatum* is present in membrane microdomain fractions resistant to treatment with non-ionic detergent at 4°C (Tagliari et al., 2012). These ergosterol-enriched membrane microdomains showed a peculiar protein distribution and a distinct degree of resistance to treatment with methyl-beta-cyclodextrin (mβCD), a sterol chelator, suggesting the existence of two populations of membrane microdomains in *H. capsulatum* yeast forms: Type 1, ergosterol-independent microdomains rich in integrin-like 50 kDa protein and GlcCer and GIPCs, possibly involved in signal transduction; and Type 2, ergosterol-dependent microdomains containing Pma1p and the 30 kDa laminin-binding protein (**Figure 4**). The type 2 microdomains were also shown to be important for infectivity of alveolar macrophage since after the treatment of yeasts forms with mβCD, the infectivity was reduced by 45%. It is worth mentioning that infectivity of mβCD-treated yeasts was completely restored by addition of exogenous ergosterol, but not cholesterol indicating that specifically ergosterol is able to restore the functionality of these fungal membrane domains.

In agreement with these findings, Singh et al. (2012) demonstrated that structural variation in sphingolipids from *C. neoformans* Δ*smt1* mutant strain (which lacks sphingolipid C9 methyltransferase activity) altered the topography of the membrane lipid affecting fungal plasma membrane rigidity, which was associated with a decrease in the fungal pathogenicity. In the same study, they described that sphingolipid microdomains in *C. neoformans* wild type are larger and more tightly packed than in Δ*smt1*. Furthermore, these authors also reported extra "soft areas" in the Δ*smt1* mutant cell membranes, which may lead to a more permeable and more fluid lipid bilayer, resulting in a less rigid conformation of selected sections of the membrane. These data also strongly suggest that the methylated forms of sphingolipids are required for a proper membrane organization associated with fungal virulence.

The dependence of the GSLs organization in plasma membrane for fungal infectivity was studied a using mAbs directed to glycan components of fungal membrane microdomains. We have demonstrated that mAb MEST-3, an IgG2a directed to Manpα3Manpα2IPC, interfered on colony formation and morphological transition from yeast to mycelium of *P. brasiliensis*, *H. capsulatum* and *S. schenckii*. Similar results were also observed when these fungi where incubated with mAb MEST-1, which reacts with GIPCs presenting terminal residues of β-D-galactofuranose linked to mannose. A possible explanation for these results could be related to the binding of these two mAbs with cell surface GIPCs thus altering the lipid bilayer organization and hindering the formation of functional membrane microdomains leading to interference in GSLs dependent signaling pathways (Toledo et al., 2010).

Membrane microdomains of host cells were also found to be involved in host cell-pathogen interaction. As demonstrated by our group (Maza et al., 2008) in experiments performed with human lung A549 epithelial cells, the membrane rafts of these cells are involved in adhesion process of *P. brasiliensis* yeast forms, promoting activation of Src-family kinases (SFKs) and extracellular signal-regulated kinase 1/2 (ERK1/2) of these epithelial

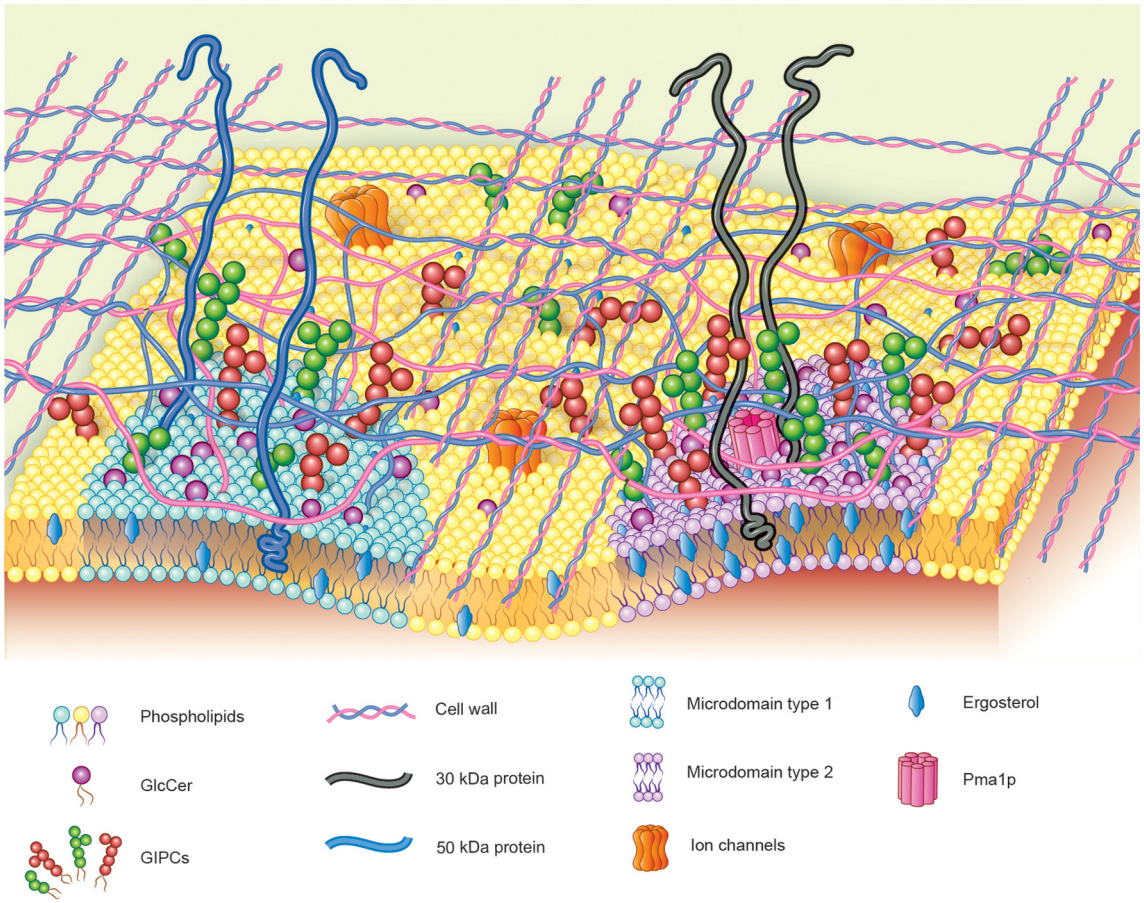

FIGURE 4 | Hypothetical model proposed for fungal membrane microdomains. Two populations of detergent-resistant membrane microdomains are displayed in fungal plasma membrane: Type-1: ergosterol-independent microdomain, rich in integrin-like proteins and GSLs, possibly involved in signal transduction; Type-2: ergosterol-enriched microdomains containing Pma1p and the 30 kDa laminin-binding protein.

cells. The activation of epithelial cells SFKs or ERK1/2 might be involved in expression of host inflammatory cytokines and therefore *P. brasiliensis* through its microdomains could be involved in modulation of host immune responses.

The concept of "glycosynapses" introduced by Hakomori (2004) helps to understand the nature and complexity of microdomains/membrane rafts interactions between host-fungal cells. The glycan moiety of fungal GIPCs and GlcCer/GalCer acting in a concerted way with carbohydrate sequences of GSLs/glycoproteins of complementary host cell microdomains (*trans*-interaction) may intermediate cell-to-cell adhesion with concurrent signal transduction.

CONCLUSIONS AND FUTURE PERSPECTIVES

Due to its importance in clinical contexts, fungal infections have raised questions regarding its biological process and the key molecules related with the infection maintenance and host cell-pathogen interaction. Considering the structural diversity and the biological roles described for fungal GlcCer and GIPCs, disruption of these biosynthetic pathways may represent an interesting/inviting approach to new vistas for fungal infection therapy. Furthermore, considering the influence of fungal GSLs in host immune response, they may also be considered as fungal biomarkers to detect and identify fungal infections, and as well as follow-up markers of a mycosis at different stages.

Taking these findings together, the next steps would include the elucidation of the biological role of GSLs in the interaction of host-fungal cells during the course of the infection. As shown by Maza et al. (2008) and Tagliari et al. (2012) membrane microdomains of both fungi and host cells play important roles in these interactions, such as observed for fungal adhesion to epithelial cells and fungal infectivity of alveolar macrophages. Additionally, other issues regarding the role of fungal cell wall in the cellular contact must also be elucidated. It is tempting to hypothesize that around the fungal membrane microdomain regions, the glycans of the cell wall could be less tight, leading to a more "coarse" organization, this hypothetical model is tentatively shown in **Figure 4**. This more loose conformation of the cell wall around the microdomain regions could expose these membrane structures such as: adhesins, glycosphingolipids and glycoproteins allowing the cross-talk of pathogen-host cell through the membrane microdomains.

Cellular membranes are not autonomous cellular structures, since they are linked to intracellular and extracellular networks

(Nicolson, 2014). One attractive hypothesis is that fungal cell wall could act as biological interface or "conduit" for the information transfer between membrane microdomain and cell wall, perhaps in a glycan-glycan communication and thus mediating the transmission of signals either on localized points or in a network at the cell wall modulating biological events in fungal biology as well as in the fungal infection.

An in-depth knowledge of fungal microdomain interactions in combination with the elucidation of the GSL glycan-dependent signaling pathways and cell wall biosynthesis regulatory steps will certainly provide an integrated view allowing to elaborate a more refined concept of the concerted membrane microdomain-GSL-cell wall involved in key events related to survival and proliferation of pathogenic/opportunistic fungi in the human host.

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Fungal glycans and the innate immune recognition

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Polysaccharides such as α- and β-glucans, chitin, and glycoproteins extensively modified with both *N*- and *O*-linked carbohydrates are the major components of fungal surfaces. The fungal cell wall is an excellent target for the action of antifungal agents, since most of its components are absent from mammalian cells. Recognition of these carbohydrate-containing molecules by the innate immune system triggers inflammatory responses and activation of microbicidal mechanisms by leukocytes. This review will discuss the structure of surface fungal glycoconjugates and polysaccharides and their recognition by innate immune receptors.

Keywords: fungal pathogens, polysaccharides, glycoconjugates, pattern recognition receptors, innate immunity

INTRODUCTION

The fungal cell wall is basically comprised of chitin and β-glucans, which form an inner rigid core, but several other structurally complex polysaccharides, glycoproteins, enzymes, and lipids have been identified as cell wall components, frequently loosely anchored in the external layer. The fungal cell wall is a dynamic structure where its polymeric constituents are under continuous chemical modification and refolding during their biosynthesis. The cell wall is an excellent target for the action of antifungal agents and also a target for the innate immune recognition, since most of its components are absent from mammalian cells (Masuoka, 2004; Latgé, 2010).

FUNGAL GLYCOCONJUGATES AND POLYSACCHARIDES

MANNANS

The cells of the majority of yeasts contain mannose-containing polysaccharides that are often covalently attached to protein. These polysaccharides are predominantly α-D-manopyranans that display a variety of sequences of linkage types in branched structures. A few of the branched mannans carry some β-D-manopyranose residues in the side chains (Gorin et al., 1969). In phosphomannans some sugar residues are attached through phosphorodiester linkages (Masuoka, 2004). The cell surface of *Candida* species is surrounded by a layer enriched in mannosylated glycoproteins and mannosylated lipids. These cell wall mannoproteins (CWMPs) are non-covalently bound to the cell wall as phosphopeptidomannan (PPM) or covalently attached to β (1 → 6) glucan through remnant glycosylphosphatidylinositol (GPI) anchors (GPI-anchored proteins), which is in turn attached to β (1 → 3) glucan or chitin, that form the inner cell wall layer (Masuoka, 2004). PPM containing *O*- and *N*-linked oligosaccharides, can be obtained by autoclaving yeast cells. *O*-linked mannose residues associated with serine/threonine consist of short chains of α-(1 → 2) or α-(1 → 3)-linked mannose. *N*-linked mannans consist of an inner core elongated by an α-(1 → 6)-linear chain with branched side chains of α-(1 → 2) or α-(1 → 3) mannose (Shibata et al., 2003). This structure is similar to that of *Saccharomyces cerevisiae*, extensively investigated (Stewart and Ballou, 1968). In addition, a number of yeast mannans contain β-D-mannopyranosyl units in the side chains. β-(1 → 2)-linked mannose residue is also present in the CWMP of some *Candida* species. *C. albicans* serotype A, *C. glabratta*, *C. tropicalis*, and *C. lusitanea* have the antigenic factor 6, which corresponds to the Man β(1 → 2)-Manα(1 → 2) Manα 1 residue (Kobayashi et al., 1992).

A cell wall lipoglycan, phospholipomannan (PLM) is another glycoconjugate of *C. albicans* that presents β-mannosides. It consists of linear chains of β-(1 → 2)-Man*p* units and inositol covalently linked through a phosphodiester bond to a lipid moiety (Trinel et al., 2002). The degree of polymerization of β-(1 → 2) mannosides chains is up to 19 in *C. albicans* serotype A strains and short β-(1 → 2) mannosides chains with predominance of mannotriose in *C. albicans* serotype B (Trinel et al., 2005). PLM is able to activate inflammasome pathway through a ROS-independent mechanism and this activity seems to be related with the lipid moiety of the molecule. On the other hand, the induction of

TNF-α production is dependent on the glycan moiety (Devillers et al., 2013). Long glycan chains in PLM favors the formation of complexes with a glycan-binding protein, galectin-3, secreted by macrophages (Fradin et al., 2000).

The role of mannosylation has been extensively investigated in glycosylation deficient mutants. The Ca^{+2}/Mn^{+2} ATPase Pmr1p is required for the transport of Mn^{+2} the Golgi apparatus, where it is necessary as a cofactor for the activity of mannosyltransferases in *S. cerevisiae* and *C. albicans*. The *pmr1* deficient *C. albicans* strain shows a strong reduction of the mannose content in the cell wall which reflects a great reduction in the *N*- and *O*-linked glycosylation and phosphomannan synthesis. The *pmr1* deficient *C. albicans* also exhibits an increased susceptibility to cell wall noxious agents, and interestingly this strain shows a constitutive activation of Mkc1p, a MAP kinase involved in the activation of signaling pathways required for the maintenance of the cell wall integrity (Bates et al., 2005). The phenotype of the *pmrp1* deficient *C. albicans* is similar to that observed in the *och1* deficient *C. albicans*, that lacks the 1,6-mannosyltransferase Och1p and, as a consequence, this strain does not form the α1,6-linked polymannose core, while the *O*-glycosylation remains functional (Bates et al., 2006). The double deficient *mnt1/mnt2 C. albicans* strain lacks the activity of the α-1,2-mannosyltransferases, Mnt1p and Mnt2p, what results in the absence of the Man_2–Man_5 residues, and therefore in the absence of *O*-linked glycosylation. As observed in the *C. albicans* mutant strains that lack the *N*-linked glycosylation, the *mnt1/mnt2* deficient strain shows an increased susceptibility to cell wall damaging agents (Munro et al., 2005).

Besides its role in the cell wall architecture, the mannosylation plays an important role in the growth and morphology of *C. albicans*. Although, mannosylation deficient *C. albicans* strains have been demonstrated to grow *in vitro*, they present morphological alterations with a deficient filamentation and formation of aggregates by the yeasts (Bates et al., 2005, 2006; Munro et al., 2005). Thus, an adequate mannosylation plays an important role in the *C. albicans* cell wall integrity and development of the morphological stages of *C. albicans*. Interestingly, while *N*-glycosylation and *O*-glycosylation are required for the cell wall protection against environmental stress, as well as for the development of *C. albicans*, the synthesis of phosphomannans is dispensable for these aspects, since the selective phosphomannan deficient strain, *mmn4*, exhibits a similar growth, morphology, and susceptibility to cell wall toxic agents (Hobson et al., 2004).

Mannosylation plays a critical role in the virulence of *C. albicans*. *N*- and *O*-linked mannans are required for the virulence of *C. albicans* in model of systemic candidiasis, while the presence of phosphomannans is dispensable (Hobson et al., 2004; Bates et al., 2005, 2006; Munro et al., 2005). Although the *N*- and *O*-glycosylation deficient *C. albicans* strains present a decreased lethality in experimental models of infection, a deficient *N*-glycosylation does not impact the fungal loads during the infection. In contrast, the *O*-glycosylation deficient *C. albicans* strain presents decreased fungal counts in the experimental infection, suggesting that while *N*- and *O*-glycosylation are required for the virulence, only *O*-glycosylation is necessary for the colonization of the organs during the systemic infections (Munro et al., 2005; Bates et al., 2006). Interestingly, the *O*-glycosylation deficient *C. albicans* strain (*mnt1*Δ*/mnt2*Δ) shows a deficiency in the adhesion to collagen matrices and epithelial cells, while *N*-glycosylation, but nor *O*-glycosylation, is required for the phagocytosis, binding and cytokine production by macrophages and dendritic cells, suggesting that *O*-linked mannans are involved in the tissue colonization, while *N*-linked mannans are major determinants of the innate immune recognition (Munro et al., 2005; Bates et al., 2006; Cambi et al., 2008). The impact of the glycosylation pathways involved in the mannosylation of fungal glycoconjugates in the biology and virulence of other pathogenic fungi is largely unknown. However, since the extensive mannosylation of glyconconjugates is a conserved pattern found in fungi, the distinct patterns of glycosylation must be determinants for the fungal pathogenesis. Thus, genetic approaches targeting glycosylation pathways in pathogenic fungi must be useful for the comprehension of the fungal virulence and biology.

HETEROPOLYSACCHARIDES WITH MANNAN MAIN CHAINS

Peptidorhamnomannans (PRMs) are common cell wall components that are distributed in species of the *Scedosporium*/*Pseudallescheria* complex and can be isolated by the methodology routinely used in our laboratory (Lopes et al., 2011). Hot aqueous extraction, followed by treatment with Cetavlon in the presence of sodium borate, provided a precipitate of peptidorhamnomannan (PRM), containing carbohydrate *N*- and *O*-linked to peptide. Methylation-gas chromatography-mass spectrometry (GC-MS) analysis and ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectroscopy of PRM showed to contain α-Rha*p*-(1 → 3)-α-Rha*p*-side-chain epitopes linked (1 → 3) to a (1 → 6)-linked α-Man*p* core (Pinto et al., 2001). Non-reducing, *O*-linked oligosaccharides were isolated from the PRMs of *P. boydii*, *S. apiospermum*, and *S. prolificans* mycelium by alkaline β-elimination under reducing conditions (Pinto et al., 2005; Barreto-Bergter et al., 2008, 2011). Three major oligosaccharides were obtained and their structures elucidated based on a combination of techniques including gas chromatography, Electrospray Ionization Mass Spectrometry (ESI-MS), ^{1}H (obs), ^{13}C Heteronuclear Multiple-Quantum Correlation-NMR (HMQC-NMR) spectroscopy and methylation analysis (Pinto et al., 2005). It is interesting to note that these different carbohydrate epitopes present an conserved α-Rha*p*-(1 → 3)-α-Man*p*-(1 → 2)-α-Man*p*-(1 → structural component. PRM *O*-linked oligosaccharides were also isolated from *Sporothrix schenckii* (Lopes-Bezerra, 2011). Besides glucuronic acid–containing oligosaccharides, a trisaccharide α-Rha*p* (1 → 3)-Man*p* (1 → 2)-Man-ol similar to a conserved structural component of the *Scedosporium*/*Pseudallescheria* complex was identified (Lopes-Bezerra, 2011). PRMs are antigenic and *O*-linked glycans may account for a significant part of PRM antigenicity, since de-*O*-glycosylation treatment has been demonstrated to decrease its antigenicity by 70–80% (Pinto et al., 2005). Similar results were obtained with *A. fumigatus* PGM (Leitao et al., 2003) and PRM from *S. schenckii* (Lopes-Bezerra, 2011). The immunodominance of the *O*-linked oligosaccharide chains was evaluated by testing their ability to inhibit reactivity between PRM and anti-*P. boydii* antiserum in an ELISA hapten system (Pinto et al., 2005). Up

to 75% inhibition occurred with both penta- and hexasaccharides from *P. boydii* PRM. Similar results were observed using penta- and hexasaccharides from *S. prolificans* (our unpublished results). These oligosaccharide alditols blocked recognition between rabbit sera and intact PRM in a dose-dependent manner. Thus, *O*-linked oligosaccharide chains, despite being the less abundant carbohydrate components of the *P. boydii* and *S. prolificans* glycocomplexes, may account for a significant part of the antigenicity associated with the rhamnomannan component of *P. boydii/S. prolificans*. Rhamnomannans were also isolated from *P. boydii* using a hot alkaline extraction and their structures determined by one-dimensional (1H and 13C) and two-dimensional Correlation Spectroscopy (COSY), Total Correlation Spectroscopy (TOCSY), and Heteronuclear Single Quantum Correlation (HSQC) experiments. The NMR data of fraction II showed at C-1 signals at δ 97.9/4.981, 101.0/4.967, 102.2/5.228, and 103.9/5.060, typical of terminal α-rhamnose units, *O*-3,6-substituted- α-mannopyranose, O-2-substituted- α-mannopyranose and α-Man*p*-3-O-substituted units, respectively. The signal at δ 79.9/4.127 confirms the 3-O-substituted α-Man*p* units (Figueiredo et al., 2010).

PRMs derived from *P. boydii* are also involved in the adhesion and infection of an epithelial cell line by *P. boydii* conidia, since the competition with soluble PRM and anti-PRM antibodies are able to inhibit the entry of conidia. Interestingly the determinants involved in the interactions mediated by PRMs seem to require the presence of terminal rhamnose residues that are eliminated by partial hydrolysis, while *O*-glycosylation, the protein portion or mannosyl residues are not necessary (Pinto et al., 2004). *P. boydii* derived rhamnomannans induce cytokine release by macrophages in a mechanism dependent on TLR4 signaling, as well as degradation of IkBα and phosphorylation of MAPKs. The induction of cytokine production by these molecules requires the presence of terminal non-reducing residues of rhamnose, since their removal by partial acid hydrolysis abolishes the ability of rhamnomannan to induce cytokine production by macrophages. Thus, structures with terminal rhamnose and mannose residues present in *P. boydii* rhamnomannans are likely structural motifs involved in TLR recognition (Figueiredo et al., 2010). Thus, rhamnomannans represent a characteristic pattern of glycosylation found on the surface of fungi of the complex *Scedosporium/Pseudallescheria*, mediating cell infection and the innate immune recognition.

Galactomannans are important structural components of the *Aspergillus* cell-wall, being widely distributed among most *Aspergillus* species. Galactomannans are mannose-containing polysaccharides containing terminal D-galactose residues in both furanose and pyranose ring forms, and for the latter both α-D and β-D configurations have been encountered (Latgé et al., 1994). A galactomannan was isolated either from a culture filtrate (Latgé et al., 1994) or from the fungal cell wall of *A. fumigatus* (Leitao et al., 2003). Its structure was elucidated by acid and enzymatic hydrolysis, partial acetolysis, methylation analysis, and ^{13}C NMR spectroscopy (Leitao et al., 2003). It consists of a main chain of (1 → 6)-linked α-D-mannopyranosyl residues substituted at *O*-2 by 1–3 consecutive α-D-mannopyranosyl units that were (1 → 2)-linked. β-D-Galactofuranosyl-containing side-chains, with (1 → 5)-links and an average length of approximately 6 units, were attached to *O*-6 of the mannan core, β-D-Gal*f*-(1 → 5)-[β-D-Gal*f*-(1 → 5)]$_{0-5}$-(1 → 6)-α-Man*p*-. Galactomannans have also been shown to exist in a glycosylinositol membrane-bound form. Chemical and enzymatic degradation and mass spectrometry analysis showed that the lipid anchor was a glycosylphosphatidylinositol (GPI), containing a C18-phytosphingosine and a monohydroxylated lignoceric acid, in the lipid portion (Costachel et al., 2005). Summarizing, galactomannans from *A. fumigatus* are found in three different forms, namely: (1) as a free polysaccharide found in the culture medium, (2) covalently linked to the β(1 → 3) glucans of the fungal cell wall, and (3) GPI-anchored to the membrane.

Besides the polysaccharides described above that are covalently interlinked to form a skeletal structure, *N*- and *O*-linked peptidogalactomannans were present in the outer layer of *A. fumigatus* cell wall and isolated by hot buffered aqueous extraction (Haido et al., 1998). *O*-linked oligosaccharides were selectively released from pGM by β-elimination under mild alkaline reductive conditions in the presence of sodium borohydride. Their primary structures were determined based on a combination of techniques including gas chromatography, ESI-QTOF-(Quadrupole followed by a time-of-flight mass analyzer) MS, ^{1}H- and TOCSY, and ^{1}H-^{13}C HMQC NMR spectroscopy and methylation analysis, to be: α-Glc*p*-(1 → 6)-Man-ol, β-Gal*f*-(1 → 6)-α-Man*p*-(1 → 6)-Man-ol, β-Gal*f*-(1 → 5)-β-Gal*f*-(1 → 6)-α-Man*p*-(1 → 6)-Man-ol and β-Gal*f*-(1 → 5)-[β-Gal*f*-(1 → 5)]$_3$-β-Gal*f*-(1 → 6)-Man-ol (Leitao et al., 2003). These *O*-linked oligosaccharides may account for a significant part of the peptidogalactomannan antigenicity, because de-*O*-glycosylation decreased by 50% its activity. The immunodominant epitopes were present in the tetra- and hexasaccharide, which contain a β-Gal*f*-(1 → 5)-β-Gal*f* terminal group (Leitao et al., 2003).

Another cell wall polysaccharide, a phosphonogalactomannan, was isolated via alkaline extraction from *A. versicolor* mycelia and its complex structure was identified by ^{31}P, ^{1}H, and ^{13}C NMR spectroscopy and methylation analysis and appeared to have similar structure as the galactomannan from *A. fumigatus* and *A. niger*, except for the presence of phosphorodiester groups (Tischer et al., 2002).

Galactomannans are major antigens produced during the infections caused by *A. fumigatus*. Galactomannans are detected in the serum and bronchoalveolar lavage of patients with invasive aspergillosis, and their detection has been used as a diagnostic marker for the infections caused by *Aspergillus spp* (Acosta et al., 2011; He et al., 2012; Teering et al., 2014). Galactomannans are secreted by *A. fumigatus* and, in association with galactosaminogalactans, are major components of the biofilm produced by the *A. fumigatus* mycelium. Interestingly, the *A. fumigatus* biofilm is found around the mycelia in aspergilomas and pulmonary lesions found in invasive infections, indicating that this structure must participate in the pathogenesis of the infections (Loussert et al., 2010). Galactomannans are able to inhibit the *A. fumigatus* conidial phagocytosis by dendritic cells, indicating that the recognition of cell surface expressed galactomannans is required for the *A. fumigatus* binding and internalization (Serrano-Gómez et al., 2004). Thus, glycoconjugates containing galactomannans must represent important antigens and targets for the immune

response, besides playing a role in the pathogenesis of aspergillosis, possibly acting as components of extracellular adhesive structures during the host tissue colonization.

α-D-LINKED GLUCANS

Another important group of polysaccharides, the α-glucans, have been isolated from several fungal cells. The fungal α-glucans described include α (1 → 3)-linked, and in some species glycogen-like α (1 → 4) and (1 → 6)-linked chains. The fungal α-glucans described show an outermost localization in the cell wall and are easily extracted by hot extraction, being soluble in alkaline conditions, in opposition to the largely insoluble β-glucans that form the rigid core of the cell wall.

Pseudonigeran, isolated following alkaline extraction of cell walls of *A. niger*, is an α (1 → 3)-linked α-D-glucopyranan as shown by methylation, periodate oxidation, and partial hydrolysis studies (Horisberger et al., 1972). It is also present in cell walls of *A. nidulans*, *A. fumigatus*, and *Cryptococcus* spp (Bacon et al., 1968; Zonneveld, 1972). An α (1 → 3)-glucan is present in the outer most layer of the *Histoplasma capsulatum* yeast cell wall (Rappleye et al., 2007), whereas the mycelial form contains none (Kanetsuna et al., 1974). In *Blastomyces dermatiditis* and *Paracocciodioides brasiliensis* the levels of the α (1 → 3)-glucans are much higher in the yeast than in the mycelial form (Kanetsuna and Carbonell, 1970, 1971; Kanetsuna et al., 1972). Another α-glucan has been isolated from *P. boydii* and its structure was determined, using a combination of techniques including gas chromatography, ^{1}H TOCSY, ^{1}H, and ^{13}C NMR spectroscopy and methylation analysis, to be a glycogen-like polysaccharide consisting of linear 4-linked α-D-Glc*p* residues substituted at position 6 with α-D-Glc*p* branches (Bittencourt et al., 2006). A similar structure was detected in *A. fumigatus* (Bahia et al., 1997).

The role of α-glucans in the fungal biology is still incompletely understood. During the germination of *A. fumigatus* conidia, the exposure of α (1 → 3)-glucans promotes the aggregation and development of the germ tubes (Fontaine et al., 2010). In *H. capsulatum*, α (1 → 3) glucans mask the β (1 → 3) glucans what results in a deficient recognition of these molecules by Dectin-1, and this has been speculated to avoid the induction of pro-inflammatory cytokines by macrophages (Rappleye et al., 2007). Glycogen-like α-glucans isolated of *P. boydii* are able to inhibit the phagocytosis of *P. boydii* conidia, furthermore these molecules induce the release of pro-inflammatory cytokines by murine macrophages, thus indicating that α (1 → 4)(1 → 6) glucans represent immunostimulatory molecules mediating the recognition of *P. boydii* by macrophages (Bittencourt et al., 2006). Thus, the investigation of the roles of α-glucans in the fungal development and interaction with immune cells must bring important insights in the fungal biology and virulence.

β-D-LINKED GLUCANS

β-D-Glucans are on interest because of their potential in modulating a wide range of innate host immune responses. They are present in virtually all fungi. A number of studies carried out on fungal polysaccharides have been demonstrating the presence of predominant 3-linked β-D-glucopyranosyl structures. A linear cell wall glucan from *S. schenckii* contains 3-*O*-, 6-*O*-, and 4-*O*-substituted β-D-glucopyranosyl units (Previato et al., 1979). Cell-wall polysaccharides, such as β (1 → 3)-glucans, have been characterized in *Aspergillus* spp (Bernard and Latgé, 2001). The β-D-glucopyranan from the mycelial form of *P. brasiliensis* contains 90% of (1 → 3) linkages. This polymer is a major constituent of the cell wall of the filamentous phase of *H. capsulatum* (Davis et al., 1977). The β-D-glucopyranans of *C. albicans* serotype B and *C. parapsilosis* are mainly linear, with only approximately 10% of branch points, and contain, principally, (1 → 6) linkages (67 and 63%, respectively) (Yu et al., 1967). *C. albicans* does not contain α-glucans. It only contains both β-1,3 and β-1,6-glucans, but no mixed intrachain β-1,3/1,6 linkages. Analysis by proton NMR spectroscopy (NMR) of glucans from yeast or hyphal forms of *C. albicans* showed that they were different from *S. cerevisiae* glucans in side-chain branching and reducing termini (Ruiz-Herrera et al., 2006).

In a recent work, Lowman et al. (2014) using a mild extraction procedure for isolation of *Candida albicans* yeast and hyphal forms showed by NMR and GC-MS analysis that the hyphal glucan has a unique cyclic structure, not found in yeast glucan. Both are branched glucans having a (1 → 3)-linked, β-D-glucopyranosyl main-chain, partially substituted at *O*-6 by (single unit) β-D-glucopyranosyl groups. However, in addition to these linkages, a 2,3 linkage was identified and this feature has not been reported previously in *C. albicans* (Lowman et al., 2014). Interestingly, the cyclic hyphal β-glucan presents a differential pattern of cytokine induction by human monocytes in relation to that promoted by linear yeast β-glucans, with cyclic β-glucans being a more potent inducer of IL-1β, TNF, and IL-6 than the linear yeast β-glucans. As observed for the linear β (1 → 3) glucans the cyclic hyphal β-glucans are recognized by Dectin-1 (Lowman et al., 2014).

Fungal β-glucans have been recognized for so long as immunomodulators (Goodridge et al., 2009b). Large β-glucan particles induce cytokine production, reactive oxygen species (ROS) production and phagocytosis by neutrophils and macrophages, while soluble β-glucans act as antagonists of these responses (Brown and Gordon, 2001; Brown et al., 2003; Gantner et al., 2003; Kennedy et al., 2007; Goodridge et al., 2011). Furthermore, β-glucans induce the activation of the alternative pathway of the complement cascade (Bose et al., 2013). β-glucans are also efficient adjuvants, promoting dendritic cell maturation and antigen loading by these cells, what triggers the activation of CD4 and cross-priming of CD8 lymphocytes (Yoshitomi et al., 2005; Leibundgut-Landmann et al., 2008; Weck et al., 2008).

Recognition of fungal β-glucans by macrophages requires the exposure of the inner layer of the fungal cell wall, as a result of the growth or germination, as observed during division and septation of *C. albicans* yeasts and hyphae, and germination of resting conidia of *A. fumigatus* (Gantner et al., 2005; Hohl et al., 2005; Steele et al., 2005; Gersuk et al., 2006). Thus, the β-glucan recognition must represent a mechanism for the detection of the growth and morphological differentiation of pathogenic fungi. Interestingly, *A. fumigatus* and other environmental fungi express a highly hydrophobic external layer formed by hydrophobin proteins which masks the β-glucans in the resting conidia, and it has been speculated that the recognition of exposed β-glucans

in the germinating conidia, but not in the resting ones, could have evolved to avoid the persistent inflammation, to the ubiquitous resting conidia, while permitting the detection of the invasive morphological stages (germ tubes and hyphae) (Aimanianda et al., 2009).

CHITIN

Chitin is an important skeletal component in most fungi. Chitin is a linear polysaccharide composed by 4-linked-2-acetamido-2-deoxy-β-D-glucopyranan (Munro and Gow, 2001). Chitin and β-glucans are the most abundant polysaccharides conserved through the evolution in the fungal cell wall and are the most common polysaccharides in fungal species. Chitin represents a small percentage in *S. cerevisiae*, but the content is higher in other yeasts and filamentous fungi (Xie and Lipke, 2010). Chitin composes the insoluble core of the fungal cell wall, either isolated or associated with β-glucans (Masuoka, 2004). The composition of the alkali insoluble core of the *A. fumigatus* cell wall has been described, and it contains pure chitin chains, as well as chitin chains associated to β-1,3 glucans and chitin/galactomannans associated to the side branching chains of β-1,3 glucans (Fontaine et al., 2000).

Chitin and chitosan, its deacetylated polymer, present several immunomodulatory effects. Exposition to chitin has been implicated in the development of allergic airway inflammation, and in an experimental model of pulmonary inflammation induced by *Aspergillus* cell wall, the digestion with chitinase, decreases the inflammation, and leukocyte recruitment (Van Dyken et al., 2011). Chitin induces cytokine production, leukocyte recruitment, and alternative activation of macrophages (Reese et al., 2007; Da Silva et al., 2009, 2010). Furthermore, macrophages promptly ingest chitin and chitosan particles (Nishiyama et al., 2006). Curiously, chitosan, but not chitin, is able to promote the activation of the inflammasome NLRP3/ASC/caspase-1, and therefore chitosan leads to the activation of caspase-1 and release of IL-1β (Bueter et al., 2011, 2014).

GALACTOSAMINOGALACTAN

Galactosamine-containing polysaccharides have been identified in *Aspergillus* species. An exocellular polysaccharide from *A. nidulans* is a linear molecule and according to methylation analysis contains 4-*O*-substituted α-D-galactopyranosyl and 4-*O*-substituted 2-acetamido-2-deoxy-α-D-galactopyranosyl units. Periodate oxidation and ^{1}H-NMR data showed their ratio to be approximately 1.8:1.0. (Gorin and Eveleigh, 1970). A heteropolysaccharide from *A. niger* has a related structure but lacks *N*-acetyl groups. It contains 4-*O*-substituted α-D-galactopyranosyl and 2-amino-2-deoxy-α-D-galactopyranosyl units in the ratio 7:2 (Bardalaye and Nordin, 1976, 1977). A galactosaminogalactan secreted by the mycelium of *A. fumigatus* was identified and the carbohydrate structure analysis showed that it is a linear heterogeneous polymer of α-1-4 galactosyl and α1-4 *N*-acetylgalactosaminyl residues (Fontaine et al., 2011). Galactosaminogalactans have been localized on the outer layer of the *A. fumigatus* cell wall and due to its localization might mask the exposure of other polysaccharides such as β-(1 → 3)-glucan (Gravelat et al., 2013). *In vitro* studies suggest that this molecule is the principal mediator of *A. fumigatus* adherence and plays a role in biofilm formation (Loussert et al., 2010; Gravelat et al., 2013). The *A. fumigatus* galactosaminogalactans are antigenic, and anti-galactosaminogalactans antibodies are present in the human serum, even in the absence of *Aspergillus* spp infections, but these polysaccharides are in fact immunosuppressive, as observed by decreased neutrophil recruitment, and higher fungal loads, during the *A. fumigatus* experimental infection following the immunization with galactosaminogalactans (Fontaine et al., 2011). The immunosuppressive properties of *A. fumigatus* galactosaminogalactans have been attributed to the inhibition of the production of IFN-γ, neutrophil chemoattractant chemokines, and the induction of IL-1Ra, an antagonist cytokine for the IL-1 receptor (Gresnigt et al., 2014).

PATTERN RECOGNITION RECEPTORS INVOLVED IN THE RECOGNITION OF FUNGAL CARBOHYDRATES

TOLL LIKE RECEPTORS (TLRs)

TLRs comprise a family of receptors that share homology with the Toll receptor (Takeuchi and Akira, 2010). The Drosophila Toll was described as a regulator of the dorsoventral differentiation in the embryo of *Drosophila*, and further pointed as a receptor required for the antifungal immune responses in *Drosophila* (Lemaitre et al., 1996). Following the characterization of the Toll receptor in *Drosophila*, 10 human TLRs, and 13 murine TLRs have been described (Takeuchi and Akira, 2010; Hidmark et al., 2012; Li and Chen, 2012; Oldenburg et al., 2012). TLRs present an amino-terminal extracellular domain containing leucine rich repeats and intracellular domains that share homology with the Toll/IL-1 receptor (TIR) domain. The TIR domains recruit adaptor proteins containing TIR domains, such as MyD88, TRIF, TRAM, and TIRAP, what leads to signaling pathways that culminate in the activation of the transcriptional complexes NF-κB, AP-1, IRFs (IRF3/7), and MAP kinases, and as consequence in the expression of cytokines and co-stimulatory molecules (Takeuchi and Akira, 2010).

TLR4 is the receptor responsible for responses to the bacterial lipopolysaccharides (LPS) (Poltorak et al., 1998). The LPS recognition by TLR4 requires the association with MD2 (Shimazu et al., 1999; Schromm et al., 2001; Akashi et al., 2003). TLR4 has been demonstrated to recognize fungal mannans. *S. cerevisiae* and *C. albicans* derived mannans induce cytokine production by human monocytes by a mechanism dependent on CD14, TLR4 and this is amplified in the presence of the Lipopolysaccharide Binding Protein (LBP) (Tada et al., 2002). Netea et al. reported, in an extensive investigation of mannosylation-defective *C. albicans* strains, that TLR4 cooperates with the Mannose Receptor (MR) in the recognition of mannans, with TLR4 detecting *O*-linked mannans, while MR is responsible for the sensing of *N*-linked mannans (Netea et al., 2006). TLR4 is also involved in the recognition of PRMs isolated from *P. boydii* (Figueiredo et al., 2010) and in detection of the glucuronoxylomannans from *C. neoformans* (Shoham et al., 2001), indicating that TLR4 acts as a receptor for the sensing of distinct mannose containing polysaccharides.

TLR2 recognizes bacterial lipoproteins and lipoteichoic acid, mycobacterial lipoarabinomannans, and GPI anchors from protozoan parasites (Takeuchi et al., 2000; Campos et al., 2001;

Sandor et al., 2003; Tapping and Tobias, 2003; Krishnegowda et al., 2005). The recognition mediated by TLR2 have been described to involve the dimerization with TLR1, for the detection of triacylated lipoproteins (Takeuchi et al., 2002; Jin et al., 2007), or TLR6, in case of diacylated lipoproteins (Takeuchi et al., 2001; Kang et al., 2009).

TLR2 has also been pointed as a receptor involved in the recognition of fungal molecules. TLR2 is the receptor responsible for the activation of NF-κB and cytokine release by macrophages in response to a PLM isolated from *C. albicans*, while TLR4 and TLR6 contribute partially to the responses evoked by this lipoglycan (Jouault et al., 2003). TLR2 has also been described as a receptor involved in the recognition of glucogen-like α-1,6-branched α-1,4-glucans, such as an enzymatically produced glycogen (Kakutani et al., 2012), and a glycogen-like α-glucan purified from *P. boydii* (Bittencourt et al., 2006). TLR2/TLR1 and TLR2/TLR6 is also involved in the recognition of the glucuronoxylomannans isolated from *C. neoformans* and *C. gatii* capsules (Fonseca et al., 2010).

The mechanisms by which TLR4 and TLR2 recognize mannose-containing glycoconjugates and other fungal polysaccharides are poorly understood. TLR4 and TLR2 have been demonstrated to bind fungal polysaccharides, and these interactions are inhibited in the presence of soluble mannans and fucose (Hsu et al., 2009). Mannans occur as polysaccharide components in the structures of many fungal glycoconjugates, such as glycoproteins and glycolipids (Masuoka, 2004). In this way, the widely employed mannan preparations are in fact highly heterogeneous mixtures of mannosylated glycoproteins, and possibly glycolipids. Thus, the use of purified mannans, as activators, ligands or competitors for TLR4 or TLR2 mediated responses, although useful in demonstrating the recognition of these fungal glycoconjugates, fails in elucidating the specific structural motifs involved in the activation of these receptors.

The structural bases for the recognition of bacterial lipoproteins and LPS, by TLR2/TLR1 and TLR2/TLR6, and TLR4/MD2, respectively, have been elucidated by crystallographic analyses (Jin et al., 2007; Kang et al., 2009; Park et al., 2009). The emerging pattern indicates that fatty acid chains in the bacterial ligands interact with hydrophobic pockets in the extracellular domain localized in the interfaces of the receptor complexes. It is unclear how TLR2 and TLR4 recognize hydrophilic ligands such as polysaccharides, but it seems reasonable that the interaction of these receptors with carbohydrates must work in a distinct way from the classical bacterial lipid ligands. In this sense, the role of TLR2 and TLR4 as receptors have been extensively expanded to the recognition of several distinct molecules, including heme (Figueiredo et al., 2007), hyaluronic acid (Termeer et al., 2002; Jiang et al., 2005; Scheibner et al., 2006), heparan sulfate (Johnson et al., 2002; Brunn et al., 2005) and biglycan (Schaefer et al., 2005). Thus, the mechanisms of recognition by these receptors seem to be wider than the previously described recognition of microbial lipid molecules.

In conclusion, it seems evident that more detailed investigations are required for the characterization of the mechanisms involved in the recognition of fungal carbohydrates by TLR2 and TLR4. Alternative approaches such as, (1) use of chemically defined oligosaccharides as ligands for TLR2 and TLR4, (2) specific enzymatic digestion of fungal polysaccharide preparations and analysis of TLR2 and TLR4 activation/binding, (3) extensive purification and elucidation of the structures of fungal polysaccharides by complementary analytic tools (mass spectrometry, RMN) and co-relation with TLR2 and TLR4 activation, and (4) TLR2 and TLR4 binding assays must be determinant for the elucidation of the recognition of fungal polysaccharides by TLR2 and TLR4.

C-TYPE LECTIN RECEPTORS (CLR)

CLR comprise an important group of proteins involved in the recognition of fungal pathogens. CLR are defined by the presence of domains composed by two loops joined by disulfide bonds, the C-type Lectin Domains (CTLDs), which contain carbohydrate recognition domains (CRD). CLR are characterized by the interaction with carbohydrates that in many cases requires the presence of Ca^{+2} (Sancho and Reis E Sousa, 2012). Two conserved motifs are present in the CRD and dictate the specificity for carbohydrates; the EPN motif confers binding to mannose, *N*-acetylglucosamine, L-fucose, and glucose, while a QPD motif determines the recognition of galactose and *N*-acetylgalactosamine (Drickamer, 1992; Kolatkar and Weis, 1996; Kolatkar et al., 1998; Zelensky and Gready, 2005; Lee et al., 2011; Sancho and Reis E Sousa, 2012).

Among the CLRs, Dectin-1, Dectin-2, MCL, Mincle, MR, and DC-specific ICAM3-grabbing non-integrin (DC-SIGN) have been implied in the recognition of fungal carbohydrates (Sancho and Reis E Sousa, 2012; Zhu et al., 2013). Dectin-1, Dectin-2, MCL, and Mincle employ the Immunoreceptor Tyrosine-based Activation Motifs (ITAM) to induce cell signaling through the activation of Src kinases, Syk, and PLCγ what leads to NF-κB and NFAT mediated transcription. Dectin-1 presents an intracellular tyrosine based motif named hemi-ITAM, since it presents only a LXXY, while a typical ITAM motif carries two similar separated tyrosine based motifs (YXXL/I). Dectin-2, Mincle, and MCL do not bear ITAM motifs, but they signal through the interaction with the ITAM containing protein, FcRγ chain. MR and DC-SIGN do not present ITAM motifs, although they are involved in the modulation of cytokine production by macrophages and dendritic cells and in the internalization of carbohydrate-carrying molecules and pathogens (Sancho and Reis E Sousa, 2012).

MANNOSE RECEPTOR (CD206)

MR is a type-I transmembrane protein presenting an *N*-terminal cysteine rich domain, a fibronectin type II domain, eight extracellular CTLDs (1–8), and an intracellular portion that contains a motif involved in the endocytic signaling, FENTLY (Martinez-Pomares, 2012). MR recognizes glycoconjugates containing mannose, fucose, *N*-acetylglucosamine (Taylor et al., 1992; Taylor and Drickamer, 1993), sulfated *N*-acetylgalactosamine or sulfated galactose (Leteux et al., 2000; Liu et al., 2000). Recognition of sulfated polysaccharides is dependent on the cysteine-rich domain, but is independent of the CLTDs (Leteux et al., 2000; Liu et al., 2000). In contrast, recognition of mannose containing glycoconjugates requires the activity of the CTLDs 4–8, as demonstrated

by binding assays employing recombinant versions of the MR CTLDs (Taylor et al., 1992).

MR is involved in the recognition of several fungal pathogens, such as *Pneumocystis carinii* and *C. albicans* (Ezekowitz et al., 1991; Netea et al., 2006; Cambi et al., 2008). MR has been described as a receptor involved in the phagocytosis of fungal pathogens (Ezekowitz et al., 1991; Cambi et al., 2008), but its role as a professional phagocytic receptor has been questioned when MR is expressed in non-phagocytic cells (Le Cabec et al., 2005). Although the ability of MR in triggering phagocytic signaling pathways has been put in check, it is well-established that MR promotes the endocytosis of mannose-containing ligands and participates in the phagocytosis of fungal pathogens (Ezekowitz et al., 1991; Burgdorf et al., 2006; Cambi et al., 2008). Thus, it is possible that MR acts as a receptor involved in the binding of mannose containing ligands what would permit the internalization mediated by another phagocytic receptor. Alternatively the phagocytic activity of MR must be a cell type specific property.

MR is also involved in the induction of signaling pathways that promote cytokine production in response to fungal pathogens and mannans (Netea et al., 2006; Tachado et al., 2007; Van De Veerdonk et al., 2009). TNF release by macrophages in response to *C. albicans* requires the recognition mediated by MR and TLR4 for *N*-linked mannans and *O*-linked mannans, respectively (Netea et al., 2006). MR has been demonstrated to recognize mannosylated glycoconjugates, such as phosphatydil-myo-inositol mannosides (PIMs) and mannose-capped lipoarabinomannan (LAM) from mycobacteria, and *O*-linked recombinant proteins expressed in *Pichia pastoris*. Thus, while *N*-linked mannoproteins seems to be the major ligands for MR in *C. albicans*, it is possible that other mannosylated glycoconjugates could be recognized by MR in fungal pathogens (Kang et al., 2005; Torrelles et al., 2006; Lam et al., 2007). MR cooperates with TLR2 in the induction of cytokines in response to *P. jirovecci*, and upon the *P. jirovecci* stimulation these receptors physically interact (Tachado et al., 2007). How MR contributes to signaling pathways involved in cytokine production is still unknown. MR has been described to promote the $PPAR_{\gamma}$ expression and activation and this pathway is required for the induction of cytokines by mycobacterial ManLAM (Rajaram et al., 2010). However, MR has a short intracellular domain that lacks known signaling motifs involved in the gene expression of cytokines. Thus, it seems probable that MR works in cooperation with other receptors which are able to trigger the expression of cytokines, such as TLRs and CLRs.

DECTIN-2

Dectin-2 was initially characterized as a CLR expressed in a cell line derived from Langerhans cells. Dectin-2 is a type II transmembrane protein with one CLTD present in the COOH-terminal region and a short cytoplasmic tail. Dectin-2 activation is able to induce the production of cytokines and eicosanoids (Sato et al., 2006; Barrett et al., 2009; Saijo et al., 2010). Dectin-2 is expressed in macrophages, some populations of dendritic cells and in IL-6/IL-23 stimulated neutrophils (Ariizumi et al., 2000a; Taylor et al., 2005, 2014; Barrett et al., 2009; Robinson et al., 2009).

Dectin-2 has been described to recognize α-mannans (Saijo et al., 2010). Dectin-2 has an EPN motif in the extracellular domain, which has been demonstrated to be involved in the binding to mannose/fucose containing glycoconjugates (Ariizumi et al., 2000a). Dectin-2 binds to zymosan in a Ca^{+2} dependent mechanism that is inhibited by mannose, fucose, and in higher concentrations, *N*-acetylglucosamine, glucose, and galactose. Dectin-2 binds efficiently to extensively mannosylated synthetic carbohydrates, such as $Man_9GlcNac_2$, while binding decreases deeply with the reduction in the mannose residues (McGreal et al., 2006). Binding assays have demonstrated the Dectin-2 binding to BSA conjugated to different monosaccharides. Dectin-2 shows maximal binding to mannosylated and fucosylated-BSA, while the binding to BSA conjugated to *N*-acetylglucosamine, glucose or *N*-acetylgalactosamine is greatly reduced (Lee et al., 2011). Dectin-2 is a receptor involved in the recognition of *Malassezia spp* and *C. albicans*, and the Dectin-2 ligands have demonstrated to be a glycoprotein containing *O*-linked α-1,2-mannobiose residues, for *Malassezia* (Ishikawa et al., 2013), and α-mannans, for *C. albicans* (Saijo et al., 2010; Zhu et al., 2013).

Recently, Dectin-2 has been demonstrated to cooperate with MCL for the recognition of fungal mannans. Dectin-2 and MCL bind to mannans, and also form heterodimeric complexes. Although the expression of each receptor can promote the recognition of *C. albicans* hyphae and mannans, their association confers a higher sensitivity to the recognition of mannans and *C. albicans* (Zhu et al., 2013). Thus, the cooperation between MCL and Dectin-2 must represent a general mechanism of interaction of CLRs, extending and amplifying leukocyte responses to fungal carbohydrates.

DECTIN-1

Dectin-1 was first identified by means of the isolation of mRNA selectively expressed in a Langerhans cell derived line (Ariizumi et al., 2000b). Dectin-1 is a type II transmembrane protein and its structure comprises one CTLD in the extracellular portion, and a cytoplasmic region that presents the signaling motif, hemi-ITAM (Sancho and Reis E Sousa, 2012). Dectin-1 is expressed in macrophages, dendritic cells, neutrophils, and eosinophils (Brown et al., 2002; Taylor et al., 2002; Willment et al., 2005). Dectin-1 mediated signaling promotes cytokine production (Brown et al., 2003; Rogers et al., 2005; Rosas et al., 2008; Goodridge et al., 2009a), generation of ROS (Gantner et al., 2003; Kennedy et al., 2007), phagocytosis (Brown and Gordon, 2001; Brown et al., 2002) and dendritic cell maturation (Yoshitomi et al., 2005). Thus, Dectin-1 acts as a PRR connecting the recognition of fungal exposed β-glucans to the leukocyte activation and adaptive immunity induction.

Differently from many CLRs, Dectin-1 binding to β-glucans does not require Ca^{+2}, although it occurs by means of the interaction of the extracellular CTLD with β1,3-glucans (Brown and Gordon, 2001; Adams et al., 2008). Surface plasmon resonance binding assays have demonstrated that Dectin-1 shows an extraordinary specificity to β1,3-glucans containing β1,6-branches, in contrast Dectin-1 do not bind mannans, pullulans, β1,6-glucans or β1,3/β1,4-glucans (Adams et al., 2008).

The minimal structure recognized by Dectin-1 is a β1,3-heptasaccharide of glucose with a terminal β1,6-glucose branch, and higher polymerization degrees increase the affinity of Dectin-1 by β1,3-glucose oligosaccharides (Adams et al., 2008).

The mechanism of activation of Dectin-1 involves the clustering of the receptor by aggregates of β-glucans (Rosas et al., 2008; Goodridge et al., 2011). Although Dectin-1 shows a high affinity for soluble β-glucans, for example glucan phosphate presents an IC50 for the competition of the Dectin-1 binding to glucans of about 2 pmol L^{-1} (Adams et al., 2008), soluble β-glucans act as antagonists of the activation induced by β-glucan particles (Brown et al., 2003; Gantner et al., 2003). The activation of Dectin-1 requires its own clustering by β-glucan particles and the exclusion of the tyrosine phosphatases CD45 and CD148. CD45 and CD148 play a dual role in the Dectin-1 signaling, promoting the basal activity of Src kinases through the remotion of an inhibitory phosphotyrosine, while upon the clustering of Dectin-1/β-glucan particles, they become excluded from the signaling cluster, what must permit the ITAM signaling pathway to proceed (Goodridge et al., 2011). Thus, the activation of Dectin-1 is regulated by the physical nature of its ligands, with β-glucan particles of 0.5 μm or larger being strong activators of Dectin-1 (Goodridge et al., 2011).

Dectin-1 mediated responses are specific for some myeloid cells. Bone marrow derived macrophages and elicited peritoneal macrophages do not present Dectin-1 mediated responses, in contrast, alveolar macrophages, resident peritoneal macrophages and dendritic cells show Dectin-1 dependent responses to β-glucans (Rosas et al., 2008; Goodridge et al., 2009a). GM-CSF and IFN-γ can promote Dectin-1 responsiveness to non-responding cells, such as bone marrow macrophages, indicating that the responses mediated by Dectin-1 are flexible, according the program of differentiation of myeloid cells (Rosas et al., 2008; Goodridge et al., 2009a).

MINCLE

Mincle was firstly identified as a macrophage expressed gene dependent on the activity of the transcriptional factor NF-IL6 (Matsumoto et al., 1999). Mincle is a type II transmembrane protein containing a short intracellular tail and a CLTD in the extracellular domain. Mincle induces cell signaling through the interaction with the FcRγ chain which contains ITAM motifs and thus promotes Syk activation, and the NF-κB and NFAT mediated transcription of cytokines (Yamasaki et al., 2008).

Mincle has been demonstrated to be involved in the fungal recognition (Bugarcic et al., 2008; Wells et al., 2008; Yamasaki et al., 2009). Soluble recombinant Mincle binds to *C. albicans* (Bugarcic et al., 2008). Mincle is required for the TNF production by macrophages in response to *C. albicans* and *Clec4e*$^{-/-}$ mice show a deficient clearance of *C. albicans* in an experimental model of infection (Wells et al., 2008). Mincle also recognizes the human commensal fungi, *Malassezia spp*. Mincle is able to confer NFAT activation by cell lines in response to *Malassezia spp*, besides *Clec4e*$^{-/-}$ macrophages shows impaired cytokine production in response to *Malassezia spp* stimulation. Supporting the *in vitro* data, *Clec4e*$^{-/-}$ mice show impaired leukocyte recruitment and cytokine production in a model of peritonitis induced by *Malassezia* challenge (Yamasaki et al., 2009). Recently, Ishikawa et al. have demonstrated that two *Malassezia* derived glycolipids are ligands for Mincle, a glyceroglycolipid containing the disaccharide gentiobiose joined to a glycerol backbone, which is acylated with C14 and C18 fatty acids, and a polar glycolipid composed by two mannosyl-10-hydroxy-octadecanoic acids and one dimannosyl-10-hydroxy-octadecanoic acid which are esterified to a mannitol core (Ishikawa et al., 2013). Although the ligands for Mincle have not been identified in other fungi, it seems reasonable that Mincle must recognize fungal glycolipids in other fungal pathogens, for example *C. albicans* (Bugarcic et al., 2008; Wells et al., 2008).

DC-SIGN (CD209)

DC-SIGN is a type II transmembrane protein and its extracellular domain carries one CRD in the COOH-terminal portion and seven repeats which form an extracellular stalk that has been implied in the oligomerization of DC-SIGN (Geijtenbeek et al., 2000; Mitchell et al., 2001). The DC-SIGN CRD presents an EPN motif, and as expected DC-SIGN binds mannose-containing glycoconjugates, as well fucosylated carbohydrates, such as Lewis antigens, in a Ca^{+2} dependent mechanism (Appelmelk et al., 2003; Guo et al., 2004). The intracellular amino-terminal domain bears motifs involved in the internalization, such as triacidic and di-leucine sequences and non-ITAM/ITIM tyrosine based motifs (Van Kooyk and Geijtenbeek, 2003). DC-SIGN is an endocytic receptor involved in the binding and internalization of many pathogens, and it is expressed by dendritic cells and macrophages (Kwon et al., 2002; Geijtenbeek et al., 2003; Tailleux et al., 2003; Tassaneetrithep et al., 2003), as well as mannosylated and fucosylated antigens (Appelmelk et al., 2003; Frison et al., 2003; Guo et al., 2004). Coherently with its role as an endocytic receptor, the DC-SIGN mediated binding is decreased in the low pH values found during endosomal acidification, what indicates that, upon internalization by DC-SIGN, the cargo is released in the endosomal vesicles (Guo et al., 2004). DC-SIGN mediated recognition of mannose containing ligands has been described to amplify the cytokine production induced by TLR activation, while fucosylated ligands inhibit the induction of pro-inflammatory cytokines, but amplify the expression of IL-10 (Gringhuis et al., 2009). Furthermore, DC-SIGN activation by mannosylated lipoarabinomannans (ManLAM) has been demonstrated to inhibit the dendritic cell maturation by LPS (Geijtenbeek et al., 2003). Thus, DC-SIGN triggering has complex effects, promoting the entry of pathogens, inducing the production of cytokines, particularly IL-10, while inhibiting dendritic maturation, and it is speculated that DC-SIGN targeting by some pathogens could promote immune evasion during the infection of dendritic cells (Van Kooyk and Geijtenbeek, 2003).

DC-SIGN is involved in the fungal recognition. Expression of DC-SIGN in non-phagocytic cells is able to promote binding and phagocytosis of *C. albicans*. Besides, DC-SIGN is recruited to the dendritic cell phagosomes containing *C. albicans,* and its blockade decreases the *C. albicans* binding and internalization (Cambi et al., 2003, 2008). *N*-linked mannans have been described as the glycoconjugates responsible for *C. albicans* binding and internalization by dendritic cells, since a decreased

binding of *C. albicans* is observed with deficient *N*-linked mannosylation strains (Cambi et al., 2008). In contrast, dendritic cells do not show any deficiency in the binding of *C. albicans* strains showing deficient *O*-linked mannosylation, lacking phosphomannans or terminal β1,2-mannoses. Interestingly, DC-SIGN mediated binding of *C. albicans* requires *N*-linked mannans, while intact *O*-linked mannans, phosphomannans or terminal β1,2 mannosides are dispensable. Although DC-SIGN is clearly involved in the recognition of *C. albicans* by dendritic cells, it cooperates with MR that makes the greatest contribution for the *C. albicans* binding to dendritic cells (Cambi et al., 2008). Altogether, these results indicate that MR and DC-SIGN mediate the recognition of *C. albicans N*-linked mannans promoting binding and internalization. DC-SIGN is also a receptor for the recognition of *A. fumigatus* conidia by human dendritic cells and macrophages. In contrast to *C. albicans*, DC-SIGN, but not MR receptor, is required for the binding of *A. fumigatus* conidia by human dendritic cells. *A. fumigatus* recognition by DC-SIGN is inhibited by purified mannans and galactomannans, and since *A. fumigatus* presents galactomannans as major mannose containing glycoconjugates, it must be an important target for DC-SIGN recognition by *A. fumigatus* (Serrano-Gómez et al., 2004).

The structural determinants for the DC-SIGN binding are the presence of terminal mannose or fucose residues. Binding assays have demonstrated that the soluble DC-SIGN CRD binds to mannans, mannose-containing oligosaccharides, and Lewis antigen structures, in a Ca^{+2} dependent mechanism (Appelmelk et al., 2003; Guo et al., 2004; Van Liempt et al., 2004). DC-SIGN binding to mannosylated proteins is inhibited by soluble fucose- and mannose-conjugated proteins, and in a lower extension by glucose-conjugates, while *N*-acetylgalactosamine- and *N*-acetylglucosamine-conjugates are ineffective as competitors (Lee et al., 2011). Crystallographic analyses demonstrate that mannose and fucose residues interact with the primary binding site in the CRD domain, in coordination with Ca^{+2} that interacts with two hydroxyl groups, while adjacent residues, such as galactose or mannose, present, respectively in high mannose containing structures, interact with secondary binding sites (Guo et al., 2004). Recognition of fucosylated Lewis structures requires strict interaction of a galactose with the secondary binding site, and the sialylation of the adjacent galactose abolishes the DC-SIGN binding to $Lewis^X$ and $Lewis^A$ ligands while sulfation reduces the binding (Appelmelk et al., 2003; Guo et al., 2004).

Although DC-SIGN has been pointed as a receptor involved in the binding and internalization of fungal pathogens, the roles of DC-SIGN in modulating dendritic cell and macrophage responses to fungi are still unknown. DC-SIGN has been implied in the induction of IL-10 and inhibition of dendritic cell maturation, so it would be interesting to evaluate the impact of DC-SIGN mediated recognition in the responses of dendritic cells and induction of T cell mediated responses to fungal pathogens. Alternatively, DC-SIGN could cooperate with other PRR, amplifying the pro-inflammatory induction in response to fungi, as previously observed with LAMs and TLR agonists.

CD11b/CD18 (MAC-1, CR3)

CD11b/CD18 is a member of the leukocyte specific integrins that share the β_2 common chain, also identified as CD18. CD11b/CD18 is a heterodimeric complex composed of the non-covalently associated type I proteins, the αM chain (CD11b) and the common chain CD18. It is expressed by leukocytes, including neutrophils, monocytes, macrophages, eosinophils, and NK cells (Ross, 2000; Hynes, 2002).

Besides its roles as a mediator of the leukocyte adhesion to the activated endothelium and the phagocytic receptor for iC3b opsonized particles (Holers, 2014), CD11b/CD18 has also been described to recognize β1,3-glucans. The αM chain presents two distinct domains involved in the recognition of the ligands by CD11b/CD18, the I-domain that is involved in the binding to iC3b, ICAM-1, and fibrinogen, while a distinct lectin domain has been implied in the recognition of β1,3-glucans, *N*-acetyl-*D*-glucosamine, glucose, and mannose (Thornton et al., 1996).

CD11b/CD18 has been demonstrated to be the major receptor involved in the binding of zymosan and *S. cerevisiae* yeasts, as well as the ROS production by these stimuli, by human neutrophils and macrophages, while Dectin-1 was demonstrated to be dispensable for these responses (Van Bruggen et al., 2009). Otherwise, the cooperation of Dectin-1 and CD11b/CD18 is necessary for neutrophil responses to zymosan and β-glucans, while macrophage recognition of β-glucans relies only in the Dectin-1 mediated recognition (Li et al., 2011). This mechanism requires the inside-out activation of CD11b/CD18 by Dectin-1 mediated recognition of β-glucans which promotes Vav1,3 activation and thus enables CD11b/CD18 binding and internalization of β-glucans by neutrophils, as well as ROS production (Li et al., 2011). In contrast with the recognition of β-glucan particles, the binding of soluble β-glucans by human monocytes and neutrophils has been demonstrated to be dependent on the β-glucan opsonization by iC3b which promotes CD11b/CD18 binding (Bose et al., 2013). Interestingly the responses of blood mononuclear cells to β-glucans particles requires the Dectin-1 mediated recognition, while soluble β-glucans require the CD11b/CD18 mediated detection (Bose et al., 2014). A similar role for Dectin-1 recognition of β-glucan particles, in contrast to a role for the complement for soluble β-glucans, has been observed for the antitumoral responses induced by the treatment with these β-glucans in murine experimental models (Qi et al., 2011).

While CD11b/CD18 has been convincingly demonstrated to be a receptor for β-glucans, there is still a considerable controversy about its role in the recognition of β-glucans, and this seems to extend in some experimental settings to the Dectin-1 mediated responses. Differences in the experimental settings must be responsible for the observed disparities, including (1) use of distinct β-glucan structures (particulated vs. soluble) (Rosas et al., 2008; Qi et al., 2011; Bose et al., 2013), heterogeneity and purity of the β-glucan particles, since zymosan and fungi are in fact highly heterogeneous and contains mannans, as well as chitin and lipids, furthermore zymosan is also a TLR activator (Van Bruggen et al., 2009), (3) presence of serum (a source of C3 that may trigger CD11b/CD18 activation even in situations where β-glucans were not recognized) (Bose et al., 2013), and (4) differences in the

cell types investigated (neutrophils vs. macrophages) (Qi et al., 2011). Thus, CD11b/CD18 and Dectin-1 are receptors involved in the recognition of β-glucans and their activities must dictate the outcome of immune responses during the fungal infections. Besides that, while CR3 has been demonstrated to recognize fungal β-glucans, it has also been described to be a receptor for the internalization of mycobacterial glycoconjugate-coated beads, such as mycobacterial PIM_2 or a succinylated glycopeptidolipid (Villeneuve et al., 2005). Thus, considering the ability that CR3 presents as a lectin receptor, it must also work as a receptor for fungal glycoconjugates other than β-glucans.

CD14

CD14 is a glycosylphosphatidylinositol (GPI) anchored membrane protein presenting an extracellular amino-terminal portion containing leucine rich repetitions that assume a horseshoe-like conformation carrying a large hydrophobic pocket (Kim et al., 2005; Kelley et al., 2013; Zanoni and Granucci, 2013). CD14 has been demonstrated to be a receptor for bacterial LPS, and although CD14 lacks an intracellular signaling region, it cooperates with the TLR4/MD2 complex, conferring a highly sensitive recognition of LPS (Akashi et al., 2003). CD14 has been also described to work as a co-receptor for the recognition mediated by TLR2 (Schröder et al., 2004), TLR3 (Lee et al., 2006), TLR7 and TLR9 (Baumann et al., 2010).

CD14 is also involved in the recognition of fungal carbohydrates and glycoconjugates. Recognition of *C. albicans* and *S. cerevisiae* mannans is dependent on CD14, LBP, and TLR4 which shows similarities with the LPS detection, thus suggesting that LBP mediates the transfer of soluble mannans to CD14 and TLR4 (Tada et al., 2002). CD14 has also been demonstrated to be required for the recognition of fungal α-glucans (Bittencourt et al., 2006). Coherently with the observations of the CD14 mediated recognition of fungal carbohydrates, CD14 is also involved in the recognition of the fungi *A. fumigatus* (Wang et al., 2001; Mambula et al., 2002) and *P. boydii* (Figueiredo et al., 2010).

The structural bases of the CD14 interactions with fungal carbohydrates are still unknown. Crystallographic analyses of CD14 have revealed a NH_2-terminal hydrophobic region buried in a horseshoe structure. Based on the proposed structure of CD14 and the structure of LPS, the large hydrophobic pocket seems to be site interacting with the lipid portion of LPS (Kim et al., 2005; Kelley et al., 2013). Otherwise, the hydrophilic O-antigen, a long and variable polysaccharide chain present in LPS, as well as peptidoglycan, have been described to interact with CD14 (Kitchens and Munford, 1995; Schwandner et al., 1999). The neighbor grooves and edges around the hydrophobic pocket are speculated to support hydrophilic interactions with polysaccharides (Kim et al., 2005; Kelley et al., 2013). Thus, although direct CD14 binding to mannans or α-glucans has not been evaluated, CD14 must

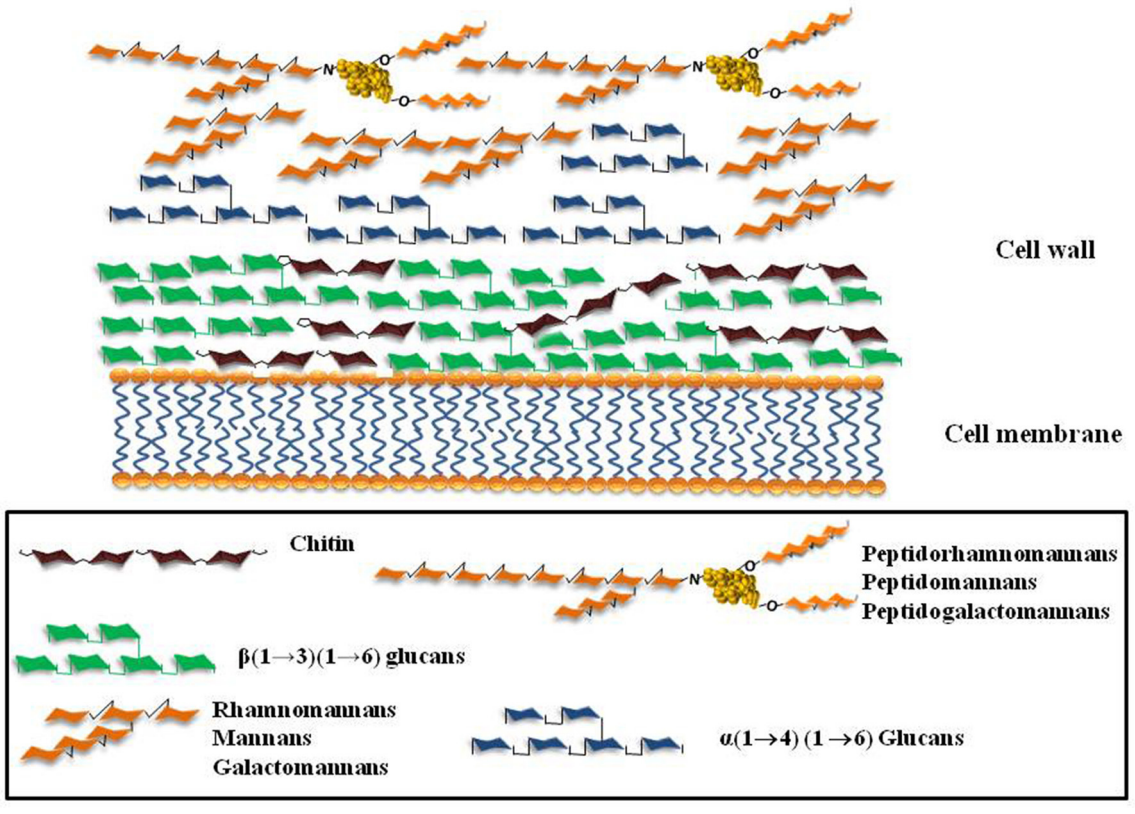

FIGURE 1 | Schematic representation of the major cell wall components of the fungi *A. fumigatus, C. albicans, Scedosporium/Pseudallescheria* complex.

be able to bind fungal polysaccharides using hydrophilic clefts. Based on the role of CD14 for the recognition of TLR ligands, it is probable that CD14 must bind fungal polysaccharides and load them into TLR4 or TLR2, thus promoting the signaling by these receptors.

THE RECOGNITION OF CHITIN, A PUZZLE INCLUDING SEVERAL CANDIDATES AND RESPONSES

While chitin and chitosan are clearly recognized by leukocytes, there is still a great controversy about the mechanisms of recognition of chitin. Silva et al. have demonstrated that chitin induces cytokine production by macrophages by a mechanism involving the recognition mediated by TLR2, MR, and Dectin-1 (Da Silva et al., 2009). In contrast, other studies have failed to demonstrate that highly purified chitin particles can induce pro-inflammatory cytokine production by peripheral mononuclear blood cells (Mora-Montes et al., 2011). Instead, *C. albicans* purified chitin has been demonstrated to inhibit the *C. albicans* induced cytokine production by blood mononuclear leukocytes (Mora-Montes et al., 2011). Highly purified chitin particles induce IL-10 production by macrophages and this requires the TLR9, MR, and NOD2 mediated recognition (Wagener et al., 2014). Chitin is able to reduce the inflammatory cell recruitment induced the *in vivo* LPS challenge. Corroborating the anti-inflammatory properties of chitin particles, a chitin deficient *C. albicans* strain induces an increased TNF release, what is mimicked by the inhibition of chitinases during the stimulation with the wild-type *C. albicans* strain (Wagener et al., 2014).

Many questions remain about the chitin recognition by leukocytes. The commonly used commercial sources of chitin have been demonstrated to carry contaminants of glucose, mannose, and undefined molecules what may contribute for some responses, such as cytokine induction by macrophages (Mora-Montes et al., 2011; Wagener et al., 2014). Furthermore, although macrophages are able to phagocytose chitin particles, the mechanisms involved are still unknown. The direct interaction of proposed receptors with chitin particles has also not been demonstrated. Even so TLR2 and TLR9 have been pointed as receptors for chitin induced cytokine release (Da Silva et al., 2009; Wagener et al., 2014), the eosinophil recruitment induced by chitin is MyD88 independent (Reese et al., 2007), thus excluding a role for TLR2 and TLR9 in the chitin induced eosinophilic inflammation. Besides, many aspects may influence the results obtained with chitin particles, such as (1) the presence of contaminants,

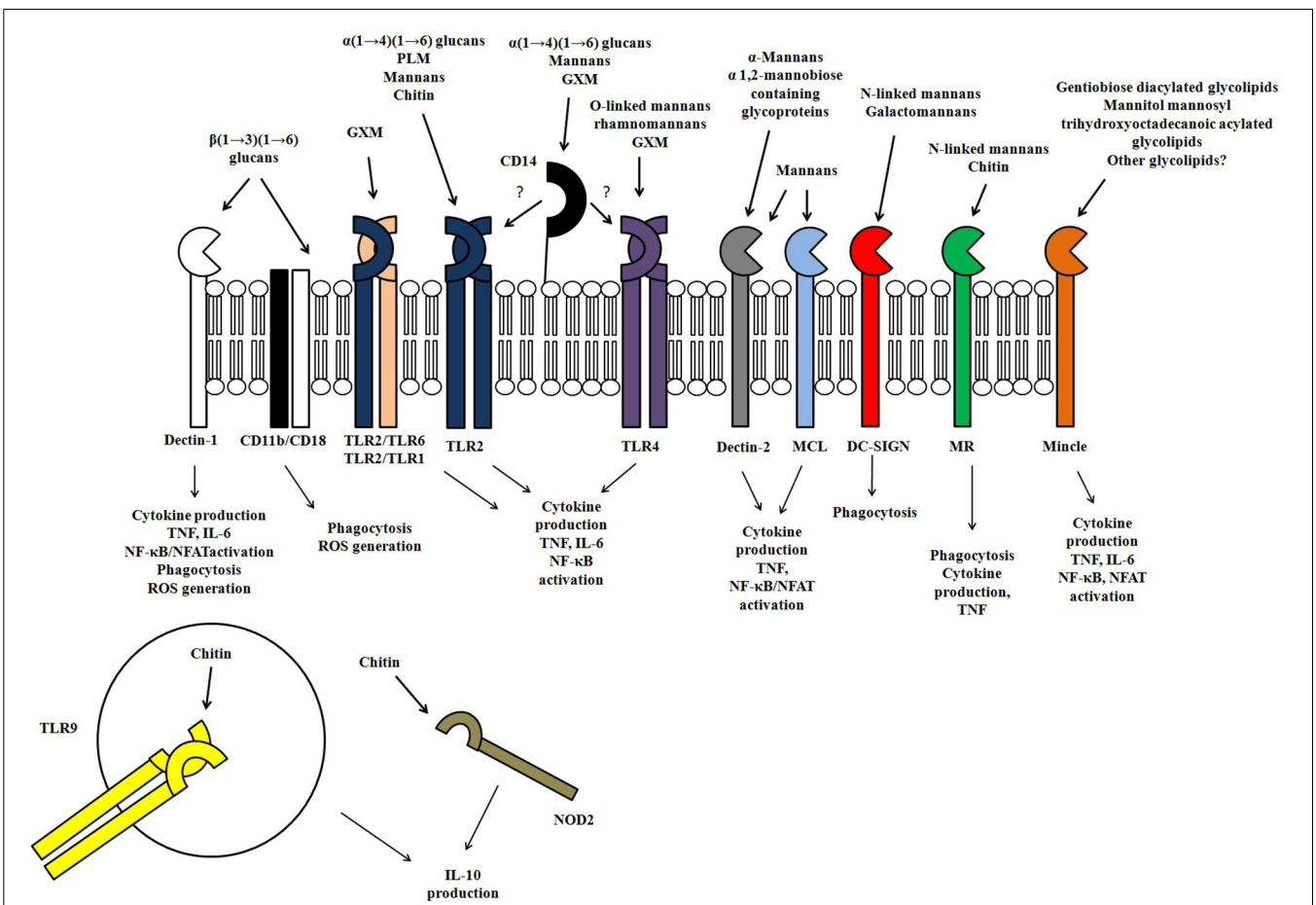

FIGURE 2 | Representation of the receptors involved in the innate immune recognition of fungal polysaccharides and glycoconjugates, as well the responses evoked in response to fungal polysaccharides.

(2) responses to distinct cell types (blood mononuclear cells, peritoneal macrophages, macrophage cell lines), (3) size and acetylation of chitin particles. Thus, although chitin represents a target for the innate immune recognition, more extensive analyses are required to characterize the immune responses to chitin and the receptors involved.

CONCLUSIONS

Polysaccharides and glycoconjugates are the major components of the fungal surface. Their structures vary among filamentous fungi and yeast and also among fungal species (**Figure 1**). The variety of carbohydrate structures present in the different fungal pathogens offers exceptional targets for the innate immune recognition which has evolved to recognize specific fungal glycans through a plethora of different receptors (**Figure 2**). Thus, the identification of fungal carbohydrates and their recognition by pattern recognition receptors must bring important contributions to the comprehension of the pathogenesis and immunity to fungal infections, and this must reveal new opportunities for the development of new classes of immunomodulators, antigens, and adjuvants.

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Glycan analysis of *Fonsecaea monophora* from clinical and environmental origins reveals different structural profile and human antigenic response

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Dematiaceous fungi constitute a large and heterogeneous group, characterized by having a dark pigment, the dihydroxynaftalen melanin—DHN, inside their cell walls. In nature they are found mainly as soil microbiota or decomposing organic matter, and are spread in tropical and subtropical regions. The fungus *Fonsecaea monophora* causes chromoblastomycosis in humans, and possesses essential mechanisms that may enhance pathogenicity, proliferation and dissemination inside the host. Glycoconjugates confer important properties to these pathogenic microorganisms. In this work, structural characterization of glycan structures present in two different strains of *F. monophora* MMHC82 and FE5p4, from clinical and environmental origins, respectively, was performed. Each one were grown on Minimal Medium (MM) and Czapeck-Dox (CD) medium, and the water soluble cell wall glycoconjugates and exopolysaccharides (EPS) were evaluated by NMR, methylation and principal component analysis (PCA). By combining the methylation and 2D NMR analyses, it was possible to visualize the glycosidic profiles of the complex carbohydrate mixtures. Significant differences were observed in β-D-Gal*f*-(1→5) and (1→6) linkages, α- and β-D-Glc*p*-(1→3), (1→4), and (1→6) units, as well as in α-D-Man*p*. PCA from ^{1}H-NMR data showed that MMHC82 from CD medium showed a higher variation in the cell wall carbohydrates, mainly related to O-2 substituted β-D-Gal*f* (δ 106.0/5.23 and δ 105.3/5.23) units. In order to investigate the antigenic response of the glycoconjugates, these were screened against serum from chromoblastomycosis patients. The antigen which contained the cell wall of MMHC82 grown in MM had β-D-Man*p* units that promoted higher antigenic response. The distribution of these fungal species in nature and the knowledge of how cell wall polysaccharides and glycoconjugates structure vary, may contribute to the better understanding and the elucidation of the pathology caused by this fungus.

Keywords: glycan, PCA, dematiaceous fungi, NMR, antigenic response

INTRODUCTION

The family Herpotrichiellaceae contains a large group of fungi whose main characteristic is the presence of melanized hyphae and conidia. Some dematiaceous fungi belonging to this family are pathogenic to vertebrate hosts. Pathogenic black yeasts are part of this group and during a period of their life cycle they show a yeast-like development. These black fungi are able to withstand adverse growth conditions and cause a variety of infections (Vicente et al., 2001, 2008; López-Ribot et al., 2004; Cunha et al., 2010; Sun et al., 2011). Most dematiaceous fungi are present in soil and found as decomposing vegetation-inhabiting saprobes. Secondary host infection is dependent mainly on three factors: host resistance, amount of inoculum and fungal virulence (Alviano et al., 2003). Those factors are influenced by the composition of the culture media (Viccini et al., 2009).

The principal subcutaneous mycoses caused by these fungi are chromoblastomycosis and phaeohyphomycosis. Chromoblastomycosis is a chronic mycotic infection with a slow evolution that affects skin and subcutaneous cell tissue (Esterre and Queiroz-Telles, 2006; Ameen, 2009; Queiroz-Telles et al., 2009; Sharma et al., 2010; Sugiyama et al., 2011). Phaeohyphomycosis was proposed by Ajello (Ajello et al., 1974) as cutaneous and subcutaneous infections, the most frequent ones and the deepest (systemic), acute or chronic infections, caused by a wide variety of dematiaceous fungi (Revankar et al., 2004).

Cell wall structure is vital for all fungi and is the first cell barrier with its surrounding environment, protecting it from environmental stress (Pinto et al., 2008). Polysaccharides are associated with polypeptides, constituting the cell wall glycoproteins, forming the external layers that are involved in different types of interactions with the extracellular environment. Several of

these molecules are immunostimulatory compounds with a great potential, such as pathogenicity regulators and determinants for the host immune response (Silva et al., 2008).

Some of these glycan-containing molecules can be recognized by antibodies from patients and can be used to diagnose fungal infections. Proteins and glycoproteins exposed on the external layers are involved in various types of fungal interactions. These glycoproteins are known to influence the immune response (Alviano et al., 2004; López-Ribot et al., 2004; Pinto et al., 2008).

In this context, we evaluated the growth of strains from clinical and environmental origins of the fungus *Fonsecaea monophora* and studied the water-soluble polysaccharides in two different culture media. Principal component analysis (PCA) and immunological assays were performed with the exopolysaccharides (EPS) and soluble cell wall polysaccharides. The differences between these strains could lead to the understanding of the physiological and virulence mechanisms involved regarding both saprobe and pathogenic strains.

MATERIALS AND METHODS

FUNGAL STRAINS

Two strains of *F. monophora*, of clinical and environmental origins, were used: strain MMHC82 (CBS 102248), isolated from patients with chromoblastomycosis lesions, was kindly furnished by the laboratory of Micology of the Universidade Federal do Paraná Clinical Hospital (HC-UFPR/Curitiba-PR, Brazil), and strain FE5p4 (CBS 102225/DH 11584) isolated from decomposing wood of the Centro Nacional de Pesquisas de Florestas (CNPF/EMBRAPA Colombo-PR, Brazil). These samples have already been morphologically and genetically characterized by CBS (Central Bureau Voor Schimmelcutures, Institute of the Royal Netherlands Academy of Arts and Science), (De Hoog et al., 2004; Vicente et al., 2008).

GROWTH CURVES AND EPS PRODUCTION

Each strain was grown in Sabouraud medium for 48 h at 37°C, 5 mL of each pre-inoculum with the strain MMHC82 and FE5p4 was inoculated in 250 mL of either Czapeck-Dox (CD) and Minimum Medium (MM), incubated at 37°C and kept at constant agitation for 48 h. An aliquot (19 mL) was removed from each culture and 1 mL was used to measure the sugar consumption. The remaining sample (18 mL) was used for EPS and cell wall polysaccharide isolation. The solution was filtered (Whatman filter paper grade 1) and the supernatant precipitated with 3 volumes of ethanol. The process was monitored every 48 h for 20 days. The growth and EPS production patterns were assembled based on the biomass and EPS (dry weight, obtained after freeze-drying). In addition to the growth curves and the EPS production, measurements were also obtained on the consumption of sucrose in the CD medium and glucose on the MM medium. Glucose was measured based on glucose oxidase method (DiaSys Diagnostic Systems GMBH & Co.KG), which forms gluconic acid and H_2O_2. The latter reacts with the phenol and 4-amineantipyrine, giving rise to p-benzoquinone-monoimine antipyrine (red color) and the concentration of residual glucose determined, in mg/ml, by the absorbance measured at 500 nm (Viccini et al., 2009). Sucrose consumption was determined by Silica-Gel 60G TLC plates (Merck), with sucrose solutions in different concentrations as standard (2.5–10 $mg.ml^{-1}$). Plates were developed with AcOEt:*n*-PrOH:AcOH:H_2O (4:2:2:1, v/v), and detection of carbohydrates was performed with orcinol-H_2SO_4 treatment (Sassaki et al., 2011). The plates were analyzed by densitometry using the Scion Image Program (Scion Corporation, Maryland, USA) and the results plotted in Microsoft Excel 2007 (Sassaki et al., 2005).

EPS AND MYCELIUM POLYSACCHARIDE EXTRACTION

Cell free medium was obtained after centrifugation at 12.000 × g for 20 min and the supernatant was submitted to cold ethanolic precipitation (3:1, v/v) and left overnight at −20°C. The latter gave yield to the native exopolysaccharide, which was centrifuged at 8.400 × g for 10 min. EPS purification was conducted after dialyzed against tap water using membranes with 16 kDa cut-off for 48 h, followed by lyophilization to measure the EPS dry weight production. Mycelia polysaccharides were obtained by aqueous extraction conducted in an autoclave under a pressure of 1 atm at 120°C for 40 min, which assures fungi inactivation and extraction of water-soluble polysaccharides in one step (Farres et al., 1996). The polysaccharides were obtained after precipitation with ethanol (3:1, v/v) of the supernatant solution resulted after removing the mycelia by centrifugation.

NMR SPECTROSCOPY

NMR spectra were obtained with a Bruker 400 MHz AVANCE *III* NMR spectrometer with a 5 mm inverse Z gradient probe. The samples were deuterium exchanged by repeated dissolution in D_2O and freeze-drying, finally the samples were dissolved in D_2O solution containing Na^0 1 mM. ^{1}H-NMR chemical shifts of signals are expressed in δ (ppm), relative to trimethylsilyl propionate sodium salt TMSP (δ = 0) at 70°C. Spectra were acquired using 256 scans to give a S/N ratio of 300 (90° pulse, relaxation delay = 4.0 s, number of time domain points = 65476 and acquisition time = 7.7 s). Integration of H-1 area was performed without tube rotation and respecting a HDO signal with a medium half line width varying from 2.5 to 3.5 Hz after Lorentzian deconvolution and post Fourier transformation. 2D NMR experiment was carried out using HSQC, heteronuclear correlation via double inept transfer with decoupling during acquisition, using trim pulses in inept transfer (hsqcetgpsi) program recorded for quadrature detection in the indirect dimension and acquired using 32 scans per series of 2 K × 400 data points, with zero filling in F1 (4 K) prior to Fourier transformation (Sassaki et al., 2011).

METHYLATION ANALYSIS

Per-*O*-methylation of each isolated polysaccharide fraction (10 mg each) was carried out using NaOH-Me_2SO-MeI (Ciucanu and Kerek, 1984). After 30 min at 25°C with vigorous stirring, the mixture was maintained overnight at 25°C. The reaction was interrupted by the addition of water, neutralized with AcOH, dialyzed against distilled water and freeze-dried. The products were submitted to one more cycle of methylation, followed by partition between $CHCl_3$ and water. The per-*O*-methylated derivatives were hydrolyzed with 50% v/v aq. H_2SO_4 (0.5 ml v/v, 1 h, 0°C), followed by a dilution until it reached 5.5% (addition of 4.0 ml of

distilled H_2O). The solution was kept at 100°C for 12 h and was then neutralized with $BaCO_3$, filtered and the filtrate evaporated to dryness. The resulting mixture of *O*-methylaldoses was reduced with $NaBD_4$ and acetylated with Ac_2O-pyridine to give a mixture of partially *O*-methylated alditol acetates (PMAAs) which were analyzed on a GC-MS Varian Saturn 2000R using a DB-225 capillary column (30 m × 0.25 mm i.d.), held at 50°C during injection and then programmed at 40°C.min^{-1} to 215°C (constant temperature). PMAAs electron impact (EI) spectra were obtained at 70 eV at 200°C, and post-run analysis was performed with a Saturn Workstation 5.1, the identification of PMAAs being confirmed using selected PMAAs mixtures, according to Sassaki et al. (2005).

MONOSACCHARIDE ANALYSIS

Cell-wall glycoconjugates (0.1 mg) were hydrolyzed with 2 M trifluoroacetic acid (1 mL) at 100°C for 8 h. After evaporating the acid, the resulting monosaccharides were converted to their alditol acetates through successive $NaBH_4$ reduction and acetylation with Ac_2O-pyridine and then analyzed by GC-MS. Monosaccharide analyses were performed using a Varian 4000 MS equipped with 30 m × 0.25 mm low bleed/MS capillary columns. The alditol-acetates were submitted to GC-MS analysis using capillary columns of CP-Sil-5CB programmed as follows: injector 250°C, oven start at 50°C (hold 2 min) to 90°C (20°C.min^{-1}, then held for 1 min), 280°C (5°C.min^{-1}, then held for 2 min) and to 310°C (3°C.min^{-1}, then held for 5 min) (Sassaki et al., 2008). EI spectra were obtained at 70 eV at 200°C. Post-run analysis was performed with a Saturn Workstation.

PRINCIPAL COMPONENT ANALYSIS

Chemometric analysis, chemometrics or multivariate analysis is a valuable mathematical statistics tool, which along with different analytical techniques allows several variables to be analyzed in a single sample. In many cases, the visual inspection of NMR data reveals only a small amount of information. Thus, statistical methods have been used to extract the maximum of information from these data.

The principal component analysis (PCA) is the most widely used method among multivariate techniques. PCA is an additive linear method in the sense that each principal compound has a share of importance in the data set. Generally, a small quantity of principal components totalizes over 90% of the total variance, and in these cases, data can be resized in small PCs, thus reducing the dataset dimension.

PCA analysis from NMR data was performed on AMIX v 3.8.3 (Bruker) and input variables were obtained directly from the raw NMR data. The buckets were built using a width of 0.01 ppm, ranging from 5.50 to 4.70 ppm. The method of integration was scaling in relation to the total intensity of signals.

HUMAN SERUM

For the immunological assays 12 sera samples obtained from chromoblastomycosis patients were used. Negative control was performed in parallel using six sera samples from disease-free individuals. All samples were furnished by the Laboratory of Micology of the HC-UFPR. The use of all biological material obtained from patients was approved by the Human Research Ethics Committee of the HC-UFPR, registry number 2126.021/2010-01. The inclusion criteria used by the serum bank of the Clinical Hospital was the confirmation for the diagnosis of chromoblastomycosis by performing direct mycological, culture and/or histopathological examination. The exclusion criteria used were negative results of the above examinations.

ANTIBODY PRODUCTION AND EVALUATION

Sera samples from chromoblastomycosis patients were evaluated in regard to their capacity to form immune complexes by the reaction of each serum antibody and glycoconjugates obtained from fungal cell wall of *F. monophora* strains. ELISA tests were performed with the glycoconjugate antigens present in *F. monophora*. 96-well plates were coated with 100 μL of a 10 μg.ml^{-1} carbohydrate-containing antigens for 12–16 h at 4°C. After the coating period, the polystyrene plate was rinsed thrice with a wash buffer (0.05% Tween saline) and blocked with a 2% casein solution diluted in PBS for 2 h at 37°C. The plate was rinsed again as described above and incubated with sera from the patients, diluted into incubation buffer (0.25% casein diluted in PBS, 0.05% Tween 20, Bio-Rad, Hercules, CA). As a negative control, sera samples from disease-free individuals were used. The second antibody added to the plate consisted of anti-human IgG immune Fc specific immunoglobulins with peroxidase (Sigma, St. Louis, MO) diluted 1:1000 into incubation buffer. The enzymatic activity was evaluated using ortophenilen-diamine (OPD) solution; after 15 min of incubation, the reaction was halted by the addition of 20 μL of diluted sulfuric acid (1:20) and the absorbance was measured at 490 nm.

RESULTS AND DISCUSSION

GROWTH EVALUATION AND EPS PRODUCTION FOR FE5p4 AND MMHC82 STRAINS

Biomass increased significantly after the eighth day of culture, and the best medium to obtain biomass, for both FE5p4 and MMHC82 strains, was MM (**Figures 1A,B**, respectively). It was observed that the addition of an organic substrate to the medium (sucrose or glucose), increases biomass proportionally, as well as EPS production, mainly after the fourth day.

Also, glucose levels on MM decreased throughout the 20 days of culture (**Figures 1A,B**), and was nearly depleted at the twentieth day. In CD medium, sucrose consumption was also assessed, and similarly decreased throughout the 20 days. The lack of a carbon source in the medium may explain the decrease in the amount of EPS recovered from the cultures (**Figures 1A,B**), probably due to the use of the polysaccharide as a carbon source.

A higher production of EPS was obtained for MM cultures when compared to CD. According to Silva and coworkers (Silva et al., 2006), the carbon source can determine the amount of polysaccharides formed, as well as the glycosidic composition of the synthesized polymer.

Since the sugar sources did not deplete completely, as shown on **Figures 1A,B**, the EPS production was maintained for both strains during their exponential growth phases. The highest amounts were found in the sixteenth, seventeenth, and eighteenth days. However, after the twentieth day, EPS production decreased

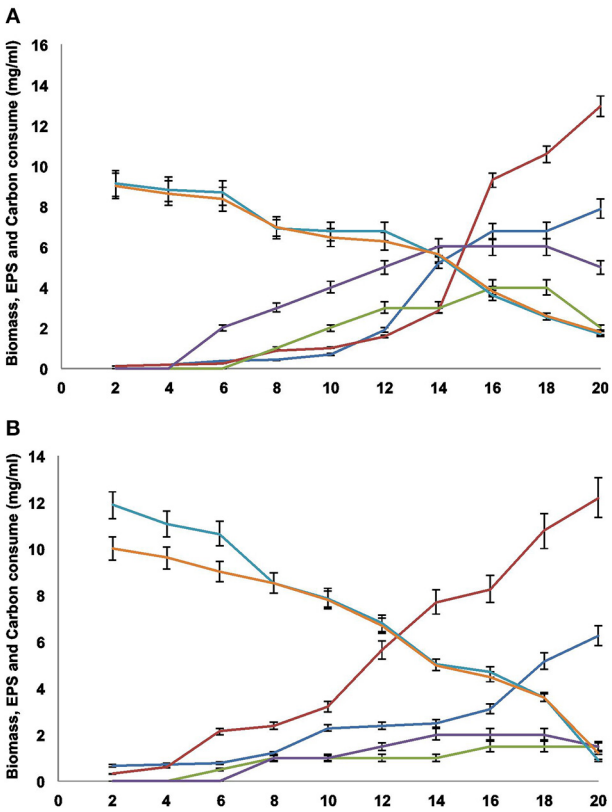

FIGURE 1 | (A) FE5p4 and **(B)** MMHC82. Profiles for (■) biomass MM, (♦) biomass CD, (X) EPS MM, (▲) EPS CD, (●) sucrose consumption and (×) glucose consumption. The results are expressed as the average of triplicates±SE.

significantly, probably due to degradation by the fungi and the use of EPS as carbon source in response to low sugar levels in the media, as discussed previously. This physiological behavior may be described as a survival strategy for these microorganisms, since EPS may act as an energy reserve during cellular stress. As reported by Yurlova and de Hoog (Yurlova and De Hoog, 2002; De Hoog et al., 2004), differences on EPS production using different culture media were found, however timed data was only acquired by the end of the fifth day of culture, not throughout the entire growth period.

MONOSACCHARIDE COMPOSITION OF EPS AND CELL WALL POLYSACCHARIDES

The EPS obtained from both strains showed high amounts of mannose, galactose, and glucose either for MM and CD cultures. The main differences observed were the higher relative amount of galactose found for strain MMHC82 in comparison to glucose when growth on both CD and MM medium. The EPS produced by strain Fe5p4 showed a higher amount of glucose when cultivated on CD medium compared to MM medium. For both strains, independent of growth condition, the most abundant monosaccharide found on EPS composition was mannose.

In previous studies performed by Alviano et al. (1991, 2003), which evaluated melanin and carbohydrate composition of *F. monophora* exopolysaccharides, similar results were found. According to Barreto-Bergter et al. (2008) carbohydrates from *Scedosporium prolificans*, an opportunist pathogen that causes a localized infection in tissues and bones of immunocompromised patients, were compared to those from *Pseudallescheria boydii* and they observed as primary monosaccharides mannose and rhamnose, followed by galactose and glucose (Barreto-Bergter et al., 2008)

Cell wall polysaccharides produced by fungal strains used in this work, grown on both MM and CD medium, had a higher relative amount of glucose when compared to mannose and galactose (Table 1 in Supplementary Material). A similar composition has been previously reported for the cell wall of other species of pathogenic fungi, such as *Histoplasma capsulatum* (Gorocica et al., 2009). However, for *Aspergillus fumigatus, A. wentii* and *Chaetosartory chrysella*, mannose-containing molecules were also found forming cell walls as galactomannans with distinct chemical and physical properties (Gomez-Miranda et al., 2003).

NUCLEAR MAGNETIC RESONANCE AND METHYLATION ANALYSIS OF THE CELL WALL POLYSACCHARIDES

Besides the determination of the monosaccharide composition, methylation and NMR analyses were performed in order to determine the structural characteristics of the cell wall polysaccharides obtained from both strains grown on MM and CD cultures (Table 2 in Supplementary Material, **Figures 2A–D**).

The anomeric region for the polysaccharide of strain MMHC82 grown on CD medium showed characteristic signals with chemical shifts attributed to α-linked monosaccharides at δ 101.3/5.21 and δ 98.4/5.01 as well as β-linked units at δ 108.4/5.10, δ 106.0/5.23, δ 105.3/5.21, δ 103.5/4.73, and δ 103.0/4.50 (**Figure 2A**) (Gorin and Mazurek, 1975). Similar signals were found for this strain when grown on MM, however with different relative intensities among the peaks (**Figure 2B**).

The main difference found for this strain when comparing growth condition is the presence of signals corresponding to β-D-Gal*f* units (δ 106.0/5.23 and δ 105.3/5.23), on CD medium, in contrast to the signals attributed to α-D-Man*p* (δ 99.1/5.11) and β-D-Man*p* (δ 99.1/4.82 and 101.3/5.11) (Viccini et al., 2009) found for the MM samples (**Figures 2A,B**).

The spectrum obtained for strain FE5p4 grown in CD medium had H1/C1 cross peaks corresponding to α-anomeric configuration at δ 101.1/5.22 and δ 98.4/5.01, and also signals attributed to β-linked units at δ 108.3/5.10, δ 105.9/5.23, and δ 103.0/4.59, which shows a similar profile to the spectrum obtained for strain MMHC82, also with great differences on the peaks relative intensities (**Figure 2C**). Examination of the 2D NMR spectrum profile for MM-grown samples showed major differences especially regarding the presence of anomeric signals attributed to α-D-Man*p* units, at δ 101.1/5.22 (**Figures 2A,C**) (Barreto-Bergter et al., 2008). On CD medium, the differentiation of the strains were related to α-D-Man*p* (1→6) and β-D-Man*p* (1→2) (**Figures 2B,D**), (Viccini et al., 2009).

Biomolecules containing D-Gal*f* residues have been described as important antigens among several human pathogenic fungi. Since Gal*f* is not found in human hosts, these molecules could be

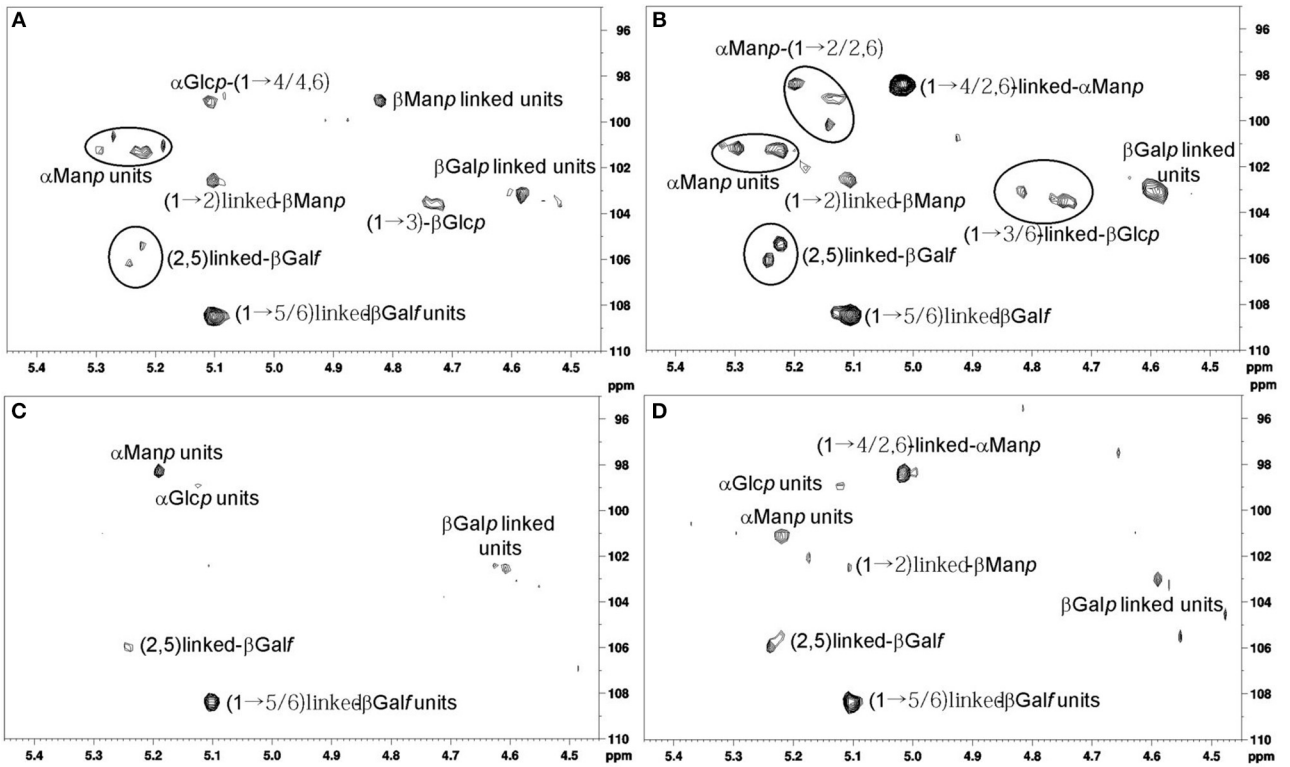

FIGURE 2 | (A) Anomeric region analysis of strain MMHC82 spectrum for MM. **(B)** Anomeric region analysis of strain MMHC82 spectrum for CD medium. **(C)** Anomeric region analysis of strain FE5p4 spectrum for MM. **(D)** Anomeric region analysis of strain FE5p4 spectrum for CD medium.

a differential antigen between fungus and host, allowing cytokine induction and immunological activation. Monoclonal antibodies tested against these structures have shown to be effective on the detection of this type of antigen. The same has been reported for biomolecules containing D-Man*p* units, since it can stimulate antibody production and T-cell activation thus preventing the development of the infection process by this fungus (Stynen et al., 1992).

Bittencourt et al. (2006) characterized the α-D-glucan structure of a polysaccharide found on the cell wall of *Pseudallescheria boydii*, and assessed its role in the innate immune response, stating that the lack of such structures diminishes phagocytosis. This suggests that α-D-glucan plays an important role in macrophage and dendritic cell stimulation for the immune system. It has also been reported that pathogenicity in fungi is dependent on β-D-glucans (Stahl and Ezekowitz, 1998).

Methylation analysis of the cell wall polysaccharides obtained from the strain FE5p4 showed a backbone containing D-Man*p* (Barreto-Bergter et al., 2008; Gorin et al., 2010) and D-Gal*p* residues, both C6-linked (Leal et al., 2008), with branching units of D-Man*p,* D-Gal*f*, and D-Glc*p* at O-3. For the same strain grown on MM medium, the cell wall polysaccharides showed similar structural characteristics, with backbone units being 3-*O*-substituted by D-Man*p*, D-Gal*f*, and D-Glc*p* in different ratios.

Strain MMHC82 produced a mannan as the main cell wall component, with a backbone predominantly composed of D-Man*p* (1→2) and D-Man*p* (1→6)-linked when grown on CD medium, and D-Man*p* (1→2) and D-Man*p* (1→4)-linked on MM culture. Regarding the 3-*O*-substitutions in D-Man*p*, D-Gal*f*, and D-Glc*p* units were most present in the cell wall polysaccharide produced by this strain on either medium, with 6-*O*- substitutions appearing for the MM condition.

CHEMOMETRIC ANALYSIS

Principal component analysis (PCA) was performed using the acquired NMR data from cell wall samples. PCA results suggest that the culture conditions promote differentiation of the polysaccharide structures on both environmental and pathogenic strains. PC1 of the NMR data explains approximately 81.6% of the total variance and PC2 explain 17.5% of the variance (**Figure 3**).

The ^{1}H-NMR results showed that both strains, when grown on MM medium, shows a significant structural variability on cell wall polysaccharide structures. As shown on **Figure 3**, strain FE5p4 (non-pathogenic), seems to be related to the pathogenic strain when the water soluble cell wall polysaccharides, identified from growth on CD, are compared, suggesting that MM medium is more suitable to show the differences between the polysaccharide synthesized by each strain.

ANTIBODIES EVALUATION

ELISA testing was performed using sera from patients with chromoblastomycosis, as well as from disease-free individuals as control to check for reactivity against the water-soluble cell wall

polysaccharides (antigens) of strains MMHC82 and FE5p4 of the fungus *F. monophora*.

Antigens obtained from both strains grown on each condition were denominated as follows: Antigen 1 (Ag1), obtained from strain MMHC82 grown on MM; Antigen 2 (Ag2), from strain MMHC82 on CD medium; Antigen 3 (Ag3), from strain FE5p4 grown on MM; and Antigen 4 (Ag4), from strain FE5p4 on CD medium (**Figure 4** and **Figure S1**).

The results for the antigen-antibody reaction using sera from chromoblastomycosis patients showed a great significance. Polysaccharide antigen Ag1 showed the best antigenic response to sera from patients with chromoblastomycosis, giving rise to a 91% increase on absorbance when compared to results from the control group. Ag2, Ag3, and Ag4 were shown to be much less reactive to the antibodies present in the sera of infected patients, and no significant difference was observed on absorbance in comparison to the non-chromoblastomycosis control groups. The majority of sera samples obtained from patients showed markedly reactivity against the antigens, particularly against Ag1, although there were some sera from patients that showed low reactivity to all the antigens (P5, P11, and P12), which can be a consequence of many factors, such as geographic prevalence of the fungal strains, antigenic variation and individual-specific differences in immune response (Vera-Cabrera et al., 2012; Wevers et al., 2014).

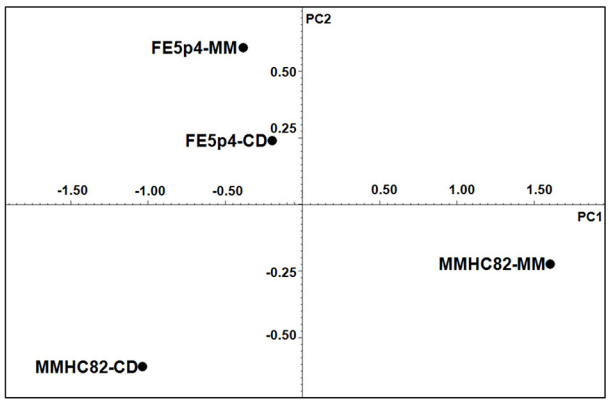

FIGURE 3 | Chemometric analysis from ^{1}H NMR spectra.

CONCLUSIONS

When grown on MM and CD medium, the fungus *F. monophora* produces an EPS containing mannose, galactose and glucose in variable proportions depending on the strain. MM has shown to be the best of the tested conditions to produce these polymers and also greater amount of biomass.

Water soluble cell wall polysaccharides from both strains also had glucose, mannose and galactose in their composition. Methylation analysis and NMR of the polysaccharides showed a backbone with D-Man (1→6) and D-Gal*f* (1→6) units with 3-*O*-substitutions, indicating the presence of a galactofuranan, together with the presence of a (1→4)-linked α-D-glucan branched at O-6, a "glycogen-like" polysaccharide, a β-D-glucan (1→3), (1→6)-linked and also the presence of α-D-mannans (1→6) substituted at O-2 and O-4.

Chemometric analysis of the ^{1}H-NMR data showed that strain MMHC82 growth in MM medium, had great variation in its biochemical profile regarding cell wall polysaccharides, discriminating the clinical from the environmental strain as observed in PC1 which explains 81.6% of the total variance.

The antigen from water-soluble cell wall polysaccharide obtained from the strain MMHC82 (pathogenic) when grown on MM was the only sample that showed high antigenic response against the antibodies found in the sera from patients with chromoblastomycosis. All other cell wall polysaccharides tested were undifferentiated from the control experiment. These results were attributed to the presence of β-D-Man*p* in Ag1 which is not found in the other antigen samples, as supported by NMR and methylation analysis.

The results presented in this manuscript highlight the importance to study macromolecules from clinical and environmental fungal strains. The PCA results proved useful, as they could discriminate between strains with different ecological

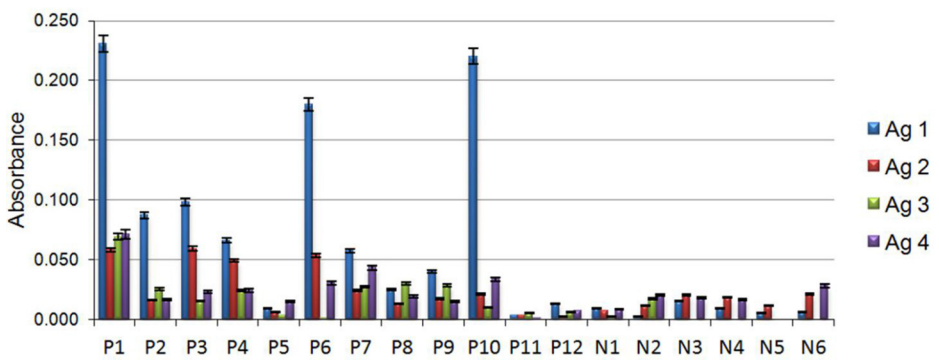

FIGURE 4 | Individual data from immunoassays with antigens existing on the serum of patients with chromoblastomycosis (P), patients without chromoblastomycosis (N). Antigens were assessed to determine their capacity of reaction in the formation of immune complexes with wall polysaccharides of the fungus *F. monophora*. Antigen 1 (Ag1), belonging to the cell wall of strain MMHC82 drawn from MM, antigen 2 (Ag2), cell wall antigen of strain MMHC82 from CD medium, antigen 3 (Ag3), cell wall antigen from strain FE5p4 from MM and antigen 4 (Ag4), cell wall antigen from strain FE5p4 from CD medium.

characteristics using the data obtained from ^{1}H-NMR analysis. Another important conclusion of this study was to show that the β-D-Man*p* seems to play an important role on the antigenic response. The structural diversity of the polysaccharides found for both strains using two media, supports the use of these macromolecules as physiological fingerprints, aiming toward both taxonomic identification and antigenic studies.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fcimb.2014.00153/abstract

Figure S1 | Immunoassay with antigens existing on the serum of patients with chromoblastomycosis (black bars), patients without chromoblastomycosis (white bars). Antigens were assessed to determine their capacity of reaction in the formation of immune complexes with wall polysaccharides of the fungus *F. monophora*. Antigen 1 (Ag1), belonging to the cell wall of strain MMHC82 drawn from MM, antigen 2 (Ag2), cell wall antigen of strain MMHC82 from CD medium, antigen 3 (Ag3), cell wall antigen from strain FE5p4 from MM and antigen 4 (Ag4), cell wall antigen from strain FE5p4 from CD medium. Results correspond to the medium ± E.P.M of 6–12 samples. $^{***}p < 0.001$ in relation to the control group, serum of non-chromoblastomycosis patient (ANOVA of a pathway followed by *t*-test with a Bonferroni adjustment).

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Glycolipids from seaweeds and their potential biotechnological applications

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Marine macroalgae, or seaweeds, are a formidable source of natural compounds with diverse biological activities. In the last five decades it has been estimated that more than 3000 natural compounds were discovered from these organisms. The great majority of the published works have focused on terpenoids. In comparison, glycolipids are a neglected class of macroalgal secondary metabolites therefore remaining as a largely unknown reservoir of molecular diversity. Nevertheless, the interest regarding these compounds has been growing fast in the last decades as activities of ecological or pharmaceutical interest have been highlighted. This paper will review recent work regarding isolation and structural characterization of glycolipids from seaweeds and their prospective biological activities.

Keywords: seaweeds, glycolipids, MGDG, DGDG, SQDG, biological activity, marine macroalgae

INTRODUCTION

For the last five decades, both chemists and biologists have carried out an intense effort regarding marine natural products (Bhakuni and Rawat, 2005; Blunt et al., 2014). Such dedication has resulted in the discovery of more than 20,000 compounds from marine microorganisms, invertebrates, and macroalgae (Hu et al., 2011). Marine organisms appear then as a formidable source of natural products. While products of primary metabolism like amino acids, carbohydrates and proteins, are vital for maintaining life processes, others such as alkaloids, phenolics, steroids, terpenoids, are secondary metabolites that have ecological, toxicological, and pharmacological significance (Maschek and Baker, 2008). Bioactivities such as antiherbivory, antifoulant, antifungal, antitumor, antimicrobial, and antiparasitic effects have been highlighted for marine natural products (Noda et al., 1990; Deal et al., 2003; Bhadury and Wright, 2004; Cheung et al., 2014).

With an estimation of more than 30,000 species identified around the world, marine macroalgae, or seaweeds, constitute a huge source of natural compounds with diverse biological activities (Guiry, 2012). In the last 50 years it has been estimated that more than 3000 natural compounds were discovered from these organisms (Leal et al., 2013). The great majority of the published works have focused on terpenoids, phenolic compounds, or polysaccharides. Glycolipids represent a less studied class of metabolites with recently growing interest. Seaweeds biosynthesize three major types of glycolipids: monogalactosyldiacylglycerides (MGDGs), digalactosyldiacylglycerides (DGDGs), and sulfoquinovosyldiacylglycerides (SQDGs) (**Figure 1**).

These glycoglycerolipids are present in chloroplasts of eukaryotic algae where MGDGs and DGDGs are the most abundant lipids of the thylakoid membrane and appear to play a crucial role in photosynthesis (Hölzl and Dörmann, 2007).

This work will present a concise review of studies from the last 15 years regarding the isolation and structural characterization of bioactive glycolipids from marine macroalgae.

OCHROPHYTA

The MGDG isolated from *Petalonia binghamiae* was characterized as a potent inhibitor of the activities of mammalian DNA polymerase α (Mizushina et al., 2001).

SQDG and DGDG from the Japanese macroalga *Sargassum horneri* were found to induce apoptosis of the human colon carcinoma Caco-2 cell when associate with sodium butyrate (Hossain et al., 2005).

The acetone fraction of *Sargassum thunbergii* extract exhibited moderate antifungal effects on *Candida albicans*. Further investigation of this extract led to the isolation of four MGDGs, two of them newly described as (2S)-1-*O*-(5Z,8Z, 11Z,14Z,17Z-eicosapentaenoyl)-2-*O*-(9Z,12Z,15Z-octadecatrienoyl)-3-*O*-β-D-galactopyranosyl- sn-glycerol and (2S) -1-*O*-(9Z,12Z, 15Z-octadecatrienoyl)-2-*O*-(6Z,9Z,12Z,15Z-octadecatetraenoyl)-3-*O*- β -D-galactopyranosyl-sn-glycerol (Kim et al., 2007).

Two glucopyranosyldiacylglycerols were isolated from *Sargassum fulvellum*. The two compounds were identified to be 1-*O*-palmitoyl-2-*O*-oleoyl-3-*O*-(α-D-glucopyranosyl)-glycerol

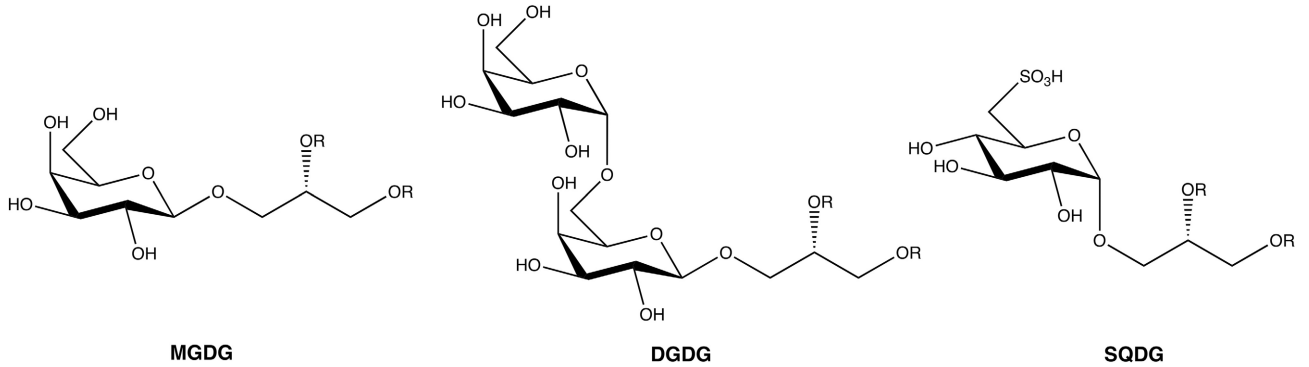

FIGURE 1 | General structure of the three main glycoglycerolipids from seaweeds. R, acyl chain.

and 1-*O*-myristoyl-2-*O*-oleoyl-3-*O*-(α–glucopyranosyl)-glycerol and showed fibrinolytic activity in the reaction system of single chain urokinase-type plasminogen activator and plasminogen (Wu et al., 2009).

The dichloromethane-methanol (7/3) extract of *Lobophora variegata* from the Yucatan coast (Mexico) demonstrated activity against the protozoa *Trichomonas vaginalis*, with an IC_{50} value of 3.2 μg/ml (Cantillo-Ciau et al., 2010). Further fractionation of that extract was undergone and led to a chloroform fraction that showed activity against the protozoa *T. vaginalis*, *Giardia intestinalis*, and *Entamoeba histolytica,* with good selectivity ($SI > 10$). Purification of this fraction allowed the isolation of three SQDGs: the major compound 1-*O*-palmitoyl-2-*O*-myristoyl-3-*O*-(6-sulfo-α-D-quinovopyranosyl)-glycerol, along with small amounts of 1,2-di-*O*-palmitoyl-3-*O*-(6-sulfo-α-D-quinovopyranosyl)-glycerol and a new compound identified as 1-*O*-palmitoyl-2-*O*-oleoyl-3-*O*-(6-sulfo-α-D-quinovopyranosyl)-glycerol.

Plouguerné et al. (2010) isolated MGDGs in a fraction obtained from *Sargassum muticum* collected from the coast of Britanny (France), that inhibited the bacteria *Shewanella putrefaciens* and *Polaribacter irgensii* and the fungi *Halosphaeriopsis mediosetigera*, *Asteromyces cruciatus*, *Lulworthia uniseptata*, *Monodictys pelagica*, all involved in marine microfouling. The inhibitory activity was reported for a concentration of 0.75 mg/l.

The crude ethyl acetate extract of *Fucus evanescens,* collected on the Arctic coast of Ungava Bay, Nunavik (Canada), showed strong antibacterial activity (≥4 $\log_{10}$ colony-forming units (cfu) against *Hemophilus influenza*, *Legionella pneumophila*, *Propionibacterium acnes*, and *Streptococcus pyogenes*, when tested at 100 μg/ml. This glycolipid rich extract also inhibited by 3 $\log_{10}$ cfu the bacteria *Clostridium difficile* and *Staphylococcus aureus* (Amiguet et al., 2011). Further purification of the glycolipid rich extract led to the isolation and identification of the main compound as the MGDG 2′, 3′-propyl dilinolenate-β-D-galactopyranoside.

El Baz et al. (2013) investigated the structures and biological activities of sulfolipids from the Mediterranean macroalgae *Dilophus fasciola* and *Taonia atomaria*. The authors highlighted antibacterial and antiviral activities from sulfolipids extracts. The major compounds were identified as SQDG and SQMG (sulfoquinovosylmonoacylglyceride).

SGDGs were identified in fractions obtained after the purification of the organic extract of the Brazilian macroalga *Sargassum vulgare*. The main SQDG responsible for the anti-HSV1 and anti-HSV2 activities highlighted was characterize as 1, 2-di-*O*-palmitoyl-3-*O*-(6-sulfo-α-D-quinovopyranosyl)-glycerol (Plouguerné et al., 2013). The structure of the SQDG was determined using Electrospray Ionization Mass Spectrometry (ESI-MS) (**Figure 2A**) and the configuration of the anomeric carbon was confirmed by ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) analysis, based on Heteronuclear Single Quantum Coherence (HSQC) fingerprints (**Figure 2B**).

Imbs et al. (2013) isolated a highly unsaturated monogalactosyldiacylglycerol (MGDG) from the ethanol extract of *Fucus evanescens*, collected from the west coast of the Iturup Island of the Sea of Okhotsk (Russia). This compound, identified as 1-*O*-(5Z,8Z,11Z,14Z,17Z-eicosapentanoyl)-2-*O*-(6Z,9Z,12Z,15Z-octadecatetraenoyl)-3-*O*-β-D-galactopyranosyl-sn-glycerol, inhibited the growth of human melanoma cells with an IC_{50} = 104 μM.

RHODOPHYTA

Ohta et al. (1998) isolated the SQDG KM043 from *Gigartina tenella*, collected at Sagami Bay, Kanagawa, Japan, that demonstrated inhibition of DNA polymerase α, DNA polymerase β and HIV-reverse transcriptase type 1, with respective IC_{50} values of 0.25, 3.6, and 11.2 μM. The structure of the SQDG was identified as 1-(1′-*O*- α-D-sulfoquinovosyl)-2-palmitoyl-3-[5″ (E), 8″ (E), 11″ (E), 14″ (E), 17″ (E)-eicosapentaenyl]-syn-glycerol.

Al-Fadhli et al. (2006) isolated three distinct fractions containing polar glycolipids from the soluble fraction of crude methanolic extract of *Chondria armata*. The structure of the glycolipids was elucidated using multidimensional NMR techniques and ESI-MS in the positive ion mode. The most active fraction showed significant growth inhibition of the bacteria *Klebsiella* sp., the yeast *Candida albicans* and the fungus *Cryptococcus neoformans* when tested at the concentration of 130 μg/disc. The main compound present in the fraction was identified as the MGDG 1-eicosapentanoyl-2-palmitoyl-3-*O*-galactopyranosyl-glycerol.

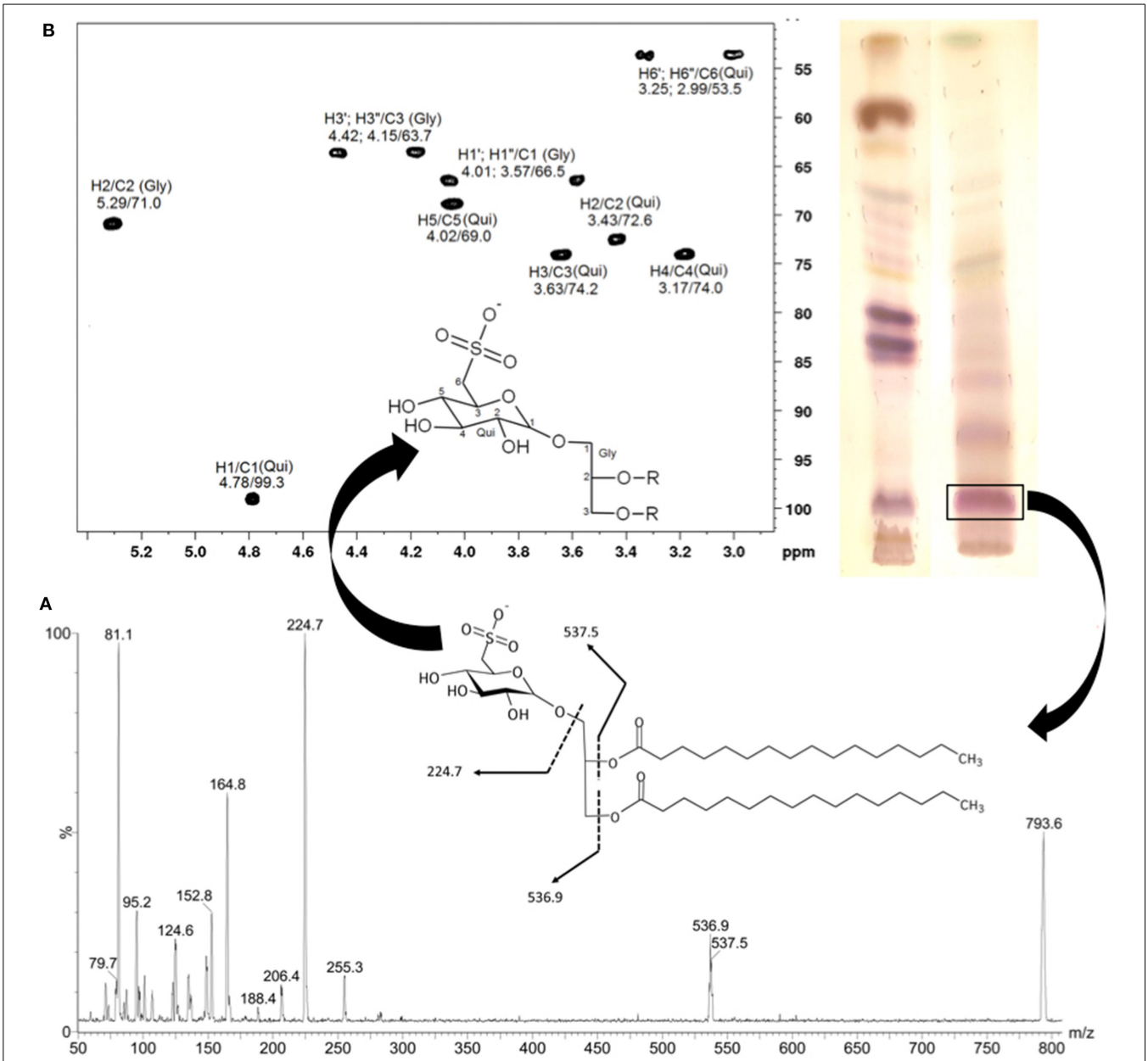

FIGURE 2 | Electrospray ionization mass spectrum (ESI-MS) of main SQDG from *Sargassum vulgare* (negative mode). The fragmentation pathway of the ion at m/z = 794 is compatible with the structure of 1,2-di-*O*-palmitoyl-3-*O*-(6-sulfoquinovopyranosyl)-glycerol **(A)**. The configuration of the anomeric carbon was confirmed analyzing the partial fingerprint spectrum 2D-^{1}H/^{13}C-HSQC of the polar head group of SQDG **(B)**. Gly, glycerol; Qui, quinovose. (Adapted from Plouguerné et al., 2013).

The MGDG lithonoside isolated from the cytotoxic hexane-soluble extract of the Fijian coralline macroalga *Hydrolithon reinboldii* demonstrated moderate growth inhibitory activity against cancer cell lines with a mean IC_{50} value of 19.8 μM (Jiang et al., 2008).

de Souza et al. (2012) isolated an anti-HSV (herpes simplex virus) glycolipid-enriched fraction from the Brazilian macroalga *Osmundaria obtusiloba*. The major compound of the active fraction was identified as the SQDG 1,2-di-*O*-acyl-3-*O*-(6-deoxy-6-sulfo-α-D-glucopyranosyl)-sn-glycerol.

Tsai and Pan (2012) isolated SQDGs from *Porphyra crispata* collected from northeastern Taiwan that inhibited the growth of human hepatocellular carcinoma cell line (HepG2), with an IC50 of 126 μg/ml.

El Baz et al. (2013) investigated the structures and biological activities of sulfolipids from *Laurencia papillosa* and *Galaxaura cylindrica* collected from the Red Sea. The authors highlighted antibacterial and antiviral activities from sulfolipids extracts. The major compounds were identified as SQDG and SQMG.

Anti-inflammatory activity was highlighted for two SQDGs isolated from *Palmaria palmata* (Banskota et al., 2014). The bioactive compounds were identified as (2S)-1-*O*-eicosapentaenoyl-2-*O*-myristoyl-3-*O*-(6-sulfo-a-D-quinovopyranosyl)-glycerol and (2S)-1-*O*-eicosapentaenoyl-2-*O*-palmitoyl-3-*O*-(6-sulfo-α-D-quinovopyranosyl)-glycerol and demonstrated nitric oxide inhibitory activity with IC_{50} values of 36.5 and 11.0 μM, respectively.

CHLOROPHYTA

Wang et al. (2007) isolated a SQDG from the n-butanol fraction of the invasive *Caulerpa racemosa* collected from the South China Sea. The SQDG compound was characterized using spectroscopic methods as (2S)-1,2-di-*O*-palmitoyl-3-*O*-(6′-sulfo-α-D-quinovopyranosyl) glycerol, and was active against HSV-2, with a 50% inhibitory concentration (IC_{50}) of 15.6 mg ml^{-1} against both standard and clinical strains of HSV-2.

MGDGs capsofulvesin A and B, along with the MGMG capsofulvesin C, isolated from *Capsosiphon fulvescens* collected from the southern coastal area of Wando (Korea), demonstrated cholinesterase inhibitory activity (Fang et al., 2012).

Islam et al. (2014) revealed for the first time the aldose reductase inhibitory activity of the capsofulvesin A and capsofulvesin B.

Such results highlighted the potential health benefits of *C. fulvescens* in improving neurotransmission as well as in preventing diabetic complications.

El Baz et al. (2013) investigated the structures and biological activities of sulfolipids from the Mediterranean macroalga *Ulva fasciata*. The authors highlighted antibacterial and antiviral activities from sulfolipids extracts, and the major compounds were identified as SQDG and SQMG.

CONCLUSION

Among the three phyla of marine macroalgae, Ochrophyta appears as the main source of recently reported bioactive glycolipids, followed by Rhodophyta and Chlorophyta. Within Ochrophyta, the order Fucales was the most reported, followed by Dictyotales.

Among bioactive glycolipids isolated from marine macroalgae, SQDGs, and MGDGs dominated the reports for the last 15 years. Khotimchenko (2003) studied the distribution of glyceroglycolipids in marine macroalgae and highlighted the predominance of SQDG as a characteristic of brown seaweeds from the order Fucales. It would then be logical to expect such order as a major source of bioactive SQDGs. However, according to the recent literature, bioactive SQDGs were more abundant in Dictyotales.

The most reported biological activities for glycolipids from seaweeds were antibacterial, antitumor, and antiviral activities, enhancing the pharmacological potential of these compounds. Less reported, but of significant interest, is the ecological role that may assume glycolipids biosynthetized by seaweeds. Antifouling, and antiherbivory activities were already reported for glycolipids from *Sargassum muticum* and *Fucus vesiculosus*, respectively (Deal et al., 2003; Plouguerné et al., 2010).

Concerning the mechanism of action of glycolipids, it remains until today relatively obscure. Recent studies about synthesis and structural modification of glycoglycerolipids helped to understand the structure-activity relationship. On general point of view, the bioactivities of glycoglycerolipids are related to the sugar moiety, the position of the glycerol linkage to the sugar, the length and location of the acyl chain, and the anomeric configuration of the sugar (Zhang et al., 2014). Polyunsaturated fatty acids (PUFAs) are biologically active compounds, which are abundant components of macrophytic glycolipids (Khotimchenko, 1993a,b, 2003; Sanina et al., 2000, 2004; Raposo et al., 2013). Therefore, it has been hypothesize that fatty acids may be responsible for biological activities highlighted from macrophytic glycolipids (Tsai and Pan, 2012). As the distribution of PUFAs in glycolipids from seaweeds depends from their taxonomic position, it may then be logical to expect some taxa to be more active than others (Sanina et al., 2004). Among glycolipids, the degree of saturation of fatty acid increased in the lines of MGDG->DGDG->SQDG. Concerning SQDGs, the antiviral activity may be related to the presence of the sulfonate group (Plouguerné et al., 2013). The sulfate moiety along with the nature of the fatty acids of the SQDGs are also important for the inhibition of DNA polymerase α and β (Mizushina et al., 1996, 1997; Hanashima et al., 2000, 2001).

Glycoglycerolipids from seaweeds are compounds with both biotechnological potential and ecological interest. Further studies are needed to extend knowledge concerning the mechanism of action of these molecules as well as their distribution between macroalgal species.

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