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Research paper

Crystal structure of a pro-inflammatory lectin from the seeds of *Dioclea wilsonii* Standl

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ABSTRACT

The crystal structure and pro-inflammatory property of a lectin from the seeds of *Dioclea wilsonii* (DwL) were analyzed to gain a better understanding of structure/function relationships of Diocleinae lectins. Following crystallization and structural determination by standard molecular replacement techniques, DwL was found to be a tetramer based on PISA analysis, and composed by two metal-binding sites per monomer and loops which are involved in molecular oligomerization. DwL presents 96% and 99% *grandiflora* bohydrate parison to other Diocleinae lectins can be related to the differences in the dose-dependent pro-inflammatory effect elicited in Wistar rats, probably via specific interactions with mast cells complex carbohydrate, resulting in significant paw edema. DwL appears to be involved in positive modulation of mast cell degranulation via recognition of surface carbohydrates. Since this recognition is dependent on site volume and CRD configuration, edematogenesis mediated by resident cells varies in potency and efficacy among different Diocleinae lectins.

1. Introduction

Lectins are ubiquitous proteins or glycoproteins with at least one non-catalytic domain binding reversibly to a specific mono- or oligosaccharide [1]. Due to their ability to decipher membrane glycocodes, lectins participate in numerous cellular processes, such as cell communication, host defense, fertilization and development [2].

Diocleinae lectins exhibit glucose/mannose-monosaccharide binding specificity. Studies of their chemical and physicochemical properties have revealed a high degree of similarity in amino acid sequence and three-dimensional structure [3]. A comparative study of the primary structure of lectins from the genera *Canavalia*, *Dioclea* and *Cratylia* (subtribe Diocleinae) indicated the existence of regions specific to each genus, evidencing different branches in the evolutionary tree [4]. Despite great structural similarities, these lectins express a wide range of biological activities with varying potency and efficacy [5].

Concanavalin A (ConA) is the most studied Diocleinae lectin. Thus, many of its structures have already been deposited in the PDB with different ligands (mono- and oligosaccharides). The observed differences in interactions within the carbohydrate-binding site of Diocleinae lectins may help clarify the correlation between structure and physiological function [5,6]. The spatial distribution of carbohydrate-binding sites in oligomeric lectins determines their

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ability to distinguish and crosslink multivalent saccharide ligands or specific cell-surface arrays of glycoconjugates [6,7].

Information on carbohydrate-binding site recognition and specific interactions with different sugars and glycans obtained by X-ray crystallography can help establish correlations between cell-surface binding activity and biological activities in different physiological systems [8].

Diocleinae lectins are dimeric or tetrameric structures of dome-shaped monomers consisting of jelly-roll motifs interconnected by turns and loops [9]. According to Rangel and coworkers [10], DwL weighs 25.6 kDa and features 237 residues per monomer. The amino acid sequence identity between DwL (UniProtKB, with accession P86624) [10] and DRL is 96%. Despite the considerable similarity in primary and three-dimensional structure, the proteins in this group differ substantially with regard to biological properties, making them excellent models for the study of structure/function relationships [5,11].

Diocleinae lectins may produce either anti-inflammatory [12,13] or pro-inflammatory effects [14,15], depending on the route of administration. Sugar residues also play an important role, as shown by the reversal of biological activities when lectins are associated with their specific binding sugars. Several reports have described pro-inflammatory activities (mast cell stimulation) for Diocleinae lectins [16–18]. Mast cells are intimately involved in the pathophysiology of allergic diseases and inflammation and their activation promotes the release of chemical mediators responsible for important tissue changes such as vasodilatation, increased vascular permeability, bronchoconstriction and neutrophil or eosinophil chemotaxis [19].

Thus, the objective of this study was to evaluate the pro- and anti-edematogenic activity of a lectin isolated from the seeds of *Dioclea wilsonii* and the correlation between crystal structure and biological properties, including the participation of resident cells.

2. Materials and methods

2.1. Isolation of D. wilsonii lectin (DwL)

DwL was purified in two steps by dextran affinity chromatography on a Sephadex G-50 column followed by ion exchange chromatography on HiTrap SP XL 01, as described by Rangel and colleagues [10]. The apparent molecular weight of DwL and fragments (β and γ) was estimated by SDS-PAGE in the presence of β-mercaptoethanol [20].

2.2. Crystallization and X-ray data collection

DwL was diluted homogenously to a concentration of 10.0 mg mL^{-1} in ultrapure water and incubated during 1 h with 3 mM X-Man (5-bromo-6-chloro-3-indolyl-alpha-D-mannopyranoside) for all crystallization experiments. Crystallization conditions for DwL were screened using the hanging-drop vapor diffusion method with Hampton Research Crystal Screens I and II (Hampton Research, Riverside, CA, USA) [21] at room temperature (293 K). Crystals were obtained using condition #41 of Crystal Screen II (0.01 M Nickel (II), 0.1 M Tris pH 8.5, 1.0 M lithium sulfate monohydrate). Crystal improvement was achieved by lowering the pH value to 8.0 of the condition #41 [10].

X-ray data were collected from a single orthorhombic crystal (point group I222) cooled to 100 K. Crystals were soaked in a cryoprotectant solution containing 30% glycerol to avoid ice formation and data were collected using synchrotron radiation (MX1 station, National Laboratory of Synchrotron Light–LNLS, Campinas, Brazil) of 1.47 Å wavelength. A complete data set was obtained using a CCD detector (MAR Research) with 120 frames with 40 s of exposure per frame and a 1° oscillation range. Data had to be cut at 2.3 Å due to ice ring issues. *I*/sigma(*I*) was 0.0 in the next shell (2.23 Å). The data set extending to a maximum resolution of 2.3 Å was indexed and integrated using XDS [22] and scaled with SCALA [23].

2.3. Molecular replacement and refinement

The primary sequence of DwL was aligned with that of other ConA-like lectins. Thus, the lectins of *Dioclea violacea* (DVL), *Dioclea guianensis* (Dgui) and *Dioclea rostrata* (DRL) presented 96%, 95% and 96% identity with DwL, respectively [10]. DRL (PDB code: 2ZBJ) was the best model in the molecular replacement study using Phaser [24]. After this, it was observed $R_{factor} = 0.3565$ and $R_{free} = 0.356$.

Several rounds of iterative refinement were performed with the PHENIX suite [25], adding water molecules and defining the optimal X-ray weight. The residues GLY58, GLY70, VAL129, ASP192 and SER202 were replaced and the side chain conformations were roughly adjusted to satisfy the electron density map. A total of 92 water molecules were added. Manual adjustments in Coot [26] were made after each refinement step and X-Man molecules were manually added to the model. The structure was validated using the MolProbity server [27]. The temperature factors for ligands were 24.71 (X-Man) and 23.46 (Abu).

2.4. Biological assays

2.4.1. Animals

Wistar rats (180–250 g) were kept in cages (6 in each) in a controlled environment (circadian cycle, 25 °C, food and water *ad libitum*). The experimental protocols were approved by the Institutional Animal Care and Use Committee of the State University of Ceará (UECE, Fortaleza, Ceará, Brazil) under No. 10130208-8/40, following the recommendations of the Guide for the Care and Use of Laboratory Animals of the US Department of Health and Human Services (NIH publication No. 85–23, revised 1985).

2.4.2. Drugs and reagents

α-methyl-D-mannoside (α-CH_3), D-glucose, indomethacin, $N^{(\omega)}$-nitro-L-arginine methyl ester (L-NAME), pentoxifylline, pyrilamine, thioglycolate, compound 48/80 and Evans blue were purchased from Sigma (St. Louis, MO, USA). All drugs were solubilized directly in sterile saline, except for indomethacin, which was initially dissolved in dimethyl sulfoxide up to 10% of the total volume, then in saline.

2.4.3. Rat paw edema model

Paw volume was measured with a hydroplethysmometer at baseline (immediately before s.c. injection of inflammatory stimulants into the hind paw) and at 0.5, 1, 2–8 and 32 h. The results were expressed as paw volume variation (mL) in relation to baseline values. The area under the time-course curve (AUC) was expressed in arbitrary units [28].

2.4.4. Evaluation of edematogenic activity

In order to evaluate the edematogenic effect of DwL, paw edema was induced by s.c. injection of DwL (0.01, 0.1, 1 mg/kg) in a final volume of 0.1 mL/100 g body mass. The increase in vascular permeability was indirectly measured by quantifying protein leakage from inflamed paws [29] induced with 1 mg/kg DwL s.c. The animals were injected i.v. with Evans blue (25 mg/kg) 1 h before euthanasia, coinciding with the peak of edematogenic activity. The paws were then sectioned at the ankle, weighed and incubated with 2 mL formamide for 72 h at 37 °C. The optical

density (A600 nm) of the extracted dye was expressed as mean μg Evans blue/g tissue ± SEM.

To measure the anti-edematogenic effect of DwL, the most pro-inflammatory concentration of DwL was injected i.v. 30 min prior to s.c. administration of carrageenan (300 μg/paw), a well-known phlogistic agent [30]. Following the same protocol, positive controls received carrageenan while negative controls received sterile saline only. An additional group of animals received the stardard anti-inflammatory, dexamethasone (1 mg/kg, i.p.).

2.4.5. Modulation of edematogenic activity

The mechanism of edematogenesis was investigated by administering indomethacin (5 mg/kg; s.c.), L-NAME (30 mg/kg; i.v.) pyrilamine (10 mg/kg; i.p.) or pentoxifylline (90 mg/kg; s.c) 30 min prior to injection with 1 mg/kg DwL. The controls received the same volume of sterile saline.

Compound 48/80 was injected i.p. over 4 days (on the first 3 days at 0.6 mg/kg, then at 1.2 mg/kg) to achieve systemic mast cell depletion. The animals also received thioglycolate i.p. for 4 days (on the first day at 3%/10 mL, then at 1.5%/10 mL) to induce systemic increase in macrophage population [31,32]. The paws were injected s.c. with DwL (1 mg/kg) 24 h after subchronic treatment with compound 48/80 or thioglycolate. Controls were injected with saline.

2.4.6. Investigation of the lectin domain involvement

DwL at the most pro-inflammatory dose was incubated with its binding sugars glucose (0.5 M) or α-CH_3 (1 M) for 1 h at 37 °C to allow for lectin–sugar interaction before the experiment. Lectin and sugar controls were incubated individually under similar conditions.

2.4.7. Statistical analysis

Results were expressed as mean values ± SEM for each group of 5–7 animals. Differences were analyzed with ANOVA or Student's *t* test, as appropriate. The level of statistical significance was set at $p < 0.05$.

3. Results

3.1. Purification and crystallization of DwL

DwL was purified and crystallized as described by Rangel and coworkers [10]. SDS-PAGE revealed the presence of DwL (25 kDa) and fragments (β and γ: 12 kDa).

The crystals belonged to point group I222. The Matthews coefficient [33] indicated the presence of a monomer in the asymmetric unit. X-ray data collection statistics are shown in Table 1.

3.2. Overall structure of DwL

The structure of DwL was obtained by molecular replacement at 2.3 Å using the structure of DRL (PDB code: 2ZBJ) as search template [34]. The structure was solved using the program Phaser [24] with a maximum likelihood gain of 1299.86, RFZ of 15.0 and TFZ of 30.1 for the best solution. The refined structure of tetrameric DwL was determined by PISA, and presents 92 water molecules, one calcium ion and one manganese ion per monomer. An X-Man molecule was modeled into the carbohydrate recognition domain (CRD) (Fig. 1) while a co-purified α-aminobutyric acid (Abu) molecule was observed in the hydrophobic pocket, both according to the Fo–Fc electronic density map. After the last refinement of the structure, R_{factor} was 0.21 and R_{free} was 0.26. The stereochemistry of the structure was monitored with a Ramachandran plot, analyzing the φ and ψ angles and the root mean square deviation of the bonds.

Table 1
Statistics of data collection, refinement and structure quality.

Parameters	Values
Data collection	
Beamline wavelength	1.42 Å
Space group	I222
Unit cell parameters (Å)	
a	58.8
b	66.8
c	107.3
Total reflections	45,720 (6659)[d]
Number of unique reflections	9392 (1381)[d]
Molecules per asymmetric unit	Monomer
Resolution limits (Å)	19.4–2.3
R_{merge}[a] (%)	0.046 (0.099)[d]
Completeness (%)	97.0 (99.0)[d]
Multiplicity	4.9 (4.8)[d]
I/σ (I) (Average)	9.0 (7.8)[d]
Molecular replacement	
Log-likelihood gain	1299.86
RFZ	15.0
TFZ	30.1
Refinement	
Resolution range (Å)	19.4–2.3
R_{factor}[b] (%)	21.3
R_{free}[c] (%)	26.1
B factor (Å^2)	24.3
Number of residues in asymmetric unit	237
Number of water molecules	92
RMS deviations	
Bond length (Å)	0.009
Bond angle (degree)	1.357
Ramachandran plot	
Residues in most favored regions (%)	96.9
Residues in additional allowed regions (%)	3.1

[a] $R_{merge} = \frac{\sum_{hkl}\sum_i |\bar{I}(hkl) - \langle I(hkl)i\rangle|}{\sum_{hkl}\sum_i \langle I(hkl)i\rangle}$ where $I(hkl)i$ is the intensity of ith measurement of the reflection h and $I(hkl)$ is the mean value of the $I(hkl)i$ for all I measurements.

[b] $R_{factor} = \frac{|F_{obs}| - |F_{calc}|}{|F_{obs}|}$.

[c] Calculated with 5% of the reflections omitted from refinement.

[d] Values in parenthesis represent the high-resolution shell (2.4–2.3 Å).

The graph confirmed the absence of residues in disallowed regions and the deviations were within the normal range. Molecular replacement and refinement data are shown in Table 1. The structure factors and coordinates were deposited in the Protein Data Bank under accession code number 3SH3.

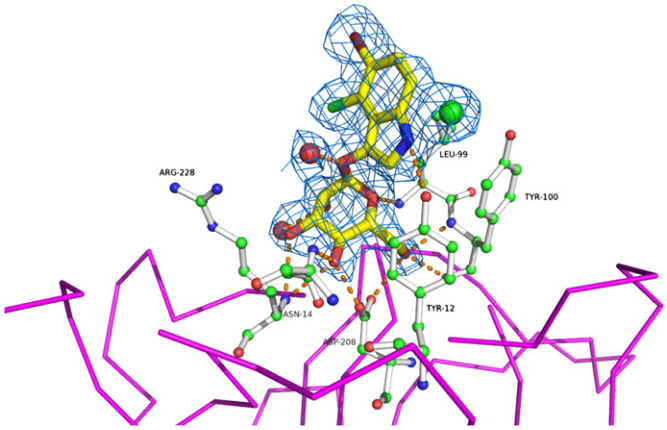

Fig. 1. Interaction between X-Man and the residues of the carbohydrate-binding domain of *Dioclea wilsonii* lectin. The figure shows 2fo–fc map countered at 1σ for X-Man and dash lines represent polar contacts between X-Man with CRD of DwL.

The structure of DwL includes a metal-binding site containing the conserved residues ASN14 and TYR12 (which interact with calcium), GLU8 and HIS24 (which interact with manganese) and ASP10 and ASP19 (which interact with both). In addition, the peptide bond of ALA207 and ASN208 in the *cis* configuration is isomerized due to the presence of divalent metals changing the side chain orientation of ASN14 and ASP208 [7].

The difference electron density map shows a feature in the vicinity of LEU115, LEU126 and VAL179, corresponding to a non-protein amino acid (Abu) commonly co-purified with Diocleinae lectins [35]. The hydrophobic pocket shows a region accessible to the solvent surface, allowing hydrophilic interactions to occur. These hydrophilic interactions occur through hydrogen bonds between the nitrogen atom in Abu and the nitrogen atom in HIS180 (main chain) at 2.7 Å, and between the oxygen atom in Abu and the oxygen atom in SER125 (main chain) or the nitrogen atom in SER125 (peptide bonds) at 3.1 Å. Abu also interacts with two water molecules, involving oxygen and nitrogen at 2.7 Å and 2.8 Å, respectively. In addition, Abu mediates between the components of the canonical dimer, establishing hydrogen bonds with ASP139 and SER129 (Fig. 2).

The carbohydrate-binding site is occupied by X-Man. DwL interacts with mannose in much the same way as other ConA-like lectins (Table 2). A structural comparison between DwL and DVL (PDB code: 3AX4) can help clarify the interaction between DwL and X-Man. By superimposing these structures, differences may be observed in the interaction between X-Man and the hydrophobic sub-site (Fig. 3). Thus, in DwL the side chain of LEU99 is positioned at an angle preventing hydrophobic interaction. This could lead to interaction with more complex carbohydrates — a process requiring the involvement of this sub-site.

3.3. Biological activities

DwL evoked dose-dependent paw edema at all doses tested, beginning at 30 min, peaking at 4 h and subsiding at 24 h (Fig. 4A). Injection of 0.01, 0.1 and 1 mg/kg DwL increased paw volume 4.8 times (AUC: 7.61 ± 0.93), 5.7 times (AUC: 9.01 ± 0.87) and 7 times (AUC: 11.21 ± 0.8), respectively, compared to saline (AUC: 1.58 ± 0.63 arbitrary units) (Fig. 4B). The highest peak of paw edema (1 mg/kg at 5 h) was paralleled by a 3-fold increase in vascular permeability (67.2 ± 3.5 μg) compared to saline (21.29 ± 10.9 μg Evans blue/g paw wet weight) (Fig. 4C). On the

Table 2
Van der Waals interaction and polar contacts between lectins of *Dioclea wilsonii* and *Dioclea violacea*.

Amino acids	X-Man	*D. wilsonii* PDB: 3SH3	*D. violacea* PDB: 3AX4
Van der Waals interactions			
TYR 12 CD2	O4	3.44	–
TYR 12 CD2	C6	–	3.43
TYR 12 OH	C11	2.88	2.88
GLY 98 CA	O6	3.21	2.96
GLY 98 C	O6	3.39	3.28
LEU 99 CD2	C9	–	3.48
LEU 99 CD2	N1	–	3.40
ALA 207 CB	C6	3.40	–
ALA 207 CB	O6	3.04	3.26
ASP 208 CG	O4	3.25	–
ASP 208 CG	C6	–	3.49
ASP 208 OD1	C4	3.23	3.13
ASP 208 OD1	C6	3.39	3.07
ASP 208 OD2	C6	3.31	3.25
GLY 227 CA	O3	–	3.25
ARG 228 CG	O3	3.18	–
ARG 228 CB	O4	3.27	3.27
Polar contacts			
TYR 12 OH	N1	3.23	3.06
ASN 14 N	O4	2.82	–
ASN 14 ND2	O4	–	2.96
GLY 98 N	O6	3.34	3.06
LEU 99 N	O5	3.17	3.08
LEU 99 N	O6	3.12	3.04
TYR 100 N	O6	2.85	2.94
ASP 208 OD1	O4	–	2.63
TYR 100 O	O6	3.12	3.04
ASP 208 OD1	O4	2.33	–
ASP 208 OD2	O6	2.70	–
ASP 208 OD2	O6	–	2.92
ARG 228 N	O3	3.08	3.18
ARG 228 N	O4	3.43	3.23

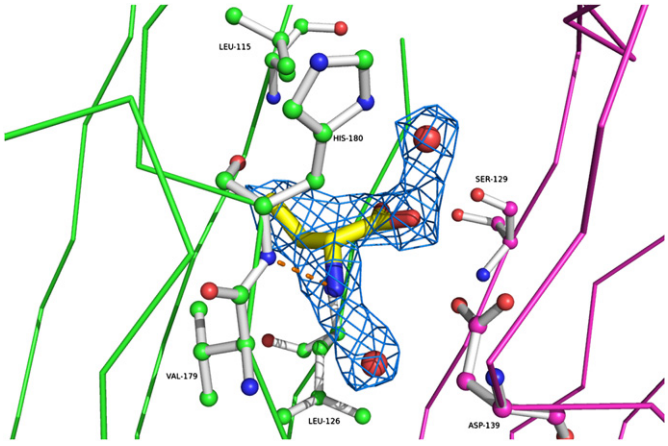

Fig. 2. α-Aminobutyric acid (Abu) binding site. The electron density shows 2fo–fc map countered at 1σ for Abu and the amino acid residues involved in the site are also demonstrated.

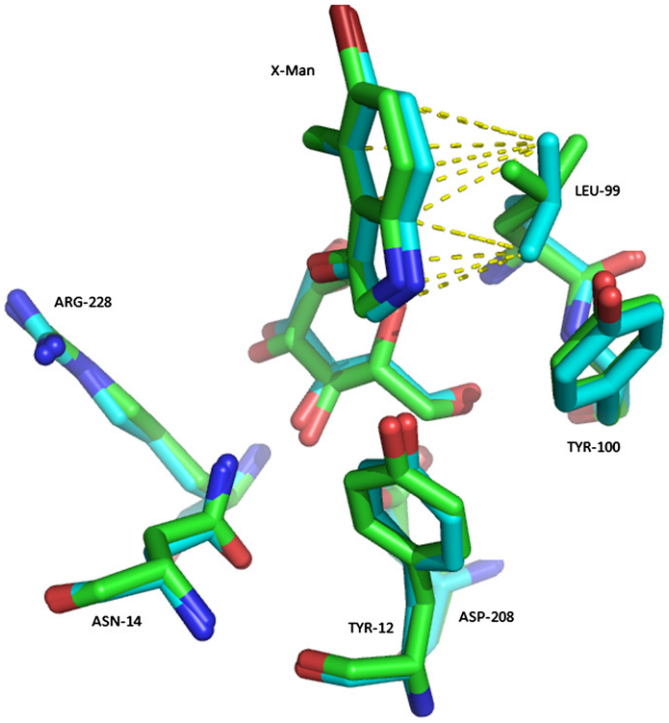

Fig. 3. Structural comparison between DwL (green) and DVL (PDB code: 3AX4, in blue). Differences in the interaction between X-Man and the hydrophobic sub-site may be observed after structures superimposition. In DwL, the side chain of Leu99 is positioned at an angle preventing hydrophobic interactions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

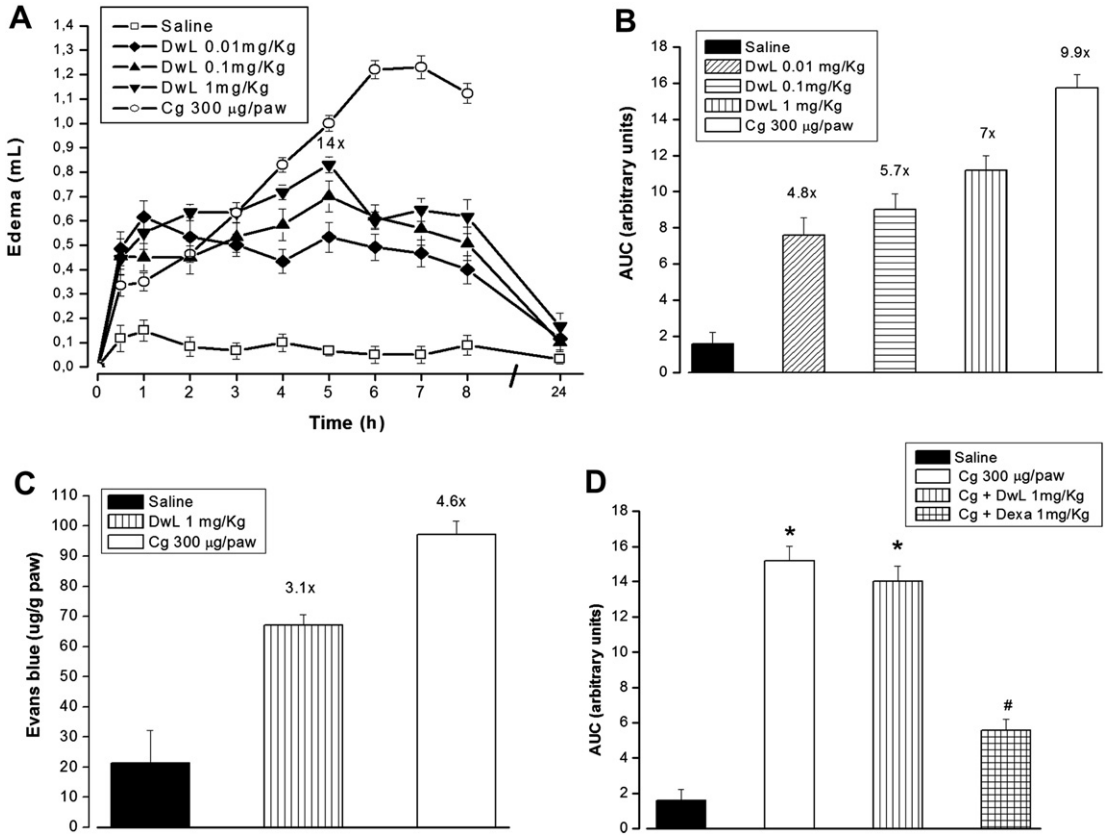

Fig. 4. *Dioclea wilsonii* lectin induces paw edema and increase vascular permeability in rats. Animals were injected s.c. with DwL (0.01; 0.1; 1 mg/kg), carrageenan or saline (0.01 mL/paw) and data expressed as the increase in paw volume (mL) subtracted from zero time (Panel A) or area under curve (arbitrary units) (Panels B and D). Animals received DwL s.c. (1 mg/kg) 4 h before Evans blue (25 mg/kg, i.v.), sacrificed 1 h later for paw processing and data expressed as µg of Evans blue/g paw wet weight (Panel C). Animals were injected i.v. with DwL (1 mg/kg) or saline (0.01 mL/paw) or i.p. with dexamethasone (1 mg/kg) before s.c. injection of carrageenan (300 µg/pata) (Panel D). Edema was measured before (time zero) and at ½, 1, 2–8 and 24 h after stimulus. Mean ± S.E.M. ($n = 6$). $^*p < 0.05$ compared to saline; $\#p < 0.05$ compared to DwL. DwL: lectin from *Dioclea wilsonii*; Cg: carrageenan.

other hand, i.v. injection of DwL (AUC: 14.01 ± 0.87) did not inhibit carrageenan-induced edema (AUC: 15.2 ± 0.8) (Fig. 4D).

The edematogenic effect of saturated solution of DwL with glucose (AUC: 3.43 ± 0.1) or α-CH_3 (AUC: 3.72 ± 0.2) was similar to that of DwL (AUC: 3.31 ± 0.14) (Fig. 5A) per se. Neither glucose (AUC: 0.20 ± 0.05) nor α-CH_3 (AUC: 0.22 ± 0.03) induced edema when administered alone compared to saline (AUC: 0.19 ± 0.08 arbitrary units).

The administration of pyrilamine (histamine antagonist) reduced the edematogenic effect of DwL (AUC: 2.71 ± 0.19) by 47% (AUC: 1.44 ± 0.29); indomethacin (non-specific enzyme cyclooxygenase inhibitor) by 32% (AUC: 1.85 ± 0.18); L-NAME (non-selective NOS inhibitor) by 31% (AUC: 1.99 ± 0.17) and pentoxifylline (non-specific cytokine inhibitor) by 53% (AUC: 1.28 ± 0.21 arbitrary units) (Fig. 5B), suggesting an edematogenesis mediated by mechanisms involving histamine, prostaglandins, nitric oxide, IL-1 and TNF-α.

The edematogenic effect of DwL was reduced about 65% (AUC: 3.29 ± 0.18) by mast cell depletion evoked with compound 48/80 (AUC: 1.02 ± 0.08), but not by thioglycolate-induced macrophage accumulation (AUC: 2.85 ± 0.17) (Fig. 5C).

4. Discussion

Like most legume lectins, DwL has a protein chain in the asymmetric unit featuring 237 residues. Diocleinae lectins consist of one intact subunit (α) and two fragments (β and γ) originated from the post-translational processing of the lectin precursor during seed development [10]. The mannose moiety of X-Man binds to the CRD, stabilizing the protein. This leads to a more highly ordered crystal arrangement allowing the formation of better-quality crystals, as observed for other legume lectins such as *Parkya plathycephala* lectin – PPL [36].

Being a tetramer, DwL is formed by the intersection of two perpendicular crystallographic two-fold axes and consists of two dimers in canonical β-sheet conformation. Referred to as "jelly-roll", this quaternary arrangement is found in all ConA-like lectins. Abu has previously been identified in lectins, including CGL [35] and ConBr [37]. It has been suggested this interaction is part of a plant defense mechanism.

In respect to lectin effect in the inflammatory process, DwL displayed edematogenic activity paralleled by increased vascular permeability, but failed to produce anti-inflammatory effects. Diocleinae lectins may produce either anti-inflammatory [12,13] or pro-inflammatory effects [14,15,38] depending on the route of administration. However, for some lectins, such as DwL, no anti-inflammatory activity has yet been observed. The same is observed for the lectins of *Canavalia brasiliensis* (ConBr) and *Dioclea grandiflora* [13], the later shows 99% of identity with DwL. The edematogenic effect of DwL was of short duration, representing a typical manifestation of acute inflammation like other Diocleinae lectins.

Several studies have shown that resident cells may participate in leukocyte recruitment via synthesis of inflammatory mediators [39–41]. In our study, DwL-induced edema was reversed by treatment with compound 48/80, but not with thioglicolate, which

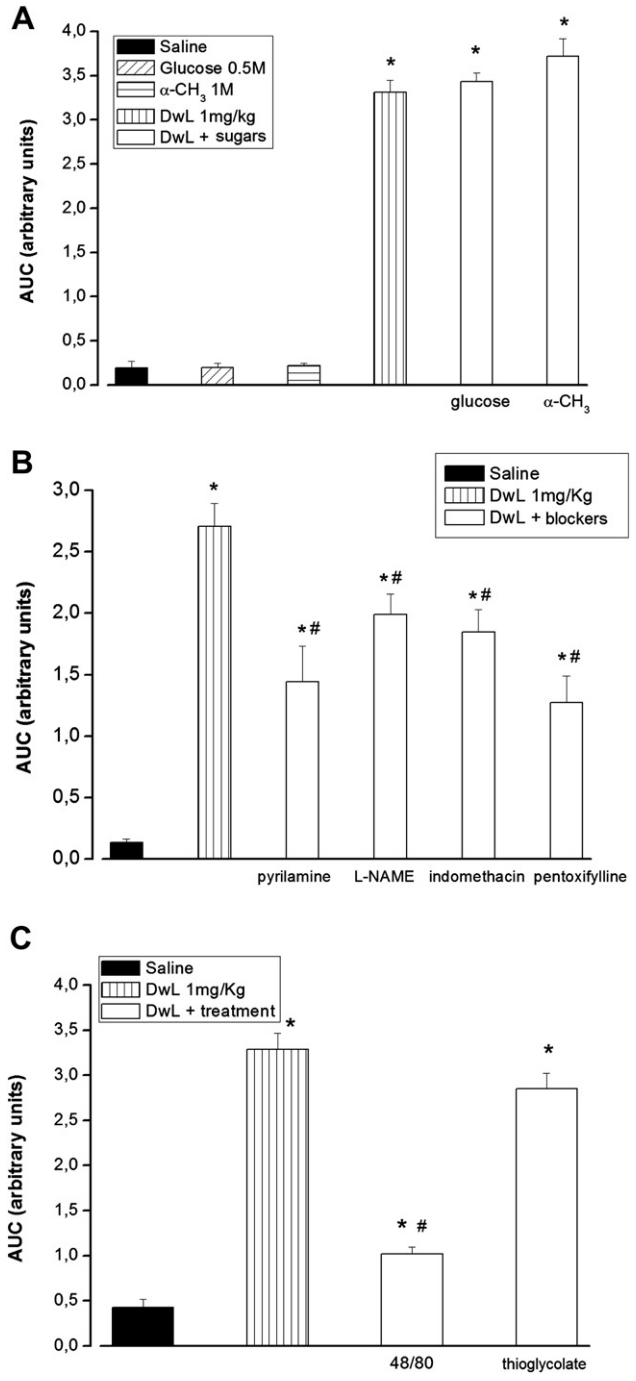

Fig. 5. Mechanisms involved in the edema induction by *Dioclea wilsonii* lectin. DwL (1 mg/kg) alone or associated with 0.5 M Glucose or 1 M α-CH3 was administered s.c. (Panel A). Pyrilamine (10 mg/kg; i.p.), L-NAME (30 mg/kg; i.v.), indomethacin (5 mg/kg; s.c.) or pentoxifylline (90 mg/kg; s.c) were injected in single doses 30 min before DwL (Panel B). Animals received i.p. compound 48/80 (at 0.6 mg/kg on the first 3 days and at 1.2 mg/kg on the day 4) or thioglycolate (in the first day at 3% and in the following days at 1.5%) over 4 days. 24 h later, normal or pre-treated rats were injected with DwL (1 mg/kg; s.c.) (Panel C). Negative controls received saline (0.1 ml/100 g body weight; s.c.); positive controls received DwL s.c. only. Edema was measured before (time zero) and at ½, 1, 2, 3, 4, 5, 6 after DwL. Means ± SEM ($n = 6$) as area under curve (AUC-arbitrary units). *$p < 0.05$ compared to saline; #$p < 0.05$ compared to DwL.

is evidence of the influence of mast cells in the lectin effect. In fact, mast cell activation could be implied in the neutrophil recruitment to rat peritoneal cavity induced by DwL [10]. Our findings are supported by several studies on mast cell recruitment promoted by plant lectins: *Araucaria angustifola* lectin, a GlcNAc-binding protein, produced edematogenic effect via activation of inflammatory mediators from resident mast cells [42]; the plant lectin KM+ was observed to activate and degranulate mast cells, providing an amplification loop for the neutrophil recruitment induced by this lectin [43]; and Diocleinae lectins (ConM, ConA, ConBr, DvirL, DRL, DVL, Dgui and CRL I) have been observed to induce histamine release in rat peritoneal mast cells [16–18,44].

Carbohydrate recognition is the main activity and potential biotechnological application of Diocleinae lectins. The importance of carbohydrate sites has also been demonstrated for a range of effects produced by Diocleinae lectins in inflammation models [12,13,15,38]. However, saturated solution of ConA and ConBr with glucose also released histamine from Wistar rat peritoneal mast cells [16]. Likewise, our results show that the edematogenic activity of DwL was not reversed by its binding sugars, suggesting that mast cell activation involves interactions with complex glycans that commonly depend on nonpolar interactions (Table 2). The observed interaction between DwL and X-Man sheds light on some important aspects of carbohydrate recognition by ConA-like lectins in general. The number of hydrogen bonds in DwL is similar to that of previously described members of the genus *Dioclea* (Table 2), but a comparison of the structures of DwL and DVL reveals important differences in hydrophobic and Van der Waals interactions (Table 2) (Fig. 1). Different from lectins of *Canavalia* genus, which show a peak of edema at the first half an hour, DwL showed a later peak of edematogenic activity, in accordance with other lectins of the same genus (Table 3). Differences in interaction patterns between the X-Man indolyl group and LEU99 are due to differences in the LEU99 side chain conformation of DVL and DwL. Thus, the two interactions with CD2 and the binding-enhancing hydrophobic contacts observed in DVL are absent in DwL (Fig. 3). This can explain why despite high identity of DwL with DVL (99% – SwissProt accession code: P58909) and Dgui (95% – SwissProt accession code: P81637) [10], DwL do not present anti-inflammatory effect [13] (Table 3). Comparing DwL edematogenic effect with other Diocleinae lectins, it can be observed that DwL show the best potency, increasing the edema in 12 times in relation to controls (Table 3). The conformation of the hydrophobic sub-site in DwL together with lack of affinity for monosaccharides may explain why this lectin interaction with complex glycans in mast cells lead to better efficacy for edematogenic activities and why the monosaccharides were unable to prevent the edematogenic effect of this lectin.

DVL and DwL also differ in the position 161. DVL contains a serine, and DwL contains an asparagine, although it is located about 24 Å of the monosaccharide binding site. This difference might be important when it comes to interaction with complex carbohydrates, since the activities seem to be caused by interactions with complex carbohydrates, as indicated by the reverse assays. It is

Table 3
Comparison between anti and pro-inflammatory effects of Diocleinae lectins.[a]

Lectin	Time of edema peak	Increase of edema	Anti-inflammatory effect
ConBr	½ h	8×	No
CGL	½ h	6×	N.I.
ConM	½ h	5×	N.I.
ConA	½ h	3×	N.I.
CRL I	4th h	6×	Yes
DVL	N.I.	N.I.	Yes
D. virgata	1st h	4×	Yes
Dgui	N.I.	N.I.	Yes
DGF	5th h	5×	No
DwL	5th h	12×	No

N.I.: not investigated.
[a] Adapted from [6,11,12,15,39].

Table 4
Distances between residues of the carbohydrate-binding domain of lectins from genus *Dioclea*.

Residues	Distance (Å)		
	DWL	DVL	DRL
ARG 228 N – TRY 12 OH	9.91	9.55	9.00
ARG 228 N – ASN 14 ND2	4.69	4.86	3.79
TYR 100 N – TYR 12 OH	7.23	6.91	7.30
TYR 100 N – LEU 99 N	2.71	2.65	2.78
TYR 12 OH – ASN 14 ND2	5.32	5.36	5.77
ARG 228 N – LEU 99 N	9.37	10.25	9.80
ARG 228 N – TYR 100 N	11.51	11.82	11.07

known that single mutations in amino acids at key positions close to the monosaccharide binding site drastically affects biological activities in Diocleinae lectins [15]. In this study, we report the same feature but occurring relativity distant of the monosaccharide binding site, which goes with the fact that the biological activities presented here are caused by complex carbohydrates.

DwL seems to interact with complex carbohydrates in mast cells surface, which in turn release mediators producing symptoms of inflammation, including vasodilatation, plasma extravasation and neutrophil migration. In this study, pre-treatment of rats with a prostaglandin and thromboxane blocker – indomethacin [45], an NOS inhibitor – L-NAME [46], an H_1 histamine receptor antagonist – pyrilamine [47] and a pro-inflammatory cytokine expression down-regulator – pentoxifylline [48] reduced the edematogenic effect of DwL, evidencing the involvement of prostaglandin, NO, histamine and cytokines (TNF-α and IL-1β). Upon being activated, mast cells possess the ability to release mediators – both preformed and newly synthesized – which serve to alert the immune response or amplify an existing response. Granule-associated mediators, including proteases, histamine, proteoglycans and cytokines (*eg* TNF-α) are released immediately upon activation, being critical for initiating mast cell-mediated innate immune responses. Newly synthesized mediators include lipids, cytokines and chemokines [19,49,50]. Lipid mediators such as leukotriene C4, leukotriene B4 and prostaglandin D2 are involved in various allergic and pro-inflammatory responses [49,50]. Mast cells produce various cytokines ranging from pro-inflammatory cytokines (*eg* TNF-α, IL-6 and IL-1β) to several chemokines (*eg* CCL5 and CXCL8), which recruit immune cells to the site of infection. Finally, mast cells are phagocytic and produce nitric oxide, superoxide radicals and antimicrobial peptides, such as cathelicidins [19,51].

Conformational changes in the hydrophobic sub-site have consistently been associated with the strengthening or weakening of interactions between glycans and lectins [3,6]. Another interesting structural aspect reported for legume lectins is the CRD conformation in lectins of the genus *Canavalia*. It has been suggested that certain geometric aspects of the CRD are related to cell recognition and the effect of these lectins on endothelial cells [52]. A comparison of three lectins of the genus *Dioclea* revealed discrepancies (Table 4) similar to those previously described for DVL and DRL using the same experimental model. However, the present study is to our knowledge the first to establish three-dimensional structural/functional relationships using a model of edematogenesis. These discrepancies in geometric aspects of the CRD can be involved in differences in inflammation induced by lectins of the *Dioclea* genus. For example, it should explain why DRL induces neutrophil migration via macrophage activation [14] while DwL via activation of mast cells.

5. Conclusion

DwL differs structurally from DVL and DRL with regard to the conformation of the carbohydrate recognition domain and related biological activities. The mechanism of edematogenic activity in DwL appears to involve positive modulation of mast cell degranulation via recognition of surface carbohydrates. Since this recognition is dependent on site volume and CRD configuration, edematogenesis mediated by resident cells varies in potency and efficacy among different Diocleinae lectins.

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