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G_2/M cell cycle arrest by an *N*-acetyl-D-glucosamine specific lectin from *Psathyrella asperospora*

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Abstract A new *N*-acetyl-D-glucosamine (GlcNAc) specific lectin was identified and purified from the fruiting body of the Australian indigenous mushroom *Psathyrella asperospora*. The functional lectin, named PAL, showed hemagglutination activity against neuraminidase treated rabbit and human blood types A, B and O, and exhibited high binding specificity towards GlcNAc, as well as mucin and fetuin, but not against asialofetuin. PAL purified to homogeneity by a combination of ammonium sulfate precipitation, chitin affinity chromatography and size exclusion chromatography, was monomeric with a molecular mass of 41.8 kDa, was stable at temperatures up to 55 °C and between pH 6–10, and did not require divalent cations for optimal activity. *De novo* sequencing of PAL using LC-MS/MS, identified 10 tryptic peptides that revealed substantial sequence similarity to the GlcNAc recognizing lectins from *Psathyrella velutina* (PVL) and *Agrocybe aegerita* (AAL-II) in both the carbohydrate binding and calcium binding sites. Significantly, PAL was also found to exert a potent anti-proliferative effect on HT29 cells (IC_{50} 0.48 μM) that was approximately 3-fold greater than that observed on VERO cells; a difference found to be due to the differential expression of cell surface GlcNAc on HT29 and VERO cells. Further characterization of this activity using propidium iodine staining revealed that PAL induced cell cycle arrest at G_2/M phase in a manner dependent on its ability to bind GlcNAc.

Keywords *Psathyrella asperospora* · *N*-Acetyl-D-glucosamine (GlcNAc) specific lectin · Mushroom lectin · Anti-proliferation · G_2/M cell cycle arrest

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Introduction

Lectins are proteins, non-immunoglobulin in nature, capable of specific recognition and reversible binding to the carbohydrate moiety of glycoconjugates on cell surface, resulting in cell agglutination and subsequent precipitation in solution [1]. The specificity of lectins makes them an important tool in glycoprotein purification, identification and glycan analysis [2]. Lectins are ubiquitous in nature, occurring in plants, humans, animals, fungi, bacteria, viruses, and also in foods, with their abundance being wider in mushrooms compared to plants [3]. Over the past few decades, a number of lectins have been isolated from mushrooms, and they have attracted considerable interest due to their various bioactive properties, including anti-proliferative [4–6], anti-tumour [7–9], mitogenic [4, 8, 10], immunomodulatory [7, 11, 12], hypotensive and vasorelaxing [13], and antiviral [6, 8, 14] activity.

Australia has a spectacular biodiversity including animal, plants and fungi. Indigenous Australians have been using fungi traditionally as medicines and in religious practice for thousands of years [15]. A large number of different mushrooms species are present in Australia that are poorly explored and catalogued. Importantly, very little is known about the extent and diversity of lectins from Australian mushroom species [16]. *Psathyrella asperospora* (Family: Psathyrellaceae) (Syn.: *Lacrymaria asperospora*) is an Australian indigenous mushroom, not used for food, that we recently reported expresses an *N*-acetyl-D-glucosamine (GlcNAc) specific lectin

activity [16]. Another species (*Psathyrella velutina*) of the same genus has been reported to express a GlcNAc/*N*-acetylneuraminic acid (Neu5Ac) specific lectin, referred to as PVL [17, 18]. PVL has now been well characterized with respect to specificity and interaction kinetics [18, 19], and a 1.5 Å crystal structure is also available [20]. However, until now there has been no report of its pharmacological activity including cytotoxic or anti-proliferative activity.

Lectins with high affinity towards GlcNAc have been isolated and characterized from both vertebrates and invertebrates [1], and have been found to be potent and selective inhibitors of human immunodeficiency virus and cytomegalo virus replication *in vitro* [21] as well as other human pathogens [22], and are known to be cytotoxic towards human hepatocellular carcinoma, human placenta choriocarcinoma and rat osteosarcoma cell [23].

Here we report the isolation and characterization of a new GlcNAc specific lectin from the fruiting body of *P. asperospora* named PAL that exhibits potent anti-proliferative activity against colon adenocarcinoma (HT29) cells. Further characterization of PAL's anti-proliferative activity showed that HT29 cells are arrested at G_2/M phase of the cell cycle, and that this effect can be halted through the addition of free GlcNAc.

Materials and methods

Mushroom collection and reagents

The fruiting bodies of *P. asperospora* (Accession no. MEL 2061945) were collected in Melbourne, Australia, identified at the Royal Botanic Gardens, Melbourne and immediately frozen at −20 °C. Unless otherwise stated all the reagents were purchased from Sigma. Fructose was obtained from Ajax chemicals, lactose from OXOID Ltd, Neu5Ac from Jülich Chiral Solutions GmbH, chitin affinity sepharose from New England BioLabs, rabbit erythrocytes from IMVS Veterinary Services Division, and cell culture medium and reagents from GIBCO.

PAL purification, molecular mass determination and protein estimation

Apart from the final size exclusion chromatography step all other purification steps were carried out at 4 °C. Thawed fruiting bodies (40 g) was suspended in 80 mL PBS (pH 7.4) homogenized using a Waring blender and left overnight at 4 °C with gentle shaking. The resulting homogenate was filtered through cotton gauze and centrifuged twice, first for 20 min at 1,500×g, followed by another 20 min at 10,000×g. Solid $(NH_4)_2SO_4$ was added to the resulting supernatant (crude homogenate) to a final concentration of 80 % and allowed to fully dissolve for 45 min. Following centrifugation at 12,000×g for 25 min, the resulting 80 % pellet was re-suspended in a minimal volume of 20 mM Tris buffered saline (TBS) (pH 8.5) and extensively dialyzed against the same buffer. Subsequently, the dialyzed 80 % $(NH_4)_2SO_4$ precipitate was loaded onto a 1 mL chitin sepharose column equilibrated with TBS (pH 8.4) and the affinity adsorbed PAL eluted with TBS (pH 8.5) containing 50 mM GlcNAc and 10 % (v/v) glycerol. The GlcNAc eluted fraction was extensively dialyzed against TBS (pH 8.5), concentrated using an Amicon ultrafiltration device (MWCO 10 kDa) and applied to a Superdex 75 GL column (GE healthcare) (10×300 mm; bed volume 24 mL) equilibrated with TBS (pH 8.5). The purification of PAL was monitored at each step using a hemagglutination assay with rabbit erythrocytes as described in [16]. PAL purified in this manner was stored at −20 °C in TBS (pH 8.5) containing 10 % (v/v) glycerol. Protein estimation was performed using the BCA (Bicinchoninic acid) Protein Quantitation Kit as described by the manufacturer (Thermo scientific). Standard curves were prepared using bovine serum albumin (BSA) concentrations between 0 and 2 mg/mL. Samples and standards were read on a Viktor3 1420 Multilabel counter (PerkinElmer) at 595 nm.

The molecular mass of purified PAL was determined using SDS-PAGE, size exclusion chromatography (SEC), and dynamic light scattering (DLS). SDS-PAGE was performed on a 10 % (w/v) acrylamide gel as described by Laemmli, 1970 [24], and gels stained with coomassie brilliant blue R-250. SEC was performed on a Superdex 75 GL column calibrated with Conalbumin (75 kDa), Ovalbumin (43 kDa), Carbonic anhydrase (29 kDa), Ribonuclease-A (13.7 kDa) and Aprotinin (6.5 kDa). DLS analysis of purified PAL (34.34 μg) was performed on a Enterprise^{MDP} PD2100 Refractometer (Precision Detectors) connected to a BioSep SEC-S 2000 LC column (300×7.80 mm; bed volume 24 mL, Phenomenex) controlled through an Aglient 1100 series HPLC (Aglient Technologies). The SEC-S 2000 column was equilibrated with PBS (pH 7.4) and calibrated with BSA (5 mg/mL).

PAL *de novo* peptide sequencing and database searches

The band corresponding to PAL was excised from coomassie brilliant blue stained SDS-PAGE gel and tryptically digested according to standard protocols at the Australian Proteome Analysis Facility (APAF). Tryptic peptides were extracted and analysed with Q-Star Elite (AB Sciex, Framingham, MA) using a 150 μm × 10 cm ProteCol C18 column (SGE Analytical Science). Each peptide type was subject to positive ion nanoflow electrospray (ESI) MS analysis on QSTAR that was operated in an information dependent acquisition mode (IDA). In IDA mode a TOFMS survey scan was acquired (m/z 400–1600, 0.5 s), with the three largest multiply charged

ions (counts >50) in the survey scan sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 2 s (m/z 100–1600).

Database similarity searches with LC-MS/MS data were performed using National Center for Biotechnology Information (NCBI) database to identify the lectin from non-redundant fungi entries by Mascot software (Matrix Science, London, UK), and for *de novo* sequencing the raw data was processed using the peptide sequencing tool Analyst QS 2.0 software (AB Sciex). The resulting peptide sequences were analyzed by BLAST (using non-redundant fungi database) and aligned using Clustalw 2.

Hemagglutination inhibition and biochemical characterization

Hemagglutination inhibition assays were performed as described previously [16] using 13 different saccharides and 7 different glycoproteins. The pH and temperature optimum of PAL was established by determining the hemagglutination titer at pH's between 6 and 10, and following incubation of lectin at temperatures between 25 °C and 85 °C for 1 h. The effect of mono- and di-valent cations on PAL's hemagglutination activity was determined in the absence and presence of 1 mM $CaCl_2$, $MgCl_2$ and KCl after dialysis against 40 mM EDTA.

Analysis of GlcNAc expression on HT29 and VERO cells

HT29 (human colon adenocarcinoma) and VERO (African green monkey kidney) cells were cultured in DMEM containing 10 % fetal bovine serum and 1 % Penicillin-Streptomycin (10,000 U/mL and 10,000 μg/mL, respectively) at 37 °C in a humidified atmosphere with 5 % CO_2 in air. HT-29 and VERO cells were seeded in 12-well plates (5×10^4 to 1×10^5 per well) and cultured until 70 % confluence was reached after which they were harvested by cell scrapping. Cells were washed with PBS and processed for lectin staining with 80 μg/mL WGA-FITC for 15 min in the dark. Cells were then fixed with 2 % formaldehyde and then immediately analysed using CyAn™ ADP Analyzer (Beckman Coulter). Using De novo FCS express 4 software the results of cell fluorescence were displayed as histograms. Cells with no staining served as a negative control.

Analysis of anti-proliferation using MTT assay

The anti-proliferative effect of PAL on the growth and viability of HT29 and VERO cells were determined using tetrazolium dye (MTT) as previously described by Horiuchi *et al*. [25]. Briefly, 5×10^4 cells/well in a 24-well plate were cultured with different final concentrations of PAL (ranging from 0.15 to 2.4 μM) in growth medium for 24 h and then incubated with MTT reagent for 2 h. The resulting purple formazan dye was solubilized using 0.1 % SDS and 0.04 M HCl in absolute isopropanol and the absorbance measured at 595 nm using a Viktor3 1420 Multilabel counter. Negative controls were afforded by growing cells under the same conditions in the absence of PAL. The ability of GlcNAc to protect cells from PAL's anti-proliferative effect was evaluated by incubating HT29 and VERO cells in the presence and absence of 0.48 μM and 0.6 μM PAL respectively, at GlcNAc concentrations between 0.2 mM and 3.13 mM. The protective effect of Neu5Ac, galactose (Gal), lactose (Lac) and L-Fucose (L-Fuc) was determined at a final saccharide concentration 1.56 mM.

Cell cycle analysis by flow cytometry

HT29 cells (1×10^5/well) in 12-well plates were cultured in the presence and absence of 0.48 μM PAL for 24 h, after which they were harvested by trypsinization, washed with PBS and fixed with 70 % ice cold ethanol. Following centrifugation, the fixed cells were incubated with RNase A solution (0.2 mg/mL RNase and 0.05 % Triton X-100) in PBS and resuspended in 1 mg/mL propidium iodide. Cell cycle distribution was analyzed using BD LSR Fortessa Cell Analyzer (BD Biosciences) and 10,000 cells were used for each analysis. The results were displayed as histograms, data was recorded using BD FACS Diva software and was analyzed latter using *De novo* FCS express 4 Flow Cytometry software. Cell cycle assays were also carried out in presence of GlcNAc as described above.

Results

Purification and *de novo* sequencing of *Psathyrella asperospora* lectin (PAL) tryptic peptides

We previously showed that the crude homogenate from *P. asperospora* was able to hemagglutinate both rabbit and human blood types A, B and O, with hemagglutination activity being enhanced following neuraminidase treatment of human blood types A, B and O [16]. Neuraminidase treatment of rabbit blood had no effect on activity. Importantly, regardless of the blood types used and treatment prior to activity assays the only saccharide able to inhibit hemagglutination was GlcNAc, with minimum inhibitory concentrations (MICs) in the low mM range [16]. Therefore, in this study due to its high hemagglutination titer, untreated rabbit blood was used to monitor PAL activity during purification.

PAL was purified to homogeneity in three steps; 80 % ammonium sulfate $(NH_4)_2SO_4$ precipitation, chitin affinity chromatography and Superdex S75 size exclusion chromatography (SEC). From 40 g of frozen *P. asperospora* fruiting bodies 3.5 mg of PAL was purified at a purification fold of

21.1, and a recovery of 19.2 % (Table 1). The molecular mass of PAL as determined by SEC (Fig. 1a) was approximately 36.0 kDa, which correlated well with that determined by SDS-PAGE (Fig. 1b) under reducing (Fig. 1b, lane 5) and non-reducing (Fig. 1b, lane 6) conditions of approximately 40 kDa. Accurate determination of molecular mass was afforded by DLS (dynamic light scattering) analysis, with PAL determined to have a molecular mass of 41.8 kDa. PAL was further characterized by *de novo* sequencing using LC-MS/MS, with 10 tryptic peptides identified (Fig. 1c). Mascot analysis revealed the 10 peptides to share sequence similarity to the *P. velutina* lectin (PVL, gi:78057570) and *Agrocybe aegerita* lectin 2 (AAL-II, gi:375333787), both of which are GlcNAc specific [17, 26]. An NCBI-BLASTP search and ClustalW2 analysis of a 43 amino acid peptide sequence constructed from peptides 4, 6 and 7 (Fig. 1c), TVALAD LVGEGTGGVYLLRGGSLLLQVVKVLDNFGYNA-GGSVR, exhibited high identity to both PVL (70 % identity) and AAL-II (45 % identity) (Fig. 2). Similarly, PAL peptides 1, 2, 3, 5, 8, 9 and 10 also showed high identity to PVL and AAL-II. Significantly, a number of these peptides overlapped with known carbohydrate (peptides 2, 3, 5, 7, 8, 9 and 10) and calcium (peptides 3 and 5) binding domains. Figure 2 shows the multiple sequence alignment of PVL, AAL-II and tryptic peptides of PAL, with the carbohydrate and calcium binding domains as determined through the X-ray crystal structure of PVL being highlighted. Although highly conserved, some differences between PAL, PVL and AAL-II are evident. In particular, a number of PVL residues known to be involved in carbohydrate recognition and binding, Trp54, Glu191, Asn214, Asp270 and Try306 are, based on *de novo* sequencing of tryptic peptides, substituted in PAL with Ser, Val, Pro, Phe and Leu respectively. However, given the high affinity and strict specificity for GlcNAc that PAL exhibits, it would appear that these substitutions have no dramatic effect on the structure of the carbohydrate-binding sites. In addition, Leu183 in the PVL Ca^{2+} binding domain is conservatively substituted with Ala in PAL (Fig. 2), and a two-residue insertion (an Arg and Leu) was found in PAL between PVL residues Leu183 and Leu184. However all other residues in the PVL Ca^{2+} binding consensus sequence, Asp-h-Thr-Gly-Asp-Gly-h-h-Asp, are conserved.

PAL carbohydrate specificity and biochemical characterization

As previously observed for the crude homogenate from *P. asperospora* [16], among the saccharides tested GlcNAc was the only capable of inhibiting the hemagglutination activity of purified PAL, with a MIC of 0.78 mM. All other saccharides were unable to inhibit PAL activity even at 50 mM (Table 2). Of particular interest was the lack of PAL hemagglutination inhibition exhibited by free Neu5Ac. The closely related PVL is known to bind free Neu5Ac in addition to GlcNAc, but only very weakly ($K_d < 10^{-3}$ M) [20]. However, PAL did show high binding affinity for mucin (MIC 0.002 mg/mL) and fetuin (MIC 0.0078 mg/mL), but not for asialofetuin even at 1 mg/mL concentration (Table 2), suggesting that sialoglycoconjugates may be a ligand for PAL.

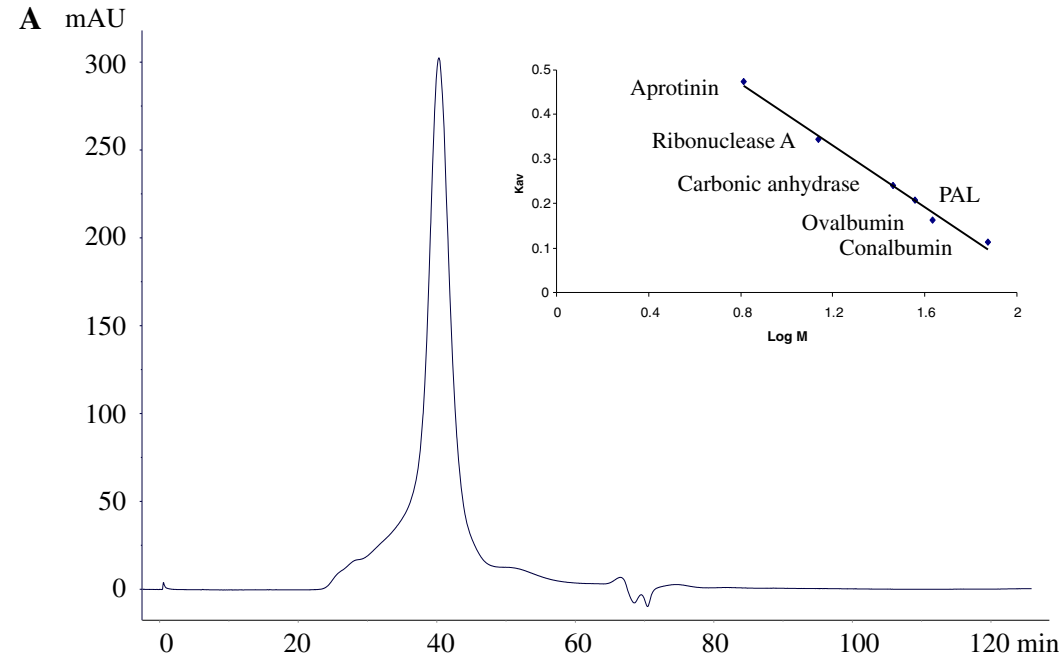
Further characterization of PAL's hemagglutination activity revealed complete stability up to 55 °C, which was reduced to 50 % at 65 °C, and no longer detectable at 75 °C. PAL's hemagglutination activity was also unaffected by changes in pH, with no change in activity observed between pH 6.0 and 10.0, as well as by the addition of EDTA or various mono- and di-valent cations including KCl, $CaCl_2$ and $MgCl_2$.

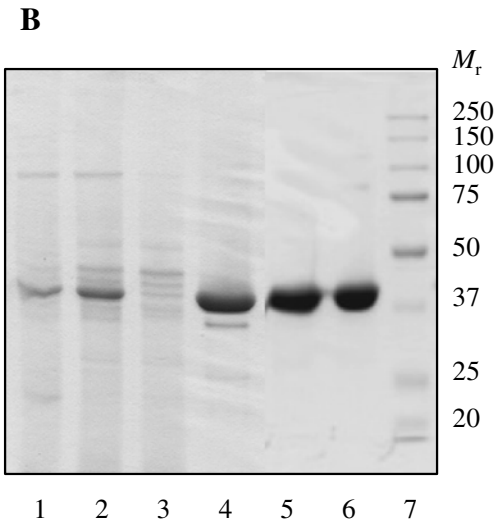
PAL exhibits anti-proliferative activity and induces G_2/M arrest

The effect of PAL on the proliferation of human colon adenocarcinoma HT29 and monkey kidney VERO cells was assessed initially using the MTT assay. Figure 3a shows a dose dependent anti-proliferative effect of PAL up to a concentration of 0.6 μM, following which point anti-proliferation activity plateaued, thus only permitting an IC_{50} determination against HT29 cells (IC_{50} 0.43 μM or 20 μg/mL). However, at a PAL concentration of 0.6 μM anti-proliferative activity was almost 3-fold greater against HT29 compared to VERO cells. Interestingly, visual examination and trypan blue staining of cells following PAL treatment suggested that the lectin was cytostatic rather than cytotoxic (data not shown). The addition of GlcNAc to the media was able to protect both HT29 and VERO cells from the anti-proliferative effect of PAL in a dose-dependent manner, with full protection afforded at 1.56 mM GlcNAc (Fig. 3b). The protective effect of

Table 1 Purification of *P. asperospora* lectin (PAL)

Fraction	Total protein (mg)	Total HA-A (HA-U)	Specific activity (HA-U/mg)	Purification fold	% Recovery
Crude homogenate	391.8	208256	531.6	1	100
80 % $(NH_4)_2SO_4$precipitate	86.1	129331	1501.5	2.8	62.1
Chitin affinity chromatography	11.8	90112	7664.7	14.4	43.3
Size exclusion chromatography	3.5	39813	11240.4	21.1	19.1





De novo sequencing

C

No.	*De novo* peptide sequence
1	**FMADMR**
2	**FDGVASGWR**
3	**LLDGTGGGVSK**
4	**GGSLLLQVVK**
5	**VLADLTGDGAR**
6	**TVALADLVGEGTGGVYLLR**
7	**VLDNFGYNAGGSVR**
8	**ETQVLSQAVVVPSR**
9	**ADLVGFGEPGSLCPR**
10	**MVLADFSYGGAGPFDMHK**

Fig. 1 **a** Partially purified PAL following $(NH_4)_2SO_4$ precipitation (80 % w/v) and chitin affinity chromatography was purified to homogeneity by size exclusion chromatography (SEC) on Superdex 75 GL column. The calculated molecular weight by SEC of purified PAL was 35.96 KDa (insert). **b** SDS-PAGE of purified PAL; lane 1, crude homogenate; lane 2, 80 % $(NH_4)_2SO_4$ precipitation; lane 3, chitin affinity unbound; lane 4, chitin affinity eluent; lane 5, SEC Superdex 75 GL eluent (non-reducing conditions); lane 6, SEC Superdex 75 GL eluent (reducing conditions); and lane 7 Molecular weight markers. **c** Amino acid sequence analysis of purified PAL. Ten PAL tryptic peptides were identified through *de novo* sequencing by MS/MS

additional saccharides (Neu5Ac, Gal, Lac and L-Fuc) was also investigated, however none were able to protect HT29 and VERO cells from the anti-proliferative activity of PAL, even at 1.56 mM.

In order to further explore the mechanism by which PAL inhibits cell proliferation a cell cycle analysis by propidium iodide (PI) staining was performed. Figure 3c clearly shows that in the presence of 0.48 μM PAL there was a significant increase in the percentage of HT29 cells arrested in G_2/M phase (33.9 %), with a corresponding decrease in the percentage of cells observed in the S phase (17.3 %), compared to PAL untreated cells (13.6 % and 35.5 %, respectively). No change in the percentage of HT29 cells in G1 phase of the cell cycle was observed. Similar to the ability of GlcNAc to protect cells from the anti-proliferative effect of PAL as observed using the MTT assay (Fig. 3b), the addition of GlcNAc to the culture

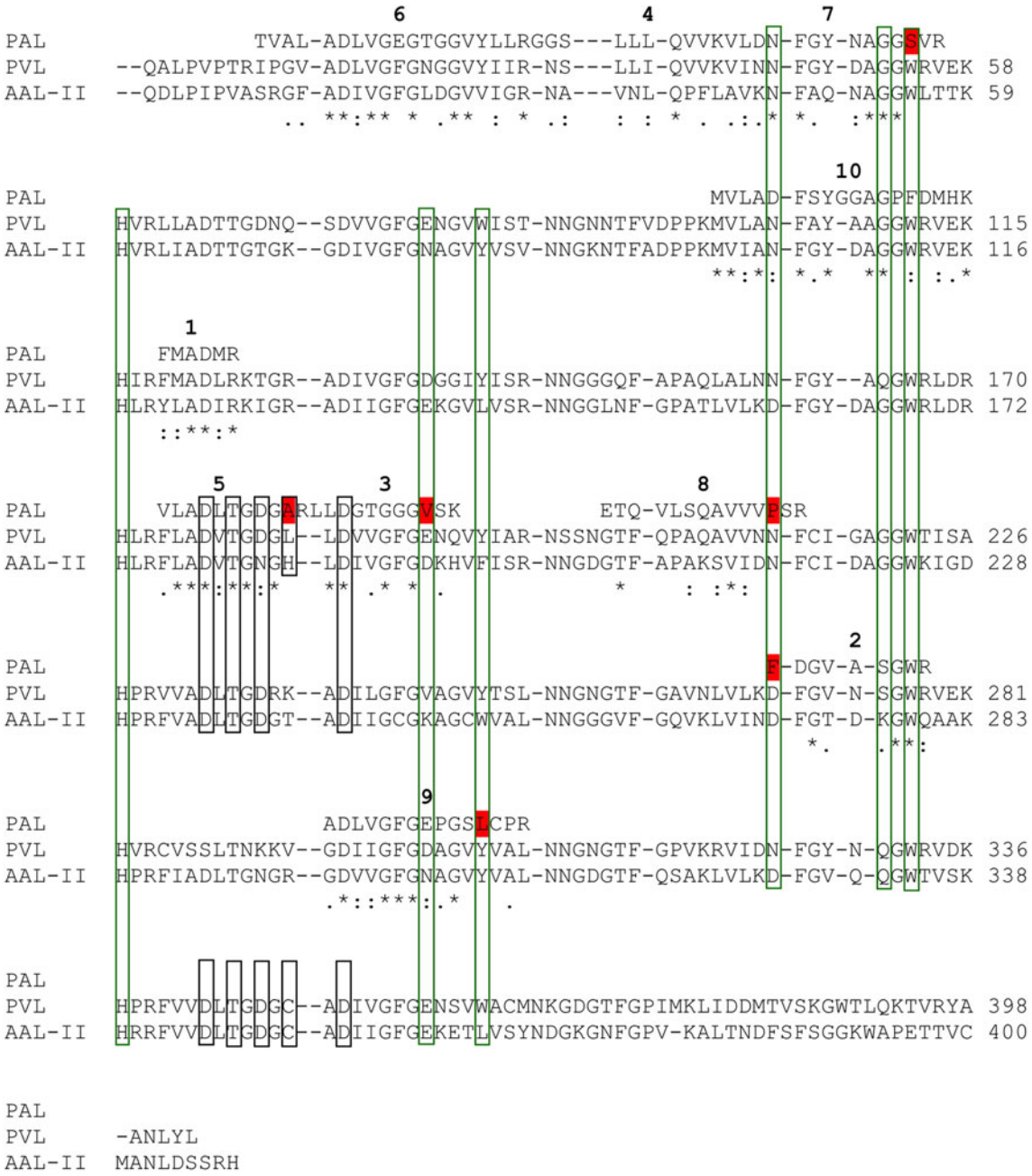


Fig. 2 Cluster W2 sequence alignment of 10 PAL tryptic peptide sequences (numbered), PVL (gi:78057570) and AAL-II (gi:375333787). Residues involved in carbohydrate and Ca^{2+} binding in PVL are boxed in green and black respectively. Amino acids highlighted in red represent PAL residues not conserved in either PVL or AAL-II

media and subsequent PI staining following the addition of PAL lead to a significant decrease in cells arresting in G_2/M (Fig. 3c). Presumably the addition of GlcNAc to the culture media inhibits binding of PAL to cell surface glycoconjugates thus protecting cells from PAL's anti-proliferative effects.

Taken together, our data suggests that the observed differences in PAL anti-proliferative activity between HT29 and VERO cells, and the ability of both cell lines to be protected by exogenous GlcNAc, may be due to differential GlcNAc expression. To assess this the expression of GlcNAc on HT29 and VERO cells was evaluated by flow cytometry using FITC-labelled WGA (Wheat Germ Agglutinin). Figure 4 clearly shows that HT29 expresses approximately 2-fold more GlcNAc on the cell surface than VERO cells.

Discussion

Mushroom lectins exhibit a broad specificity varying from simple sugars to complex saccharides and glycoproteins [6].

Table 2 Inhibition of hemagglutination activity associated with purified PAL

	Minimum inhibitory concentration (MIC)
Saccharides*	
Galactose	–
D-Glucose	–
GlcNAc	0.78 mM
GalNAc	–
Lactose	–
D-Mannose	–
L-Arabinose	–
D-Ribose	–
D-Xylose	–
L-Fucose	–
D-Maltose	–
Neu5Ac#	–
Sucrose	–
Glycoproteins**	
α-acid glycoprotein	0.125 mg/mL
Fetuin	0.0078 mg/mL
Asialofetuin	–
Mucin	0.002 mg/mL
BSA	0.5 mg/mL
Conalbumin	1.0 mg/mL
Fibrinogen	1.0 mg/mL

*Saccharide concentration range from 0.2 to 50 mM, # Maximum final concentration was 25 mM, ** Glycoprotein concentration range from0.0005 to 1.0 mg/mL

PAL showed strict specificity for GlcNAc among the saccharides tested, but also exhibited high binding affinity towards fetuin and mucin, which possess terminal Neu5Ac. Similarly, the closely related PVL preferential binds free and oligosaccharides bearing non-reducing terminal GlcNAc structure [17] as well as terminal Neu5Ac residues on glycoproteins and oligosaccharides [18, 27]. The sequence alignment of PVL, AAL-II and tryptic peptides of PAL reveals high sequence conservation in regions known to be involved in carbohydrate recognition and binding. However, a number of residues involved in PVL's recognition of GlcNAc [20], Trp54, Glu191, Asn214, Asp270 and Try306 are, based on *de novo* sequencing of tryptic peptides, substituted in PAL with Ser, Val, Pro, Phe and Leu respectively. Although, given the high affinity and strict specificity for GlcNAc that PAL exhibits, it

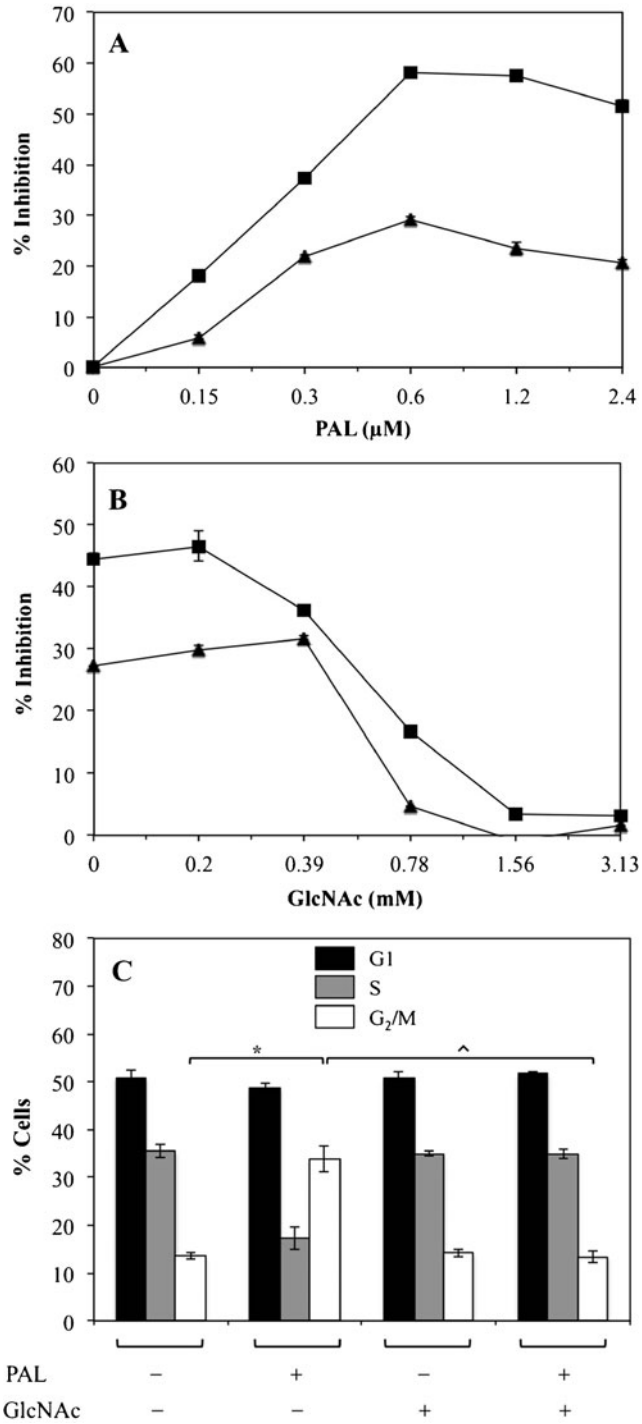


Fig. 3 **a** *In vitro* anti-proliferative effect of PAL was found to be approximately 3-fold greater against HT29 (*black square*) compared to VERO (*black up-pointing triangle*) cells. DMEM was used as negative control and results are stated as mean ± SEM (standard error of the mean; $n=2$, each in triplicate). **b** The addition of GlcNAc to the media was able to protect both HT29 (*black square*) and VERO (*black up-pointing triangle*) cells from the anti-proliferative effect of PAL in a dose-dependent manner. DMEM was used as negative control and results are stated as mean ± SEM ($n=2$, each in triplicate). **c** Cell cycle progression was examined by flow cytometry following PI staining. PAL (0.48 μM) mediated a statistically significant ($p<0.05$) increase in HT29 cells arresting in G_2/M phase (*white bars*) of the cell cycle. HT29 cell arrest in G_2/M phase mediated by PAL was found to be GlcNAc dependent, with the addition of 1.56 mM GlcNAc to the culture media protecting cells from the anti-proliferative effect of PAL. The addition of GlcNAc in the absence of PAL had no effect on cell cycle progression. Results are stated as mean ± SEM, $*P<0.05$ ($n=2$, each in triplicate)

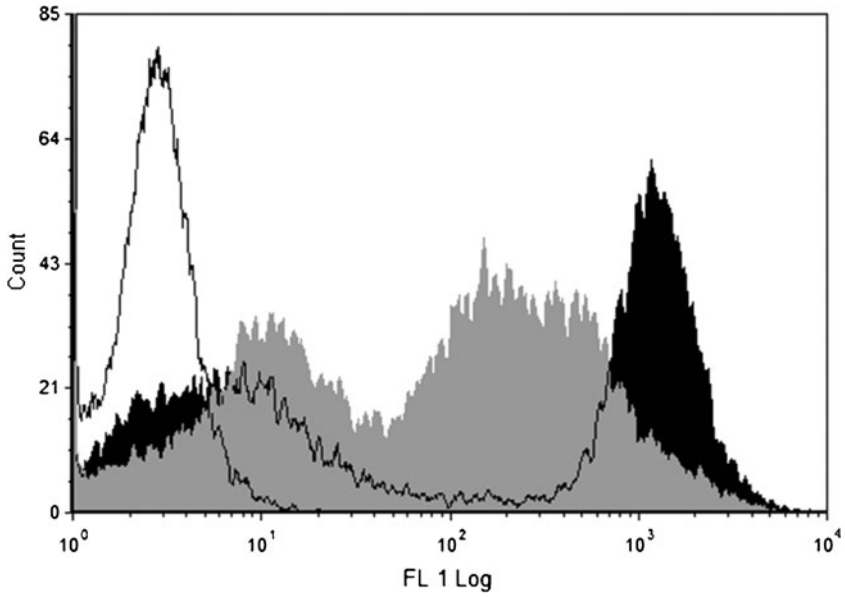


Fig. 4 Flow cytometry analysis of HT29 (*black shading*) and VERO (*grey shading*) cells labelled with FITC conjugated WGA. Unstained cells served as a negative control (*white shading*)

would appear that these substitutions have no dramatic effect on the structure of the carbohydrate-binding sites.

PAL, AAL-2 [26] and PVL [17] all bind GlcNAc in a divalent cation independent manner, however the crystal structure of PVL revealed two Ca^{2+} binding sites consisting of nine residue loops with the consensus sequence Asp-h-Thr-Gly-Asp-Gly-h-h-Asp (where h is an hydrophobic residue) [20]. It is still unclear as to the exact function of Ca^{2+}in PVL for mushroom metabolism, although it has been postulated that Ca^{2+} binding may play a role in lectin stabilization in a similar way to that seen in integrins [20]. A sequence alignment of PVL, AAL-II and tryptic peptides of PAL revealed a high conservation of residues in Ca^{2+} binding site 1, with only Leu183 substituted with an Ala in PAL, and a His in AAL-II. PAL sequence information was not obtained for Ca^{2+} binding site 2, however this site is completely conserved between PVL and AAL-II. Therefore it would seem highly probable that the ability to bind Ca^{2+} is a conserved feature of this family of GlcNAc specific lectins.

Lectins are well known to possess cytotoxicity and/or anti-proliferative activity against cultured cells [4–6]. To assess the anti-proliferative activity of PAL, MTT assays were performed on human colon cancer HT29 and monkey kidney VERO cells. In addition to possessing complex carbohydrates, and a small proportion of Gal and GalNAc, the cell surface of HT29 also possesses a high proportion of GlcNAc-terminated glycans ([28] and this study). In addition GlcNAc is abnormally expressed in several carcinomas, with uncontrolled and aberrant expression of GlcNAc present on cancer cell surfaces [29, 30]. Although, PAL showed anti-proliferative activity against both cell lines, the effect on HT29 cells was 3-fold greater than that observed on VERO cells, primarily due to different levels of GlcNAc expressed on the cells (Fig. 4). In both cases, this activity was only abolished in the presence of 1.56 mM GlcNAc, with the addition of other saccharides notably Neu5Ac having no effect on PAL's anti-proliferative activity. To our knowledge PAL represents the first reported GlcNAc-specific mushroom lectin with potent (IC_{50} 0.43 μM) anti-proliferative activity. The vast majority of mushroom lectins thus far reported to possess anti-proliferative activity exhibit IC_{50} ranging from 2 to 20 μM [6]. The two most potent mushroom lectins so far identified are the Gal specific PEL from the Basidiomycete *Pleurotuseous* and the xylose-specific lectin from the Ascomycete *Xylariahypoxylon* that inhibit proliferation of cancer cell lines MCF-7, K562 and HEP-2 with an IC_{50} of 2 μg/mL (approx. 0.125 μM) [31], and M1 and HepG2 cells with an IC_{50} of approximately 1 μM [32], respectively.

Further analysis of PAL's anti-proliferative activity on HT29 cells using PI staining revealed a significant accumulation of cells in G_2/M phase, a phenomenon that was also blocked by the addition of free GlcNAc to the cell culture media prior to adding PAL. The entry of cells into M phase of the cell cycle is carefully regulated by the cell cycle checkpoint at the late G_2 phase. The G_2 checkpoint allows the cell to repair DNA damage before entering mitosis. This checkpoint is controlled by a family of protein kinases that are regulated by a complex group of proteins including the cyclins [33]. A previous study has shown that WGA induces G_2/M phase arrest in mouse L929 fibroblasts after one cell cycle through activation of Chk1 and suppression of the cdc2-B1 complex. Even though pro-apoptotic proteins Bax and caspase 3 are up-regulated and the anti-apoptotic Bcl-2 protein down-regulated, no apoptotic bodies upon treatment of L929 cells with WGA were observed. However, strong cytotoxicity and induction of apoptotic bodies in HT29 cells by WGA was noted [34]. The fact that PAL appears to be cytostatic rather than cytotoxic suggests that the accumulation of cells in G_2/M

phase may not be a consequence of the invocation of some aspect of the apoptotic pathways.

Taken together our data shows that the potent anti-proliferative activity of PAL is mediated through binding of GlcNAc residues on the cell surface, with differences in the observed cytotoxicity of PAL towards HT29 and VERO cells being due to differences in GlcNAc expression. To our knowledge PVL has never been analyzed for cytotoxic and/or anti-proliferative activity, however the cytotoxic activity of the GlcNAc/Neu5Ac specific lectin WGA has been relatively well characterized. As opposed to PAL where anti-proliferative activity was mediated through binding GlcNAc residues on the cell surface, WGA cytotoxicity was found to predominantly result from its Neu5Ac binding activity [34]. Liu *et. al*. showed that the cytotoxic activity of WGA towards L929 fibroblast cells was suppressed following succinylation, which specifically eliminates WGA's Neu5Ac binding activity [34]. More recently the GlcNAc specific lectin AAL-II was found to induce apoptosis in hepatoma cells, and inhibit hepatoma growth and extend the survival time of tumour bearing mice [26]. It was suggested that AAL-II's specific anti-tumour activity was a direct consequence of AAL-II's selective binding of GlcNAc on the hepatoma cell surface, however this was not directly shown. Given GlcNAc is known to be aberrantly expressed on the surface of cancer cells, our cytostatic highly selective GlcNAc specific PAL might have a potent application in cancer diagnosis or therapy.

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